In vitro antifungal activity of N-3-(1,2,4-dithiazole-5-thione)-β--resorcylcarbothioamide

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Abstract

The aim of the study was the determination of antifungal activity of N-3-(1,2,4-dithiazole-5-thione)-ß-resorcylcarbothioamide (DTRTA) against *Candida albicans*, *non-Candida albicans*, dermatophytes and molds and evaluation of the enzymatic activity *C. albicans* strains. We used reference strains *C. albicans* 10231 ATCC, 200 of *C. albicans* strains, 7 of *non-C. albicans*, 12 dermatophytes strains and 20 molds strains isolated from different ontocenoses from patients. DTRTA was synthesized at Department of Chemistry University of Agriculture in Lublin was used to tests. The mean MIC of DTRTA against *C. albicans* strains isolated from patients was 22.01 mg/L, for reference *C. albicans* 10231 ATCC – 12.5 mg/L on Sabouraud's medium (SB). The mean MIC of isolates from patients was 17.8 mg/L, and reference strains – 6.25 mg/L on YNB medium, respectively. The MICs of DTRTA against 7 *non-C. albicans* was 33 mg/L on SB and 18.2 mg/L on YNB. The MICs of DTRTA against dermatophytes ranged from 3 to 50 mg/L. The MICs of DTRTA against molds were 25 mg/L and 100 mg/L, respectively. *C. albicans* strains had the enzymatic activity of 16 among 19 hydrolases, after exposure to DTRTA, 15 among 19 enzymes, respectively. *Non-C. albicans* isolates had the enzymatic activity of 13 among 19 hydrolases, after exposure to DTRTA, 11 among 19 enzymes, respectively. This findings indicate hat DTRTA exerts a potent antifungal activity against the yeast-like fungi strains, dermatophytes and molds in vitro and.

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Introduction

The incidence of nosocomial infections by the yeast-like fungi strains has surged over the past decade from the eighth to the fourth most common cause of nosocomial bloodstream infection in the general hospital population [1]. In surgical patients, the incidence of Candida infections has increased from 2.5 to 5.6 per 1000 discharges with mortality rates of 30% to 75% [2,3]. In these reports the predisposing factors included: hematological malignancy, solid organ tumors, neutropenia, intravascular devices, hyperalimentation, antimicrobial therapy and corticosteroid therapy [4]. However, one result of widespread use of powerful antifungal drugs administered for increasingly broader indications has been a dramatic increase in the isolation of *non-Candida albicans* and resistant forms of Candida [5-7].

The discovery of the azole antifungal compounds, ketoconazole, itraconazole, and fluconazole, allowed for a broader spectrum of antifungal treatment and a shorter treatment duration [8]. These drugs act by inhibiting cytochrome P450-dependent ergosterol synthesis and cytochrome c oxidative and peroxidative enzymes. This disruption of enzymic processes ultimately leads to fungal cell death [8]. Advances made during the 1990s led to the introduction of a new allylamine, terbinafine, for the treatment of dermatophytoses [9,10]. However, the resistance of the yeasts to fungal agents is increasing. This still need to develop new antimycotics [11].

During the researches of the substances of fungistatic action the synthesis conditions of a new group of compounds with meta-substituted dihydroxybenzthioacyl moiety: 2,4-dihydroxythiobenzanilides modified in aniline ring and N-heterocyclic derivatives of 2,4-dihydroxythiobenzamide has been elaborated [12]. It has been suggested that these the

compounds exert and activity against the fungi and bacteria [13-16]. The compounds show a relatively wide range of fungistatic action. Depending on the type of modification of N-aryl fragment can act against the dermatophytes [14,15], yeasts [16,17] and molds [16,17]. The microbiological tests also show that these compounds act mainly against the Gram positive cells [18]. In search for new compounds with β-resorcylcarbothionyl moiety N-3-(1,2,4-dithiazole-5-thione)-β-resorcylcarbothioamide – DTRTA as a substance with expected antifungal activity was obtained. The aim of the study was the determination of antifungal activity of a new DTRTA against *Candida albicans*, *non-Candida albicans*, dermatophytes and molds and evaluation of the enzymatic activity *C. albicans* strains.

Materials and methods

N-3-(1,2,4-dithiazole-5-thione)-β-resorcylcarbothioamide (DTRTA) 0.025 mol of 3-amino-1,2,4-dithiazole-5-thione (2) and 0.01 mol of bis-(β-resorcylcarbothioyl)thionyl (1) was added into 50 ml of methanol and heated to boiling (3 hrs). After reaction completed, the mixture was hot filtered and added with 100 ml of water. Separated compound was filtered, washed with water and recrystallized from dilute (2:1) methanol (60 ml). bis-(β-resorcylcarbothioyl)thionyl as the starting material was prepared according to patent [12].

In order to define the antifungal activity of DTRTA, we tested against *Candida albicans* reference strains 10231 ATCC, 200 fresh clinical isolates of *C. albicans*, 7 isolates of *non-C. albicans*, 12 dermatophytes and 20 strains of molds (details are not shown).

The yeasts were identified to the species level by the CandiSelect (Bio-Rad), Fungiscreen 4H (Bio-Rad), Auxacolor (Bio-Rad) tests. Dermatophytes and molds were identified by standard methods. Prior to antifungal susceptibility testing, each isolate was passaged on SB or YNB medium to ensure optimal growth characteristics.

DTRTA was used in the tests. It was dissolved in 1% DMSO. Susceptibility testing was performed by the agar dilution method. For yeasts, dermatophytes and molds MICs were determined according to National Committee for Clinical Laboratory Standards (NCCLS) reference document M27 [19].

Sabouraud's medium – SB (Bio-Rad) and YNB (Dom Handlowy Nauki PAN in Cracow) were used. Starting inocula were adjusted by the spectrophotometric method densitometr (BioMerieux) to 1x 10^5 CFU/ml. Concentrations of DTRTA were ranging from 0.025 to 200 mg/L. Plates were incubated at 37°C and read after 24 h incubation. A solvent control was included in each set of assays; the DMSO solution at maximum final concentration of 1% had no effect on fungal growth. Concentrations of DTRTA were ranging from 0.025 to 200 mg/L. Dermatophytes inocula were prepared from 3 weeks colonies, cultured on SB medium. Control plates with SB medium without DTRTA or with 1% DMSO were prepared. Microcultures were incubated at 27°C, and MICs values read after 5 and 15 days. Molds inocula contained 1 x 10^5 CFU/ml. Petrie plates with tested media and serial dilutions of DTRTA were inoculated with 20 µl of molds suspension. Control plates were also prepared. Plates were incubated at 27°C and read after 5 days.

The enzymatic activity of the yeast-like fungi was performed by API ZYM test (BioMeriux). API ZYM is a semi-quantitative micromethod designed for the research of enzymatic activities. This method is applicable to all specimens (tissues, cells, biological fluids, microorganisms, washings, soil, oil, etc.). It allows the systematic and rapid study of 19 enzymatic reactions using only very small sample quantities. The API ZYM strip is composed of 20 microtubes where the bottom forms a sort of support especially designed to contain the enzymatic substrate and a buffer. This support allows for contact between the enzyme and the general insoluble substrate. All procedures were done according to the manufacturer's instructions. The results were determined by using the API ZYM color scale ranging from 0 (negative) to 5 (maximum), depending on the amount of substrate metabolized where: 1 – corresponds to 5 nmol, 2 – to 10 nmol, 3 – to 20 nmol, 4 – to 30 nmol and 5 – to >40 nmol.

According to The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology: the following enzyme nomenclature [20] is valid 1. esterases (hydrolase esters of the carboxyl group – lipase and phospholipase A2 hydrolases, monoester phosphoric acid – alkaline phosphatase and acid hydrolases ester sulphuric – sulphatase), 2. glucosidases (α-glucosidase; β-glucosidase; α-mannosidase; N-acetyl-β-glucosaminidase), 3. peptidases (aminopeptidases, arylamidases, proteinases, elastases, collagenases, keratinases), and 4. ureases. We evaluated the enzymatic activity of the yeast-like fungi strains, before and after addition of DTRTA.

Student-t (two-tailed) test was used to compare mean MIC values, Wilcoxon's paired test was used to compare enzymatic activity before and after exposure of sample in sore scale. Significance was defined as a P value of 0.05.

Results

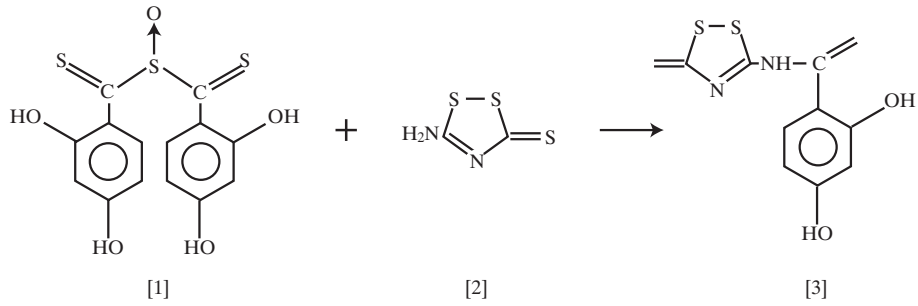
N-3-(1,2,4-dithiazole-5-thione)-β-resorcylcarbothioamide (DTRTA) was obtained in the reaction according to *Fig. 1*. The analytical data of compound were in agreement with the proposed structure. The purity was confirmed by HPLC and HPTLC chromatography in reversed-phase system (RP-8, RP-18, methanol-water).

DTRTA had a mean MIC of 12.5 mg/L for reference *C. albicans* 10231 ATCC on SB, 6.25 mg/L on YNB, respectively. DTRTA had MIC over the test range of 3-50 mg/L for *C. albicans* isolates on SB. A mean MIC for *C. albicans* isolates was 22.01 mg/L on SB, and 17.8 mg/L on YNB. Significant differences between reference *C. albicans* 10231 ATCC MICs on SB and YNB was found ($p<0.001$), between DTRTA MICs on SB and YNB was found ($p<0.05$).

DTRTA had MIC over the test range of 6.25 - 50 mg/L for *non-C. albicans* clinical isolates on SB and 3-25 mg/L on YNB (*Tab. 1*). The mean MICs of DTRTA against 7 *non-Candida* albicans isolates were 33 mg/L on SB, and 18.3 mg/L on YNB. Mean MIC for *non-C. albicans* strains for DTRTA was 19.5 mg/L.

Testing of the dermatophytes clinical isolates confirmed some antifungal activity of DTRTA against T. mentagrophytes

Figure 1. **Synthetic route and the structure of N-3-(1,2,4-dithiazole-5-thione)-β-resorcylcarbothioamide (DTRTA)**



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v. interdigitale with a MIC at which 100% of the isolates are inhibited of 13.3 mg/L after 5 days of incubation, and 18.8 mg/L after 15 days, respectively (*Tab. 2*). DTRTA had a mean MIC of 10.9 mg/L for T. mentagrophytes v. granulosum after 5 days of incubation, and 21.9 mg/L after 15 days of incubation. A mean MIC of DTRTA against E. floccosum isolates was 12.5 mg/L after 5 and 25 mg/L after 15 days of incubation. DTRTA had a mean MIC of 25 mg/L for T. rubrum isolates and 50 mg/L for T. tonsurans isolates after 5 and 15 days of incubation.

DTRTA had MIC over the test range of 25-100 mg/L for molds, after 5 days of incubation, as listed in *Tab. 3*. A mean MIC of DTRTA against S. brevicaulis isolated was 25 mg/L, A. nidulans isolated was 100 mg/L, Aspergillus species was 25 mg/L, Mucor species was 62.5 mg/L, Rhizopus was 50 mg/L, Trichoderma was 100 mg/L, Alternaria species was 100 mg/L, Penicillium species was 58.3 mg/L, Cladosporium species was 75 mg/L, Monilia species was 100 mg/L and Acremonium species was 50 mg/L after 5 days isolates.

The reference *C. albicans* strains had enzymatic activity of 14 enzymes. The highest enzymatic activity had esterase, esterase lipase, leucine and valine arylamidase and N-acetyl-β-glucosaminidase. Exposure to DTRTA inhibited the enzymatic activity of 10 enzymes. Before exposure to DTRTA, *C. albicans* isolates had enzymatic activity of 16 enzymes, after exposure to DTRTA 4 enzymes were inhibited. The highest enzymatic activity had leucine arylamidase, esterase, esterase lipase, α-glucosidase and N-acetyl-β-glucosaminidase (data are not shown).

Significant decrease of the enzymatic activity in 70 *C. albicans* strains was noted among following enzymes: phosphatase alkaline, lipase, tripsin, chymotripsin, phosphatase acid, naphtol-AS-BI-phosphohydrolase, β-glucosidase, N-acetyl-β-glucosamindase, α-mannosidase ($P<0.001$) and esterase, valine arylamidase, α-glucosidae ($p<0.01$) and esterase lipase ($p<0.05$) after exposure of DTRTA (data are not presented).

Before exposure to DTRTA, *non-C. albicans* isolates had enzymatic activity of 13 enzymes, after exposure to DTRTA 8 enzymes were inhibited. The highest enzymatic activity had leucine arylamidase, esterase lipase, phosphatase alkaline and esterase (data are not shown).

Table 1. **MICs DTRTA values against strains *non-Candida albicans* on Sabouraud's and YNB medium**

Number of strains	Sabouraud's medium	YNB medium
Candida krusei n=1	25	25
Candida tropicalis n=2	37.5	18.6
Candida paratropicalis n=1	50	25
Candida glabrata n=1	6.25	3
Rhodotorula rubra n=1	50	25
Candida species n=1	25	12.5
Mean MIC value of total 7 strains non-Candida albicans	33.0 ± 17.2	18.3 ± 9.0

Discussion

In this study, we demonstrated antifungal activity of new benzothiazole derivatives against *C. albicans*, dermatophytes and molds in vitro. Furthermore, *C. albicans* strains used in this study were resistant to several commonly used antimycotics.

In mycological literature there are numerous studies on the resistance of yeast-like fungi to currently used antifungal agents [20-22]. A correlation has been demonstrated between the amount of phospholipase produced and virulence in *C. albicans* strains and other yeast species. Certain fungi such as: Mucor, Rhizopus, Aspergillus, Penicillium and Candida, have the ability of releasing hydrolytic enzymes into environment, which break down multimolecular compounds – polysaccharides, proteins, lipids, hydrocarbons [1].

Our results are in accordance with the previous reports [24-26] Bujadakova et al. assessed anti-Candida activity of 6-amino-2-n-pentylthiobenzothiazole, benzylester of (6-amino-2-benzothiazolylthio) acetic acid and of 3-butylthio-(1,2,4-triazolo)-2,3-benzothiazole. The compounds were active against Candida strains. First compound exhibited inhibitory activity on germ-tube formation and mycelial growth in the *C. albicans* strains, while others were not active in these tests. All the compounds tested were highly active on a nystatin-resistant *C. albicans* strains [24]. Similar findings were also obtained by [25].

Azolium salts and neutral 2-aryl derivatives of benzimid-

Table 2. **MICs DTRTA values against 12 dermatophytes strains on Sabouraud's medium**

Dermatophytes strains	Number of strains	DTRTA	
		MIC (mg/L)	Mean MIC (mg/L)
Reading after 5 days			
Trichophyton mentagrophytes varietas interdigitale	4	12.5	13.3 ± 9
Trichophyton mentagrophytes varietas interdigitale		3	
Trichophyton mentagrophytes varietas interdigitale		25	
Trichophyton mentagrophytes varietas interdigitale		12.5	
Trichophyton mentagrophytes varietas granulosum	4	12.5	10.9 ± 3.1
Trichophyton mentagrophytes varietas granulosum		12.5	
Trichophyton mentagrophytes varietas granulosum		12.5	
Trichophyton mentagrophytes varietas granulosum		6.25	
Epidermophyton floccosum	2	12.5	12,5 ± 0
Epidermophyton floccosum		12.5	
Trichophyton rubrum	1	25	25
Trichophyton tonsurans	1	50	50
Reading after 15 days			
Trichophyton mentagrophytes varietas interdigitale	4	12.5	18.8 ± 7.2
Trichophyton mentagrophytes varietas interdigitale		12.5	
Trichophyton mentagrophytes varietas interdigitale		25	
Trichophyton mentagrophytes varietas interdigitale		25	
Trichophyton mentagrophytes varietas granulosum		25	
Trichophyton mentagrophytes varietas granulosum	4	25	21.9 ± 6.25
Trichophyton mentagrophytes varietas granulosum		25	
Trichophyton mentagrophytes varietas granulosum		12.5	
Epidermophyton floccosum	2	25	25 ± 0
Epidermophyton floccosum		25	
Trichophyton rubrum	1	25	25
Trichophyton tonsurans	1	50	50

Table 3. **MICs DTRTA values against molds strains on Sabouraud's medium**

Molds strains	Number of strains	DTRTA	
		MIC Mg/L	Mean MIC Mg/L
Scopulariopsis brevicaulis	1	25	25±0
Aspergillus species	1	25	25±0
Aspergillus nidulans	2	100	100±0
Aspergillus nidulans		100	
Mucor species	2	25	62.5±53
Mucor species		100	
Rhizopus	2	50	50±0
Rhizopus		50	
Trichoderma	1	100	100±0
Alternaria species	1	100	100±0
Penicillium species	6	100	58.3±34.2
Penicillium species		25	
Penicillium species		50	
Penicillium species		25	
Penicillium species		50	
Penicillium species		100	
Cladosporium species	2	100	75±35.4
Cladosporium species		50	
Monilia sitophila	1	100	100±0
Acremonium species	1	50	50±0

azole, benzothiazole and benzoxazole were synthesized by Cetinkaya et al. [26]. The salts 1 and the neutral compounds 2 were evaluated for their in vitro antimicrobial activity against standard strains: Enterococcus faecalis (ATCC 29212), Staphylococcus aureus (ATCC 29213), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), *C. albicans* and *Candida tropicals*. The compounds 1f, 1g, 1l, 1m, 1n, 2a, 2b, 2c, 2e, 2f showed antimicrobial activity against E. faecalis (ATCC 29212), S. aureus (ATCC 29213), Escherichia coli (ATCC 25922), P. aeruginosa (ATCC 27853), *C. albicans* and *C. tropicals*, with minimum inhibitory concentrations (MICs) ranging between 50 to 200 mg/mL. Compounds 1f, 1g, 1l, 1m, 2b, 2c showed the highest activity. Benzothiazolium and benzoxazolium salts were more active than 1.3-disubstituted benzimidazolium salts and neutral 2-substituted benzimidazole, benzothiazole and benzoxazole derivatives.

In our opinion, the new DTRTA exerts the potent antifungal activity against the yeast-like fungi strains, dermatophytes and molds in vitro.

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