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SYNTHESIS AND EVALUATION OF THIAZOLE – PYRIMIDINE DERIVATIVES AS NEW ANTICANDIDAL AND CYTOTOXIC AGENTS

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The aim of this study was to describe the synthesis of four new 2-(3,4-diphenyl-3*H*-thiazol-2-ylidene)amino-4,6-dimethylpyrimidine derivatives, which were screened for their anticandidal activity and cytotoxicity. The title compounds **(2a – 2d)** were synthesized via the reaction of 1-phenyl-3-(4,6-dimethylpyrimidin-2-yl)thiourea with phenacyl bromides. Anticandidal activity of the synthesized compounds was evaluated using microbroth dilution method. All compounds were also investigated for their cytotoxic effects on A549 and NIH3T3 cell lines. Compound **2c** can be considered as the most promising anticandidal agent due to its inhibitory effects on *Candida albicans*, *C. glabrata*, *C. tropicalis* with a MIC value of 125 μg/mL and low toxicity to NIH3T3 cells (IC_{50} = 193.32 μg/mL). Although compound **2a** was the most effective derivative against A549 cells with an IC_{50} value of 0.0623 mM, it is not a good candidate for cancer treatment because of its high toxicity against NIH3T3 cells (IC_{50} = 0.00316 mM).

Keywords: anticandidal activity, cytotoxicity, pyrimidine, thiazole

INTRODUCTION

Invasive fungal infections are becoming increasingly implicated as a cause of crucial and fatal diseases. This is especially the case in immunocompromised patients, who have a tendency to infections caused by opportunistic fungal pathogens that are normally kept in check by a functioning immune system [1 – 3]. Although amphotericin B, fluocytosine and azoles such as ketoconazole, fluconazole or itraconazole have been considered efficient for the treatment of fungal infections, some of these drugs show toxicity, produce recurrence because they are fungistatic rather than fungicidal, or lead to development of resistance due in part to the intensive prophylactic use of antifungal drugs [4].

There is, therefore, a clear need for the discovery of new antifungal agents, which could constitute alternatives for the management of fungal infections [5].

As known, not only biochemical similarity of the human cell and fungi forms is a handicap for selective activity, but also the easily gained resistance is the main problem encountered in developing safe and effective antifungals. The incidence of systemic fungal infections has been increasing. The choice of suitable antifungal agents remains relatively limited, although the advent of the new echinocandin class is a welcome development as the expansion of the group of azole antifungals as heterocyclic compounds [6, 7].

To pursue this goal, our research efforts are directed to finding new chemical classes of antifungal agents. The methods of investigation using structure – activity relationships (SAR) enabled us to find some new pharmacophores of the above-mentioned activity. Many studies have been carried out on thiazole and pyrimidine as antifungal pharmacophores [8 – 11].

On the basis of these findings, we became interested in biological evaluation of thiazole – pyrimidine derivatives as anticandidal and anticancer agents. Herein, we describe the synthesis of a novel series of thiazole – pyrimidine derivatives (compounds **2a – 2d,** Table 1) and focus the attention on their potential anticandidal activity and cytotoxicity with respect to A549 and NIH3T3 cell lines.

EXPERIMENTAL PART

Chemistry

All reagents were purchased from commercial suppliers and were used without further purification. Melting points

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were determined on an Electrothermal 9100 melting point apparatus (Weiss-Gallenkamp, Loughborough, UK) and are uncorrected. ^{1}H NMR spectra of the synthesized compounds were recorded on a Bruker 400-MHz spectrometer (Bruker, Billerica, USA). Mass spectra (MS) were recorded on a VG Quattro Mass spectrometer (Agilent, Minnesota, USA). Elemental analyses were performed on a Perkin-Elmer EAL 240 elemental analyzer (Perkin-Elmer, Norwalk, USA). Elemental analysis data were consistent with the data calculated for the synthesized compounds.

General Procedure for the Synthesis of Compounds (Scheme 1)

1-Phenyl-3-(4,6-dimethylpyrimidin-2-yl)thiourea (1). A mixture of (4,6-dimethylpyrimidin-2-yl)amine (0.1 mol) and phenyl isothiocyanate (0.1 mol) in ethanol was refluxed for 2 h. The solid that separated upon cooling was filtered, dried, and recrystallized from ethanol.

2-(3,4-Diphenyl-3H-thiazol-2-ylidene)amino-4,6-dimethylpyrimidine derivatives (2a – 2d). A mixture of 1-phenyl-3-(4,6-dimethylpyrimidin-2-yl)thiourea **1** (0.002 mol) and appropriate phenacyl bromide (0.002 mol) was refluxed in ethanol (15 mL) for 3 h. After cooling, the resulting solid was collected by filtration, dried, and recrystallized from ethanol.

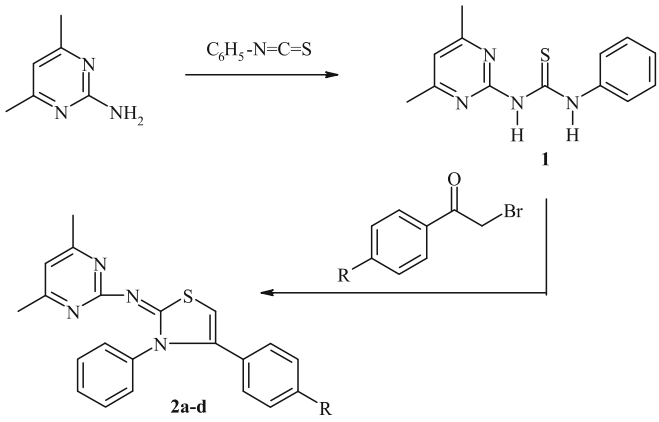
2-[3-Phenyl-4-(4-nitrophenyl)-3H-thiazol-2-ylidene]amino-4,6-dimethylpyrimidine (2a). ^{1}H NMR (400 MHz, DMSO-d_6) (δ, ppm): 2.22 – 2.42 (m, 6H, 2CH_3), 7.01 – 7.05 (m, 2H, aromatic protons), 7.21 – 7.25 (m, 1H, aromatic proton), 7.37 – 7.43 (m, 3H, aromatic protons), 7.64 – 7.77 (m, 3H, aromatic protons), 8.02 (d, J = 4.5 Hz, 1H, aromatic proton), 8.26 (d, J = 4.5 Hz, 1H, aromatic proton); MS $[M+1]^+$: *m/z* 404.

2-[3-Phenyl-4-(4-methylphenyl)-3H-thiazol-2-ylidene]amino-4,6-dimethylpyrimidine (2b). ^{1}H NMR (400 MHz, DMSO-d_6) (δ, ppm): 2.23 – 2.46 (m, 9H, 3CH_3), 6.99 – 7.03 (m, 3H, aromatic protons), 7.18 – 7.44 (m, 3H, aromatic protons), 7.64 – 7.80 (m, 5H, aromatic protons); MS $[M+1]^+$: *m/z* 373.

2-(3,4-Diphenyl-3H-thiazol-2-ylidene)amino-4,6-dimethylpyrimidine (2c). ^{1}H NMR (400 MHz, DMSO-d_6) (δ, ppm): 2.25 – 2.49 (m, 6H, 2CH_3), 6.96 – 7.04 (m, 2H, aromatic protons), 7.30 – 7.45 (m, 6H, aromatic protons), 7.60 – 7.82 (m, 4H, aromatic protons); MS $[M+1]^+$: *m/z* 359.

TABLE 1. Some Properties of Compounds **2a – 2d**

Compound	R	Yield, %	Molecular Formula	Molecular Weight	Melting Point
2a	NO_2	87	$C_{21}H_{17}N_5O_2S$	403	126 – 127
2b	CH_3	90	$C_{22}H_{20}N_4S$	372	254 – 255
2c	H	75	$C_{21}H_{18}N_4S$	358	208 – 209
2d	Cl	88	$C_{21}H_{17}ClN_4S$	392	237 – 238



Scheme 1. The synthetic route for preparation of the target compounds (**2a – 2d**).

2-[3-Phenyl-4-(4-chlorophenyl)-3H-thiazol-2-ylidene]amino-4,6-dimethylpyrimidine (2d). ^{1}H NMR (400 MHz, DMSO-d_6) (δ, ppm): 2.27 – 2.44 (m, 6H, 2CH_3), 7.00 – 7.05 (m, 2H, aromatic protons), 7.30 – 7.39 (m, 2H, aromatic protons), 7.41 – 7.47 (m, 4H, aromatic protons), 7.62 – 7.67 (m, 2H, aromatic protons), 7.75 – 7.80 (m, 1H, aromatic proton); MS $[M+1]^+$: *m/z* 393.

Microbiology

Anticandidal assays and microorganisms. The activity of compounds **2a – 2d** were first screened including the standard antifungal ketoconazole by an agar diffusion method using two strains of *Candida albicans* (ATCC 90028 and a clinical isolate Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskişehir, Turkey), *C. utilis* (NRRL Y-900), *C. tropicalis* (NRRL Y 12968), *C. krusei* (NRRL Y-7179), and two clinical isolates of *C. glabrata* (Clinical isolate-Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskişehir, Turkey and Anadolu

TABLE 2. Antifungal Activity of Compounds **2a – 2d** (MIC, μg/mL)

Candida strains	2a	2b	2c	2d	Ketoconazole
C. albicans (Clinical isolate)*	125	250	125	250	3.9
C. albicans (ATTC 90028)	125	125	125	125	3.9
C. glabrata (Clinical isolate)*	125	250	125	125	1.9
C. glabrata (Clinical isolate)**	125	125	125	125	3.9
C. utilis (NRRL Y-900)	125	125	125	125	1.9
C. tropicalis (NRRL Y 12968)	125	125	125	250	3.9
C. krusei (NRRL Y-7179)	125	250	250	125	3.9

* Obtained from Osmangazi University, Faculty of Medicine, Eskişehir, Turkey.

** Obtained from Anadolu University, Faculty of Science, Eskişehir, Turkey.

University, Faculty of Science, Department of Biology, Eskişehir, Turkey) and all active compounds (inhibition zones 9 – 11 mm, at 2 mg/mL concentration) were further evaluated using the broth microdilution method to identify the minimum inhibitory concentrations (MIC) against all *Candida* spp [12, 13].

Microorganisms were obtained from ATCC, NRRL and clinical isolates (Faculty of Medicine, Eskisehir Osmangazi University, Turkey) and were stored in 15% glycerol containing micro-test tubes at –86°C (strain numbers of microorganisms are given in Table 2). All *Candida* strains were inoculated on Sabouraud Dextrose Agar (SDA) prior to experiments at 37°C. After sufficient growth, *Candida* spp. were transferred to Mueller Hinton Broth (MHB) for further incubation under the same conditions for another 24 h.

Broth microdilution assay. The test compounds (**2a – 2d**) and ketoconazole were first dissolved in DMSO which was used to prepare the stock solutions at an initial concentration of 2 mg/mL. Serial dilution series were prepared in 100 μL MHB with an equal amount of the test samples. The last row was filled only with water as growth control for microorganisms. Overnight grown microorganism suspensions were first diluted in double-strength MHB and standardized to 10^8 CFU/mL (using McFarland No: 0.5) under sterile conditions. Then each microorganism suspension was pipetted into each well and incubated at 37°C for 24 h. Ketoconazole was used as a standard antifungal agent against *Candida* spp. Sterile distilled water and medium served as a positive growth control. The first well without turbidity was assigned as the minimum inhibitory concentration (MIC, μg/mL). Average results of three separately performed experiments are given in Table 2.

Cytotoxicity evaluation. NIH3T3 (mouse embryonic fibroblast cell line- ATCC® number CRL-1658™) and A549 (human lung carcinoma epithelial cell line- ATCC® number CCL-185™) cells were used for cytotoxicity tests. NIH3T3 and A549 cells were incubated in RPMI 1640 (Hyclone, Thermo Scientific, USA) supplemented with 10% fetal calf serum (Hyclone, Thermo Scientific, USA) and 1% penicillin/streptomycin (Hyclone, Thermo Scientific, USA) at 37°C, 5% CO_2. A549 and NIH3T3 cells were seeded at 20000 cells into each well of 96-well plates. After 24 h incubation period, the culture medium was removed and the compounds were added to culture medium at 0.00316 – 10 mM doses. After next 24 h incubation, cytotoxicity test was performed using the In Cytotox-XTT 1 Parameter Cytotoxicity Kit (Xenometrix AG, Gewerbertrasse 25, Switzerland), which measures mitochondrial activity (tetrazolium hydroxide (XTT)) in NIH3T3 and A549 cell cultures. Firstly, the cells were washed with phosphate buffer saline (PBS) and were added 200 μL/well of fresh culture medium. XTTI and XTTII solutions were mixed at 1 : 100 ratio. Then, 50 μL of this mixture was added to all wells. The plate was incubated for 3 h at 37°C, 5% CO_2. After 3 h, the content of the well was mixed by pipetting up and down. Then, optical density (OD) of the plate was read at 480 nm with a reference wavelength at 680 nm. Percentage inhibition was calculated for each concentration of compounds. IC_{50} values were estimated by non-linear regression analysis.

RESULTS AND DISCUSSION

The synthesis of thiazole-pyrimidine derivatives (**2a-d**) was carried out according to the steps shown in Scheme 1. 1-Phenyl-3-(4,6-dimethylpyrimidin-2-yl)thiourea (**1**) was synthesized via the reaction of (4,6-dimethylpyrimidin-2-yl)amine with phenyl isothiocyanate. The ring closure reaction of 1-phenyl-3-(4,6-dimethylpyrimidin-2-yl)thiourea (**1**) with phenacyl bromides afforded 2-(3,4-diphenyl-3*H*-thiazol-2-ylidene)amino-4,6-dimethylpyrimidine derivatives (**2a – 2d**). The structures of compounds **2a – 2d** were confirmed by ^{1}H NMR, mass spectroscopy, and elemental analysis. The general structure of the synthesized compounds is shown in Scheme 1.

All compounds were tested *in vitro* against various *Candida* species. Data on the antifungal activity of all compounds and ketoconazole are given in Table 2.

Compound **2c** can be identified as the most promising anticandidal agent due to its inhibitory effects on *C. albicans* (ATCC 90028 and Clinical isolate), *C. glabrata* (Clinical isolate-Anadolu University, Faculty of Science), *C. tropicalis* (NRRL Y 12968) and low toxicity to NIH3T3 cells. Compound **2c** exhibited the inhibitory activity against *C. albicans*, *C. glabrata*, *C. tropicalis* with a MIC value of 125 μg/mL, whereas ketoconazole showed the inhibitory activity with a MIC value of 3.9 μg/mL.

The IC_{50} values of compounds **2a**, **2b**, **2c** and **2d** for A549 cell line were 0.0623 mM, 0.658 mM, 0.559 mM and 0.1 mM, respectively. The IC_{50} values of all compounds with

TABLE 3. Cytotoxic Activity of Compounds **2a –2d** against A549 Cell Line

Compound	IC_{50}, mM	IC_{50}, μg/mL
2a	0.0623	25.11
2b	0.658	244.78
2c	0.559	200.12
2d	0.1	39.2
Cisplatin	0.0386	11.58

TABLE 4. Cytotoxic Activity of Compounds **2a –2d** against NIH3T3 Cell Line

Compound	IC_{50}, mM	IC_{50}, μg/mL
2a	0.00316	1.27
2b	0.01	3.72
2c	0.54	193.32
2d	0.0316	12.39

respect to A549 cells are presented in Table 3. According to our results, compound **2a** is the most effective agent against A549 cell line.

The IC_{50} values of compounds **2a**, **2b**, **2c** and **2d** for NIH3T3 cell line were 0.00316 mM, 0.01 mM, 0.54 mM and 0.0316 mM, respectively. The IC_{50} values of all compounds with respect to NIH3T3 cells are presented in Table 4. According to our results, compound **2a** is the most toxic compound against NIH3T3 cell line.

Small differences in IC_{50} values of the compounds between A549 cell line and NIH3T3 cell line may be accepted probably meaningless, but larger differences could be due to some specific cytotoxicity mechanism. According to the IC_{50} values obtained, the inhibitory effects of the compounds on A549 cells were not determined to be selective when compared with their effects on NIH3T3 cells.

CONCLUSION

In the present work, we synthesized a new series of thiazole – pyrimidine derivatives and investigated their anticandidal activity. All compounds were also investigated for their cytotoxicity with respect to A549 and NIH3T3 cell lines.

In particular, compound **2c** was the most promising antifungal derivative against *C. albicans* (ATCC 90028 and Clinical isolate), *C. glabrata* (Clinical isolate-Anadolu University, Faculty of Science), *C. tropicalis* (NRRL Y 12968) with a MIC value of 125 μg/mL and low toxicity to NIH3T3 cells (IC_{50} = 193.32 μg/mL). Other derivatives (**2a**, **2b**, and **2d**) are not good candidates for *Candida* infections and cancer treatment because of their high toxicity.

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