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Antifungal Activity, Cytotoxicity and Mechanism of Action of Nitroheteroaryl-1,3,4-thiadiazole Containing N-benzyl and N-methoxyethyl Substitution Against *Aspergillus fumigatus*

N-benzil ve N-metoksietil İçeren Nitroheteroaril-1,3,4-tiyadiazolün *Aspergillus fumigatus*'a Karşı Antifungal Aktivitesi, Sitotoksisitesi ve Etki Mekanizması

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Abstract

Introduction: This study aimed to evaluate antifungal activity and cytotoxicity of two new nitroheteroaryl-1,3,4-thiadiazole derivatives containing N-methoxyethyl (9) and N-benzyl (10) moiety against *Aspergillus fumigatus* with a special focus on their mechanism of action at cellular level.
Materials and Methods: The fungal growth rate was evaluated by microbioassay technique. Ergosterol content of the cell membrane was determined, and morphological changes of fungal compartments were assessed by electron microscopy. Cytotoxicity against Vero and Hep2 cell lines was determined by cell culture technique.
Results: Based on the data obtained, compound 9 showed 31.90-100% inhibition at concentrations of 25-800 µM, while compound 10 presented 24.26-100% inhibition at higher concentrations (25-3200 µM). Compounds 9 and 10 showed minimum fungicidal concentration of 500 and 8000 µM and IC_{50} values of 43.39 and 1008.67 µM, respectively. Ergosterol content was not meaningfully affected by both compounds. Electron microscopy showed deformation of *A. fumigatus* hyphae, depletion of hyphae contents, and destruction of cell membrane and membranous organelles in the fungus exposed to both compounds. Compounds 9 and 10 had no obvious cytotoxicity against Vero and Hep2 cell lines *in vitro*.
Conclusion: Taken together, our results showed that both compounds, especially compound 9, could be considered as potential candidates for developing antifungal drugs against *A. fumigatus* as the main etiologic agent of life-threatening invasive aspergillosis.
Keywords: Thiadiazole, *Aspergillus fumigatus*, ergosterol, antifungal activity, electron microscopy, cytotoxicity

Öz

Giriş: Bu çalışma, N-metoksietil (9) ve N-benzil (10) parçalarını içeren iki yeni nitroheteroaril-1,3,4-tiyadiazol türevinin *Aspergillus fumigatus*'a karşı antifungal aktivitesini ve sitotoksisitesini, özellikle de hücresel düzeydeki etki mekanizmalarına odaklanarak değerlendirmeyi amaçlamıştır.
Gereç ve Yöntem: Mantar büyüme hızı mikrobiyoassay tekniği ile değerlendirildi. Hücre zarının ergosterol içeriği belirlendi ve mantar bölmelerinin morfolojik değişiklikleri elektron mikroskobu ile değerlendirildi. Vero ve Hep2 hücre hatlarına karşı sitotoksisite, hücre kültürü tekniği ile belirlendi.

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Öz

Bulgular: Elde edilen verilere dayanarak, bileşik 9 25-800 µM konsantrasyonlarda %31,90-100 inhibisyon gösterirken, bileşik 10 daha yüksek konsantrasyonlarda (25-3200 µm) %24,26-100 inhibisyon gösterdi. Bileşik 9 ve 10'un minimum fungusit konsantrasyonları sırasıyla 500 ve 8000 µM; IC_{50} değerleri ise 43,39 ve 1008.67 µM idi. Ergosterol içeriği, her iki bileşikten de anlamlı şekilde etkilenmedi. Elektron mikroskopisi; *A. fumigatus* hyphae'nin deformasyonunu, hif içeriklerinin tükenmesini ve her iki bileşiğe maruz kalan mantarda hücre zarı ve zarlı organellerin yok oluşunu gösterdi. Bileşikler 9 ve 10, *in vitro* olarak Vero ve Hep2 hücre hatlarına karşı belirgin bir sitotoksisiteye sahip değildi.
Sonuç: Birlikte ele alındığında, sonuçlarımız her iki bileşiğin, özellikle bileşik 9'un, yaşamı tehdit eden invaziv aspergillozun ana etiyolojik ajanı olan *A. fumigatus*'a karşı antifungal ilaçlar geliştirmek için potansiyel adaylar olarak kabul edilebileceğini göstermiştir.
Anahtar Kelimeler: Tiyadiazol, *Aspergillus fumigatus*, ergosterol, antifungal aktivite, elektron mikroskobu, sitotoksisite

Introduction

Aspergillus is a genus of filamentous fungi found ubiquitously worldwide. *Aspergillus fumigatus* (*A. fumigatus)* is known as a common species of *Aspergillus* and a major clinical invasive fungus in humans[1,2]. This invasive species has a high mortality rate in patients with immune deficiency[3,4]. Conidia of this fungus can disperse easily in the air and cause multiple infections, from life-threatening invasive aspergillosis to allergic bronchopulmonary aspergillosis, severe asthma, acute bronchitis, acute sinusitis and aspergilloma[5-7].

Enhancement of antifungal drug resistance remains a serious concern, especially azole-resistant *A. fumigatus* with high prevalence and worrying mortality rates worldwide[8]. The most important drug resistances are related to itraconazole and posaconazole, and voriconazole with triazole structure. In this situation, finding and developing new alternative antifungal drugs are critical[9-11], and researchers have attempted to look for new natural and synthetic products with favorable efficiency and high safety.

Compounds containing a heterocyclic ring play a key role in antimicrobial drugs. The 1,3,4-thiadiazole scaffold is one of the rings that have attracted the attention of various researchers owing to its special structure and high therapeutic potential. Diuretic[12], anticancer[13], antiglaucoma[14], antimicrobial and antifungal[13-15], anti-*Helicobacter pylori*[16], analgesic and anti-inflammatory[17], antituberculosis[18], and antiparasitic effects[19-22] are several pharmacological activities of 1,3,4-thiadiazole derivatives reported until now. Essentially, this ring with a mesoionic characteristic can easily pass through biological membranes and perfectly interact with biological targets[19]. Antiglaucoma (acetazolamide and methazolamide), antiparasitic (megazol), and antimicrobial (sulfamethizole and cefazolin) drugs with an active core of 1,3,4-thiadiazole are available in the global pharmaceutical market (Figure 1). The presence of various substituents on the thiadiazole ring has resulted in its diverse biological effects. The 2-position of this ring is the most flexible site for chemical changes. This position also affects the potency and physicochemical properties of thiadiazole derivatives[20].

In this study, initial screening of fifty 1,3,4-thiadiazole derivatives was performed against *A. niger* by the disk diffusion method. These compounds had previously been synthesized and indicated for anti-leishmaniasis activity[20]. Two nitroheteroaryl-1,3,4-thiadiazole compounds containing N-methoxyethyl (9) and N-benzyl (10) substitution with the inhibition zone of 30 mm were selected, and their antifungal activity against *A. fumigatus* and their cytotoxicity were evaluated (Figure 1). To our knowledge, no documentary information has been available on the antifungal activity of these compounds; hence, the evaluation of fungicidal activity and inhibitory mechanisms of such compounds against *A. fumigatus is* of paramount importance.

Materials and Methods

Materials and Fungal Strain

The RPMI-1640 was purchased from Gibco (Ireland), 3-(N-Morpholine) propane sulfonic acid (MOPS) was obtained from Sigma (USA), and potato dextrose broth (PDB) was obtained from QUELAB (Canada). Other solvents and reagents were of analytical grade and acquired from Sigma and Merck (Germany). *A. fumigatus* PTCC 5009 was obtained from Iranian Research Organization for Science and Technology and cultured on Sabouraud dextrose agar (Merck) slants at 28 °C for 7 days[23,24].

Synthesis of Studied Compounds

The preparation of compounds N-(2-methoxyethyl)-5-(5-nitrofuran-2-yl)-1,3,4-thiadiazol-2-amine(9) and N-benzyl-5-(5-nitrothiophen-2-yl)-1,3,4-thiadiazol-2-amine (10) was carried out in accordance with a previous method[20]. The synthetic route of compounds 9-10 is outlined in Figure 2. The key intermediate compound (B) was obtained from 5-nitro heteroaryl diacetate (A) in three steps. The reaction of 2-chloro-5-(5-nitro heteroaryl)1,3,4-thiadiazole (B) with appropriate primary amine in absolute ethanol was referred to as compounds 9 and 10.

Measurement of Minimum Inhibitory Concentration and Minimum Fungicidal Concentration of Thiadiazole Derivatives

Minimum inhibitory concentrations (MICs) of compounds 9 and 10 against *A. fumigatus* were determined by the broth microdilution method (CLSI M38-A2 guideline)[25]. Antifungal stock solutions were prepared by DMSO and 2-propanol. Using RPMI-1640 media buffered with MOPS at pH 7.0, the serial dilution of compound 9 (7.81-1000 µM) and compound 10 (62.5-8000 µM) were cultured in a 96-well microdilution plate. The isolate of *A. fumigatus* was grown on potato dextrose agar slants at 35 °C for a period of 7-10 days. Spores were collected using a cotton swab and suspended in 0.1% Tween 80. The suspensions were adjusted to 5×10^4 spores/ml by counting fungal spores with a light microscope. Subsequently, 100 µl of fungus stock in the RPMI media was added to the wells. Amphotericin B (positive) as well as fungal and medium (negative) were considered the control groups. Each experiment was performed in triplicate. Microdilution plates were sealed and incubated at 35 °C for 48 h. Following incubation, MIC endpoints were interpreted using a reading mirror. Only wells that showed no growth (optically clear; amphotericin B) were recorded as the MIC and compared with drug-free controls. The minimum fungicidal concentration (MFC) was determined by culturing 50 µl of fungal samples (based on the MIC test), which had a drug concentration equal to or higher than MIC, on the culture medium (Sabouraud dextrose agar) together with chloramphenicol[26,27]. The plates were incubated at 35 °C for 48 h. The MFC was defined as the lowest concentration if no fungal growth on subculture was observed.

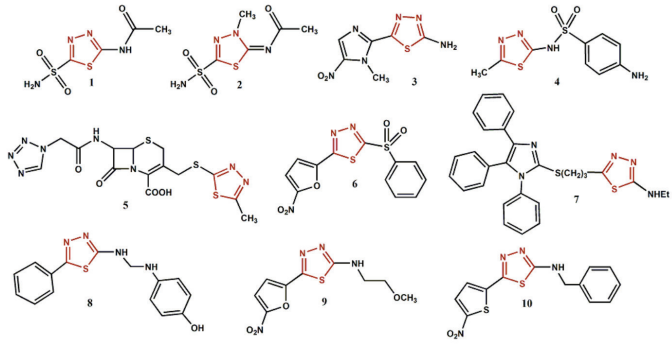

Figure 1. Chemical structure of known drugs with thiadiazole rings: (1) acetazolamide; (2) methazolamide; (3) megazole; (4) sulfamethizole; (5) cefazolin; (6) 2-(5-nitrofuran-2-yl)-5-(phenylsulfonyl)-1,3,4-thiadiazole; (7) 5-(3-(1,4,5-triphenyl-1H-imidazol-2-ylthio)propyl)-N-ethyl-1,3,4-thiadiazol-2-amine; (8) 4-((5-phenyl-1,3,4-thiadiazol-2-ylamino)methylamino)phenol; (9 and 10) studied compounds

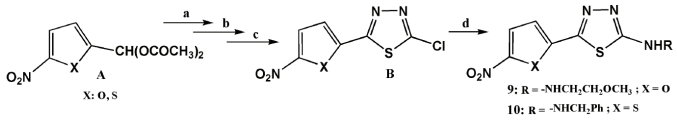

Figure 2. Synthesis of compounds 9-10. Reagents and conditions: (a) thiosemicarbazide, HCl, EtOH, reflux; (b) $NH_4Fe(SO_4)_2.12H_2O$, H_2O, reflux; (c) $NaNO_2$, HCl, Cu, 0 °C→r.t (d) RNH_2, EtOH, reflux

Assessment of Fungal Growth Inhibition by Microbioassay

The fungus was cultured on PDB medium in six-well flat bottom microplates in the presence of serial two-fold concentrations of studied compounds (25-3200 µM) using the microbioassay method[23]. The culture medium (5 ml/well) was added to the microplates and inoculated with fungal spore suspension (10^6 spores/well) prepared by the aqueous solution of 0.1% Tween 80. Afterwards, 50 µl of the prepared concentrations was added to the test wells. The solvent dimethyl sulfoxide (DMSO) and fungus were considered the control groups, and experiments were carried out in triplicate. Fungal dry weights were determined after filtering the total well contents (culture media and fungal biomass) by multilayer cheesecloth and thoroughly washed with distilled water. The fungal mycelia were separated from liquid culture medium, transferred to small aluminum foils, and then weighed and oven-dried at 80 °C until a constant weight was obtained. After 3 h, the foils were removed, and mycelia were reweighted to achieve the dry weight of fungal growth and the growth inhibition. Finally, IC_{50} values of compounds 9 and 10 were reported against *A. fumigatus.*

Ergosterol Quantitation Assay

The effect of the test compounds on ergosterol biosynthesis in *A. fumigatus* cells was evaluated by a previously described method[28]. Different concentrations of compounds were incubated with 200 µl of *Aspergillus* cells (0.5 McFarland) at 28 °C for four days. The fungus was then separated from the culture medium and placed in a 60 °C oven for 3 h. Dried samples were completely powdered inside a vial, weighted, and kept for measurement of the ergosterol content. Potassium hydroxide 25% (3 ml) was added to each sample and vortexed for 1 min. Suspension was placed in borosilicate glass tubes and incubated at 80 °C water bath for 1 h. After reaching room temperature, sterol was extracted by the addition of 3 ml of n-hexane and 1 ml of distilled water. The mixture was vigorously vortexed for 3 min and allowed to stand for 30 min in room temperature. The *n*-hexane layer was separated and transferred to glass tubes and incubated at -20 °C for 18-24 h. Then, fivefold absolute ethanol was added to the glass tubes containing the hexane phase. Sterol aliquot was scanned using a UV/visible spectrophotometer (Perkin Elmer EZ301) between 200 and 300 nm. The presence of ergosterol and 24(28)-dehydroergosterol resulted in a characteristic four-peaked curve in the extracted sample. The ergosterol content was calculated using the following formula:

Ergosterol (%) = $[(A_{281.5}/290 \times F)/\text{sample weight}] - [(A_{230}/518 \times F)/\text{sample weight}]$

where $A_{281.5}$ and A_{230} are optical absorption of samples at the wavelengths of 281.5 and 230 nm, respectively. F is the dilution factor in ethanol, and numbers 290 and 518 are E values (in percentages per centimeter) for crystalline ergosterol and 24(28)-dehydroergosterol, respectively. The fungal sample without a synthetic compound was regarded as the control.

Transmission Electron Microscopy

Structural changes of the fungal cell exposed to test compounds were evaluated using transmission electron microscopy (TEM)[23,29]. The *Aspergillus* strain was treated with sub-inhibitory concentrations of compound 9 (400 µM) and compound 10 (800 µM) for four days. The control sample did not receive any treatment. Samples were washed in 0.1 M phosphate buffer (pH 7.2) and fixed in a solution containing 3% glutaraldehyde at room temperature for 3 h. After three washing with phosphate buffer and infiltration with melt agar 2%, samples were post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7.2) at room temperature for 1 h. Samples were then dehydrated with increasing acetone concentrations (10% for 30-90% every 60 min, 100% for 180 min, and 100% overnight), embedded in Epon812, and finally polymerized in spur resins at 45 °C for 24 h and 65 °C for 72 h; Epon 812 was composed of 1.50% hardening agent DMP-30. Ultrathin sections (80 nm thickness) were prepared by Leica Ultracut UCP on 100-mesh grids and viewed under a Zeiss-EM 900 (50 KVL) TEM after staining with Uranyl acetate for 20 min and with lead citrate for 5 min.

Measurement of Cell Cytotoxicity

The cytotoxicity of the synthetic compounds were assessed on the normal cell line (Vero) and on cancer cell line (Hep2) using the MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) assay[30]. Cells (1×10^5/well) were counted and poured into 96-well plates, which were incubated in 5% CO_2 at 37 °C for 24 h. High concentration (800 µM) of compounds (9 and 10) were added to the cells and incubated at 37 °C for 24 h. After incubation, the MTT stain (10 µl) was added to each well at 37 °C for 3-4 h. Then, the cells were dissolved in 100 µl of DMSO, and their optical density (OD) values were read at 570 nm using an enzyme-linked immunosorbent assay plate reader (Epoch 2 microplate; BioTek Instruments Inc., USA). Fungal cells (without synthetic compounds) containing DMSO were considered the control group.

Cell viability (%) = mean OD/control OD × 100%.

Statistical Analysis

All results were subjected to one-way analysis of variance (ANOVA) and Tukey's range test using a Statistical Package for the Social Sciences 10.0 program for Windows. Differences with $p<0.05$ were considered significant.

Results

Antifungal Activity of Thiadiazole Derivatives

Based on Table 1, the MICs of compounds 9 and 10 were 125 and 4000 µM, respectively, and both compounds had complete fungicidal effect on *A. fumigatus* at concentrations of 500 and 8000 µM, respectively. IC_{50} values of compounds 9 and 10 were 43.39 and 1008.67 µM, respectively. As indicated in Table 2, compound 9 inhibited the fungal growth in a dose-dependent manner. Growth inhibitions were 31.90-100% in serial dilutions of 25-800 µM for compound 9, while compound 10 had a constant range of fungal growth in concentrations of 25-400 µM independent of the dose (Table 2). Compound 10 showed 24.26% increase in the lowest concentration of 25 µM and reached 100% in the concentration of 3200 µM (Table 2). In addition, compounds 9 and 10 showed a significant statistical analysis at concentrations higher than 25 µM and at all concentrations, respectively, as compared with the control (ANOVA, $p<0.05$).

Inhibition of Membrane Ergosterol Biosynthesis

As shown in Table 2, results of the inhibition of membrane ergosterol indicated that the two compounds neither play significant role in inhibiting the ergosterol biosynthesis nor stimulate the production of membrane ergosterol at higher concentrations.

Cytotoxicity of Compounds

Cytotoxicity of compounds 9 and 10 were examined on normal cell line (Vero) and cancer cells (Hep2) at high concentration (800 µM). Compounds 9 and 10 showed viability ≥85% and ≥96% on the studied cells, respectively.

Table 1. Minimum inhibitory concentration, minimum fungicidal concentration, and IC_{50} of compounds 9 and 10 against *Aspergillus fumigatus*

Compound	MIC (µM)	MFC (µM)	IC_{50} (µM)	% Viability in 800 µM	
				Vero	Hep2
9	125	500	43.39	85	100
10	4000	8000	1008.67	100	96

IC_{50}: Half maximal inhibitory concentration, Vero: Normal cell line, Hep2: Cancerous cells, MICs: Minimum inhibitory concentrations, MFC: Minimum fungicidal concentration

TEM Observations

The structural and morphological characteristics of hyphae compartments in *A. fumigatus* in a control sample and samples exposed to synthetic compounds 9 and 10 are shown in Figure 3. The cell wall was completely uniform and surrounded by a healthy fiber layer in the control hyphae (Figure 3A-C). The plasma membrane was non-folding and fully uniform in all areas. The septum and all other cytoplasmic organelles such as the nuclei and mitochondria were thoroughly normal. With synthetic compound 9 at concentration of 400 µM (Figure 3D-F), the fungus showed the highest fungal growth inhibition (96.70%). In addition, morphological changes were observed in organelles such as the mitochondria, the plasma membrane folds inward, which detached from the cell wall in some areas, the cell content evacuates, and the hyphae are deformed. The fungus in synthetic compound 10 at the concentration of 800 µM (Figure 3G-I) could inhibit 35.75% of fungal growth, and severe folds of plasma membrane and its detachment from the cell wall and deformation of hyphae were observed. Autolysis and disruption of hyphae and cytoplasmic membrane structures such as the mitochondria and evacuation of their content were observed.

Discussion

To our knowledge, this study is the first to investigate the antifungal effect of new compounds N-(2-methoxyethyl)-5-(5-nitrofuran-2-yl)-1,3,4-thiadiazol-2-amine (9) and N-benzyl-5-(5-nitrothiophen-2-yl)-1,3,4-thiadiazol-2-amine (10) containing a special core of 1,3,4-thiadiazole, which verified the inhibition ability of the compounds in the growth of *A. fumigatus* in a dose-dependent manner. In addition to MIC and MFC, cellular changes of *A. fumigatus* samples exposed to the aforesaid compounds were assessed at the electron microscopy level.

The biological activity of compounds containing 1,3,4-thiadiazole scaffold has been evaluated in various pathogens[14-20]. Previously, leishmanicidal activities of compounds 9 and 10 has been reported by Tahghighi et al.[20], particularly on the promastigote form of *Leishmania* with IC_{50} values of 28 and 115.6 µM, respectively. They also affirmed the good anti-amostigote activity of compound 9 and low cytotoxicity (CC_{50}=131.64 µM) against mouse peritoneal macrophages. Moreover, compound 9 presented a more favorable antifungal activity relative to compound 10 in the present study. Foroumadi et al.[31] reported a series of broad-spectrum antifungal agents from 1,3,4-thiadiazole series and indicated that substituted arylsulfonyl-1,3,4-thiadiazoles have

Table 2. Inhibitory effects of compounds 9 and 10 on the growth of *Aspergillus fumigatus* and ergosterol contents in the fungal cell membrane

Compound	Concentration (µM)	Fungal growth (dry weight; mg)		Ergosterol content (Per gr fungal dry weight)	
		Mean±SEM	Inhibition (%)	Mean±SEM	Inhibition (%)
9	0	34.26±2.32	0.00	0.512±0.212	0.00
	25	23.33±1.85	31.90*	0.501±0.069	2.14
	50	14.90±1.17	56.50*	0.582±0.259	0.00
	100	10.46±2.69	69.46*	0.562±0.528	0.00
	200	9.63±1.68	71.89*	1.080±0.461	0.00
	400	1.13±0.26	96.70*	ND	ND
	800	0.00	100*	ND	ND
	1600	0.00	100*	ND	ND
	3200	0.00	100*	ND	ND
10	0	35.69±2.07	0.00	0.799±0.064	0.00
	25	27.03±1.02	24.26*	0.778±0.051	2.62
	50	27.36±0.42	23.33*	0.757±0.035	5.25
	100	26.56±0.40	25.58*	0.730±0.057	8.63
	200	27.13±0.12	23.98*	0.721±0.091	9.76
	400	27.46±0.74	23.05*	0.682±0.208	14.64
	800	22.93±0.76	35.75*	1.144±0.224	0.00
	1600	3.43±3.28	90.38*	1.344±0.010	0.00
	3200	0.00	100*	ND	ND

*Statistically significant difference with the control (ANOVA, $p<0.05$).

ND: Not determined, SEM: Standard error of the mean

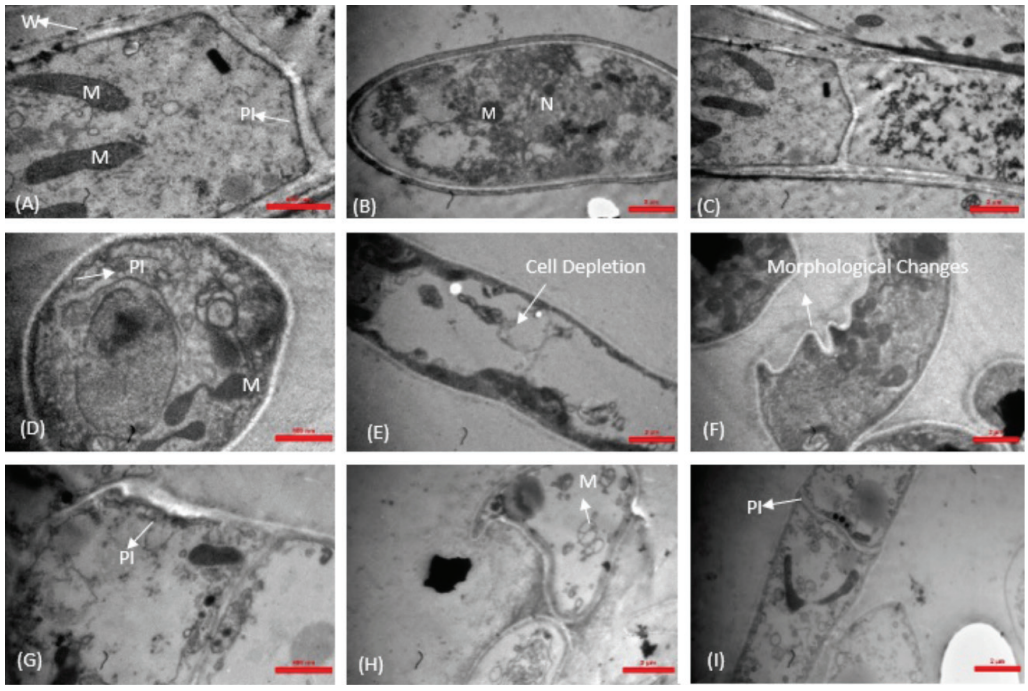

Figure 3. Electron microscopic images of *Aspergillus fumigatus* examined in PDB medium at 28 °C for 96 h in both controlled state and adjacent to synthetic compounds. (A, B, C) Cell wall (w) and homogeneous control mycelia, plasma membrane (PI), and all organelles such as the mitochondria (M) and nucleus (N) have normal structures. (D, E, F) Transmission electron microscopy of *A. fumigatus* adjacent to (D, E, F) 400 µM of compound 9 and (G, H, I) 800 µM of compound 10. Plasma membrane damage (D), cellular evacuation (E), hypha deformation, and folding of wall and membrane inwards (location of the arrow, F). Damage and disruption of cellular organization including destruction and evacuation of membrane organelles such as mitochondria, autolysis of cytoplasm, cellular deformation, and extensive damage of plasma membrane were observed

antifungal activity against various fungal strains. Among them, the compound 2-(5-nitrofuran-2-yl)-5-(phenylsulfonyl)-1,3,4-thiadiazole (6) had MIC of 6.25 µg/ml against *A. fumigatus*, even better than fluconazole. This compound contained an active moiety of 2-(5-nitrofuran-2-yl)-1,3,4-thiadiazole similar to compound (9), which is responsible for their antifungal activity (Figure 1).

In another study[32], compound (7) with structure of 5-[(1,4,5-triphenylimidazol-2-yl)thiopropyl]-2-(N-ethyl)-1,3,4-thiadiazoles was studied and exhibited a zone of inhibition of 19.4 mm against *A. fumigatus* (Figure 1). Compound 7 has been also indicated to be active against other fungal strains such as *Candida albicans* and *Geotrichum candidum* with zone inhibition values of 18.2 and 20.6 mm, respectively. Raj et al.[33] reported antifungal activity of compound 4-((5-phenyl-1,3,4-thiadiazol-2-ylamino)methylamino)phenol (8) with MICs of 18 and 16 µg/ml for *A. fumigatus* and *A. niger*, respectively (Figure 1). Based on the structure-activity profile of substituted 1,3,4-thiadiazole derivatives, minor structural variation induces different effects on their antimicrobial activity. Nitrofuran substitution of the studied compounds can lead to strong antifungal effects of these compounds because it can be metabolized and converted into an active intermediate, thereby damaging the DNA of microorganisms, affecting other intracellular macromolecules, or inducing oxidative stress[34].

The present study proved that the potency of compound 9 with substituents of nitrofuran in position 2 and N-methoxyethyl in position 5 of the 1-3-4-thiadiazole ring was higher than that of compound 10 with substituent of N-benzyl in position 5 of the thiadiazole ring. Indeed, this linear alkyl chain increased the antifungal activity, whereas an alkyl aryl substituent produced moderate activity, which may be a result of the steric hindrance around position 5 of the thiadiazole ring. Compound 9 demonstrated low cytotoxicity against Vero cells, and in a previous investigation, it demonstrated little cytotoxicity against peritoneal macrophages[20]. By contrast, the destructive effects of this compound on the cellular membrane and wall resemble its similar compounds at the electron microscopy level.

The limitation of the study was the lack of *in vivo* evaluation of the effect of thiadiazole derivatives in an animal model.

Conclusion

With regard to antifungal drug resistance and multiple side effects of available drugs, research and development of new

antifungal medication are always necessary. Based on this study, N-(2-methoxyethyl)-5-(5-nitrofuran-2-yl)-1-3-4-thiadiazole-2-amine (9) has the potential for further investigation such as *in vitro* test against resistant strains, *in vivo* experiments, formulation strategies, and application of drug delivery systems to improve their pharmacokinetics.

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Ethics

Ethics Committee Approval: *In vitro* study with no human and/or animal sampling and sample analysis.

Informed Consent: *In vitro* study with no human and/or animal data analysis.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: P.M., A.T., M.R-A., Design: A.T., M.S-G., M.R-A., Data Collection or Processing: P.M., A.T., L.J-B., A.F., M.R-A., Analysis or Interpretation: A.T., L.J-B., A.F., M.S-G., M.R-A., Literature Search: P.M., A.T., M.R-A., Writing: P.M., A.T., M.S-G., M.R-A.

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