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Original article

Synthesis and anticandidal activity of new triazolothiadiazine derivatives

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ABSTRACT

New triazolothiadiazine derivatives were synthesized via the ring closure reaction of 4-amino-5-substituted-2,4-dihydro-3H-1,2,4-triazol-3-thiones with phenacyl bromides. The compounds were tested *in vitro* against various *Candida* species and compared with ketoconazole. Among these compounds, the compound bearing cyclohexyl moiety and *p*-chlorophenyl substituent on triazolothiadiazine ring (**2i**) was found to be the most potent derivative against *Candida albicans* (ATCC 90028). It is clear that there is a positive correlation between anticandidal activity and two functional moieties, namely cycloaliphatic group and *p*-chlorophenyl substituent on triazolothiadiazine ring. The compounds were also investigated for their cytotoxic effects using MTT assay. Compound **2a** exhibited the highest cytotoxic activity, whereas compound **2f** possessed the lowest cytotoxic activity against NIH/3T3 cells.

1. Introduction

Candida species have emerged as the most common cause of systemic fungal infections worldwide over the last two decades. A large number of studies have attempted to assess risk factors for candidaemia. The most frequently implicated risk factors include treatment with broad-spectrum antibiotics, use of central venous catheters and implantable prosthetic devices, parenteral nutrition, prolonged intensive care unit stay, hemodialysis and immunosuppression (including HIV infection, neutropenia, use of glucocorticosteroids, chemotherapeutic agents, and immunomodulators) [1–7].

Azoles, especially triazole antifungal agents, play a leading role in the treatment of systemic fungal infections owing to their broad spectrum and improved safety profile. The prominent drugs bearing triazole ring are fluconazole, itraconazole, voriconazole, and posaconazole, all of which are widely used antifungal drugs for the treatment of systemic fungal infections [8,9].

The widespread use of these agents has led to the development of resistance in recent years. Several researchers have extensively studied the mechanisms of resistance to azoles in *Candida* species. At the molecular level, *Candida* species can develop resistance to azoles via decreased accumulation of the drug resulting from enhanced efflux, interference of their action on lanosterol 14α-demethylase, alterations in other enzymes of the biosynthetic pathway of ergosterol, and decreased permeability of the fungal membrane to the drug. As a consequence of this situation, medicinal chemists have focused on the development of new effective anticandidal agents [3].

Among triazole derivatives, 1,2,4-triazoline-3-thiones and their fused heterocyclic derivatives including triazolothiadiazines have received considerable attention due to their biological importance [10–12]. Some studies have confirmed that triazolothiadiazine derivatives possess anticandidal activity [13–17].

In continuation of our previous work on the synthesis and antimicrobial evaluation of triazolothiadiazine derivatives [15], herein we described the discovery of new triazolothiadiazine derivatives, which were tested *in vitro* against various *Candida* species and investigated for their cytotoxic effects.

2. Chemistry

The compounds possessing triazoline-3-thione structure (**1a**–**b**) were previously synthesized via the one-pot reaction of thiocarbohydrazide with appropriate propionic acids under solvent-free conditions [18].

New triazolothiadiazine derivatives (**2a**–**j**) were obtained by the ring closure reaction of 1,2,4-triazoline-3-thiones (**1a**–**b**) with

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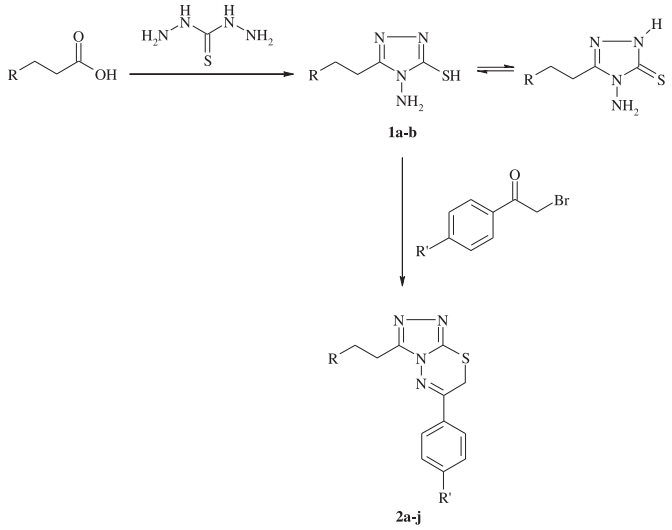

Scheme 1. The synthesis of the compounds (**2a**–**j**).

phenacyl bromides [19]. These reactions are summarized in Scheme 1 and some properties of the compounds are given in Table 1.

3. Results and discussion

The structures of all compounds (**2a**–**j**) were confirmed by ^{1}H-NMR, ^{13}C-NMR, mass spectral data and elemental analysis.

In the ^{1}H-NMR spectra of the previously synthesized compounds **1a**–**b**, we observed NH_2, NH protons at 5–6 ppm and 13–14 ppm, respectively [18].

In the ^{1}H-NMR spectra of all compounds (**2a**–**j**), the absence of the peaks corresponding to NH_2 and NH protons of compounds **1a**–**b** clearly confirmed the formation of triazolothiadiazine ring.

In the ^{1}H-NMR spectra of all compounds (**2a**–**j**), the signal due to the $-S-CH_2-$ protons of triazolothiadiazine ring gave rise to a singlet peak at 4.3–4.45 ppm.

In the ^{1}H-NMR spectra of compounds **2a**–**e**, the methylene protons directly attached to phenol and triazolothiadiazine rings were observed at 2.90–2.94 ppm and 3.08–3.12 ppm, respectively. The protons of phenol ring were observed in the region 6.62–7.01 ppm. The O–H proton of phenol appeared at 9.20 as a singlet peak.

Table 1
Some properties of the compounds (**2a**–**j**).

Compound	R	R′	Yield (%)	M.p. (°C)	Molecular formula	Molecular weight
2a	4-(OH) C_6H_5	H	75	220	$C_{18}H_{16}N_4OS$	336
2b	4-(OH) C_6H_5	NO_2	80	244–245	$C_{18}H_{15}N_5O_3S$	381
2c	4-(OH) C_6H_5	F	65	206–211	$C_{18}H_{15}FN_4OS$	354
2d	4-(OH) C_6H_5	Cl	65	274–275	$C_{18}H_{15}ClN_4OS$	370.5
2e	4-(OH) C_6H_5	CH_3	60	234–236	$C_{19}H_{18}N_4OS$	350
2f	C_6H_{11}	H	70	168–170	$C_{18}H_{22}N_4S$	326
2g	C_6H_{11}	NO_2	75	153–156	$C_{18}H_{21}N_5O_2S$	371
2h	C_6H_{11}	F	65	183–185	$C_{18}H_{21}FN_4S$	344
2i	C_6H_{11}	Cl	65	201–202	$C_{18}H_{21}ClN_4S$	360.5
2j	C_6H_{11}	CH_3	55	253	$C_{19}H_{24}N_4S$	340

In the ^{1}H-NMR spectra of compounds **2f**–**j**, the cyclohexyl protons and the methylene protons directly attached to cyclohexane ring were observed at 0.84–1.77 ppm. The methylene protons directly attached to triazolothiadiazine appeared at 2.87–2.97 ppm as a triplet peak. All other aromatic and aliphatic protons were observed at expected regions.

In the ^{13}C-NMR spectra of all compounds (**2a**–**j**), the peaks belonging to triazolothiadiazine carbons except C-7 appeared at 140–160 ppm. All other aromatic and aliphatic carbons were observed at expected regions.

In the mass spectra of all compounds (**2a**–**j**), M + 1 peaks were consistent with their molecular weights. All compounds gave satisfactory elemental analysis.

All compounds were tested *in vitro* against *Candida* species and compared with ketoconazole (Table 2). Among these compounds, compounds **2a**, **2b**, **2f**, **2g** and **2j** exhibit the highest inhibitory activity against *Candida albicans* (Clinical Isolate). This outcome confirms that phenyl and *p*-nitrophenyl groups on triazolothiadiazine ring may have a considerable influence on antifungal activity against *C. albicans* (Clinical Isolate). Although compounds **2e** and **2j** possess 4-methylphenyl moiety on triazolothiadiazine ring, they show different levels of anticandidal activity. The former bearing 4-hydroxyphenyl group exhibits the lowest antifungal activity, whereas the latter bearing cyclohexyl moiety exhibits the highest antifungal activity against *C. albicans* (Clinical Isolate). This could result from increased lipophilicity associated with cyclohexyl group.

The compound bearing cyclohexyl moiety and *p*-chlorophenyl substituent on triazolothiadiazine ring (**2i**) was found to be the most potent derivative against *C. albicans* (ATCC 90028), *Candida parapsilosis*, *C. albicans* (NRRL Y-12983), *Candida glabrata* (Clinical Isolate, Anadolu University, Faculty of Science). Among *Candida* species, *C. albicans* (ATCC 90028) is the most susceptible fungus to compound **2i**. Compound **2i** exhibits the inhibitory activity against *C. albicans* (ATCC 90028) with a MIC value of 0.03 mg/mL, whereas ketoconazole exhibits the inhibitory activity with a MIC value of 0.04 mg/mL. It is apparent that there is a positive correlation between anticandidal activity and two functional moieties, namely cycloaliphatic group and *p*-chlorophenyl substituent on triazolothiadiazine ring. It can be attributed to $+\pi$ effect of chloro substituent and cyclohexyl group.

The compound possessing 4-hydroxyphenyl moiety and phenyl substituent on triazolothiadiazine ring (**2a**) is the most effective derivative against *C. glabrata* (Clinical Isolate, Osmangazi University, Faculty of Medicine).

The biological results indicate that *Candida tropicalis* is more susceptible to compounds **2a**, **2b** and **2i**.

Compound **2h** is the most potent derivative against *Candida krusei*. It is obvious that cyclohexyl moiety and 4-fluorophenyl group on triazolothiadiazine ring have an important impact on antifungal activity against *C. krusei*.

All compounds were also evaluated for their cytotoxic properties using MTT assay. The biological study indicated that compound **2a** possessed the highest cytotoxicity, whereas compound **2f** exhibited the lowest cytotoxicity against NIH/3T3 cells among the title compounds (Table 3).

4. Conclusion

In conclusion, we focused on the synthesis of new triazolothiadiazine derivatives, which were tested *in vitro* against various *Candida* species.

Generally the compounds bearing cyclohexyl moiety (**2f**–**j**) are more effective than the compounds bearing 4-hydroxyphenyl

Table 2
Anticandidal activities of the compounds (**2a**–**j**) as MIC values (mg/mL).

	2a	2b[a]	2c	2d	2e	2f	2g[a]	2h	2i	2j	Ketoconazole
A	0.09	0.09	0.18	0.75	0.75	0.09	0.09	0.37	0.18	0.09	0.03
B	0.08	0.09	1.5	0.75	0.75	0.09	0.09	0.09	0.04	0.18	0.03
C	0.04	0.09	1.5	0.75	0.75	0.09	0.09	0.37	0.09	0.09	0.015
D	0.09	0.09	1.5	0.75	1.5	0.18	0.18	0.75	0.09	0.75	0.003
E	0.37	0.18	0.18	0.75	0.37	0.18	0.18	0.09	0.37	0.18	0.003
F	0.18	0.04	1.5	0.37	0.37	0.37	0.18	0.37	0.02	0.37	0.007
G	0.37	0.75	1.5	1.5	1.5	0.18	0.09	0.09	0.04	0.09	0.015
H	0.18	0.09	0.37	0.75	0.75	0.09	0.09	0.09	0.04	0.18	0.007

Ketoconazole: 16 mg/1 mL
A: *C. albicans* (Clinical Isolate, Osmangazi University, Faculty of Medicine, Eskişehir, Turkey), **B:** *C. albicans* (ATCC 90028), **C:** *C. glabrata* (Clinical Isolate, Osmangazi University, Faculty of Medicine, Eskişehir, Turkey), **D:** *C. tropicalis* (NRRL Y-12968), **E:** *C. krusei* (NRRL Y-7179), **F:** *C. parapsilosis* (NRRL Y-12696), **G:** *C. albicans* (NRRL Y-12983), **H:** *C. glabrata* (Clinical Isolate, Anadolu University, Faculty of Science, Department of Biology, Eskişehir, Turkey).
[a] The compounds were solved in 12 mg/2 mL DMSO, other derivatives were solved in 24 mg/2 mL DMSO.

moiety (**2a**–**e**) against *Candida* species. It can be attributed to increased lipophilicity associated with cyclohexyl group.

Compound **2i** is the most effective compound against *C. albicans* (ATCC 90028), *C. parapsilosis*, *C. albicans* (NRRL Y-12983), *Candida glabrata* (Clinical Isolate, Anadolu University, Faculty of Science). Among *Candida* species, *C. albicans* (ATCC 90028) is the most susceptible fungus to compound **2i**. Compound **2i** exhibits the inhibitory activity against *C. albicans* (ATCC 90028) with a MIC value of 0.03 mg/mL, whereas ketoconazole exhibits the inhibitory activity with an MIC value of 0.04 mg/mL. This outcome confirms that cycloaliphatic group and *p*-chlorophenyl substituent on triazolothiadiazine ring may have a considerable influence on anticandidal activity.

The cytotoxic effects of the compounds were also investigated and compound **2a** possessed the highest cytotoxicity, whereas compound **2f** exhibited the lowest cytotoxicity against NIH/3T3 cells.

5. Experimental

5.1. Chemistry

All reagents were purchased from commercial suppliers and were used without further purification. Melting points (m.p.) were determined on an Electrothermal 9100 melting point apparatus (Weiss-Gallenkamp, Loughborough, UK) and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on Bruker 400 MHz spectrometer (Bruker, Billerica, MA, USA). Carbon nuclear magnetic resonance (^{13}C-NMR) spectra were recorded on Bruker 300 MHz spectrometer (Bruker, Billerica, MA, USA). Chemical shifts were expressed in parts per million (ppm) and tetramethylsilane was used as an internal standard. Mass spectra were recorded on a VG Quattro Mass spectrometer (Agilent, Minnesota, USA). Elemental analyses were performed on a Perkin Elmer EAL 240 elemental analyser (Perkin Elmer, Norwalk, CT, USA).

Table 3
In vitro cytotoxicity of the compounds (**2a**–**j**).

Compound	IC_{50} (μg/mL)[a]
2a	31.6 + 2.8
2b	306.7 + 27.5
2c	366.7 + 28.9
2d	86.7 + 10.4
2e	400 + 100
2f	433 + 28.8
2g	210 + 50
2h	106 + 28.8
2i	103 + 32.14
2j	198 + 41.9

[a] Cytotoxicity of the compounds to mouse fibroblast (NIH/3T3) cell line. Incubation for 24 h. IC_{50} is the drug concentration required to inhibit 50% of the cell growth. The values represent mean ± standard deviation of triplicate determinations.

5.1.1. General procedure for the synthesis of the compounds

*5.1.1.1. 4-Amino-5-(2-cyclohexyl/(4-hydroxyphenyl)ethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (**1a**–**b**).* A mixture of 3-cyclohexyl/(4-hydroxyphenyl)propionic acid (0,1 mol) and thiocarbohydrazide (0,1 mol) was heated in an oil bath at 160–170 °C for 2 h. It was then dispersed with hot water and collected by filtration. The product was crystallized from ethanol.

*5.1.1.2. 3-[2-Cyclohexyl/(4-hydroxyphenyl)ethyl]-6-aryl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazines (**2a**–**j**).* A solution of triazole (0.005 mol) and appropriate phenacyl bromide (0.005 mol) in absolute ethanol (30 mL) was heated under reflux for 1 h, cooled to room temperature and then neutralized with ammonium hydroxide. The product thus obtained was recrystallized from ethanol.

*5.1.1.2.1. 3-[2-(4-Hydroxyphenyl)ethyl]-6-phenyl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine (**2a**).* ^{1}H-NMR (400 MHz, DMSO-d_6): 2.90–2.94 (2H, t, phenol-C**H$_2$**), 3.08–3.12 (2H, t, C**H$_2$**-triazole), 4.32 (2H, s, S-CH_2), 6.62–6.65 (2H, d, $J = 8.44$ Hz, phenol-H_3, H_5), 6.99–7.01 (2H, d $J = 8.44$ Hz, phenol-H_2, H_6), 7.63–7.66 (3H, m, phenyl H_3, H_4, H_5), 7.98–8.00 (2H, dd, $J = 1.55$, $J = 1.50$, phenyl H_2, H_6), 9.20 (1H, s, O–H).

^{13}C-NMR (300 MHz, DMSO-d_6): 22.91 (CH_2), 26.35 (CH_2), 31.63 (CH_2), 115.08 (2CH), 127.39 (2CH), 129.01 (2CH), 129.24 (2CH), 130.48 (CH), 131.80 (C), 133.55 (C), 140.04 (C), 152.38 (C), 154.63 (C), 155.65 (C).

For $C_{18}H_{16}N_4OS$, calculated: C, 64.26; H, 4.79; N, 16.65; found: C, 64.25; H, 4.80; N, 16.65.

MS (FAB) $[M + 1]^+$: *m*/*z* 337.

*5.1.1.2.2. 3-[2-(4-Hydroxyphenyl)ethyl]-6-(4-nitrophenyl)-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine (**2b**).* ^{1}H-NMR (400 MHz, DMSO-d_6): 2.90–2.94 (2H, t, phenol-C**H$_2$**), 3.08–3.12 (2H, t, C**H$_2$**-triazole), 4.40 (2H, s, S-CH_2), 6.62–6.65 (2H, d, $J = 8.43$ Hz, phenol-H_3, H_5), 6.99–7.01 (2H, d, $J = 8.42$ Hz, phenol-H_2, H_6), 8.23–8.26 (2H, d, $J = 8.94$ Hz, *p*-nitrophenyl H_2, H_6), 8.39–8.41 (2H, d, $J = 8.96$ Hz, *p*-nitrophenyl H_3, H_5), 9.20 (1H, s, O–H).

^{13}C-NMR (300 MHz, DMSO-d_6): 22.98 (CH_2), 26.35 (CH_2), 31.63 (CH_2), 115.08 (2CH), 124.07 (2CH), 128.73 (2CH), 129.24 (2CH), 133.55 (C), 139.52 (C), 139.87 (C), 149.08 (C), 152.93 (C), 154.01 (C), 155.65 (C).

For $C_{18}H_{15}N_5O_3S$, calculated: C, 56.68; H, 3.96; N, 18.36; found: C, 56.69; H, 3.96; N, 18.35.

MS (FAB) $[M + 1]^+$: *m*/*z* 382.

*5.1.1.2.3. 3-[2-(4-Hydroxyphenyl)ethyl]-6-(4-fluorophenyl)-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine (**2c**).* ^{1}H-NMR (400 MHz, DMSO-d_6): 2.90–2.94 (2H, t, phenol-C**H$_2$**), 3.08–3.12 (2H, t, C**H$_2$**-triazole), 4.36 (2H, s, S-CH_2), 6.62–6.65 (2H, d, J = 8.45 Hz, phenol-H_3, H_5), 6.99–7.01 (2H, d J = 8.45 Hz, phenol-H_2, H_6), 7.42–7.45 (2H, m, *p*-fluorophenyl H_3, H_5), 8.05–8.10 (2H, dd, J = 5.38, J = 5.41, *p*-fluorophenyl H_2, H_6), 9.20 (1H, s, O–H).

^{13}C-NMR (300 MHz, DMSO-d_6): 22.75 (CH_2), 26.33 (CH_2), 31.68 (CH_2), 115.07 (2CH), 129.00 (2CH), 129.19 (2CH), 129.26 (2CH), 130.44 (C), 132.36 (C), 136.63 (C), 139.93 (C), 152.99 (C), 153.56 (C), 155.66 (C).

For $C_{18}H_{15}FN_4OS$, calculated: C, 61.00; H, 4.27; N, 15.81; found: C, 61.00; H, 4.28; N, 15.80.

MS (FAB) $[M+1]^+$: *m*/*z* 355.

*5.1.1.2.4. 3-[2-(4-Hydroxyphenyl)ethyl]-6-(4-chlorophenyl)-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine (**2d**).* ^{1}H-NMR (400 MHz, DMSO-d_6): 2.90–2.94 (2H, t, phenol-C**H$_2$**), 3.08–3.12 (2H, t, C**H$_2$**-triazole), 4.32 (2H, s, S-CH_2), 6.62–6.65 (2H, d, J = 8.45 Hz, phenol-H_3, H_5), 6.99–7.01 (2H, d, J = 8.46 Hz, phenol-H_2, H_6), 7.63–7.66 (2H, d, J = 8.72 Hz, *p*-chlorophenyl H_3, H_5), 7.98–8.00 (2H, d, J = 8.69 Hz, *p*-chlorophenyl H_2, H_6), 9.20 (1H, s, O–H).

^{13}C-NMR (300 MHz, DMSO-d_6): 22.75 (CH_2), 26.33 (CH_2), 31.68 (CH_2), 115.07 (2CH), 129.00 (2CH), 129.19 (2CH), 129.26 (2CH), 130.44 (C), 132.36 (C), 136.63 (C), 139.93 (C), 152.99 (C), 153.56 (C), 155.66 (C).

For $C_{18}H_{15}ClN_4OS$, calculated: C, 58.30; H, 4.08; N, 15.11; found: C, 58.32; H, 4.08; N, 15.10.

MS (FAB) $[M+1]^+$: *m*/*z* 371.

*5.1.1.2.5. 3-[2-(4-Hydroxyphenyl)ethyl]-6-(4-methylphenyl)-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine (**2e**).* ^{1}H-NMR (400 MHz, DMSO-d_6): 2.40 (3H, s, CH_3), 2.92–2.95 (2H, t, phenol-C**H$_2$**), 3.09–3.12 (2H, t, C**H$_2$**-triazole), 4.33 (2H, s, S-CH_2), 6.64–6.66 (2H, d, J = 8.41 Hz, phenol-H_3, H_5), 7.01–7.03 (2H, d, J = 8.42 Hz, phenol-H_2, H_6), 7.38–7.40 (2H, d, J = 8.15 Hz, *p*-methylphenyl H_2, H_6), 7.89–7.91 (2H, d, J = 8.27 Hz, *p*-methylphenyl H_3, H_5), 9.19 (1H, s, O–H).

^{13}C-NMR (300 MHz, DMSO-d_6): 21.00 (CH_3), 23.00 (CH_2), 27.00 (CH_2), 32.00 (CH_2), 115.50 (2CH), 128.00 (2CH), 129.90 (2CH), 130.90 (2CH), 131.00 (C), 132.36 (C), 140.60 (C), 142.90 (C), 153.50 (C), 155.00 (C), 156.20 (C).

For $C_{19}H_{18}N_4OS$, calculated: C, 65.12; H, 5.18; N, 15.99; found: C, 65.10; H, 5.18; N, 16.00.

MS (FAB) $[M+1]^+$: *m*/*z* 351.

*5.1.1.2.6. 3-[2-Cyclohexylethyl]-6-phenyl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine (**2f**).* ^{1}H-NMR (400 MHz, DMSO-d_6): 0.84–0.95 (2H, m, cyclohexyl-H), 1.05–1.31 (4H, m, cyclohexyl-H), 1.60–1.77 (7H, m, cyclohexyl-H, cyclohexyl-C**H$_2$**), 2.87–2.91 (2H, t, C**H$_2$**-triazole), 4.41 (2H, s, S-CH_2), 7.57–7.62 (3H, m, phenyl H_3, H_4, H_5), 8.00–8.02 (2H, m, phenyl H_2, H_6).

^{13}C-NMR (300 MHz, DMSO-d_6): 21.41 (CH_2), 22.98 (CH_2), 25.68 ($2CH_2$), 26.06 (CH_2), 32.43 ($2CH_2$), 33.88 (CH_2), 36.37 (CH), 127.39 (2CH), 129.01 (2CH), 130.48 (CH), 131.80 (C), 140.04 (C), 152.38 (C), 154.63 (C).

For $C_{18}H_{22}N_4S$, calculated: C, 66.22; H, 6.79; N, 17.16; found: C, 66.20; H, 6.80; N, 17.15.

MS (FAB) $[M+1]^+$: *m*/*z* 327.

*5.1.1.2.7. 3-[2-Cyclohexylethyl]-6-(4-nitrophenyl)-7H-1,2,4-triazolo [3,4-b]-1,3,4-thiadiazine (**2g**).* ^{1}H-NMR (400 MHz, DMSO-d_6): 0.90–0.93 (2H, m, cyclohexyl-H), 1.04–1.30 (4H, m, cyclohexyl-H), 1.59–1.77 (7H, m, cyclohexyl-H, cyclohexyl-C**H$_2$**), 2.87–2.91 (2H, t, C**H$_2$**-triazole), 4.46 (2H, s, S-CH_2), 8.23–8.26 (2H, d, J = 8.95 Hz, *p*-nitrophenyl H_2, H_6), 8.39–8.41 (2H, d, J = 8.93 Hz, *p*-nitrophenyl H_3, H_5).

^{13}C-NMR (300 MHz, DMSO-d_6): 21.41 (CH_2), 22.98 (CH_2), 25.68 ($2CH_2$), 26.06 (CH_2), 32.43 ($2CH_2$), 33.88 (CH_2), 36.37 (CH), 124.07 (2CH), 128.73 (2CH), 133.55 (C), 139.52 (C), 139.87 (C), 152.93 (C), 154.01 (C).

For $C_{18}H_{21}N_5O_2S$, calculated: C, 58.20; H, 5.70; N, 18.85; found: C, 58.20; H, 5.69; N, 18.85.

MS (FAB) $[M+1]^+$: *m*/*z* 372.

*5.1.1.2.8. 3-[2-Cyclohexylethyl]-6-(4-fluorophenyl)-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine (**2h**).* ^{1}H-NMR (400 MHz, DMSO-d_6): 0.90–0.93 (2H, m, cyclohexyl-H), 1.15–1.30 (4H, m, cyclohexyl-H), 1.59–1.77 (7H, m, cyclohexyl-H, cyclohexyl-C**H$_2$**), 2.87–2.90 (2H, t, C**H$_2$**-triazole), 4.40 (2H, s, S-CH_2), 7.43–7.47 (2H, m, *p*-fluorophenyl H_2, H_6), 8.07–8.10 (2H, m, *p*-fluorophenyl H_3, H_5).

^{13}C-NMR (300 MHz, DMSO-d_6): 21.41 (CH_2), 22.98 (CH_2), 25.68 ($2CH_2$), 26.06 (CH_2), 32.43 ($2CH_2$), 33.88 (CH_2), 36.37 (CH), 129.00 (2CH), 129.26 (2CH), 130.44 (C), 131.80 (C), 140.04 (C), 152.99 (C), 153.56 (C).

For $C_{18}H_{21}FN_4S$, calculated: C, 62.76; H, 6.15; N, 16.27; found: C, 62.75; H, 6.15; N, 16.25.

MS (FAB) $[M+1]^+$: *m*/*z* 345.

*5.1.1.2.9. 3-[2-Cyclohexylethyl]-6-(4-chlorophenyl)–7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine (**2i**).* ^{1}H-NMR (400 MHz, DMSO-d_6): 0.90–0.95 (2H, m, cyclohexyl-H), 1.05–1.31 (4H, m, cyclohexyl-H), 1.59–1.77 (7H, m, cyclohexyl-H, cyclohexyl-C**H$_2$**), 2.90–2.94 (2H, t, C**H$_2$**-triazole), 4.44 (2H, s, S-CH_2), 7.67–7.69 (2H, d, J = 8.55 Hz, *p*-chlorophenyl H_2, H_6), 8.04–8.06 (2H, d, J = 8.55 Hz, *p*-chlorophenyl H_3, H_5).

^{13}C-NMR (300 MHz, DMSO-d_6): 21.41 (CH_2), 22.98 (CH_2), 25.68 ($2CH_2$), 26.06 (CH_2), 32.43 ($2CH_2$), 33.88 (CH_2), 36.37 (CH), 129.00 (2CH), 129.26 (2CH), 130.44 (C), 131.80 (C), 140.04 (C), 152.99 (C), 153.56 (C).

For $C_{18}H_{21}ClN_4S$, calculated: C, 59.90; H, 5.86; N, 15.52; found: C, 59.90; H, 5.85; N, 15.50.

MS (FAB) $[M+1]^+$: *m*/*z* 361.

*5.1.1.2.10. 3-[2-Cyclohexylethyl]-6-(4-methylphenyl)-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine (**2j**).* ^{1}H-NMR (400 MHz, DMSO-d_6): 0.90–0.95 (2H, m, cyclohexyl-H), 1.05–1.31 (4H, m, cyclohexyl-H), 1.61–1.77 (7H, m, cyclohexyl-H, cyclohexyl-C**H$_2$**), 2.40 (3H, s, CH_3), 2.94–2.97 (2H, t, C**H$_2$**-triazole), 4.45 (2H, s, S-CH_2), 7.40–7.42 (2H, d, J = 8.13 Hz, *p*-methylphenyl H_2, H_6), 7.94–7.96 (2H, d, J = 8.27 Hz, *p*-methylphenyl H_3, H_5).

^{13}C-NMR (300 MHz, DMSO-d_6): 20.97 (CH_3), 21.41 (CH_2), 22.98 (CH_2), 25.68 ($2CH_2$), 26.06 (CH_2), 32.43 ($2CH_2$), 33.88 (CH_2), 36.37 (CH), 127.39 (2CH), 129.01 (2CH), 130.48 (C), 131.80 (C), 140.04 (C), 152.38 (C), 154.63 (C).

For $C_{19}H_{24}N_4S$, calculated: C, 67.02; H, 7.10; N, 16.45; found: C, 67.00; H, 7.10; N, 16.46.

MS (FAB) $[M+1]^+$: *m*/*z* 341.

5.2. Microbiology

5.2.1. Anticandidal evaluation

A modified microbroth dilution method was carried out according to the procedure and a previous work [20,21]. Ketoconazole was used as a reference drug.

The compounds (**2a**–**j**) were tested *in vitro* against *C. albicans* (Clinical isolate, Osmangazi University, Faculty of Medicine, Eskişehir, Turkey), *C. albicans* (ATCC 90028), *C. glabrata* (Clinical isolate, Osmangazi University, Faculty of Medicine, Eskişehir, Turkey), *C. tropicalis* (NRRL Y-12968), *C. krusei* (NRRL Y-7179), *C. parapsilosis* (NRRL Y-12696), *C. albicans* (NRRL Y-12983), *C. glabrata* (Clinical Isolate, Anadolu University, Faculty of Science, Department of Biology, Eskişehir, Turkey).

5.3. Toxicity

The level of cellular MTT (Sigma) reduction was quantified as previously described in the literature with small modifications [22,23].

5.3.1. Cell culture and drug treatment

NIH/3T3 cells were obtained from the American Type Culture Collection (ATCC, USA). The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Life Technologies, UK), 100 IU/mL penicillin (Gibco, Paisley, Scotland) and 100 mg/mL streptomycin (Gibco) at 37 °C in a humidified atmosphere of 95% air and 5 % CO_2. Exponentially growing cells were plated at 2×10^4 cells/mL into 96-well microtiter tissue culture plates (Nunc, Denmark) and incubated for 24 h before the addition of the drugs (the optimum cell number for cytotoxicity assays was determined in preliminary experiments). Stock solutions of compounds were prepared in dimethyl sulphoxide (DMSO; SigmaeAldrich, Poole, UK) and further dilutions were made with fresh culture medium (the concentration of DMSO in the final culture medium was <0.1% which had no effect on the cell viability).

5.3.2. MTT assay for cytotoxicity of the compounds

It is widely used as a measure of cytotoxicity. After 24 h of preincubation, the tested compounds were added to give final concentration in the range 0,5–500 μg/mL and the cells were incubated for 24 h. At the end of this period, MTT was added to a final concentration of 0,5 mg/mL and the cells were incubated for 4 h at 37 °C. After the medium was removed, the formazan crystals formed by MTT metabolism were solubilized by addition of 200 μL DMSO to each well and absorbance was read at 540 nm with a microtitre plate spectrophotometer (Bio-Tek plate reader). Every concentration was repeated in three wells and IC_{50} values were defined as the drug concentrations that reduced absorbance to 50% of control values.

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