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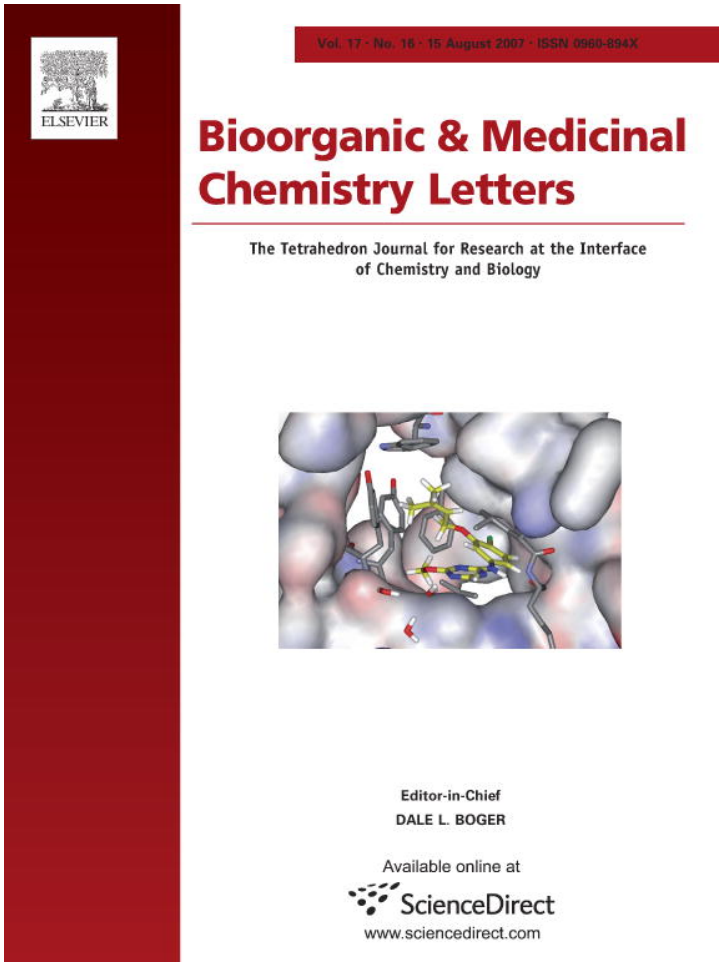

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Synthesis and in vitro activity of 2-thiazolylhydrazone derivatives compared with the activity of clotrimazole against clinical isolates of *Candida* spp.

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Abstract—In this paper, we report on the synthesis of a novel series of 2-thiazolylhydrazone derivatives and the influence of the substituents on the thiazole ring on antifungal activity. All synthesized compounds were screened for their in vitro activities against 22 clinical isolates of *Candida* spp., representing six different species, compared to clotrimazole as a reference compound. Some of the tested compounds were found to possess significant antifungal activity when compared to clotrimazole, in particular compound **14** which exhibited higher potency against most of the *Candida* spp. considered. The compounds that were most active as anti-*Candida* agents were also submitted to cytotoxic screening by the Trypan Blue dye exclusion assay and in general they were shown to induce low cytotoxic effects.

Although *Candida* spp. are present as commensal flora in 30–60% of healthy individuals,[1] they can become infective depending on predisposing conditions related to the host, such as systemic disease leading to immunosupression, and local conditions.[2] As a matter of fact, the pathogenicity of *Candida* spp. is affected by several virulence factors, such as the ability to adhere to epithelial and endothelial cells,[3] germination, extracellular proteinases and phospholipases, and phenotypic switching.[4]

Over the past few decades, the frequency of systematic fungal infections has progressively increased, due to the larger population of immunologically compromised hosts (AIDS, cancer, and transplant), thanks to advances in supportive therapy and an increasing elderly population.

In addition, the widespread use of antimicrobial agents for prophylaxis and treatment has led to less effective antibiotics, not only because many induced side effects but also due to the emergence of drug resistant microorganisms.[5–7]

Large-scale surveillance for fungal bloodstream infections has been performed worldwide by a number of organizations, including the European Confederation of Medical Mycology (ECMM),[8] the Centres for Disease Control and Prevention (CDC),[9] and the National Epidemiology of Mycoses Survey (NEMIS).[10] These studies have demonstrated an increasing incidence of drug-resistant fungal pathogens. As a matter of fact, a significant number of yeast species (*Candida glabrata*, *Candida krusei*, *Candida guilliermondii*, *Candida lusitaniae*) have been shown to exhibit primary resistance to amphotericin B, while *C. glabrata* and *C. krusei* have

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been found to be intrinsically less susceptible to triazoles than the same *Candida albicans*. It has also been found that *Candida dubliniensis* can rapidly develop in vitro stable resistance to fluconazole.[11,12] Furthermore, *C. albicans* intrinsically resistant strains have been observed as part of a commensal microflora or due to acquisition from the environment or from other individuals.[13]

In addition, despite advances in therapy thanks to the introduction of fluconazole and itraconazole, the development of new, more potent azoles, such as voriconazole and posaconazole, and the discovery of a new class of echinocandins,[14] mortality rates for invasive fungal infections today can reach 90% in immunocompromised patients especially.[15]

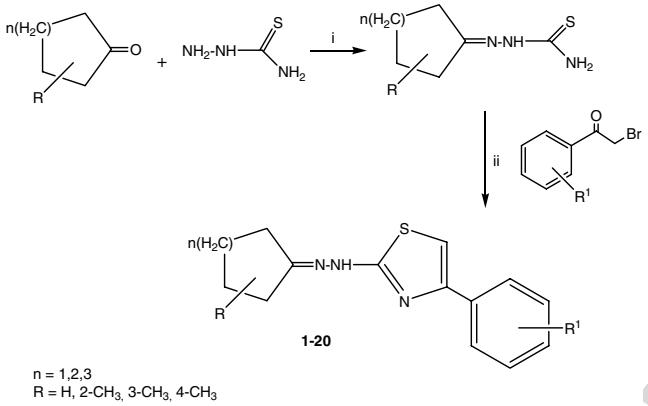
Apart from this, antifungal chemotherapy could be associated with a low toxicological profile, determining termination of the treatment.[16]

Another important issue is the clinical outcome due to several factors such as host defenses, the virulence of the fungal pathogen, drug efficacy/toxicity, and drug interactions.[17,18] Besides, pharmacokinetic studies have shown a variability of at least 50% between individuals;[19,20] in particular, plasma levels for voriconazole can vary from 74% to 100%.[21] These variations could account for the lack of efficacy and the toxicity.

As a consequence of the toxicity of the currently used polyene antifungal drugs and the emergence of candidal species resistant to azole-based agents, there is an urgent need for investigating alternative drug therapies.

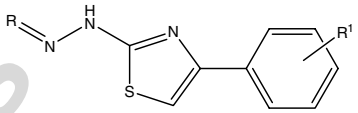
It has recently been reported in the literature that phenyl-thiazole analogues can be used as efficient antifungal agents.[22] Moreover, a large number of substituted benzothiazole,[23] thiazole derivatives of triazoles,[24] thiazolylhydrazones[25] have been shown to exhibit significant antimicrobial activity against a variety of fungal strains.

Moving from these literature indications and pursuing our research in the field,[26,27] in this report we describe the synthesis and antimicrobial evaluation of a new series of 2-thiazolylhydrazone derivatives.



Scheme 1. Synthesis of 2-thiazolylhydrazone derivatives **1**–**20**. Reagents: (i) isopropyl alcohol, AcOH; (ii) isopropyl alcohol.

Table 1. Chemical and physical data of derivatives **1**–**20**



Compound	R	R^1	M.W.	Mp (°C)	% Yield
1	Cyclopentyl	4-CH_3	352.27[a]	218–220	71
2	Cyclopentyl	4-OCH_3	368.27[a]	214–217	88
3	Cyclopentyl	3-NO_2	383.27[a]	187–190	69
4	Cyclopentyl	4-NO_2	383.27[a]	219–220	80
5	Cyclopentyl	4-CN	363.28[a]	207–208	67
6	Cyclopentyl	4-Cl	372.70[a]	223–225	58
7	Cyclopentyl	4-Br	417.16[a]	213–215	66
8	Cyclopentyl	4-C_6H_5	414.35[a]	235–237	58
9	Cyclohexyl-2-CH_3	4-CH_3	380.34[a]	160–162	64
10	Cyclohexyl-2-CH_3	4-OCH_3	396.34[a]	135–137	94
11	Cyclohexyl-2-CH_3	3-NO_2	411.31[a]	165–166	60
12	Cyclohexyl-2-CH_3	4-Br	445.21[a]	130–133	71
13	Cyclohexyl-2-CH_3	4-C_6H_5	442.41[a]	150–152	87
14	Cyclohexyl-3-CH_3	4-CH_3	380.34[a]	171–174	95
15	Cyclohexyl-3-CH_3	4-OCH_3	396.34[a]	162–165	84
16	Cyclohexyl-3-CH_3	4-CN	391.32[a]	189–190	98
17	Cyclohexyl-3-CH_3	4-Cl	400.76[a]	195–199	84
18	Cyclohexyl-3-CH_3	4-F	339.85[b]	192–195	77
19	Cyclohexyl-4-CH_3	4-Cl	400.76[a]	177–179	92
20[28]	Cycloheptyl	H	285.41	184–185	97

[a] Chloridrate.
[b] Bromidrate.

The cytotoxic activity of selected compounds that showed good activity against *Candida* species was evaluated: we incubated them in the presence of an immortalized hybrid cell line displaying an endothelial phenotype, EAhy 926, derived from the fusion of human umbilical vein endothelial cells (HUVEC) with a lung carcinoma cells and determined their cell viability by the Trypan Blue dye exclusion assay.

Table 2. ^{1}H NMR data of derivatives **1**–**20**

Compound	^{1}H NMR δ (ppm)
1[a]	1.80 (q, 2H, cyclopentyl), 1.92 (q, 2H, cyclopentyl), 2.35 (s, 3H, 4′-CH_3-phenyl), 2.50 (t, 2H, cyclopentyl), 2.62 (t, 2H, cyclopentyl), 6.67 (s, 1H, C_5H-thiaz.), 7.22 (d, J = 7.9 Hz, 2H, Ar), 7.55 (d, J = 7.9 Hz, 2H, Ar), 12.13 (br s, 1H, NH, D_2O exch.)
2[a]	1.85 (q, 2H, cyclopentyl), 1.90 (q, 2H, cyclopentyl), 2.50 (t, 2H, cyclopentyl), 2.63 (t, 2H, cyclopentyl), 3.83 (s, 3H, 4′-OCH_3-phenyl), 6.51 (s, 1H, C_5H-thiaz.), 6.96 (d, J = 7.9 Hz, 2H, Ar), 7.63 (d, J = 7.9 Hz, 2H, Ar), 12.13 (br s, 1H, NH, D_2O exch.)
3[a]	1.90 (q, 2H, cyclopentyl), 1.97 (q, 2H, cyclopentyl), 2.58 (t, 2H, cyclopentyl), 2.68 (t, 2H, cyclopentyl), 6.96 (s, 1H, C_5H-thiaz.), 7.77 (t, 1H, Ar), 8.20 (t, 1H, Ar), 8.31 (d, J = 7.9 Hz, 1H, Ar), 8.53 (d, J = 1.68 Hz, 1H, Ar), 12.13 (br s, 1H, NH, D_2O exch.)
4[a]	1.91 (q, 2H, cyclopentyl), 2.01 (q, 2H, cyclopentyl), 2.57 (t, 2H, cyclopentyl), 2.66 (t, 2H, cyclopentyl), 6.97 (s, 1H, C_5H-thiaz.), 7.94 (d, J = 8.8 Hz, 2H, Ar), 8.35 (d, J = 8.8 Hz, 2H), 12.40 (br s, 1H, NH, D_2O exch.)
5[b]	1.83 (q, 2H, cyclopentyl), 1.87 (q, 2H, cyclopentyl), 2.37 (t, 4H, cyclopentyl), 7.66 (s, 1H, C_5H-thiaz.), 7.96 (d, J = 8.4 Hz, 2H, Ar), 8.12 (d, J = 8.4 Hz, 2H, Ar), 10.83 (br s, 1H, NH, D_2O exch.)
6[b]	1.72 (q, 2H, cyclopentyl), 1.78 (q, 2H, cyclopentyl), 2.37 (t, 4H, cyclopentyl), 7.30 (s, 1H, C_5H-thiaz.), 7.44 (d, J = 8.4 Hz, 2H, Ar), 7.84 (d, J = 8.4 Hz, 2H, Ar), 10.70 (br s, 1H, NH, D_2O exch.)
7[b]	1.72 (q, 2H, cyclopentyl), 1.80 (q, 2H, cyclopentyl), 2.37 (t, 4H, cyclopentyl), 7.31 (s, 1H, C_5H-thiaz.), 7.57 (d, J = 8.4 Hz, 2H, Ar), 7.77 (d, J = 8.4 Hz, 2H, Ar), 10.65 (br s, 1H, NH, D_2O exch.)
8[a]	1.87 (q, 2H, cyclopentyl), 1.94 (q, 2H, cyclopentyl), 2.52 (t, 2H, cyclopentyl), 2.66 (t, 2H, cyclopentyl), 6.72 (s, 1H, C_5H-thiaz.), 7.38 (d, J = 7.1 Hz, 1H, Ar), 7.47 (t, 2H, Ar), 7.59 (d, J = 7.1 Hz, 2H, Ar), 7.69 (d, J = 7.9 Hz, 2H, Ar), 7.77 (d, J = 7.9 Hz, 2H, Ar), 12.24 (br s, 1H, NH, D_2O exch.)
9[a]	1.16 (d, J = 6.3 Hz, 3H, CH_3), 1.24–1.42 (m, 1H, cyclohexyl), 1.57–1.66 (m, 2H, cyclohexyl), 1.79–1.86 (m, 1H, cyclohexyl), 1.98–2.03 (m, 2H, cyclohexyl), 2.17–2.27 (m, 1H, cyclohexyl), 2.41 (s, 3H, 4′-CH_3-phenyl), 2.43–2.49 (m, 1H, cyclohexyl), 3.04–3.10 (m, 1H, cyclohexyl), 6.66 (s, 1H, C_5H-thiaz.), 7.28 (d, J = 8.4 Hz, 2H, Ar), 7.60 (d, J = .4 Hz, 2H, Ar), 12.50 (s, 1H, NH, D_2O exch.)
10[a]	1.11 (d, J = 6.3 Hz, 3H, CH_3,), 1.17–1.37 (m, 1H, cyclohexyl), 1.48–1.61 (m, 2H, cyclohexyl), 1.75–1.81 (m, 1H, cyclohexyl), 1.95–1.99 (m, 2H, cyclohexyl), 2.11–2.21 (m, 1H, cyclohexyl), 2.35–2.44 (m, 1H, cyclohexyl), 2.97–3.03 (m, 1H, cyclohexyl), 3.81 (s, 3H, 4′-OCH_3-phenyl), 7.53 (s, 1H, C_5H-thiaz.), 6.94 (d, J = 8.8 Hz, 2H, Ar), 7.60 (d, J = 8.8 Hz, 2H, Ar), 12.37 (s, 1H, NH, D_2O exch.)
11[b]	1.08 (d, J = 6.5 Hz, 3H, CH_3), 1.16–1.26 (m, 1H, cyclohexyl), 1.37–1.59 (m, 2H, cyclohexyl), 1.69–2.02 (m, 4H, cyclohexyl), 2.31–2.42 (m, 1H, cyclohexyl), 2.91–2.96 (m, 1H, cyclohexyl), 7.56 (s, 1H, C_5H-thiaz.), 7.70 (t, 1H, Ar), 8.12 (d, J = 8.1 Hz, 1H, Ar), 8.27 (d, J = 8.1 Hz, 1H, Ar), 8.68 (s, 1H, Ar), 10.95 (s, 1H, NH, D_2O exch.)
12[a]	1.15 (d, J = 6.3 Hz, 3H, CH_3), 1.26–1.38 (m, 1H, cyclohexyl), 1.54–1.65 (m, 2H, cyclohexyl), 1.75–1.81 (m, 1H, cyclohexyl), 1.92–1.98 (m, 2H, cyclohexyl), 2.13–2.23 (m, 1H, cyclohexyl), 2.39–2.47 (m, 1H, cyclohexyl), 2.97–3.02 (m, 1H, cyclohexyl), 6.76 (s, 1H, C_5H-thiaz.), 7.59 (s, 4H, Ar), 12.41 (s, 1H, NH, D_2O exch.)
13[a]	1.13 (d, J = 6.3 Hz, 3H, CH_3), 1.26–1.39 (m, 1H, cyclohexyl), 1.54–1.61 (m, 2H, cyclohexyl), 1.79–1.83 (m, 1H, cyclohexyl), 1.89–1.97 (m, 2H, cyclohexyl), 2.17–2.22 (m, 1H, cyclohexyl), 2.38–2.41 (m, 1H, cyclohexyl), 3.01–3.05 (m, 1H, cyclohexyl), 6.75 (s, 1H, C_5H-thiaz.), 7.36 (d, J = 7.1 Hz, 1H, Ar), 7.46 (t, 2H, Ar), 7.59 (d, J = 7.1 Hz, 2H, Ar), 7.69 (d, J = 8.4 Hz, 2H, Ar), 7.78 (d, J = 8.4 Hz, 2H, Ar), 12.48 (s, 1H, NH, D_2O exch.)
14[a]	1.16 (d, J = 6.4 Hz, 3H, CH_3), 1.35 (s, 1H, cyclohexyl), 1.60–1.62 (m, 2H, cyclohexyl), 1.81 (s, 2H, cyclohexyl), 1.98–1.99 (m, 2H, cyclohexyl), 2.20–2.22 (m, 1H, cyclohexyl), 2.42 (d, J = 6.1 Hz, 3H, 4′-CH_3-phenyl), 3.03–3.04 (m, 1H, cyclohexyl), 6.63 (s, 1H, C_5H-thiaz.), 7.28 (s, 2H, Ar), 7.60 (s, 2H, Ar), 12.52 (s, 1H, NH, D_2O exch.), 13.56 (s, 1H, NH, D_2O exch.)
15[a]	1.06 (d, J = 6.2 Hz, 3H, CH_3), 1.20–1.21 (m, 1H, cyclohexyl), 1.81–1.95 (m, 6H, cyclohexyl), 2.52 (t, 1H, cyclohexyl), 3.05–3.06 (m, 1H, cyclohexyl), 3.85 (s, 3H, 4′-OCH_3-phenyl), 6.52 (s, 1H, C_5H-thiaz.), 6.99 (d, J = 8.0 Hz, 2H, Ar), 7.65 (d, J = 8.6 Hz, 2H, Ar), 12.44 (s, 1H, NH, D_2O exch.), 13.58 (s, 1H, NH, D_2O exch.)
16[a]	1.19 (d, J = 6.4 Hz, 3H, CH_3), 1.57–1.61 (m, 3H, cyclohexyl), 1.83–1.85 (m, 1H, cyclohexyl), 2.00–2.03 (m, 2H, cyclohexyl), 2.19–2.21 (m, 1H, cyclohexyl), 2.50–2.52 (m, 1H, cyclohexyl), 3.00–3.01 (m, 1H, cyclohexyl), 6.87 (s, 1H, C_5H-thiaz.), 7.79 (d, J = 7.8 Hz, 2H, Ar), 7.86 (s, 2H, Ar), 12.48 (s, 1H, NH, D_2O exch.), 14.01 (br s, 1H, NH, D_2O exch.)
17[a]	1.64 (d, J = 6.5 Hz, 3H, CH_3), 1.25–1.26 (m, 1H, cyclohexyl), 1.69–1.71 (m, 1H, cyclohexyl), 1.98–1.99 (m, 2H, cyclohexyl), 2.20–2.21 (m, 2H, cyclohexyl), 2.50–2.52 (m, 2H, cyclohexyl), 2.95–2.96 (m, 1H, cyclohexyl), 6.68 (s, 1H, C_5H-thiaz.), 7.47 (d, J = 8.6 Hz, 2H, Ar), 7.67 (d, J = 8.5 Hz, 2H, Ar), 12.50 (s, 1H, NH, D_2O exch.), 13.80 (s, 1H, NH, D_2O exch.)
18[a]	1.16 (d, J = 6.5 Hz, 3H, CH_3), 1.58–1.59 (m, 3H, cyclohexyl), 1.98–1.99 (m, 1H, cyclohexyl), 2.05–20.7 (m, 2H, cyclohexyl), 2.25–2.27 (m, 1H, cyclohexyl), 2.40–2.41 (m, 1H, cyclohexyl), 3.00–3.01 (m, 1H, cyclohexyl), 6.62 (s, 1H, C_5H-thiaz.), 7.18 (d, J = 6.1 Hz, 2H, Ar), 7.70 (d, J = 5.3 Hz, 2H, Ar), 12.71 (s, 1H, NH, D_2O exch.), 14.30 (s, 1H, NH, D_2O exch.)
19[a]	0.99 (d, J = 6.1 Hz, 3H, CH_3), 1.20–1.21 (m, 2H, cyclohexyl), 1.60–1.61 (m, 1H, cyclohexyl), 2.00–2.01 (t, 2H, cyclohexyl), 2.20–2.30 (m, 2H, cyclohexyl), 2.60–2.61 (m, 1H, cyclohexyl), 3.09–3.10 (m, 1H, cyclohexyl), 6.68 (s, 1H, C_5H-thiaz.), 7.47–7.51 (m, 2H, Ar), 7.71 (d, J = 6.6 Hz, 2H, Ar), 12.55 (s, 1H, NH, D_2O exch.), 13.75 (s, 1H, NH, D_2O exch.)
20[a]	1.58–1.63 (m, 6H, cycloheptyl), 1.70–1.71 (m, 2H, cycloheptyl), 2.55–2.56 (m, 2H, cycloheptyl), 2.70–2.72 (m, 2H, cycloheptyl), 6.80 (s, 1H, C_5H-thiaz.), 7.48–7.50 (m, 3H, Ar), 7.73 (d, J = 7.9 Hz, 2H, Ar), 12.40 (s, 1H, NH, D_2O exch.), 13.80 (br s, 1H, NH, D_2O exch.)

[a] $CDCl_3$.
[b] DMSO-d_6.

2-Thiazolylhydrazone derivatives **1**–**20** were synthesized as reported in our previous communications.[28,29]

Cyclic ketones or aryl aldehydes reacted directly with thiosemicarbazide and the obtained thiosemicarbazones subsequently reacted with α-halogenoketones to yield the 4-substituted thiazole ring derivatives as shown in Scheme 1. In the synthesis of all compounds isopropyl alcohol proved to be the best solvent for our purpose. As a matter of fact, the reaction products precipitate on cooling down and can be filtered and purified by crystallization from ethanol or ethanol/isopropanol. All synthesized compounds were fully characterized by analytical and spectral data as listed in Tables 1 and 2.

All synthesized compounds were evaluated for antifungal activity and compared with the reference compound clotrimazole (Table 3).[30,31]

The newly prepared compounds were dissolved in dimethylsulfoxide (DMSO) and their in vitro activity was evaluated against a total of 22 strains of *Candida* species. The included isolates were *Candida albicans* (8 strains), *Candida glabrata* (4), *Candida krusei* (3), *Candida tropicalis* (3), *Candida sakè* (2), and *Candida parapsilosis* (2). The isolates were collected from specimens of patients at the 'Azienda Policlinico Umberto I°' of Rome 'La Sapienza' University and were obtained from haematology/oncology and surgery departments, which also included an intensive care unit. In particular, the samples were isolated from the upper and lower respiratory tract, blood, and indwelling venous catheters; the intensive care unit accounted for 65% of the cases (15/22 isolates). The isolates were identified by conventional methodologies. Prior to testing, each isolate was subcultured on a qualified medium to ensure purity and optimal growth.

The data reported in Table 3 show that cyclopentyl derivatives **1**–**6** had good anti-*C. albicans* and anti-*C. glabrata* activity. In particular compounds **1**, **2**, and **6** were very active against both strains, while compounds **3**, **4**, and **5** showed good activity only against *C. glabrata*. Furthermore, cyclohexyl substituted derivatives **9**–**19** showed good anti-*C. albicans* activity, in particular with compounds **9**–**11** and **14**–**19**. Some derivatives, especially **9** and **10**, also showed good anti-*C. glabrata* activity. Cycloheptyl derivative **20** showed good anti-*C. albicans* activity. Among all synthesized compounds, we found the antifungal activity showed by compound **14**, 2-(3-methylcyclohexyl)-1-[4-(4-methylphenyl)-2-thiazolyl]hydrazone, particularly interesting. Compound **14** was more active than the clotrimazole reference compound against *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. sakè*.

The cytotoxic profile of the compounds showed that the derivatives were less toxic at concentrations below 0.5 μg/mL with a percentage of viable cells of 73.0 and 94.7 (Table 4).[32–34]

In conclusion, a series of novel 2-thiazolylhydrazone derivatives was assessed for antifungal activity on 22 *Candida* strains. Compound **14** was found to have good antifungal activity and may be used as a good reference for identifying features of the structure that could be important for antifungal activity.

Table 3. Minimal inhibitory concentration (MIC)[a] of compounds **1**–**20** and clotrimazole against 22 strains of *Candida* species

Compound	Tested fungi (MIC[a] μg/mL)					
	C. *albicans* (8 strains)	C. *glabrata* (4 strains)	*C. tropicalis* (3 strains)	*C. krusei* (3 strains)	*C. parapsilosis* (2 strains)	*C. sakè* (2 strains)
1	0.25–2	0.50–2	4–16	64–128	128	16–32
2	0.50–2	2–4	64–128	32–64	8–16	32
3	1–8	0.50–2	64–128	>128	>128	16–32
4	8–32	0.50–2	64–128	32–64	>128	16
5	2–8	0.50–2	32–64	32–64	128	32
6	0.25–2	0.50–2	32–64	64–128	8–16	16
7	2–8	2–8	128–>128	>128	128	32
8	16–64	>128	64–128	>128	>128	32
9	0.25–2	0.50–2	2–4	64	8	32
10	0.50–2	0.25–2	8	16	8	32
11	0.50–2	16–64	64	>128	8–16	16–32
12	4–8	2–4	32–64	64–128	8–16	16
13	32–64	64–128	64	16	16	16–32
14	0.50–2	32–64	0.50–2	0.50–2	0.50–1	2–4
15	0.50–2	>128	2–4	2–4	1–2	8–16
16	0.50–2	>128	2–4	4–8	0.50–1	4–8
17	0.50–2	>128	2–4	4–8	1	2
18	0.50–4	>128	4–16	4–16	1	8
19	0.50–2	32–64	2–4	05–1	8	2
20	0.50–2	>128	8–16	8–16	8–16	16–32
Clo[b]	0.50–4	4–8	4–8	4–8	4–8	8–16

[a] Range value.
[b] Clotrimazole.

Table 4. Cytotoxic effect of selected compounds tested on EAhy 926 cells after 24 h of incubation at 37 °C, using Trypan Blue exclusion test, expressed as cell survival fraction[a] (%)

Compound	Concentration[b] (μg/mL)			
	50	5	0.5	0.05
1	75.0 ± 3.2	80.0 ± 4.1	82.0 ± 4.6	85.4 ± 5.0
2	66.0 ± 3.2	76.6 ± 4.0	82.4 ± 2.8	85.0 ± 2.2
5	76.0 ± 4.6	81.0 ± 3.2	84.7 ± 2.8	88.9 ± 6.0
6	64.9 ± 4.2	81.0 ± 3.0	89.2 ± 2.8	86.0 ± 2.6
9	64.0 ± 3.2	63.6 ± 3.6	78.5 ± 4.2	85.7 ± 3.6
10	75.0 ± 4.0	83.3 ± 3.6	84.0 ± 3.2	86.0 ± 4.8
11	83.3 ± 3.6	85.7 ± 4.2	86.0 ± 3.8	88.8 ± 2.2
14	38.5 ± 4.3	72.7 ± 3.1	83.3 ± 3.6	84.0 ± 2.0
15	54.5 ± 3.2	72.7 ± 3.8	80.0 ± 1.9	83.3 ± 2.2
16	25.0 ± 1.9	50.0 ± 2.5	68.7 ± 2.9	68.8 ± 1.4
17	28.6 ± 2.8	50.0 ± 1.9	70.0 ± 4.2	73.0 ± 3.1
18	52.9 ± 2.7	60.0 ± 1.5	76.9 ± 1.7	80.0 ± 2.0
20	54.5 ± 5.0	80.0 ± 3.9	87.5 ± 1.8	94.7 ± 2.5

[a] Cells incubated with culture medium alone represented the controls and the cell viability was always greater than 97%.

[b] Data represent the arithmetic means ± SD of at least three independent experiments.

Moreover other tested compounds may be good candidates for further investigation.

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30. *Antifungal activity:* All synthesized derivatives **1**–**20** were evaluated for their antifungal activity dissolved in dimethylsulfoxide (DMSO). The in vitro antifungal activities of the compounds were determined with the broth microdilution method with Sabouraud dextrose broth (BBL Microbiology Systems, Cockeysville, MD) as recommended by the NCCLS.[31] Microtiter plates containing serial dilutions of each compound were inoculated with each organism to yield the appropriate density (10^3/mL) in a 100 μL final volume; each plate included positive controls (fungi without a compound) and a negative control (medium only). The plates were incubated for 24 h at 37 °C. The MIC for all isolates was defined as the lowest concentration of antifungal agent that completely inhibited growth of the organism, as detected by the unaided eye.
31. National Committee for Clinical Laboratory Standards 2003. Performance standards for antimicrobial susceptibility testing. Supplement M100-S13. National Committee for Clinical Laboratory Standards, Wayne, PA.

32. *In vitro cytotoxicity:* The cytotoxicity of the newly synthesized compounds under investigation was tested against EAhy, human cell line obtained from a hybridoma between HUVEC and epithelial cells from a lung carcinoma. The viability of cells exposed to test compounds was estimated by the Trypan Blue dye exclusion assay. Cell lines were maintained as adherent type cultures under humidified atmosphere in 5% CO_2 at 37 °C, Dulbecco's modified Eagle's culture medium (high glucose) supplemented with 2 mM L-Glutamine, HAT supplement and containing antibiotic mixture. Experiments were performed in cells grown to 60–70% confluency.[33] The stock solutions of the investigated compounds were prepared in sterile dimethylsulfoxide (DMSO) and the successive dilutions were made in culture medium; the DMSO percent present in culture medium never exceeded 0.5%. EAhy cells in the exponential phase of growth (1×10^5/mL) were seeded into 24-well microplate. and incubated for 24 h with four different concentrations of the compounds (50–0.05 μg/mL). Some plates containing cells alone or cells and DMSO represented the controls. After the incubation period, cells were mechanically scraped off from the plates and an aliquot was diluted (1:1) with a solution 0.4% Trypan Blue Stain. After few minutes at rt cells were counted under an optical microscope in a Thoma hemocytometer chamber by two different operators. On the basis that Trypan Blue is a vital dye[34] and can enter and interact with the cells unless the plasmatic membrane is damaged, blue stained cells were considered as dead. Values are expressed as % of viable cells. Cell viability in control samples was always 97–98%.

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