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Preliminary communication

Synthesis and the selective antifungal activity of 5,6,7,8-tetrahydroimidazo[1,2-a]pyridine derivatives

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ABSTRACT

Even though there are new classes of compounds now frequently used in treatment of fungal infections, the density of deeply invasive candidiasis has increased at least 10-fold during the past decade. Furthermore, many infections due to *Candida* species are actually refractory to antifungal therapy. In this present study, it was aimed to synthesize, new hydrazide derivatives of tetrahydroimidazo[1,2-a]pyridine and evaluate their anticandidal activity and cytotoxicity *in vitro*. The reaction of tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid hydrazides with various benzaldehydes gave tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid benzylidene hydrazide derivatives. The chemical structures of the compounds were elucidated and confirmed by IR, ^{1}H NMR, MS-FAB$^+$ spectroscopy and elemental analyses. Eight new tetrahydroimidazo[1,2-a]pyridine derivatives were synthesized and screened for their antifungal effects against a panel of ten human pathogenic *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Candida utilis*, and *Candida zeylanoides* using agar diffusion and broth microdilution assays, respectively. Furthermore, their cytotoxicity was tested against six mammalian cell lines. Among the analogues, the compound 5,6,7,8-tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid-(4-cyanobenzylidene) showed very strong inhibitory activity (up to MIC 0.016 mg/mL) against the screened *Candida* species. The same compound showed no *in vitro* toxicity up to 25 μg/mL concentration suggesting that its antifungal activity (MICs 0.016–1 mg/mL) is selective.

1. Introduction

Even though *Candida* spp. are commensally present in the human flora of about 30–60% of healthy individuals, where they may become pathologic depending on predisposing circumstances related to the host, as an example of systemic disease leading to immunosuppression, and local destructive conditions. In fact, the pathogenicity of *Candida* spp. is affected by several virulence factors, such as the ability to adhere to epithelial and endothelial cells, germination, extracellular proteinases and phospholipases, and phenotypic switching [1,2].

In recent years, fungal infections have increased remarkably and one of the main targets is the opportunistic pathogen *Candida albicans*. A major obstacle in the treatment of *C. albicans* infections is the spread of antifungal drug resistance mainly in patients chronically subjected to antimycotic therapy, *i.e.* those treated with broad-spectrum antibiotics, immunosuppressive agents, anti-cancer, and anti-AIDS drugs [3,4].

Azole and non-azole antifungal agents are usually used to treat *Candida* infections, but despite the good antifungal activities observed *in vitro*, candidemia is still a major cause of death [4]. Many valid therapy programs fail because of widespread secondary *C. albicans* infections. New effective anticandida agents are needed to combat the drug-resistant strains and widespread diffusion of *C. albicans*, *Candida glabrata*, *Candida utilis*, *Candida tropicalis*, *Candida krusei*, *Candida zeylanoides*, *Candida parapsilosis*.

Imidazoles, the first group to be developed in azole antifungals also inhibit the accumulation of methylated sterols disrupts the organization of the lipidic bilayer of membranes. Furthermore, some imidazole drugs, at high concentrations, could exert direct inhibitory effects on membranes, without interference with sterols and sterol esters [5,6]. The literature survey reveals that, although there are several synthetic study examples of the imidazole ring, there are none on the imidazo[1,2-a]pyridine ring as an imidazole fused six-membered ring system, to the best of our knowledge. Furthermore, it

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is well known that hydrazide-hydrazone group plays an important role for antimicrobial activity of such compounds [7–10].

In the design of new drugs, the development of hybrid molecules through the combination of different pharmacophores in one frame may lead to compounds with interesting biological and pharmacological profiles.

Prompted by these observations, in the present study, the synthesis of 8 new hydrazide-hydrazone derivatives of tetrahydroimidazo[1,2-a]pyridines as hybrid molecules including different pharmacophores were aimed initially for *in vitro* anticandidal as well as cytotoxic screenings.

2. Chemistry

The synthetic route of compounds is outlined in Scheme 1. To the best of our knowledge, in the present work, tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid hydrazide (**1**) was prepared for the first time, in accordance with the method described in literature [11,12]. The condensation of the acid hydrazides with appropriate benzaldehydes resulted in the formation of new tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid arylidene hydrazide derivatives such as **2a–h**.

3. Biology

3.1. Antifungal activity

The antifungal properties of compounds **2a–h** were evaluated by the broth microdilution method according to the NCCLS reference document M27-A2 [13] against *C. albicans* (isolate, obtained from Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskisehir, Turkey), *C. albicans* (ATCC 90028), *C. glabrata* (isolate-1 obtained from Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskisehir, Turkey), *C. utilis* (NRRL Y-900), *C. tropicalis* (NRRL Y-12968), *C. krusei* (NRRL Y-7179), *C. zeylanoides* (NRRL Y-1774), *C. parapsilosis* (NRRL Y-12696), *C. albicans* (NRRL Y-12983), *C. glabrata* (isolate-2 obtained from Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskisehir, Turkey). Ketoconazole, clotrimazole and griseofulvin were used as positive controls.

3.2. Cytotoxicity

The compounds synthesized (**2a–h**) were tested for their initial *in vitro* cytotoxicity against a panel of human solid tumor cells (SK-MEL: malignant melanoma; KB: oral epidermal carcinoma; BT-549: breast ductal carcinoma, and SK-OV-3: ovary carcinoma) as well as noncancerous kidney fibroblast (Vero) and kidney epithelial cells (LLC-PK_{11}) [14].

Table 1
Some characteristics of the tetrahydroimidazo[1,2-a]pyridine hydrazine derivatives.

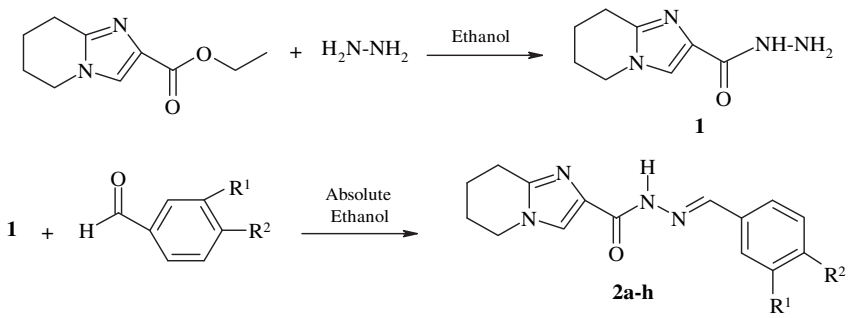
Comp.	R^1	R^2	M.p. (°C)	Yield (%)	Formula	MW
2a	H	CH_3	212–214	80	$C_{16}H_{18}N_4O$	282
2b	H	$N(CH_3)_2$	276–278	90	$C_{17}H_{21}N_5O$	311
2c	H	$CH(CH_3)_2$	188–190	77	$C_{18}H_{22}N_4O$	310
2d	$O–CH_2–O$		208–210	75	$C_{16}H_{16}N_4O_3$	312
2e	H	NO_2	266–268	92	$C_{15}H_{15}N_5O_3$	313
2f	H	Br	240–242	73	$C_{15}H_{15}BrN_4O$	347
2g	H	Cl	236–238	84	$C_{15}H_{15}ClN_4O$	302
2h	H	CN	230–232	81	$C_{16}H_{15}N_5O$	293

4. Results, discussion and conclusion

Among several clear synthetic routes to derivatives **2a–h**, we decided to explore in our synthetic approach the acylhydrazine derivative **1** as a key intermediate. This compound could be transformed in **2a–h** using classical functional group interconversion, *i.e.* $CONHNH_2 \rightarrow CONHN{=}CH\text{-}Ar$. Finally, the new target compounds **2a–h** were obtained, in good yields (73–92%), by condensing **1** with the corresponding aromatic aldehydes in ethanol, as illustrated in Table 1 and Scheme 1.

Formulas of compounds (**2a–h**) were found by elemental and spectroscopic analyses and their structures were determined by IR, ^1H NMR, and FAB^+-MS spectral data, respectively. IR data provided functional group evidence for the formation of the expected structures. In the IR spectra, some significant stretching bands due to N–H, C=O, C=N, C=N–Ph and N=C–H were at 3389–3158 cm^{-1}, 1640–1630 cm^{-1}, 1610–1590 cm^{-1}, 1549–1485 cm^{-1}, and 760–750 cm^{-1}, respectively. The ^1H NMR data were also consistent with the assigned structures. In ^1H NMR spectrum of compounds; we observed paired peaks for each of the protons N=CH (8.35–8.65 ppm), N–NH (11.10–11.75 ppm) corresponding to (*E*)- and (*Z*)-forms of the compounds, respectively. All other aromatic and aliphatic protons were observed at expected regions. The mass spectra of compounds showed [M + 1] peaks, in agreement with their molecular formula. All compounds gave satisfactory elemental analysis results in correlation with the calculations.

The antifungal activity of the compounds was studied with ten pathogenic fungi. Ketoconazole, griseofulvin and clotrimazole were used as reference agents for inhibitory activity against the tested fungi. Minimal inhibitory concentrations (MIC) were recorded as the minimum concentration of a compound that inhibits the growth of tested microorganisms. All of the compounds tested illustrated medium to very good anticandidal inhibitory activity when compared with the reference agents. The MIC values were found within the range of 0.016–1 mg/mL against all evaluated strains. Some of the compound inhibitory concentrations were expressed as >0.25 where some solubility problems were observed. The results are summarized in Table 2.



Scheme 1. The general synthetic reaction (see Table 1 for **2a–h**, R^1–R^2 details).

Table 2
Anticandidal evaluation of tetrahydroimidazo[1,2-a]pyridine hydrazine derivatives (MIC values in mg/mL).

Test substances	A[a]	B	C[a]	D	E	F	G	H	I	K[a]
2a	0.032	>0.25[b]	>0.25[b]	>0.25[b]	0.125	>0.25[b]	>0.25[b]	0.5	>0.25[b]	>0.25[b]
2b	0.125	>0.25[b]	>0.25[b]	>0.25[b]	0.125	>0.25[b]	>0.25[b]	>0.25[b]	>0.25[b]	>0.25[b]
2c	0.125	>0.25[b]	>0.25[b]	>0.25[b]	0.125	>0.25[b]	>0.25[b]	>0.25[b]	>0.25[b]	0.5
2d	0.063	>0.25[b]	>0.25[b]	0.125	0.125	>0.25[b]	>0.25[b]	>0.25[b]	>0.25[b]	>0.25[b]
2e	0.125	0.5	0.5	0.5	0.25	0.5	0.25	0.25	0.25	0.25
2f	1	0.5	0.5	1	0.5	0.25	1	1	0.125	0.25
2g	0.125	0.5	>0.25[b]	>0.25[b]	>0.25[b]	>0.25[b]	>0.25[b]	0.5	0.5	0.125
2h	0.032	0.063	0.032	0.032	0.016<	0.016<	0.032	0.032	0.032	0.032
Ketoconazole	0.063	0.063	0.125	0.125	0.125	0.125	0.5	0.032	0.032	0.032
Griseofulvin	0.25	0.25	0.25	0.5	0.125	0.25	0.5	0.5	0.5	0.125
Clotrimazole	0.08<	0.08<	0.08<	0.08<	0.032	0.08<	0.125	0.08<	0.08<	0.08<

A: *Candida albicans* (clinical isolate), B: *Candida albicans* (ATCC 90028), C: *Candida glabrata* (clinical isolate-1), D: *Candida utilis* (NRRL Y-900), E: *Candida tropicalis* (NRRL Y-12968), F: *Candida krusei* (NRRL Y-7179), G: *Candida zeylanoides* (NRRL Y-1774), H: *Candida parapsilosis* (NRRL Y-12696), I: *Candida albicans* (NRRL Y-12983), K: *Candida glabrata* (clinical isolate-2).

[a] Clinical isolates were obtained from Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskisehir, Turkey.

[b] Represents insolubility of the substance in the medium, with partial inhibition towards the microorganism.

In comparing MIC values with the standard reference agents, 5,6,7,8-tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid-(4-cyanobenzylidene) (**2h**) was in particular strong inhibitory against *C. tropicalis* and *C. krusei* (0.016 < mg/mL), respectively. Compound **2h** showed very strong inhibitory activity (0.016–0.063 mg/mL) towards all screened *Candida* species.

Strong inhibitory activity with a MIC value of 0.063 mg/mL was also observed for compound **2d** against the clinical isolate of *C. albicans*.

Compounds **2a**, **2d**, and **2h** were also effective against *C. albicans* (clinical isolate). Compounds **2a** and **2h** especially showed strong inhibitory activity against the tested microorganisms. Compound **2d** showed a similar level of activity with the reference agent whereas **2b**, **2c**, **2e** and **2g** showed moderate activity.

In comparing their MIC values with ketoconazole, compounds **2d** and **2h** were effective against *C. utilis* (NRRL Y-900). Compound **2h** especially showed high activity. Compound **2d** showed equal activity when compared with the reference agent.

On the other hand the compounds exhibited comparable activities against *C. tropicalis* (NRRL Y-12968). The compound **2h** especially showed strong activity. **2a**, **2b**, **2c**, and **2d** showed similar activity and the other compounds were found less active than ketoconazole.

From the similar results obtained with *C. zeylanoides* (NRLL Y-1774), compounds **2a**, **2b**, **2c**, **2d**, **2e**, **2g**, and **2h** showed strong activity, whereas the compound **2f** showed moderate activity when compared with ketoconazole.

When compared with their MIC values against clotrimazole, compound **2h** was effective against *C. albicans* (ATCC 90028), *C. glabrata* (isolate-1), *C. utilis* (NRRL Y-900), *C. tropicalis* (NRRL Y-12968), *C. krusei* (NRRL Y-7179), *C. zeylanoides* (NRRL Y-1774), *C. parapsilosis* (NRRL Y-12696), *C. albicans* (NRRL Y-12983), *C. glabrata* (isolate-2). Compound **2h** especially showed strong activity. The other compounds were found less active than clotrimazole.

In comparing their MIC values with clotrimazole, compounds **2a**, **2h** and **2d** were effective against *C. albicans* (isolate). Compounds **2a**, **2h** and **2d** especially showed strong inhibitory activity.

In comparing their MIC values with griseofulvin, all of compounds were effective against *C. albicans* (isolates). The compound **2f** was found inactive against *C. albicans* (isolate) when compare with griseofulvin. All of compounds were effective against *C. albicans* (ATCC 90028), *C. glabrata* (isolate-1), and *C. krusei* (NRRL Y-7179). Compound **2h** especially showed high activity. Compounds **2a**, **2b**, **2c**, **2d**, **2e**, **2f**, and **2g** showed moderate activity when compared with the reference agent.

On the other hand the compounds exhibited comparable activities against *C. utilis* (NRRL Y-900). The compound **2h**, **2d**, **2a**, **2b**, **2c**, and **2g** especially showed strong activity. Compound **2e** showed similar activity, whereas the compound **2f** showed moderate activity when compared with griseofulvin.

From the similar results obtained with *C. tropicalis* (NRRL Y–12 968). The compound **2h** especially showed strong activity. **2a**, **2b**, **2c**, and **2d** showed similar activity and the other compound **2e** indicated moderate activity with reference agent.

In comparing their MIC values with griseofulvin, all of the compounds were effective against *C. zeylanoides* (NRLL Y-1774). The compound **2h**, **2e**, **2a**, **2b**, **2c**, **2d** and **2g** especially showed strong activity. The compound **2f** showed moderate activity when compared with griseofulvin. All of the compounds were effective against *C. parapsilosis* (NRRL Y-12 696). The compound **2h**, **2b**, **2c**, **2d** and **2e** especially showed strong activity. **2a** and **2g** showed similar activity and the other compound **2f** indicated moderate activity with reference agent. All of compounds were effective against *C. albicans* (NRRL Y-12 983), Compound **2h**, **2f**, **2e**, **2a**, **2b**, **2c** and **2d** especially showed high activity. Compound **2g** showed equal activity.

In comparing their MIC values with griseofulvin, compounds **2h**, **2g**, **2e** and **2f** were effective against *C. glabrata* (isolate-2), and among them compound **2h**, especially, showed strong activity. Compound **2g** showed a similar level of inhibitory activity compared with the reference agent, whereas **2e** and **2f** showed moderate activity.

Considering all the results obtained from antifungal screen, in comparison with reference agents, it can be concluded that **2h** compound is more active than other compounds in the screen including the reference agents.

All compounds were also tested for their cytotoxicity against a panel of cancer cell lines (SK-MEL, KB, BT-549, and SK-OV-3) and noncancer cells (VERO, LLC-PK_{11}). None of the tested compounds showed any cytotoxicity to any of the cell lines up to a highest concentration of 25 μg/mL indicating a high selectivity of anticandidal activity.

Based on eight compounds evaluated, it appears that 4-cyano (**2h**) substitution on the phenyl ring has made a good contribution to the antifungal activity in this series of imidazopyridinyl–hydrazon combination without cell toxicity against selected cell lines.

It is well known that azole antifungal agents act on the synthesis of the fungal ergosterol by inhibiting the cytochrome P450-dependent enzyme lanosterol demethylase, which also plays an important role in cholesterol synthesis in mammals. In therapeutic concentrations, azole antifungal efficacy is attributed to their greater affinity and selectivity for fungal P-450_{DM} than for the mammalian enzyme. Generally, exposure of fungi to an active azole causes depletion of ergosterol and accumulation of 14α-methylated sterols, which interferes with the important functions of ergosterol

in fungal membranes. Azoles disrupt both the structure of the membrane and several of its functions such as nutrient transport and fungal chitin synthesis [3].

To the best of our knowledge tetrahydroimidazo[1,2-a]pyridine ring was investigated for the first time for *in vitro* biological activities. As the tetrahydroimidazo[1,2-a]pyridine ring in the present study is a bioisoster of the imidazole ring, which also include important azole antifungal therapeutics, it can be concluded that the compounds also display remarkable antifungal activity by using same mechanism of the current azole antifungals. It is worthwhile to extend the bioactivity evaluation from *in vitro* whole cells to a mechanistic enzymatic level as well as to further *in vivo* studies.

5. Experimental

All melting points (m.p.) were determined in open capillaries on a Gallenkamp apparatus (Weiss-Gallenkamp, Loughborough-UK), which are uncorrected, given in Table 1. The purity of the compounds was routinely checked by silica gel thin layer chromatography (TLC) plates (60 G, Merck, Darmstadt-Germany). Spectroscopic data were recorded with the following instruments: IR; Shimadzu IR-435 spectrophotometer (Shimadzu, Tokyo, Japan); ^{1}H NMR: Bruker 250 MHz spectrometer (Bruker, Billerica, Massachusetts, USA) in DMSO-d_6 using TMS as internal standard; and FAB-MS: VG Quattro Mass spectrometer (Agilent, Minnesota, USA). Elemental analyses were performed with a Leco CHNS-932 (LECO Corporation, Michigan, USA) instrument.

5.1. Chemistry

5.1.1. Preparation of tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid hydrazides (**1**)

These compounds were prepared according to the previously reported method, by reacting ethyl tetrahydroimidazo[1,2-a]pyridine-2-carboxylates with hydrazine hydrate.

5.1.2. Preparation of tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid arylidenehydrazides derivatives (**2a**–**h**)

Equimolar quantities of acid hydrazides (30 mmol) and appropriate benzaldehydes in 25 ml of absolute ethanol were refluxed for 3–5 h. The resulting solid was filtered and recrystallized from ethanol.

Some characteristics of the synthesized compounds are shown in Table 1. Analytical and spectral data (IR, ^{1}H NMR, MS-FAB$^+$) confirmed the structures of the new compounds.

*5.1.2.1. 5,6,7,8-Tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid-(4-methylbenzylidene)hydrazide (**2a**).* The physical appearance: light brown solid. IR (KBr, cm^{-1}): 3201 (NH), 1635 (C=O), 1600 (C=N), 1534, 1508 (C=N–Ph), 760 (N=C–H); ^{1}H NMR (250 MHz) (DMSO-d_6) δ (ppm): 1.85–1.90 (4H, br, C_6 and C_7 protons of tetrahydroimidazo[1,2-a]pyridine), 2.35 (3H, s, CH_3), 2.80 (2H, t, C_8 protons of tetrahydroimidazo[1,2-a]pyridine), 4.00 (2H, t, C_5 protons of tetrahydroimidazo[1,2-a]pyridine), 7.25 and 7.55 (4H, 2 d J = 8.01 Hz and J = 8.05 Hz, 1,4-disubstituted phenyl protons), 7.65 (1H, d J = 6.80 Hz, C_3 protons of tetrahydroimidazo[1,2-a]pyridine), 8.45 (1H, s, N=CH), 11.35 (1H, s, N–NH); MS-FAB$^+$: *m/z*: 282 [M], 283 [M + 1]; Anal. Calc. for $C_{16}H_{18}N_4O$:C, 68.06; H, 6.43; N, 19.84. Found:C, 68.09; H, 6.41; N, 19.88.

*5.1.2.2. 5,6,7,8-Tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid-(4-dimethylaminobenzylidene)-hydrazide (**2b**).* The physical appearance: yellow solid. IR (KBr, cm^{-1}): 3389, 3158 (NH), 1640 (C=O), 1596 (C=N), 1524 (C=C–H), 1485 (C=N–Ph), 750 (N=C–H); ^{1}H NMR (250 MHz) (DMSO-d_6) δ (ppm): 1.80–1.90 (4H, br, C_6 and C_7 protons of tetrahydroimidazo[1,2-a]pyridine), 2.75 (2H, t, C_8 protons of tetrahydroimidazo[1,2-a]pyridine), 3.00 (6H, s, N(CH_3)$_2$), 4.00 (2H, t, C_5 protons of tetrahydroimidazo[1,2-a]pyridine), 6.75 and 7.45 (4H, 2 d J = 8.60 Hz and J = 8.84 Hz, 1,4-disubstituted phenyl protons), 7.62 (1H, s, C_3 protons of tetrahydroimidazo[1,2-a]pyridine), 8.35 (1H, s, N=CH), 11.10 (1H, s, N–NH); MS-FAB$^+$: *m/z*: 311 [M], 312 [M + 1]. Anal. Calc. for $C_{17}H_{21}N_5O$:C, 65.57; H, 6.80; N, 22.49. Found:C, 65.70; H, 6.84; N, 22.57.

*5.1.2.3. 5,6,7,8-Tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid-(4-isopropilbenzylidene) hydrazide (**2c**).* The physical appearance: shining brown solid. IR (KBr, cm^{-1}): 3195 (NH), 1636 (C=O), 1590 (C=N), 1520 (C=C–H), 1495 (C=N–Ph), 753 (N=C–H); ^{1}H NMR (250 MHz) (DMSO-d_6) δ (ppm): 1.25 (6H, s, 2CH_3), 1.80–1.90 (4H, br, C_6 and C_7 protons of tetrahydroimidazo[1,2-a]pyridine), 2.73 (2H, t, C_8 protons of tetrahydroimidazo[1,2-a]pyridine), 2.80–3.00 (1H, m, $CH(CH_3)_2$), 4.05 (2H, t, C_5 protons of tetrahydroimidazo[1,2-a]pyridine), 7.30 and 7.60 (4H, 2 d J = 8.18 Hz and J = 8.19 Hz, 1,4-disubstituted phenyl protons), 7.70 (1H, d J = 6.25 Hz, C_3 protons of tetrahydroimidazo[1,2-a]pyridine), 8.45 (1H, s, N=CH), 11.35 (1H, s, N–NH); MS-FAB$^+$: *m/z*: 310 [M], 311 [M + 1]. Anal. Calc. for $C_{18}H_{22}N_4O$:C, 69.65; H, 7.14; N, 18.05. Found:C, 69.73; H, 7.19; N, 18.00.

*5.1.2.4. 5,6,7,8-Tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid-(benzo[1,3]dioxol-5-ylmethy-lene)hydrazide (**2d**).* The physical appearance: light brown powder. IR (KBr, cm^{-1}): 3183 (NH), 1631 (C=O), 1592 (C=N), 1549 (C=N–Ph), 755 (N=C–H); ^{1}H NMR (250 MHz) (DMSO-d_6) δ (ppm): 1.80–1.90 (4H, br, C_6 and C_7 protons of tetrahydroimidazo[1,2-a]pyridine), 2.75 (2H, t, C_8 protons of tetrahydroimidazo[1,2-a]pyridine), 3.95 (2H, t, C_5 protons of tetrahydroimidazo[1,2-a]pyridine), 6.05 (2H, s, O–CH2–O), 6.90–7.25 (3H, m, phenyl protons), 7.65 (1H, s, C_3 protons of tetrahydroimidazo[1,2-a]pyridine), 8.40 (1H, s, N=CH), 11.30 (1H, s, N–NH); MS-FAB$^+$: *m/z*: 312 [M], 313 [M + 1]. Anal. Calc. for $C_{16}H_{16}N_4O_3$:C, 61.53; H, 5.16; N, 17.94. Found:C, 61.33; H, 5.21; N, 17.76.

*5.1.2.5. 5,6,7,8-Tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid-(4-nitrobenzylidene)hydrazide (**2e**).* The physical appearance: fine needle shaped yellow crystals. IR (KBr, cm^{-1}): 3175 (NH), 1633 (C=O), 1610 (C=N), 1588 (C=C–H), 1545 (C=N–Ph), 1518, 1340 (N–O), 760 (N=C–H); ^{1}H NMR (250 MHz) (DMSO-d_6) δ (ppm): 1.75–1.95 (4H, br, C_6 and C_7 protons of tetrahydroimidazo[1,2-a]pyridine), 2.75 (2H, t, C_8 protons of tetrahydroimidazo[1,2-a]pyridine), 4.00 (2H, t, C_5 protons of tetrahydroimidazo[1,2-a]pyridine), 7.70 (1H, s, C_3 protons of tetrahydroimidazo[1,2-a]pyridine), 7.85 and 8.25 (4H, 2 d J = 8.86 Hz and J = 8.84 Hz, 1,4-disubstituted phenyl protons), 8.60 (1H, s, N=CH), 11.75 (1H, s, N–NH); MS-FAB$^+$: *m/z*: 314 [M + 1]. Anal. Calc. for $C_{15}H_{15}N_5O_3$:C, 57.50; H, 4.83; N, 22.35. Found:C, 57.44; H, 4.86; N, 22.38.

*5.1.2.6. 5,6,7,8-Tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid-(4-bromobenzylidene)hydrazide (**2f**).* The physical appearance: white powder. IR (KBr, cm^{-1}): 3192 (NH), 1630 (C=O), 1602 (C=N), 1543 (C=N–Ph), 750 (N=C–H), 510 (C-Br); ^{1}H NMR (250 MHz) (DMSO-d_6) δ (ppm): 1.75–1.95 (4H, br, C_6 and C_7 protons of tetrahydroimidazo[1,2-a]pyridine), 2.75 (2H, t, C_8 protons of tetrahydroimidazo[1,2-a]pyridine), 3.95 (2H, t, C_5 protons of tetrahydroimidazo[1,2-a]pyridine), 7.50–7.70 (5H, m, aromatic protons), 8.50 (1H, s, N=CH), 11.50 (1H, s, N–NH); MS-FAB$^+$: *m/z*: 346 [M − 1], 347 [M], 348 [M + 1], 349 [M + 2]. Anal. Calc. for $C_{15}H_{15}BrN_4O$:C, 51.89; H, 4.35; N, 16.14. Found:C, 52.01; H, 4.32; N, 16.02.

*5.1.2.7. 5,6,7,8-Tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid-(4-chlorobenzylidene)hydrazide (**2g**).* The physical appearance:

needle shaped white crystals. IR (KBr, cm^{-1}): 3199 (NH), 1634 (C=O), 1605 (C=N), 1539 (C=N–Ph), 750 (N=C–H); ^1H NMR (250 MHz) (DMSO-d_6) δ (ppm): 1.80–2.00 (4H, br, C_6 and C_7 protons of tetrahydroimidazo[1,2-a]pyridine), 2.80 (2H, t, C_8 protons of tetrahydroimidazo[1,2-a]pyridine), 4.00 (2H, t, C_5 protons of tetrahydroimidazo[1,2-a]pyridine), 7.50 and 7.65 (4H, 2 d $J = 8.52$ Hz and $J = 8.48$ Hz, 1,4-disubstituted phenyl protons), 7.71 (1H, s, C_3 protons of tetrahydroimidazo[1,2-a]pyridine), 8.50 (1H, s, N=CH), 11.55 (1H, s, N–NH); MS-FAB$^+$: *m/z*: 303 [M + 1], 304 [M + 2], 305 [M + 3]. Anal. Calc. for $C_{15}H_{15}ClN_4O$: C, 59.51; H, 4.99; N, 18.50. Found:C, 59.43; H, 5.00; N, 18.35.

*5.1.2.8. 5,6,7,8-Tetrahydroimidazo[1,2-a]pyridine-2-carboxylic acid-(4-cyanobenzylidene)hydrazide (**2h**).* The physical appearance: pale yellow crystals. IR (KBr, cm^{-1}): 3189 (NH), 2220 (C≡N), 1633 (C=O), 1602 (C=N), 1544 (C=N–Ph), 760 (N=C–H); ^1H NMR (250 MHz) (DMSO-d_6) δ (ppm): 1.85–2.05 (4H, br, C_6 and C_7 protons of tetrahydroimidazo[1,2-a]pyridine), 2.82 (2H, t, C_8 protons of tetrahydroimidazo[1,2-a]pyridine), 4.05 (2H, t, C_5 protons of tetrahydroimidazo[1,2-a]pyridine), 7.75 (1H, s, C_3 protons of tetrahydroimidazo[1,2-a]pyridine), 7.85 and 7.95 (4H, 2 d $J = 8.43$ Hz and $J = 8.38$ Hz, 1,4-disubstituted phenyl protons), 8.65 (1H, s, N=CH), 11.75 (1H, s, N–NH); MS-FAB$^+$: *m/z*: 294 [M + 1]. Anal. Calc. for $C_{16}H_{15}N_5O$:C, 65.52; H, 5.15; N, 23.88. Found:C, 65.33; H, 5.08; N, 23.82.

5.2. Bioassays

5.2.1. Antifungal assays and microorganisms

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 were given in Table 2). All *Candida strains* were inoculated on Sabouraud Dextrose Agar (SDA) prior the experiments at 37 °C. After sufficient growth *Candida* spp. were then transferred to Mueller Hinton Broth (MHB) for further incubation at the same conditions for another 24 h.

5.2.2. Anticandidal assay

The activity of the compounds (**1a**–**e**, **2a**–**e**) were first screened using an agar diffusion method for *C. albicans* (clinical isolate) and *C. tropicalis* and all active compounds (inhibition zones >9–11 mm, at 2 mg/mL concentration) were further evaluated using the microdilution broth method to identify the minimum inhibitory concentrations against all *Candida* spp. [13,15].

5.2.3. Agar diffusion assay

1 mL of pre-grown *Candida* suspension in MHB adjusted to McFarland No. 0.5 were inoculated to each Petri plate (9 cm and 20 ml MHA) under sterile conditions. With the aid of an sterile cork borer (8 mm) wells were formed in each Petri plate to accommodate the test samples (50 μL from a stock solution of 2 mg/mL DMSO) including the solvent and antifungal controls to incubate at 37 °C for 24 h. Inhibition zones were visualized using a tetrazolium salt (TTC, Aldrich) where clear inhibition zones ≥9 mm were considered as active, for further testing.

5.2.4. Broth microdilution assay

The test compounds and the antimicrobial standards were first dissolved in dimethyl sulfoxide (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 the microorganism. 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, in mg/mL). Average results of separately performed three experiments as given in Table 2.

5.2.5. Assay for in vitro cytotoxicity

The compounds were tested for their *in vitro* cytotoxicity against a panel of four human cancer cell lines [SK-MEL: malignant, melanoma, KB: epidermal carcinoma, BT-549: breast ductal carcinoma, SK-OV-3: ovary carcinoma] and two noncancerous cell lines [VERO: African green monkey kidney fibroblast and LLC-PK_{11}: pig kidney epithelial cells] [14]. All the cell lines were from the American Type Culture Collection (Manassas, VA). Cells (25.000 cells/well) were seeded to the wells of 96-well plate and incubated for 24 h. Samples at different concentration were added followed by an incubation for 48 h. The number of viable cells was determined by Neutral Red assay according to a modification of the procedure of Borenfreund et al. [16]. Briefly, the cells were washed with saline and incubated for 3 h with a solution of Neutral Red. The cells were washed again to remove extracellular dye. A solution of acidified ethanol was added to liberate the incorporated dye from viable cells and the absorbance was read at 450 nm. Percent cell viability was calculated in comparison to solvent control. Doxorubicin was used as the drug control.

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