Attempts to access a series of pyrazoles lead to new hydrazones with antifungal potential against *Candida species* including azole-resistant strains

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Abstract

The treatment of benzylidenemalononitriles **3a-c** with phenylhydrazines **4a-n** in refluxing ethanol did not provide pyrazole derivatives but furnished hydrazones **1a-o**. The structure of hydrazones was secured by X-Ray analysis. Newly synthesized hydrazones **1a-o** were tested against 8 *Candida spp*. strains in a dose response assay to determine the minimum inhibitory concentration (MIC_{99}). Five compounds **1c**, **1d**, **1i**, **1k** and **1l** were identified as promising antifungal agents against *Candida spp*. (*C. albicans* SC5314, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata (R azoles)*) with MIC_{99} values ranging from 16 to 32 µg/mL. To further evaluate the antifungal potential of the active compounds, they have been assayed against a mammalian cell line HEK293 to determine general cell toxicity and on NCI-60 cancer cell lines panel, demonstrating selectivity antifungal activity over cytotoxicity.

Antifungal therapies evolved slowly during the early years of the twentieth century, with the development of antifungal agents lagging that of antibacterial agents. The current therapeutic arsenal for the systemic treatment of antifungal infections mainly includes polyenes, azoles, echinocandins and pyrimidines classes of compounds.

Antifungals are now facing the same threat that antibacterial compounds: the development of antifungal resistance in medical care facilities, environments concomitant with their use. Fungal infections have emerged as an important clinical threat, with high associated morbidity and mortality rate. During the last decade, clinical needs for novel antifungal agents have altered steadily with the change in spectrum of fatal disseminated fungal infections.

In 2020, the World Health Organization (WHO) set up the "WHO antifungal expert group on identifying priority fungal pathogens" posing a high risk to human health.[1] The fungal pathogens *Candida spp*. azole-resistant were identified as preoccupying pathogens of global public health

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importance. The lack of therapeutic innovations or new chemical families in the pipe for the discovery of therapeutic alternatives reached a critical level.

Identifying new experimental drugs is more than ever expected and challenging because of the fungal diversity, as well because the drug should remain well tolerated in human subject. Up to date, there are only few different classes from a mechanistic point of view which are currently used to treat serious antifungal infections.

In this report, a new series of hydrazone compounds was identified as effective on *Candida spp*. including azole- or echinocandin-resistant strains (compounds **1a-o**, Figure 1). Hydrazone derivatives and especially acylhydrazones were described as antifungal agents on *Candida spp*. Acylhydrazone **A** (Figure 1) displayed antifungal potential on *Candida glabrata*.[2] Acylbarbituric acid hydrazone **B** inhibited the growth of *Candida albicans* and *Candida glabrata* with MIC_{80} values of 62 µg/mL and 31 µg/mL, respectively (Figure 1).[3] Hydrazone **C** was assayed for broad-spectrum antifungal activity against clinical isolates of *Candida spp*. and showed bioactivity against *Candida albicans*, *Candida glabrata* and *Candida tropicalis* with MIC values ranging from 4 to 128 µg/mL (Figure 1).[4] Moderate antifungal potency was also registered for hydrazine **D** bearing electro-withdrawing substituents on *Candida glabrata* and *Candida tropicalis* (Figure 1).[5]

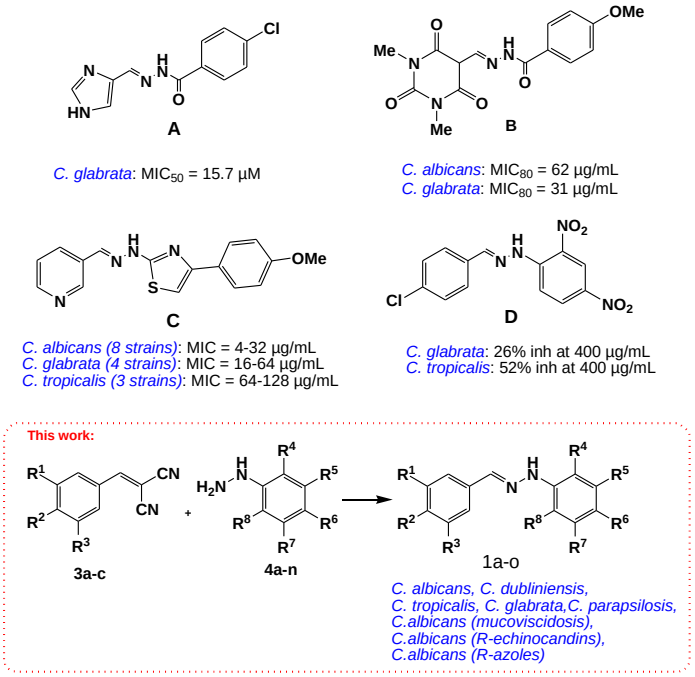

Figure 1. Structure of a selection of anti-*Candida* experimental drugs **A**-**D** and of target hydrazones **1a-o**

In the frame of an ongoing medicinal chemistry program, an attempt to access new pyrazole derivatives of general structure **5** and **6** (Scheme 2) starting from benzylidenemalononitriles **3a-c** has been tried. The latter have been easily obtained by Knoevenagel reaction of aldehydes **2a-c** with malononitrile in the presence of piperidine in refluxing ethanol (Scheme 1). They were next reacted with substituted phenylhydrazines **4a-n** in ethanol at reflux. The expected pyrazolines **5a-o** and/or **6a-o** could not be obtained. The same operatory conditions were previously described to afford aminopyrazole derivatives.[6,7] However, in our case, no trace of target heterocycle was detected in the crude by ^{1}H NMR and TLC monitoring. Instead, in all cases, a different product was detected and isolated. The structure of the final product was resolved as hydrazone derivative and secured by

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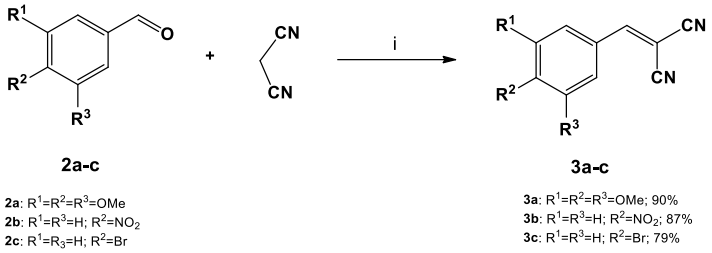
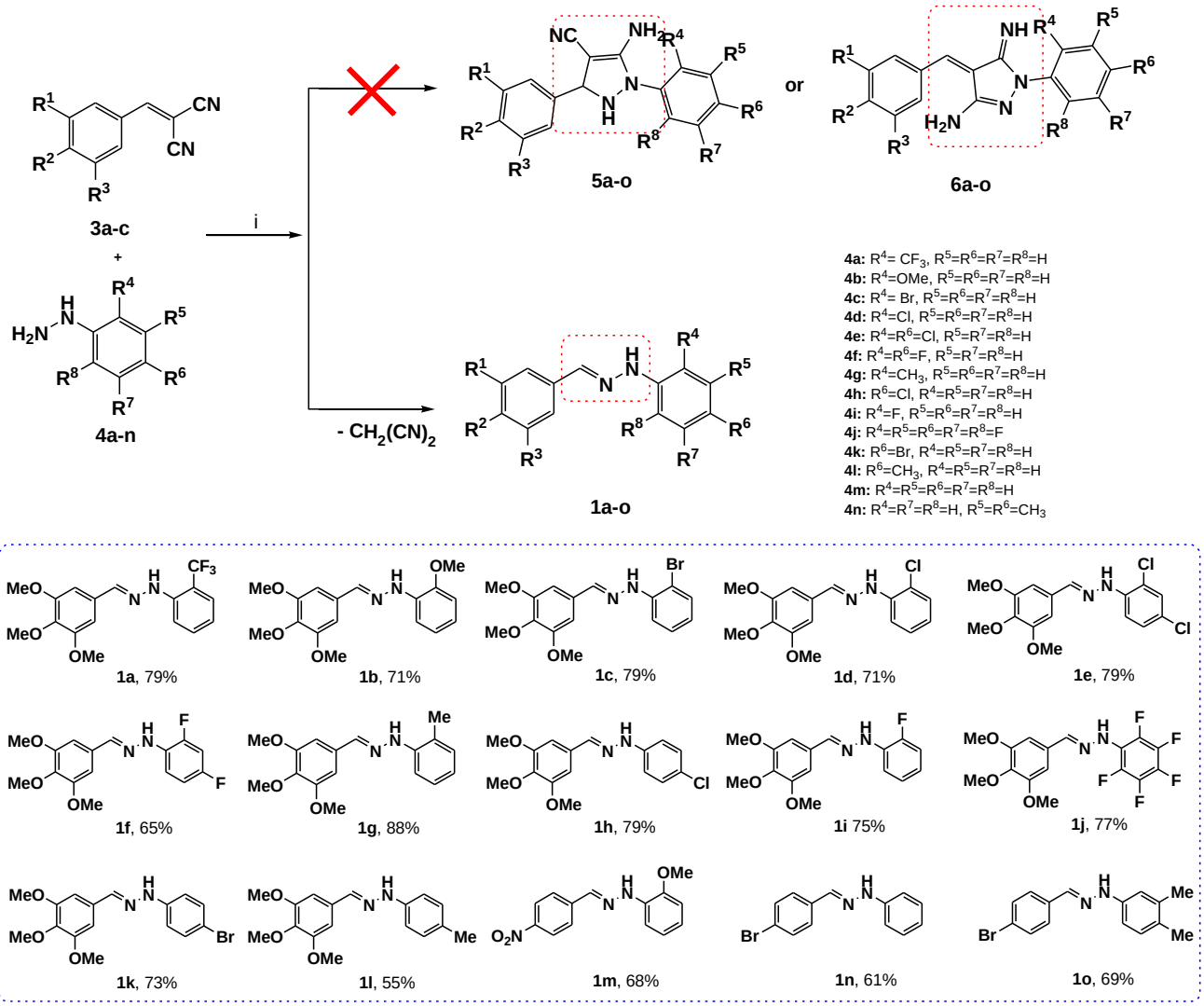
performing X-Ray on compounds **1e** and **1i** of the series (Figure 2). In addition, a chemical proof was also obtained by reacting aldehyde **2a** with hydrazine **4e** which provided the same hydrazone **1e**. This confirmed the loss of the malononitrile unit during the reaction of benzylidenemalononitriles **3a-c** with phenylhydrazines **4a-n**, explaining undoubtedly the formation of hydrazones **1a-o** (Scheme 1).



Scheme 1. *Reagents and conditions*: (i) piperidine, EtOH, reflux, 6-8 h.



Scheme 2. *Reagents and conditions*: (i) EtOH, reflux, 4-8 h.

The structure of compounds **1e** and **1i** were demonstrated by single crystal X-ray diffraction method. According to X-ray crystallography the two compounds are isostructural. They crystallize in $P2_1/c$ space group with close unit cell parameters (Table 1S, see supplementary data section for full data). The asymmetric part of the unit cell (Figure 2a, 2b) comprises one molecule of **1e** and **1i** as a

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crystallographic independent unit, respectively. There are no co-crystallized solvent molecules in both crystals.

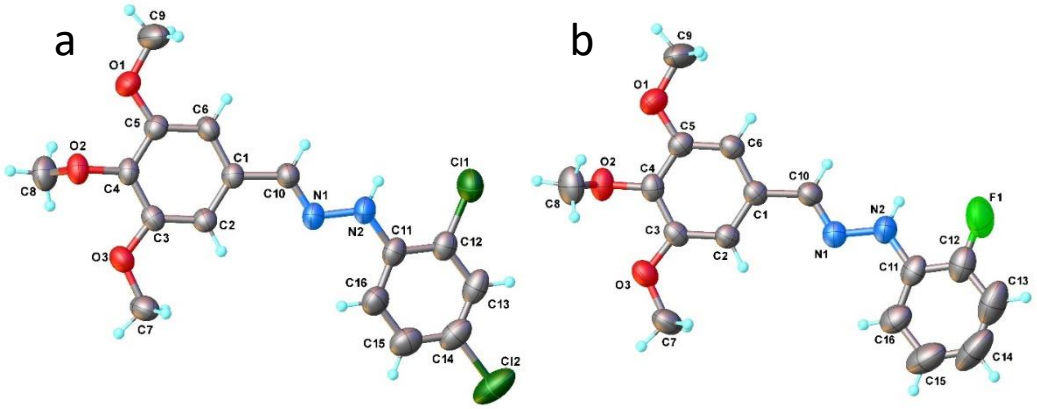

Figure 2. A view of the asymmetric part in the crystal structure of **1e** (a) and **1i** (b) with atom labeling and thermal ellipsoids at 50% level.

As expected, compounds **1e** and **1i** feature similar crystal structure packing. Indeed, for both crystals the main crystal structure motif is described as a one-dimensional supramolecular array running along *b* axis, which is formed through intermolecular NH···O and CH···O hydrogen bonding. As an example, a view of 1D architecture in the crystal structure of **1i** is shown in Figure 3.

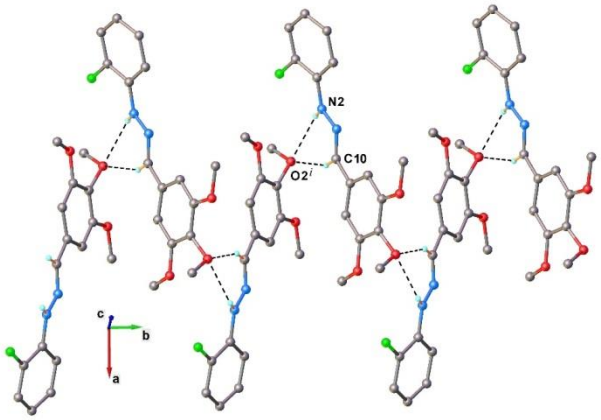

Figure 3. 1D supramolecular chain in the crystal structure of **1i**. Non-relevant hydrogen atoms are not shown. H-bonds are drawn as black-dashed lines. Symmetry code: *i*) –*x*, –0.5 + *y*, –1.5 – *z*.
H-bonds parameters: N2-H···O2 [N2-H 0.86 Å, H···O2 2.65 Å, N2···O2′ 3.432(2) Å, ∠N2HO2 151.4°]; C10-H···O2 [C10-H 0.93 Å, H···O2 2.59 Å, C10···O2′ 3.470(2) Å, ∠C10HO2 158.4°].

A mechanism was proposed for the obtention of hydrazones **1a-o** from benzylidenemalononitriles **3a-c** (Scheme 3). The first step involved the classical nucleophilic attack of the marginal nitrogen of the hydrazine on the ethylenic carbon of the 2-cyano-3-aryl-acrylonitrile. The intermediate formed underwent a proton migration 1,3. This allowed the formation of malononitrile as a leaving group and the formation of hydrazones (Schemes 2 and 3).

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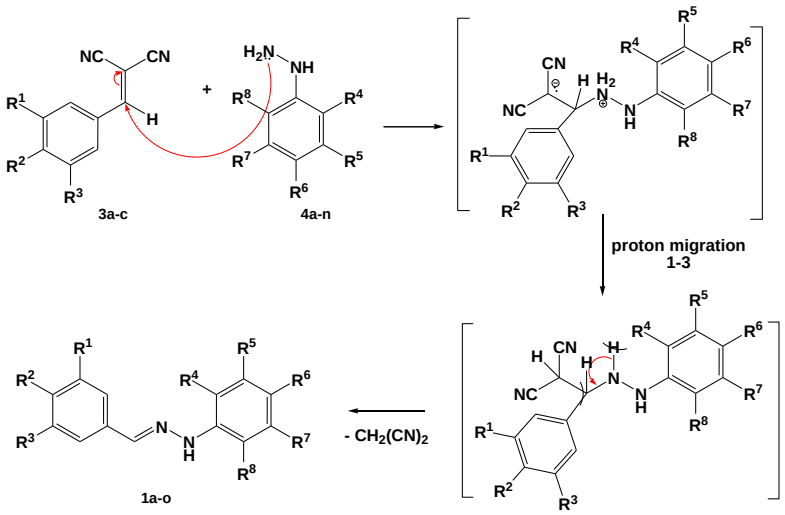

Scheme 3. Proposed mechanism for the formation of hydrazones **1a-o** from benzylidenemalononitriles **3a-c** upon reaction with hydrazines **4a-n**.

Primary antifungal screening study by whole cell growth inhibition assays, using all the synthesized hydrazones **1a-o** at a single concentration of 32 µg/mL, was realized in triplicate (n=3). Hit Confirmation of active compounds by whole cell growth inhibition assays was conducted as an 8-point dose response to determine the Minimum Inhibitory Concentration (MIC), in triplicate (n=3). The inhibition of growth was measured against 8 fungi strains: *Candida albicans* SC5314, *Candida dubliniensis*, *Candida glabrata*, *Candida parapsilosis*, *Candida albicans from mucoviscidosis patients* (*C. albicans* (*mucoviscidosis*)), *Candida albicans resistant to echinocandins* (*C. albicans* (*R echinocandins*)) and *Candida glabrata resistant to azoles* (*C. glabrata* (*R azoles*)) (Table 1). The fungal strains were obtained from Pôle de Biologie Pathologie Génétique, Centre Hospitalier Universitaire (CHU) de Lille, France. Fluconazole was used as positive reference in the assay. Samples were prepared in DMSO and water to a final testing concentration of 32 µg/mL and in triplicate (n=3) and keeping the final DMSO concentration to a maximum of 1% DMSO. All the sample-preparation were done using liquid handling robots. Only five hydrazones **1c**, **1d**, **1i**, **1k** and **1l** displayed notable antifungal activity against tested *Candida spp*. with MIC values between 16 and 32 µg/mL. The results are presented in Table 1. All other synthesized hydrazones **1a**, **1b**, **1e-h**, **1j** and **1m-o** were less active, with MIC values > 32 µg/mL (data not shown).

Table 1. MIC values of active hydrazones **1c, 1d**, **1i**, **1k** and **1l** on *Candida spp*.

Entry	Compound	MIC values (µg/mL) on *Candida spp*. [a,b,c,d]							
		C. albicans SC5314	*C. dubliniensis*	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*	*C. albicans (mucoviscidosis)*	*C. albicans (R echinocandins)*	*C. glabrata (R azoles)*
1	**1c**	32	>32[e]	32	32	>32	>32	>32	32
2	**1d**	32	>32	16	>32	>32	>32	>32	32
3	**1i**	32	>32	32	32	>32	>32	>32	32
4	**1k**	32	>32	16	32	32	>32	>32	>32
5	**1l**	32	>32	16	32	32	>32	>32	32
6	Fluconazole	0.5	0.5	0.5	0.5	0.5	0.5	0.5	>32

[a] *In vitro* inhibition percentage of pathogens. [b] Values represent mean of three experiments. [c] Compounds were tested at 32, 16, 8, 4 and 1 µg/mL concentration. [d] MIC values given in the table correspond to MIC_{99} (total growth inhibition of *Candida spp*.). [e] MIC not calculated since total inhibition of *Candida spp*. was not obtained at 32 µg/mL.

Active hydrazones **1c**, **1d**, **1i**, **1k** and **1l** share the same 3,4,5-trimethoxyphenyl unit and have the particularity of a monosubstitution on the other phenyl ring. Generally, the *ortho*-substitution by an electro-withdrawing group (F, Cl and Br) in **1i**, **1d** and **1c**, respectively was the best chemical modulation for the antifungal activity in the current work. The comparison of halogens reveals that the

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chlorine atom in hydrazone **1d** was the most active especially on *C. glabrata* (MIC=16 µg/mL) compared to fluoro and bromo congeners **1i** and **1c** (MIC=32 µg/mL) (Table 1). The trifluoromethyl substituent in hydrazone **1a** resulted in dramatical loss of the antifungal potential (MIC>32 µg/mL). The *para*-bromo substitution in hydrazone **1k** slightly decreased the antifungal potential on *C. glabrata (R azoles)* (MIC>32 µg/mL) but conserved the notable antifungal effect on *C. glabrata* (MIC=16 µg/mL) and on *C. tropicalis* (MIC=32 µg/mL) (Table 1). To be noted, only *para*-substituted compounds **1k** and **1l** displayed inhibition activity against *C. tropicalis* (MIC=32 µg/mL), while the *ortho*-substituted analogs were less active (MIC>32 µg/mL) (Table 1). The electron-donating substituents by inductive or mesomeric effect (Me or OMe) in position *ortho* of the phenyl unit were not tolerated on any of the tested fungi (MIC>32 µg/mL for hydrazones **1b**, **1g** and **1m**). The di- or polysubstitution of the same phenyl ring by both electron-withdrawing or electron-donating groups in hydrazones **1e**, **1f**, **1j** and **1o** were not favorable for antifungal activity (MIC>32 µg/mL). Finally, the replacement of the 3,4,5-trimethoxyphenyl unit by a 4-nitrophenyl or 4-bromophenyl moiety in hydrazones **1m-o** also abolished the antifungal activity against all tested *Candida spp* (MIC>32 µg/mL). The 3,4,5-trimethoxyphenyl unit seemed essential to maintain the biological activity on *C. albicans* SC5314, *C. glabrata*, *C. parapsilosis* and *C. glabrata resistant to azoles* (MIC values of 16 and 32 µg/mL, respectively). Diminished potential was registered on clinical isolates of *C. albicans (mucoviscidosis)* and *C. albicans resistant to echinocandins*. Newly synthesized hydrazones generally showed more pronounced antifungal activity on strains of *Candida glabrata* including *Candida glabrata resistant to azoles* (Table 1).

To verify the mammalian cytotoxicity of the newly identified antifungals presented herein, compounds **1c**, **1d**, **1i**, **1k** and **1l** were tested against human embryonic kidney cells (HEK293) at ten different concentrations (0.06 to 32 µg/mL) (Figure 4). The high concentration of 32 µg/mL tested corresponded to the concentration at which the compounds exhibit potent antifungal activity. Since compounds were dissolved in 0.1% DMSO in the stock solution for this assay, DMSO was used as negative reference in the same test. As depicted in Figure 2, the concentration of 0.1% of DMSO is devoid of cytotoxic effect and is safe for compounds solubilization while the concentration of 20% of DMSO displayed high toxicity. This denotes the importance of the concentration of DMSO used to dissolve the experimental drugs so as not to have distorted effects due to the solvent. All tested compounds showed no toxicity in viable kidney HEK293 cells.

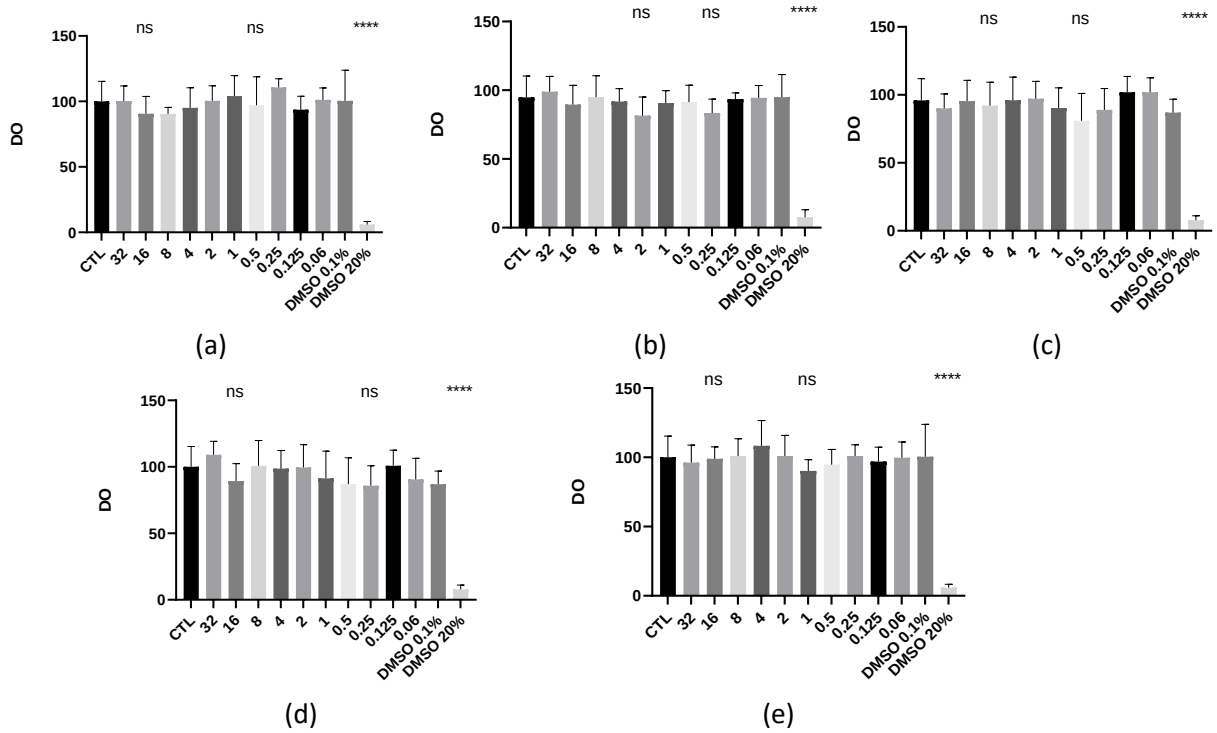

Figure 4. Evaluation of the cytotoxicity of experimental antifungals on HEK293 cells at different concentrations (32 µg/mL to 0.06 µg/mL): (a) hydrazone **1c**, (b) hydrazone **1d**, (c) hydrazone **1i**, (d) hydrazone **1k** and (e) hydrazone **1l**.

In addition, compounds **1c**, **1d**, **1i**, **1k** and **1l** have also been selected and evaluated for cell growth inhibition activity on NCI-60 cancer cell lines panel. Molecules were tested at 10 µM concentration and did not show any notable cytotoxic effect. The full One Dose Mean Graphs for antifungal agents **1c**, **1d**, **1i**, **1k** and **1l** are available in the supplementary data section associated with this article.

An attempt to isolate pyrazoles derivatives by reacting benzylidenemalononitriles **3a-c** with hydrazines **4a-n** in refluxing ethanol did not provide the target heterocyclic systems, as expected, and as previously reported, but hydrazones **1a-o** whose structure was secured by both chemical evidence and X-Ray studies. Indeed, the direct reaction of 3,4,5-trimethoxybenzaldehyde with 2,4-dichlorophenylhydrazine **4e** furnished the same hydrazone **1e** as that obtained from benzylidenemalononitrile **3a** with hydrazine **4e**. This study identified 5 hydrazones **1c**, **1d**, **1i**, **1k** and **1l** as promising antifungal agents (MIC_{99} values ranging from 16 to 32 µg/mL) against *Candida spp*. These compounds showed a CC_{50} (concentration at 50% cytotoxicity) value against HEK293 cells at >32 µg/mL and against NCI-60 cancer cell lines panel at >10 µM, demonstrating selectivity antifungal activity over cytotoxicity. Compared to known acylhydrazones and hydrazones previously reported in the literature, a selection of which is available in Figure 1, the newly synthesized hydrazones **1c**, **1d**, **1i**, **1k** and **1l** are positioned as very promising experimental molecules with antifungal activity on *Candida spp*.

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Supplementary information

Full physico-chemical characterization of newly synthesized compounds including NMR description, full description of the crystal structure of **1e** and **1i**, materials and methods for antifungal and cytotoxic evaluation and one-dose full graphs obtained for active molecules **1c**, **1d**, **1i**, **1k** and **1l** on NCI-60 cancer cell lines panel associated with this article are available at….

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