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Efficient Synthesis of Novel 3-Aryl-5-(4-chloro-2-morpholinothiazol-5-yl)-4,5-dihydro-1*H*-pyrazoles and Their Antifungal Activity Alone and in Combination with Commercial Antifungal Agents

Juan Ramírez[1], María Victoria Rodríguez[2], Jairo Quiroga[1], Rodrigo Abonia[1], Maximiliano Sortino[3], Susana A. Zacchino[3], and Braulio Insuasty[1]

[1] Heterocyclic Compounds Research Group, Department of Chemistry, Universidad del Valle, Cali, Colombia
[2] Área Biología Vegetal, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina
[3] Área Farmacognosia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

The α,β-unsaturated carbonyl compounds **5a–f** were prepared by reaction between 2-chloro-4-morpholinothiazol-5-carbaldehyde **3** and substituted acetophenones **4a–f**. Treatment of compounds **5a–f** with hydrazine hydrate employing mild reaction conditions led to the formation of 4,5-dihydro-1*H*-pyrazoles **6a–f**. Then the treatment with acetic anhydride or formic acid afforded the expected 4,5-dihydro-1*H*-pyrazoles **7a–f** and **8a–f**. The antifungal activity of each series of synthesized compounds was determined against the clinically important fungi *Candida albicans* and *Cryptococcus neoformans*. In addition, the most active compounds **7e** and **7f** were tested in combination with the commercial antifungal agents: fluconazole, itraconazole, and amphotericin B. Compound **7e** showed a synergistic effect with fluconazole against *C. albicans* while **7f** showed synergistic activities with all tested antifungal drugs against the same yeast.

Keywords: Antifungal activity / 2-Chloro-4-morpholinothiazol-5-carbaldehyde / Commercial antifungal agents / 4,5-Dihydro-1*H*-pyrazoles / Synergistic effect

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Introduction

The use of thiazole-containing compounds in organic synthesis has recently grown due to their synthetic versatility [1], and their wide spectrum of biological activities [2–10]. Regarding their antimicrobial activity, new thiazole-cyano compounds containing morpholine in their structures, showed antibacterial activity [11]; some 2,4-dichlorothiazole-5-carbaldehyde derivatives displayed antifungal activity [12]; thiazole-based chalcones showed antibacterial activity [13, 14], and hydrazinylthiazole derivatives presented antimycobacterial and antimalarial activity [15, 16]. In addition, some compounds containing thiazole and thiazolidine nucleus are in current use as therapeutic agents; abafungin, is a broad-spectrum antifungal agent formulated as a topical cream for the treatment of dermatomycoses [17], epalrestat is an aldoreductase inhibitor, approved for clinic use in Japan [18], dasatinib, was approved as a first-line treatment for chronic myelogenous leukemia by the National Cancer Institute (NCI) [19, 20] (Fig. 1).

On the other hand, five membered nitrogen-containing heterocyclic compounds like 2-pyrazolines have shown a wide spectrum of biological activities such as antidepressant [21], antifungal [22], anticancer [23–27], antimalarial [25, 28], and antimicrobial activities [29, 30].

In the course of our ongoing research on the synthesis and biological activities of pyrazolines added to our willingness to

Correspondence: Dr. Braulio Insuasty, Faculty of Science, Heterocyclic Compounds Research Group, Department of Chemistry, Universidad del Valle, A.A. 25360 Cali, Colombia
E-mail: braulio.insuasty@correounivalle.edu.co
Fax: +57 2 3392440

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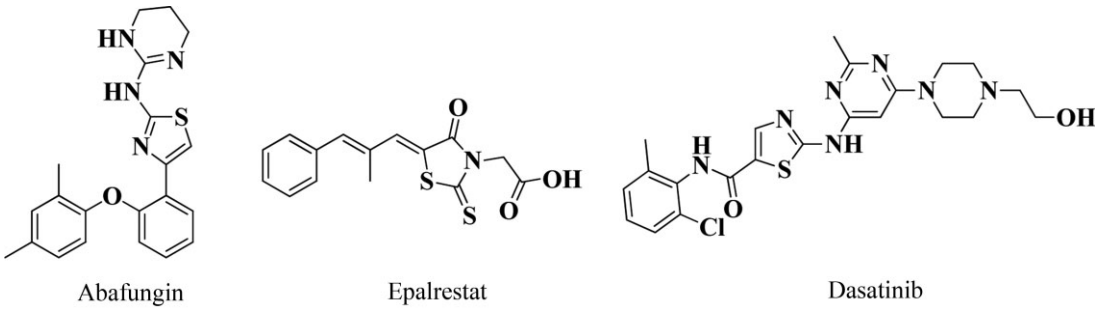

Figure 1. Chemical structures of some thiazoles and thiazolidines used as therapeutic agents.

contribute to the research on the synthesis and biological activities of new thiazole derivatives, we performed an efficient synthesis of 12 novel 3-aryl-5-(4-chloro-2-morpholinothiazole-5-yl)-4,5-dihydro-1*H*-pyrazoles containing both the pyrazole and the thiazole moieties and tested them for antifungal activities against two clinically important fungal species: *Candida albicans* and *Cryptococcus neoformans*. The selection of *C. albicans* as one of the target fungus species was due to this fungus representing over 60% of all isolates from clinical infections [31], and many treatment failures were reported in the last 10 years associated to the high appearance of resistant strains [32]. As a consequence, new structures with anti-*C. albicans* activity are very welcome. In turn, the selection of *C. neoformans* as the second fungal target is due to this fungus producing cryptococcal meningitis that has killed more than 650,000 immunocompromised patients worldwide and whose treatment is based on medications that were discovered nearly 50 years ago, since no new better drugs have been recently incorporated to clinic [33]. To solve these problems, many efforts have been undertaken into different directions. One of them was to look for new structures that, alone, possess anti-*C. albicans* and anti-*C. neoformans* activity [34]. The other one consisted in finding new structures that, acting in combination with antifungal agents [35–38], produce an improvement of the activity. In this strategy, the mixture of low doses of each component produce the same or higher activity than each one alone, showing synergism or at least additivism, avoiding the known toxicity of the antifungal drugs and decreasing the occurrence of resistant strains.

Following this trend and driven by the fact that the compounds of this series did not show much activity when acting alone (see Results and Discussion section), we tested the most active compounds in combination with amphotericin B (AmB), itraconazole (ITZ), and fluconazole (FCZ) against *C. albicans* and *C. neoformans*. These mixtures showed that some compounds were synergistic only with AmB, while other ones enhanced the activity of the three antifungal agents.

Results and discussion

Chemistry

New thiazole-based 4,5-dihydro-1*H*-pyrazoles **7a–f** and **8a–f** were prepared from α,β-unsaturated carbonyl compounds containing the thiazole ring **5a–f** employing different pathways. In this sense to obtain the aldehyde **3**, we carried out the nucleophilic aromatic substitution (S_NAr) with morpholine of chlorine in aldehyde **1**, according to the Kotlyar and coworkers methodologies [39]. The treatment of equimolar amounts of **3** with acetophenones **4a–f** under Claisen–Schmidt conditions afforded the α,β-unsaturated carbonyl compounds **5a–f** which, by treatment with hydrazine hydrate, under mild reaction conditions gave 4,5-dihydro-1*H*-pyrazoles **6a–f**. As these compounds decomposed rapidly, they were immediately isolated by filtration on vacuum and a fraction of 0.3 mmol of each compound was treated with acetic anhydride or formic acid at room temperature during 1 h to obtain the expected compounds **7a–f** and **8a–f**, respectively, in good yields (54–86%) (Scheme 1).

Structure elucidation of pyrazole derivatives **7a–f** and **8a–f** was performed based on their spectral data (FT-IR, ^{1}H NMR, ^{13}C NMR, and MS). The IR spectrum of compound **7b** as representative of the series showed absorption bands at 1672, 1595, and 1525 cm^{-1} associated with C=O, and C=N, C=C functionalities, respectively. In the ^{1}H NMR spectrum of compound **7b**, a singlet at $\delta = 2.40$ ppm indicated the presence of the acyl group and multiplets at $\delta = 3.39$–3.41 ppm and $\delta = 3.75$–3.77 ppm integrating for 4H each one assigned to methylenic protons of the morpholine ring appeared. The two methylenic protons in C-4 and the stereogenic proton in C-5 on dihydropyrazole unit form an AMX spin system; in this sense, proton 4-H_A appeared as a double-doublet at $\delta = 3.24$ ppm with coupling values $^2J_{AM} = 17.7$ Hz and $^3J_{AX} = 4.7$ Hz, proton 4-H_M exhibited a double-doublet at $\delta = 3.70$ ppm with coupling values $^2J_{AM} = 17.7$ Hz and $^3J_{MX} = 11.9$ Hz and the proton 5-H_X appeared as a double-doublet at $\delta = 5.78$ ppm with coupling constants $^3J_{AX} = 4.7$ Hz and $^3J_{MX} = 11.9$ Hz. At aromatic region, two doublets at

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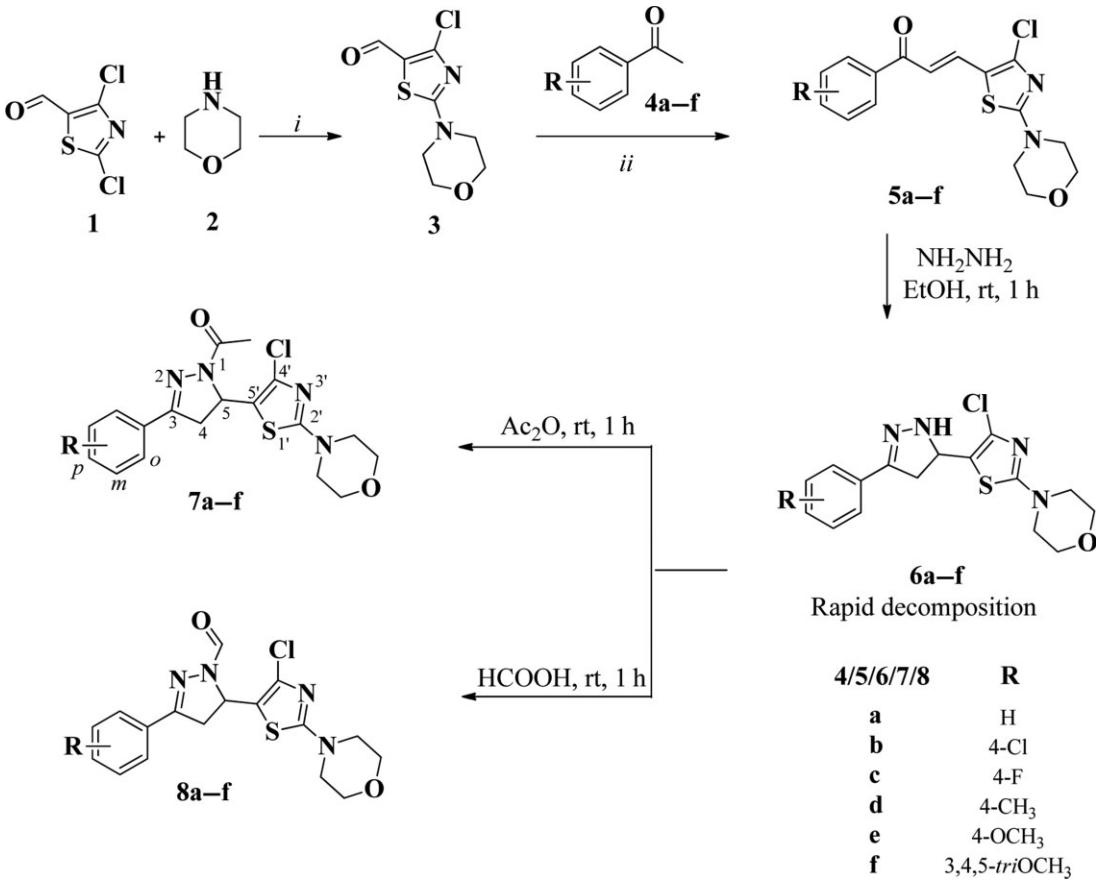

Scheme 1. General methodology for the synthesis of thiazole-based 4,5-dihydro-1*H*-pyrazoles. *i* = CH_3CN, r.t., 4 h, *ii* = substituted acetophenones, EtOH, NaOH, r.t., 8 h.

$\delta = 7.43$ and 7.69 ppm with coupling constant $J = 8.5$ Hz assigned to 4-chlorophenyl substituent were observed. The ^{13}C NMR spectrum of compound **7b** showed signals at $\delta = 21.8$ and 53.2 ppm assigned to methyl from acyl group and C-5 of the dihydropyrazole unit, respectively. Also signals at $\delta = 152.6$ and 168.9 ppm assigned to C-3 and C=O from *N*-acyl-dihydropyrazole functionality. With the help of DEPT-135 experiment, at $\delta = 40.6$, 47.6, and 65.9 ppm appeared the signals assigned to methylenic carbon atoms C-4, $-CH_2-N-CH_2-$, and $-CH_2-O-CH_2-$ of the dihydropyrazole and morpholine units. Regarding to the *N*-formyl-4,5-dihydro-1*H*-pyrazoles, the IR spectrum for compound **8b** as representative of the series showed an absorption band associated to the carbonyl group (C=O) at 1679 and absorption bands at 1596 and 1529 cm^{-1} associated with the C=N and C=C functionalities. The ^1H NMR spectrum of compound **8b** showed all signals with similar results in comparison with the ^1H NMR spectrum of compound **8b**, only appeared a new singlet at $\delta = 8.89$ ppm assigned to the proton from the formyl group. The ^{13}C NMR spectrum of compound **8b** exhibited the signal associated to carbonyl group at $\delta = 159.9$ ppm, the remaining signals appear with similar results as compound **7b** (see Experimental section).

Antifungal activity studies

All synthesized compounds **7a–f** and **8a–f** were tested alone against *C. albicans* ATCC 10231 and *C. neoformans* ATCC 32264 with the CLSI microplate design that is usually used for determining the minimum inhibitory concentration [40]. The inhibition percentages displayed by each compound at the different concentrations (Table 1) were calculated with the aid of a microplate reader, as explained in Experimental section.

As it can be observed in Table 1, **7e** and **7f** inhibited ≥50% of *C. albicans* growth at 200 μg/mL, and six of them (**7b**, **7c**, **8b**, **8d**, **8e**, and **8f**) inhibited 20–40% growth at the same concentration. Regarding *C. neoformans*, **7e**, **7f**, and **8d** inhibited ≥50% of *C. neoformans* growth at 200 μg/mL, while **7a–d**, **8a–c**, **8e**, and **8f** showed inhibition percentages between 20 and 31% at the same concentration. At lower concentrations (≤100 μg/mL), any compound overcame 30% inhibition of fungal growth.

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Table 1. Antifungal activity (% inhibition) of compounds **7a–f** and **8a–f** against *Candida albicans* ATCC 10231 and *Cryptococcus neoformans* ATCC 32264.

	Candida albicans			*Cryptococcus neoformans*		
Compound	**200 μg/mL**	**100 μg/mL**	**50 μg/mL**	**200 μg/mL**	**100 μg/mL**	**50 μg/mL**
7a	0	0	0	31.14 ± 1.9	30.33 ± 3.3	27.59 ± 1.7
7b	40.09 ± 2.5	5.80 ± 0.2	0	15.35 ± 1.2	12.04 ± 0.2	11.87 ± 1.5
7c	29.16 ± 1.8	4.70 ± 1.2	2.28 ± 0.1	27.79 ± 0.1	25.52 ± 0.3	22.32 ± 0.4
7d	0	0	0	13.60 ± 1.7	12.07 ± 2.8	10.51 ± 0.6
7e	65.90 ± 2.6	8.28 ± 0.5	0	69.97 ± 3.9	16.16 ± 1.7	10.87 ± 1.4
7f	50.85 ± 2.8	17.29 ± 1.1	3.77 ± 0.7	57.59 ± 1.2	23.59 ± 1.3	15.14 ± 2.3
8a	13.59 ± 0.4	7.44 ± 0.6	5.18 ± 0.1	20.34 ± 1.7	3.13 ± 0.4	0
8b	23.93 ± 0.5	13.00 ± 1.3	11.84 ± 1.3	22.63 ± 1.6	18.70 ± 1.3	9.29 ± 0.3
8c	16.30 ± 2.0	7.20 ± 0.3	4.26 ± 1.0	17.96 ± 2.3	13.72 ± 1.6	12.30 ± 1.3
8d	33.42 ± 1.7	21.20 ± 1.1	12.72 ± 1.4	65.35 ± 1.1	20.76 ± 2.1	19.91 ± 0.1
8e	36.20 ± 0.6	19.19 ± 1.6	8.96 ± 0.2	24.07 ± 1.5	18.42 ± 1.1	15.83 ± 1.2
8f	27.51 ± 0.5	15.02 ± 0.2	9.81 ± 1.1	24.77 ± 1.8	3.25 ± 1.1	0
AmB[a)]	100	100	100	100	100	100

[a)] Amphotericin B.

These results clearly showed that this series of compounds possess moderate antifungal activity when acting alone. So, we decided to test **7a–c**, **8a–c** in combination with the antifungal drugs FCZ, AmB, or ITZ (each combination partner at low doses) against *C. albicans* and *C. neoformans*, taking advantage of both, the new structural type of these pyrazole derivatives and the high activity of antifungal drugs. The aim was to achieve high response rates with mixtures that contain sub-inhibitory doses of antifungal drugs, thus avoiding the known toxicity of them and the generation of resistance [41].

Regarding the method of choice to test the compounds in combination with antifungal drugs, the high throughput screening synergy assay (HTSS) developed by Zhang et al. [42], was selected and applied to **7a–f**, **8a–f** at a first instance of screening. This strategy consisted in testing (in holes of a 96-well microplate) a mixture formed by both the compound and the antifungal drug, each one at a sub-optimal dose (producing 10–20% of the maximum activity) against *C. albicans* and *C. neoformans* by using FCZ as the drug partner with the aim of finding mixtures which, in combination, show an enhancement of activity, reaching ≥50% growth inhibition (Fig. 2). This simplification, previously used to screen microbial extracts [42] and plant extracts of *Baccharis* genus [43], demonstrated to help in the identification of synergistic mixtures.

In Fig. 2, bars represent the percentage of inhibition growth of: (a) each tested compound (**7a–f** or **8a–f**) at a concentration that inhibits ≤20% of either *C. albicans* or *C. neoformans* growth; (b) antifungal agent (FCZ) at a concentration that inhibits ≤20% of fungal growth; (c) a mixture containing (a) and (b). Above each bar (a), (b), or (c), it can be observed the wells after 24 h incubation at 30°C. The diminution of growth (that was quantified with the microplate reader, as explained in Experimental section) is observed in (c) when an enhancement of inhibition is observed. Only enhancements ≥50% were considered of interest.

Results of the HTSS demonstrated that any compound in combination with an antifungal drug showed enhanced activity against *C. neoformans*. Instead, **7e** and **7f** were identified as two hits against *C. albicans*. Compound **7f** alone displayed 17.29% inhibition of *C. albicans* growth (Table 1) at the concentration tested (100 μg/mL) while the activity of the mixture of this compound with sub-inhibitory doses of FCZ (0.5 μg/mL) far surpassed the activity of the single compound reaching 50% *C. albicans* growth inhibition.

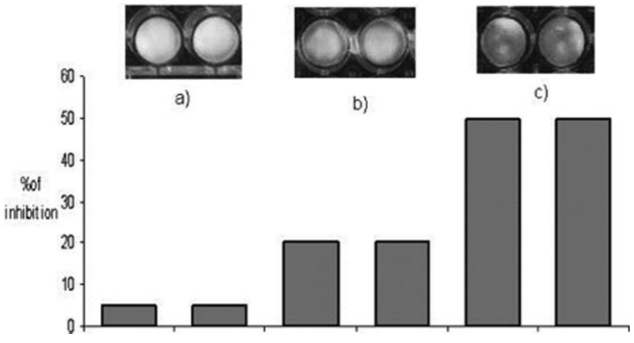

Figure 2. Top: Wells prepared as labeled, after incubation for 24 h in a moistured dark chamber at 30°C. Bars show the percentage of growth inhibition measured with a microplate reader of: (a) compound at a sub-inhibitory concentration (100 μg/mL) that inhibits only ≤20% of fungal growth; (b) antifungal agent (fluconazole) at a sub-inhibitory concentration (0.05–0.5 μg/mL) that inhibits ≤20% of fungal growth; (c) a mixture containing (a) and (b) that produces ≥50% fungal growth inhibition.

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In turn, compound **7e**, which produced an 8.28% inhibition of *C. albicans* when acting alone, showed ≥50% growth inhibition in the presence of FCZ.

To determine if the enhancement observed for **7e** and **7f** with the HTSS assay signified that the mixtures were synergistic, antagonistic, or additive, a design used by Eid et al. [44] was applied. For this study compounds **7e** and **7f** were tested in mixtures not only with FCZ but with two other antifungal drugs, ITZ and AmB against *C. albicans*. With this new design, fungal cells were incubated with serial dilutions of each antifungal drug (FCZ, ITZ, or AmB) in a 96-well microplate, and in parallel, the same series of wells were prepared with the addition of a fixed sub-inhibitory concentration (100 μg/mL) of **7e** or **7f** in each well. Dose–response curves were constructed for each antifungal drug alone and with the added compound (Fig. 3).

In the two-drug combination assays, **7e** and **7f** produced shifts of the dose–response curves of FCZ toward lower concentrations (Fig. 3A). Instead, in the dose–response curves of ITZ (Fig. 3B) and AmB (Fig. 3C), **7e** produced a shift to higher concentrations while **7f** shifted the curves to lower concentrations.

Analysis of the combination effect

The nature of the interaction (synergism, additivism, or antagonism) between **7e** or **7f** and FCZ, ITZ, or AmB, was assessed by calculating the combination index (CI) method [45]; the CI was calculated with the Eq. (1):

$$\mathrm{CI} = \frac{C_{\mathrm{AD},50}}{\mathrm{MIC}_{50,\mathrm{AD}}} + \frac{C_{\mathrm{comp},50}}{\mathrm{MIC}_{50,\mathrm{comp}}} \quad (1)$$

$C_{\mathrm{AD},50}$ is the concentration of AmB, FCZ, or ITZ in mixture that produces 50% inhibition of fungal growth, datum that was obtained from the dose–response curve of each combination. $\mathrm{MIC}_{50,\mathrm{AD}}$ is the concentration of the antifungal drug alone that produces 50% inhibition of the fungal growth. $C_{\mathrm{comp},50}$ is the concentration of compound (**7e** or **7f**) in the mixture, that produces 50% inhibition of fungal growth. $\mathrm{MIC}_{50,\mathrm{comp}}$ is the concentration of the compound alone that produces 50% inhibition of the fungal growth (see Table 2).

A $\mathrm{CI} < 1$ is indicative of synergism; $\mathrm{CI} = 1$ indicates additivism and $\mathrm{CI} > 1$ denotes antagonism [44]. Values of $\mathrm{CI} = 1$–0.3; 0.3–0.7; 0.7–0.85; and 0.85–1 indicate strong

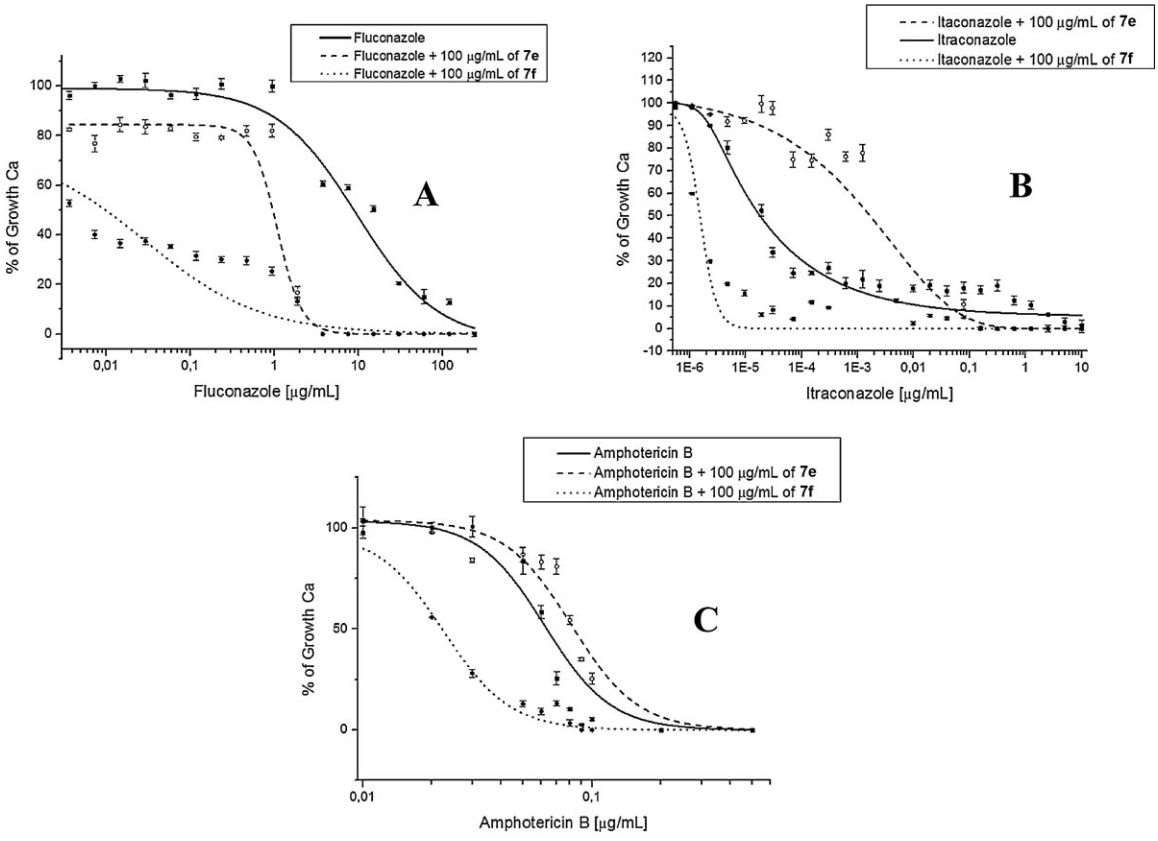

Figure 3. Dose–response curves of fluconazole (A), itraconazole (B), and amphotericin B (C) alone and in combination with 100 μg/mL of **7e** and **7f** against *C. albicans*. Results are expressed as mean ± SD ($n = 3$).

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Table 2. MIC_{50} values (μg/mL) of itraconazole (ITZ), fluconazole (FCZ), amphotericin B (AmB), **7e** or **7f** either alone or in two-drugs combination against *Candida albicans*.

Compound	MIC_{50}	CI	Effect	Potency	DRI of the AD
FCZ	2.730 ± 0.260				
ITZ	$1.780 \pm 0.30 \times 10^{-5}$				
AmB	0.071 ± 0.01				
7e	180 ± 0.61				
7f	200 ± 2.01				
FCZ + **7e**[a)]	0.972 ± 0.11	0.90	Synergistic	(+)	2.80
FCZ + **7f**[a)]	0.018 ± 0.01	0.50	Synergistic	(+++)	151.66
ITZ + **7e**[b)]	$164 \pm 0.15 \times 10^{-5}$	94.11	Antagonistic	(−−−−−)	−92
ITZ + **7f**[b)]	$0.15 \pm 0.00 \times 10^{-5}$	0.58	Synergistic	(+++)	11.86
AmB + **7e**[c)]	0.083 ± 0.003	1.71	Antagonistic	(−−−)	−1.16
AmB + **7f**[c)]	0.022 ± 0.001	0.80	Synergistic	(+++)	3.55

Data are represented as mean ± SD. The combination index (CI) and the dose reduction index (DRI) of each antifungal drug (AD) upon combination were calculated.
[a)] FCZ + 100 μg/mL of **7e** or **7f**.
[b)] ITZ + 100 μg/mL of **7e** or **7f**.
[c)] AmB + 100 μg/mL of **7e** or **7f**. Potency according to [44], (+) slight synergism; (++) moderate synergism; (+++) synergism; (++++) strong synergism; (+++++) very strong synergism; (−) slight antagonism; (−−) moderate antagonism; (−−−) antagonism; (−−−−) strong antagonism; (−−−−−) very strong antagonism.

synergism, synergism, moderate, or slight synergism, respectively. Instead, CI = 1.10–1.20; 1.20–1.45; 1.45–3.3; 3.3–10; or >10 indicate slight or moderate antagonism, antagonism, strong or very strong antagonism, respectively [45].

In addition, the dose-reduction index (DRI) is a measure of how many-fold the dose of each drug in a synergistic combination may be reduced at a given effect level, compared with the dose of each drug alone [45]. The DRI of each antifungal drug was calculated as follows (Eq. 2):

$$\mathrm{DRI} = \frac{MIC_{50}\ \text{of AD alone}}{C_{50}\ \text{of AD in combination}} \quad (2)$$

The DRI is important to visualize the extent of reduction of the dose of each partner in the mixture that would concomitantly lead to a reduced toxicity to the host while the therapeutic efficacy is retained or enhanced. However, although a DRI > 1 or higher indicates dose-reduction for a given therapeutic effect, it does not necessarily indicate synergism which, as explained above, is determined with the CI values. In Table 2, MIC_{50} values of each antifungal drug (FCZ, ITZ, or AmB) alone or in dual combinations with **7e** and **7f**, along with the CI and the DRI are showed.

Regarding the mixtures of FCZ with **7e** and **7f**, it could be observed in Table 2, columns 2 and 3, that MIC_{50} of FCZ decreased 151-fold (from 2.73 to 0.018 μg/mL) and 2.8-fold (from 2.73 to 0.97 μg/mL) when combined with **7f** and **7e**, respectively, being the CI values 0.50 (synergism) and 0.90 (slight synergism) (Table 2, columns 4–6). It is interesting to note that the DRI is very high (151.66) for the mixture with **7f**, which, along with the CI, pointed out that a very low dose of FCZ forms synergistic antifungal mixtures against *C. albicans*.

Regarding the mixtures with ITZ and AmB, **7e** showed very strong antagonism (CI = 94) or antagonism (CI = 1.71), respectively, while **7f** showed synergism (CI = 0.58) with ITZ and moderate synergism (CI = 0.80) with AmB. DRI values were 11.86 and 3.55 for the mixtures with **7f**, this compound showing again that it should be a good candidate for future research in the field of antifungal compounds.

In summary, these results demonstrated that the pyrazole-derived compounds synthesized for the first time here, could constitute alternatives for future developments in antifungal therapy as binary mixtures with an antifungal drug. In addition, it is worth to take into account that both compounds (**7e** and **7f**) that showed synergism with the tested antifungal drugs possess at least one substituent OCH_3 on their aromatic ring. This structural feature appears to be very important not only because it is present in both active compounds but because the highest synergism was shown by **7f**, which possesses three OCH_3 on the aromatic ring, reinforcing the importance of this group in the enhancement of antifungal activity of FCZ, ITZ, or AmB against *C. albicans*.

Conclusions

We performed the synthesis under mild reaction conditions, short reaction times and good yields of novel *N*-acetyl and *N*-formyl-4,5-dihydro-1*H*-pyrazoles **7a–f** and **8a–f** from α,β-unsaturated carbonyl compounds containing the thiazole

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ring **5a–f**. All compounds were tested for antifungal activity against *C. albicans* and *C. neoformans* and, of them, **7e** and **7f** showed the best activities, although moderate, when tested alone. However, when **7e** and **7f** were tested in combination with the antifungal agents FCZ, ITZ, or AmB, **7e** showed a synergistic effect with FCZ against *C. albicans* and **7f** displayed synergistic effects with all the tested antifungal agents against the same yeast. These relevant findings open new avenues for a future deepening of the antifungal properties of these novel compounds in combination with antifungal drugs.

Experimental

Reagents and solvents used were obtained from commercial sources. Melting points were measured using a Stuart SMP3 melting point device and are uncorrected. IR spectra were obtained with a Shimadzu IRAffinity-1. The ^{1}H and ^{13}C NMR spectra were run on a Bruker DPX 400 spectrometer operating at 400 and 100 MHz, respectively, using $CDCl_3$ as solvent and TMS as internal standard. The mass spectrum was obtained on a Shimadzu-GCMS-QP2010 spectrometer operating at 70 eV. Thin layer chromatography (TLC) was performed on 0.2-mm pre-coated plates of silica gel 60GF254 (Merck). The elemental analyses were obtained using a Thermo Finnigan Flash EA1112 CHN (STIUJA) elemental analyzer.

Chemistry

*General procedure for the synthesis of compounds **7a–f** and **8a–f***

A mixture of 2 mmol of aldehyde **3** (prepared previously by the methodology reported by Kotlyar et al. [39]), 2 mmol of substituted acetophenone **4a–f** and 20% NaOH solution (3 mL) in ethanol (20 mL) was stirred during 8 h at room temperature. The precipitate formed was filtered and washed with ethanol to obtain the compounds **5a–f** as pure yellow solids. Subsequently, a mixture of 1 mmol of compounds **5a–f** and 3 mmol of hydrazine hydrate in ethanol (5 mL) was stirred during 1 h at room temperature. Reaction progress was monitored by TLC and the precipitate formed was filtered and washed with water to obtain the compounds **6a–f**, which decomposed rapidly, then, 0.3 mmol of compounds **6a–f** was stirred during 1 h at room temperature in the presence of Ac_2O (1 mL) or HCOOH (1 mL) both as reagents and solvents. Water was added to reaction mixture and the precipitate formed was filtered and washed with ethanol/water 1:1 to obtain compounds **7a–f** and **8a–f** as white solids, without further purification process.

*1-Acetyl-5-(4-chloro-2-morpholinothiazol-5-yl)-3-phenyl-4,5-dihydro-1H-pyrazole (**7a**)*

White solid; 84% yield; m.p. 165–166°C; FT-IR (KBr), ν (cm^{-1}): 1673 (C=O), 1595 and 1525 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.40 (s, 3H, CH_3), 3.26 (dd, $J_{AX} = 4.7$ Hz, $J_{AM} = 17.7$ Hz, 1H, H-4_A), 3.38–3.42 (m, 4H, CH_2–N–CH_2), 3.68–3.76 (m, 5H, H-4_M, CH_2–O–CH_2), 5.78 (dd, $J_{AX} = 4.7$ Hz, $J_{MX} = 11.7$ Hz, 1H, H-5_X), 7.43–7.47 (m, 3H, H_m, H_p), 7.75 (dd, $J = 2.9$ Hz, $J = 6.6$ Hz, 2H, H_o) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8 (CH_3), 40.8 (C-4), 47.7 (CH_2–N–CH_2), 53.1 (C-5), 65.9 (CH_2–O–CH_2), 117.9 (C-5′), 126.6 (C_o), 128.8 (C_m), 130.5 (C_p), 131.1 (C_i), 132.2 (C-4′), 153.8 (C-3), 167.5 (C-2′), 168.9 (C=O) ppm; MS (IE, 70 eV) *m*/*z* (%): 392/390 [M^+] (8/20), 355 (15), 313 (14), 287 (35), 252 (100), 130 (45), 83 (60). Anal. calcd. for $C_{18}H_{19}ClN_4O_2S$: C, 55.31; H, 4.90; N, 14.33. Found: C, 55.36; H, 5.01; N, 14.37.

*1-Acetyl-3-(4-chlorophenyl)-5-(4-chloro-2-morpholinothiazol-5-yl)-4,5-dihydro-1H-pyrazole (**7b**)*

White solid; 86% yield; m.p. 179–180°C; FT-IR (KBr), ν (cm^{-1}): 1672 (C=O), 1595 and 1525 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.40 (s, 3H, CH_3), 3.24 (dd, $J_{AX} = 4.7$ Hz, $J_{AM} = 17.7$ Hz, 1H, H-4_A), 3.39–3.41 (m, 4H, CH_2–N–CH_2), 3.70 (dd, $J_{MX} = 11.8$ Hz, $J_{AM} = 17.7$ Hz, 1H, H-4_M), 3.75–3.77 (m, 4H, CH_2–O–CH_2), 5.78 (dd, $J_{AX} = 4.7$ Hz, $J_{MX} = 11.8$ Hz, 1H, H-5_X), 7.43 (d, $J = 8.5$ Hz, 2H, H_m), 7.69 (d, $J = 8.5$ Hz, 2H, H_o) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8 (CH_3), 40.6 (C-4), 47.6 (CH_2–N–CH_2), 53.2 (C-5), 65.9 (CH_2–O–CH_2), 117.6 (C-5′), 127.8 (C_o), 129.0 (C_m), 129.6 (C_i), 132.3 (C-4′), 136.5 (C_p), 152.6 (C-3), 167.5 (C-2′), 168.9 (C=O) ppm; MS (IE, 70 eV) *m*/*z* (%): 428/426/424 [M^+] (2/10/16), 389 (13), 347 (20), 287 (33), 252 (100), 130 (43), 83 (97). Anal. calcd. for $C_{18}H_{18}Cl_2N_4O_2S$: C, 50.83; H, 4.27; N, 13.17. Found: C, 50.89; H, 4.30; N, 13.28.

*1-Acetyl-5-(4-chloro-2-morpholinothiazol-5-yl)-3-(4-fluorophenyl)-4,5-dihydro-1H-pyrazole (**7c**)*

White solid; 71% yield; m.p. 154–155°C; FT-IR (KBr), ν (cm^{-1}): 1674 (C=O), 1598 and 1523 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.39 (s, 3H, CH_3), 3.23 (dd, $J_{AX} = 4.7$ Hz, $J_{AM} = 17.7$ Hz, 1H, H-4_A), 3.35–3.43 (m, 4H, CH_2–N–CH_2), 3.64–3.78 (m, 5H, H-4_M, CH_2–O–CH_2), 5.78 (dd, $J_{AX} = 4.7$ Hz, $J_{MX} = 11.7$ Hz, 1H, H-5_X), 7.11–7.15 (m, 2H, H_m), 7.74 (dd, $^{3}J_{HF} = 4.6$ Hz, $^{3}J = 8.0$ Hz 2H, H_o) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8 (CH_3), 40.7 (C-4), 47.6 (CH_2–N–CH_2), 53.1 (C-5), 65.9 (CH_2–O–CH_2), 115.9 (d, $^{2}J_{CF} = 22.0$ Hz, C_m), 117.7 (C-5′), 127.3 (d, $^{4}J_{CF} = 3.5$ Hz C_i), 128.5 (d, $^{3}J_{CF} = 8.5$ Hz C_o), 132.6 (C-4′), 152.7 (C-3), 164.0 (d, $^{1}J_{CF} = 251$ Hz, C_p), 167.5 (C-2′), 168.8 (C=O) ppm; MS (IE, 70 eV) *m*/*z* (%): 410/408 [M^+] (8/22), 373 (19), 331 (26), 287 (35), 252 (100), 189 (14), 83 (31). Anal. calcd. for $C_{18}H_{19}FClN_4O_2S$: C, 52.87; H, 4.44; N, 13.70. Found: C, 52.90; H, 4.51; N, 13.82.

*1-Acetyl-5-(4-chloro-2-morpholinothiazol-5-yl)-3-(4-methylphenyl)-4,5-dihydro-1H-pyrazole (**7d**)*

White solid; 67% yield; m.p.191–192°C; FT-IR (KBr), ν (cm^{-1}): 1672 (C=O), 1596 and 1524 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.41 (s, 3H, CH_3), 2.43 (s, 3H, $ArCH_3$), 3.24 (dd, $J_{AX} = 4.6$ Hz, $J_{AM} = 17.7$ Hz, 1H, H-4_A), 3.35–3.45 (m, 4H, CH_2–N–CH_2), 3.64–3.82 (m, 5H, H-4_M, CH_2–O–CH_2), 5.77 (dd, $J_{AX} = 4.6$ Hz, $J_{MX} = 11.7$ Hz, 1H, H-5_X), 7.26 (d, $J = 8.1$ Hz, 2H, H_o), 7.65 (d, $J = 8.1$ Hz, 2H, H_m) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8 (CH_3), 21.9 ($ArCH_3$), 40.8 (C-4), 47.6 (CH_2–N–CH_2), 53.0 (C-5), 65.9 (CH_2–O–CH_2), 118.3 (C-5′), 126.5 (C_o), 128.3 (C_i), 129.5 (C_m), 132.1 (C-4′), 140.9 (C_p), 153.8 (C-3), 167.5 (C-2′), 168.9 (C=O) ppm; MS (IE, 70 eV) *m*/*z* (%): 406/404 [M^+] (3/8), 369 (8), 327 (10), 287 (12), 252 (46), 159 (56), 83 (100). Anal. calcd. for $C_{19}H_{21}ClN_4O_2S$: C, 56.36; H, 5.23; N, 13.84. Found: C, 56.40; H, 5.28; N, 13.83.

*1-Acetyl-5-(4-chloro-2-morpholinothiazol-5-yl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazole (**7e**)*

White solid; 62% yield; m.p. 181–182°C; FT-IR (KBr), ν (cm^{-1}): 1671 (C=O), 1595 and 1520 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.39 (s, 3H, CH_3), 3.22 (dd, $J_{AX} = 4.6$ Hz, $J_{AM} = 17.6$ Hz, 1H, H-4_A),

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3.34–3.43 (m, 4H, CH_2–N–CH_2), 3.68 (dd, $J_{MX} = 11.6$ Hz, $J_{AM} = 17.6$ Hz, 1H, H-4_M), 3.73–3.80 (m, 4H, CH_2–O–CH_2), 3.87 (s, 3H, OCH_3), 5.76 (dd, $J_{AX} = 4.6$ Hz, $J_{MX} = 11.6$ Hz, 1H, H-5_X), 6.96 (d, $J = 8.7$ Hz, 2H, H_o), 7.69 (d, $J = 8.7$ Hz, 2H, H_m) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8 (CH_3), 40.8 (C-4), 47.6 (CH_2–N–CH_2), 53.0 (C-5), 55.4 (OCH_3), 65.9 (CH_2–O–CH_2), 114.2 (C_o), 118.3 (C-5′), 123.7 (C_i), 128.2 (C_m), 132.1 (C-4′), 153.8 (C-3), 161.5 (C_p), 167.5 (C-2′), 168.7 (C=O) ppm; MS (IE, 70 eV) *m/z* (%): 422/420 [M^+] (4/10), 341 (5), 256 (9), 149 (30), 137 (40), 83 (100). Anal. calcd. for $C_{19}H_{21}ClN_4O_3S$: C, 54.22; H, 5.03; N, 13.31. Found: C, 54.27; H, 5.04; N, 13.32.

*1-Acetyl-5-(4-chloro-2-morpholinothiazol-5-yl)-3-(3,4,5-trimethoxylphenyl)-4,5-dihydro-1H-pyrazole (**7f**)*

White solid; 60% yield; m.p. 182–183 °C; FT-IR (KBr), ν (cm^{-1}): 1673 (C=O), 1595 and 1525 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.41 (s, 3H, $-CH_3$), 3.24 (dd, $J_{AX} = 4.7$ Hz, $J_{AM} = 17.6$ Hz, 1H, H-4_A), 3.35–3.43 (m, 4H, CH_2–N–CH_2), 3.64–3.83 (m, 5H, H-4_M, CH_2–O–CH_2), 3.91 (s, 3H, *p*OCH_3), 3.93 (s, 6H, *m*OCH_3), 5.78 (dd, $J_{AX} = 4.6$ Hz, $J_{MX} = 11.7$ Hz, 1H, H-5_X), 6.96 (s, 2H, H_o) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.9 (CH_3), 40.8 (C-4), 47.6 (CH_2–N–CH_2), 53.1 (C-5), 56.3 (*m*OCH_3), 60.1 (*p*OCH_3), 65.9 (CH_2–O–CH_2), 103.9 (C_o), 117.9 (C-5′), 126.4 (C_i), 132.1 (C-4′), 136.4 (C_m), 140.4 (C_p), 153.5 (C-3), 167.5 (C-2′), 168.2 (C=O) ppm; MS (IE, 70 eV) *m/z* (%): 482/480 [M^+] (12/32), 445 (35), 403 (22), 287 (30), 256 (98), 173 (11), 130 (100). Anal. calcd. for $C_{21}H_{25}ClN_4O_5S$: C, 52.44; H, 5.24; N, 11.65. Found: C, 52.51; H, 5.32; N, 11.70.

*5-(4-Chloro-2-morpholinothiazol-5-yl)-1-formyl-3-phenyl-4,5-dihydro-1H-pyrazole (**8a**)*

White solid; 66% yield; m.p. 171–172 °C; FT-IR (KBr), ν (cm^{-1}): 1678 (C=O), 1597 and 1528 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.30 (dd, $J_{AX} = 4.8$ Hz, $J_{AM} = 17.8$ Hz, 1H, H-4_A), 3.36–3.45 (m, 4H, CH_2–N–CH_2), 3.69–3.86 (m, 5H, H-4_M, CH_2–O–CH_2), 5.76 (dd, $J_{AX} = 4.8$ Hz, $J_{MX} = 11.6$ Hz, 1H, H-5_X), 7.46–7.47 (m, 3H, H_m, H_p), 7.75 (d, $J = 7.5$ Hz, 2H, H_o), 8.91 (s, 1H, CHO) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 42.4 (C-4), 49.1 (CH_2–N–CH_2), 53.6 (C-5), 67.3 (CH_2–O–CH_2), 117.9 (C-5′), 128.2 (C_o), 130.3 (C_m), 132.0 (C_p), 132.3 (C_i), 134.4 (C-4′), 156.9 (C-3), 161.4 (C=O), 168.9 (C-2′) ppm; MS (IE, 70 eV) *m/z* (%): 378/376 [M^+] (25/63), 341 (34), 313 (23), 298 (22), 273 (40), 256 (43), 238 (51), 230 (22), 173 (51), 145 (58), 130 (65), 86 (100). Anal. calcd. for $C_{17}H_{17}ClN_4O_2S$: C, 54.18; H, 4.55; N, 14.87. Found: C, 54.20; H, 4.56; N, 14.70.

*5-(4-Chloro-2-morpholinothiazol-5-yl)-3-(4-chlorophenyl)-1-formyl-4,5-dihydro-1H-pyrazole (**8b**)*

White solid; 72% yield; m.p. 186–187 °C; FT-IR (KBr), ν (cm^{-1}): 1679 (C=O), 1596 and 1529 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.26 (dd, $J_{AX} = 4.9$ Hz, $J_{AM} = 17.8$ Hz, 1H, H-4_A), 3.36–3.43 (m, 4H, CH_2–N–CH_2), 3.67–3.85 (m, 5H, H-4_M, CH_2–O–CH_2), 5.75 (dd, $J_{AX} = 4.9$ Hz, $J_{MX} = 11.6$ Hz, 1H, H-5_X), 7.43 (d, $J = 8.6$ Hz, 2H, H_m), 7.68 (d, $J = 8.6$ Hz, 2H, H_o), 8.91 (s, 1H, CHO) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 40.9 (C-4), 47.7 (CH_2–N–CH_2), 52.3 (C-5), 65.9 (CH_2–O–CH_2), 116.2 (C-5′), 127.9 (C_o), 129.1 (C_i), 129.2 (C_m), 133.1 (C-4′), 136.8 (C_p), 154.4 (C-3), 159.9 (C=O), 167.7 (C-2′) ppm; MS (IE, 70 eV) *m/z* (%): 414/412/410 [M^+] (9/47/65), 375 (37), 347 (22), 332 (24), 290 (35), 273 (57), 238 (62), 230 (54), 173 (41), 130 (71), 86 (100). Anal. calcd. for $C_{17}H_{16}Cl_2N_4O_2S$: C, 49.64; H, 3.92; N, 13.62. Found: C, 49.71; H, 4.01; N, 13.65.

*5-(4-Chloro-2-morpholinothiazol-5-yl)-3-(4-fluorophenyl)-1-formyl-4,5-dihydro-1H-pyrazole (**8c**)*

White solid; 54% yield; m.p. 160–161 °C; FT-IR (KBr), ν (cm^{-1}): 1680 (C=O), 1596 and 1528 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.27 (dd, $J_{AX} = 4.9$ Hz, $J_{AM} = 17.8$ Hz, 1H, H-4_A), 3.37–3.44 (m, 4H, CH_2–N–CH_2), 3.69–3.83 (m, 5H, H-4_M, CH_2–O–CH_2), 5.75 (dd, $J_{AX} = 4.9$ Hz, $J_{MX} = 11.7$ Hz, 1H, H-5_X), 7.11–7.19 (m, 2H, H_m), 7.75 (dd, $^3J_{HF} = 4.9$ Hz, $^3J = 8.8$ Hz 2H, H_o), 8.90 (s, 1H, CHO) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 41.0 (C-4), 47.6 (CH_2–N–CH_2), 52.2 (C-5), 65.9 (CH_2–O–CH_2), 116.1 (d, $^2J_{CF} = 22.1$ Hz, C_m), 116.3 (C-5′), 126.9 (d, $^4J_{CF} = 3.5$ Hz, C_i), 128.7 (d, $^3J_{CF} = 8.5$ Hz, C_o), 133.0 (C-4′), 154.4 (C-3), 159.9 (C=O), 164.2 (d, $^1J_{CF} = 252.2$ Hz, C_p), 167.5 (C-2′) ppm; MS (IE, 70 eV) *m/z* (%): 396/394 [M^+] (10/24), 273 (28), 257 (20), 236 (40), 185 (40), 163 (60), 86 (100). Anal. calcd. for $C_{17}H_{16}FClN_4O_2S$: C, 51.71; H, 4.08; N, 14.19. Found: C, 51.73; H, 4.02; N, 14.30.

*5-(4-Chloro-2-morpholinothiazol-5-yl)-1-formyl-3-(4-methylphenyl)-4,5-dihydro-1H-pyrazole (**8d**)*

White solid; 60% yield; m.p. 177–178 °C; FT-IR (KBr), ν (cm^{-1}): 1677 (C=O), 1596 and 1527 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.43 (s, 3H, $ArCH_3$), 3.28 (dd, $J_{AX} = 4.8$ Hz, $J_{AM} = 17.8$ Hz, 1H, H-4_A), 3.38–3.44 (m, 4H, CH_2–N–CH_2), 3.71–3.83 (m, 5H, H-4_M, CH_2–O–CH_2), 5.75 (dd, $J_{AX} = 4.8$ Hz, $J_{MX} = 11.4$ Hz, 1H, H-5_X), 7.28 (d, $J = 8.1$ Hz, 2H, H_o), 7.65 (d, $J = 8.1$ Hz, 2H, H_m), 8.92 (s, 1H, CHO) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.5 ($ArCH_3$), 41.1 (C-4), 47.7 (CH_2–N–CH_2), 52.1 (C-5), 65.9 (CH_2–O–CH_2), 116.2 (C-5′), 126.8 (C_o), 127.8 (C_i), 129.6 (C_m), 132.9 (C-4′), 141.3 (C_p), 155.6 (C-3), 159.92 (C=O), 167.5 (C-2′) ppm; MS (IE, 70 eV) *m/z* (%): 392/390 [M^+] (4/10), 355 (7), 256 (8), 185 (14), 159 (17), 130 (24), 86 (100). Anal. calcd. for $C_{18}H_{19}ClN_4O_2S$: C, 55.31; H, 4.90; N, 14.33. Found: C, 55.42; H, 4.99; N, 14.31.

*5-(4-Chloro-2-morpholinothiazol-5-yl)-1-formyl-3-(4-methoxylphenyl)-4,5-dihydro-1H-pyrazole (**8e**)*

White solid; 59% yield; m.p. 213–214 °C; FT-IR (KBr), ν (cm^{-1}): 1673 (C=O), 1596 and 1527 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.25 (dd, $J_{AX} = 4.9$ Hz, $J_{AM} = 17.7$ Hz, 1H, H-4_A), 3.35–3.43 (m, 4H, CH_2–N–CH_2), 3.66–3.82 (m, 5H, H-4_M, CH_2–O–CH_2), 3.87 (s, 3H, OCH_3), 5.73 (dd, $J_{AX} = 4.9$ Hz, $J_{MX} = 10.8$ Hz, 1H, H-5_X), 6.96 (d, $J = 8.9$ Hz, 2H, H_o), 7.68 (d, $J = 8.9$ Hz, 2H, H_m), 8.89 (s, 1H, CHO) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 41.1 (C-4), 47.6 (CH_2–N–CH_2), 52.0 (C-5), 55.4 (OCH_3), 65.9 (CH_2–O–CH_2), 114.3 (C_o), 116.8 (C-5′), 123.2 (C_i), 128.3 (C_m), 132.8 (C-4′), 155.3 (C-3), 159.8 (C=O), 161.7 (C_p), 167.7 (C-2′) ppm; MS (IE, 70 eV) *m/z* (%): 408/406 [M^+] (28/75), 371 (53), 293 (69), 265 (100), 230 (43), 175 (39), 130 (65), 86 (71). Anal. calcd. for $C_{18}H_{19}ClN_4O_3S$: C, 53.13; H, 4.71; N, 13.77. Found: C, 53.17; H, 4.77; N, 13.89.

*5-(4-Chloro-2-morpholinothiazol-5-yl)-1-formyl-3-(3,4,5-trimethoxylphenyl)-4,5-dihydro-1H-pyrazole (**8f**)*

White solid; 56% yield; m.p. 223–224 °C; FT-IR (KBr), ν (cm^{-1}): 1677 (C=O), 1596 and 1524 (C=N and C=C). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.28 (dd, $J_{AX} = 4.7$ Hz, $J_{AM} = 17.6$ Hz, 1H, H-4_A), 3.34–3.49 (m, 4H, CH_2–N–CH_2), 3.67–3.85 (m, 5H, H-4_M, CH_2–O–CH_2), 3.92 (s, 3H, *p*OCH_3), 3.93 (s, 6H, *m*OCH_3), 5.77 (dd, $J_{AX} = 4.7$ Hz, $J_{MX} = 11.5$ Hz, 1H, H-5_X), 6.97 (s, 2H, H_o), 8.91 (s, 1H, CHO) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 41.4 (C-4), 47.7 (CH_2–N–CH_2), 52.3 (C-5), 56.3 (*m*OCH_3), 60.0 (*p*OCH_3), 65.9 (CH_2–O–CH_2), 104.0 (C_o), 116.4 (C-5′), 125.9 (C_i), 133.0 (C-4′), 140.6 (C_p), 153.5 (C-3), 155.2 (C_m), 159.8

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(C=O), 167.5 (C-2′) ppm; MS (IE, 70 eV) *m/z* (%):468/466 [M^+] (37/89), 431 (46), 388 (30), 346 (18), 273 (23), 130 (41), 86 (100). Anal. calcd. for $C_{20}H_{23}ClN_4O_5S$ (466.10): C, 51.44; H, 4.96; N, 12.00. Found: C, 51.48; H, 4.99; N, 12.24.

Biological evaluation

Antifungal activity

Fungal strains and inoculum preparation: For the antifungal evaluation, *C. albicans* from the American Type Culture Collection (ATCC) ATCC 10231 and *C. neoformans* ATCC 32264 were used. The strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30°C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid), and sub-cultured every 15-day to prevent pleomorphic transformations. Each inoculum of fungal cells was obtained according to reported procedures and adjusted to $1–5 \times 10^3$ cells with colony forming units (CFU)/mL [39].

High-throughput synergy screening assay (HTSS): Mixture test wells (MTWs) were prepared in microtiter trays (Greiner Bio-One Monroe, North Carolina) each one with a sub-inhibitory concentration of antifungal drug (that produced $\leq$20% inhibition of fungal growth) in DMSO (maximum concentration $\leq$ 1%), and a sub-inhibitory concentration of each of the testing compounds **7a–f** and **8a–f** (100 μg/mL). Inoculum suspension (100 μL) of either *C. albicans* or *C. neoformans* was added to each well (total 72 combinations, 36 for each fungal species). The final volume in the well was 200 μL. A growth control well (GCW) (containing medium, inoculum, the same amount of DMSO used in MTW, but mixture-free) and a sterility control well (SCW) (sample, medium, and sterile water instead of inoculum) were included in the plate for each fungus tested. Microtiter trays were incubated in a moist, dark chamber 24 or 48 h at 30°C for *C. albicans* or *C. neoformans*, respectively. Microplates were read in a VERSAMAX microplate reader (Molecular Devices, Sunnyvale, CA, USA) and the % of inhibition for each compound concentration was calculated as follows: $100 - (OD_{405}\ MTW - OD_{405}\ SCW)/(OD_{405}\ GCW - OD_{405}\ SCW)$. Tests were made in triplicate.

Statistical analysis

The effective enhancement of inhibition growth of each mixture was compared to the inhibition growth of each component alone at the same concentration as it was in mixture, by using the two-way ANOVA test for analyses of variance followed by least significant difference (LSD) of multiple comparisons; $p < 0.05$ was considered significant.

Dose–response curves for AD alone against C. albicans

In a 96-well microplate (Greiner Bio-One), serial dilutions of AmB (0.001–100 μg/mL), FCZ (1×10^{-4}–100 μg/mL), or ITZ (1×10^{-6}–10 μg/mL) were seeded with an inoculum of *C. albicans* quantified as explained above. After 24 h incubation, the inhibition percentage of fungal growth of each well was determined as above and, with these data, a dose-response curve calculated with a four-parameter logistic curve (SigmaPlot® 11.0) for each AD was constructed. From each curve, the MIC_{50} value (defined as the concentration of test compounds required to inhibit 50% of the fungal growth) of each AD alone was determined. All assays were carried out in triplicate. All data are expressed as mean ± SD.

Two-drug combination of antifungal drugs with 7e and 7f against C. albicans

Non-inhibitory concentrations of **7e** and **7f** (100 μg/mL) were combined with the same serial dilutions of AmB, FCZ, or ITZ as those used for the dose-response curve of the drug alone (see previous section) in a 96-well microplate (Greiner) to determine whether a dual combination of AD with **7e** or **7f** have additive, synergistic, or antagonistic interactions. An inoculum of *C. albicans* (1×10^3 CFU/mL) was seeded in each well and, after 24 h incubation, a new dose-response curve for each mixture was constructed and the MIC_{50} value of each mixture was determined. All assays were carried out in triplicate. All data are expressed as mean ± SD.

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