Bioorganic & Medicinal Chemistry 16 (2008) 8172–8177

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Antifungal activity of synthetic di(hetero)arylamines based on the benzo[*b*]thiophene moiety

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ARTICLE INFO

Article history:
Received 16 June 2008
Accepted 16 July 2008
Available online 20 July 2008

Keywords:
Di(hetero)arylamine
Benzo[*b*]thiophene
Antifungal activity
Flow cytometry
Germ tubes
SARs

ABSTRACT

The antifungal activity of several di(hetero)arylamine derivatives of the benzo[*b*]thiophene system was evaluated against clinically relevant *Candida*, *Aspergillus*, and dermatophyte species by a broth macrodilution test based on CLSI (formerly NCCLS) guidelines. The most active compound showed a broad spectrum of activity (against all tested fungal strains, including fluconazole-resistant fungi), with particularly low MICs for dermatophytes. Results from the inhibition of the dimorphic transition in *Candida albicans* and flow cytometry studies further confirmed their biological activity. With this study it was possible to establish some structure–activity relationships (SARs). The hydroxy groups proved to be essential for the activity in the aryl derivatives. Furthermore, the spectrum of activity in the pyridine derivatives was broadened by the absence of the ester group on position 2 of the benzo[*b*]thiophene system.

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1. Introduction

The incidence and severity of fungal diseases has increased recently, particularly in patients with impaired immunity.[1] The growing number of cases of fungi implicated in sepsis has been shown to be a consistent trend[2] and *Candida* is the third or fourth most common isolate in nosocomial bloodstream infections in the United States.[3] Furthermore, invasive candidosis is a problem of growing importance in critically ill non-immunosuppressed patients as well.[4] Besides that the mortality rate due to invasive aspergillosis has increased by 357% between 1980 and 1997 in the United States[5] and superficial mycoses, dermatophytosis (tineas), and candidosis, often recurrent, are an important cause of morbidity and are more severe in immunocompromised patients.

Together with this increase in the clinical importance of fungal infections, the small number of available antifungals, often showing only fungistatic activity, and the increase in antifungal resistance have been stimulating the search for new drugs, which are more effective and less toxic than those already in use.[6]

The diarylamine skeleton and the benzo[*b*]thiophene system[7] are often present in biologically active compounds and many examples of biological activities found for small molecules based on the benzo[*b*]thiophene moiety can be referred. Namely, they can be inhibitors of herpes simplex virus type I (HSV-1) replication, antimitotics, inhibitors of cysteine and serine proteases (importantly, thrombin), opioid receptor analgesics, and 5-HT_6 antagonists, making this a very attractive structure for medicinal chemists.[8] Its potential has, actually, already been materialized through the development of marketed drugs, like the anti-asthma drug zileuton[9] and raloxifene, a non-hormonal drug showing estrogen agonist effects on the bone and the cardiovascular system and estrogen antagonist effects on endometrial and breast tissues.[10] Benzo[*b*]thiophene is also a structural part of the commercial imidazole antifungal agent sertaconazole, an antimycotic with applications in dermatology and gynecology.[11]

For some years now we have been interested in the functionalization of either the benzene or the thiophene ring of the benzo[*b*]thiophene system, using palladium-catalyzed C–N cross-couplings to obtain the corresponding diarylamines,[12,13] and in the study of their potential antimicrobial activity.[13,14]

In this work, we present the synthesis of two new compounds and the screening of several di(hetero)arylamine derivatives of benzo[*b*]thiophenes as potential antifungal agents against dermatophytes, yeasts, and *Aspergillus* species with clinical relevance, using the macrodilution broth method. The antifungal activity was also evaluated against fluconazole-resistant *Candida albicans* strains, *Candida krusei*, *Candida glabrata*, and *Aspergillus fumigatus*, which are intrinsically resistant to fluconazole or whose resistance is easily inducible. For the compounds showing antifungal activity

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doi:10.1016/j.bmc.2008.07.042

against *C. albicans*, we have conducted a study of the effect upon germ tube formation, an important virulence factor in this yeast.[15] Further investigations by flow cytometry, using the fluorescent dyes PI (propidium iodide) and FUN-1, were also conducted. With the battery of compounds available, it was tried to establish some structure–activity relationships (SARs).

2. Results and discussion

2.1. Chemistry

The di(hetero)arylamines **1**, **3**, **4**, and **6** had previously been prepared by palladium-catalyzed C–N cross-coupling, as had the diarylamine **2**, in this case by demethylation of compound **1** using BBr_3 (Scheme 1).[13] Here, we present the synthesis of compound **5** by demethylation of compound **4** and of the diheteroarylamine **7** by palladium-catalyzed C–N cross-coupling of 3-bromobenzo[*b*]-thiophene and 3-aminopyridine under the conditions required for the coupling of heteroarylamines[13] (Scheme 1). The synthesis of diarylamines **5** and **7** allowed the enlargement of the SARs study.

2.2. Antifungal activity

Compounds **1**–**7** were screened for antifungal activity against clinical isolates and reference strains of *Candida* species (*C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis*), *Aspergillus* species (*A. fumigatus*, *A. niger*, and *A. flavus*), and dermatophytes (*Microsporum canis*, *Microsporum gypseum*, *Thichophyton mentagrophytes*, *Thichophyton rubrum, and Epidermophyton floccosum*). The MICs and MFCs of the tested and reference (amphotericin B and fluconazole) compounds are presented in Table 1.

No activity was detected against any of the tested strains for compounds **1**, **3**, and **4** at concentrations up to 200 μg/mL. Dermatophytes were found to be sensitive to compounds **2** and **6**, whereas these compounds exhibited no antifungal activity against *Aspergillus* and *Candida* species. Compounds **5** and **7** exhibited a larger spectrum of activity than compounds **2** and **6**, particularly compound **7**, additionally showing moderate activity against both *Candida* and *Aspergillus* species.

The presence of a free OH group (compound **2**) instead of an OMe group (compound **1**) is responsible for the antidermatophyte activity. The presence of two OH groups (compound **5**) broadens the spectrum of activity to include yeasts, but with higher MICs (25–50 μg/mL) than those for dermatophytes (6.25–12.5 μg/mL). The hydroxylated compounds **2** and **5** are actually fungicidal for dermatophytes, presenting the same or similar MICs and MFCs.

Comparing compounds **6** and **7** (the pyridine derivatives), it is possible to conclude that the absence of the ester group in the latter widens the spectrum of activity. Nevertheless, the MICs for dermatophytes remain lower (3.13–12.5 μg/mL) than those for *Candida* (50–100 μg/mL) and *Aspergillus* (25–50 μg/mL).

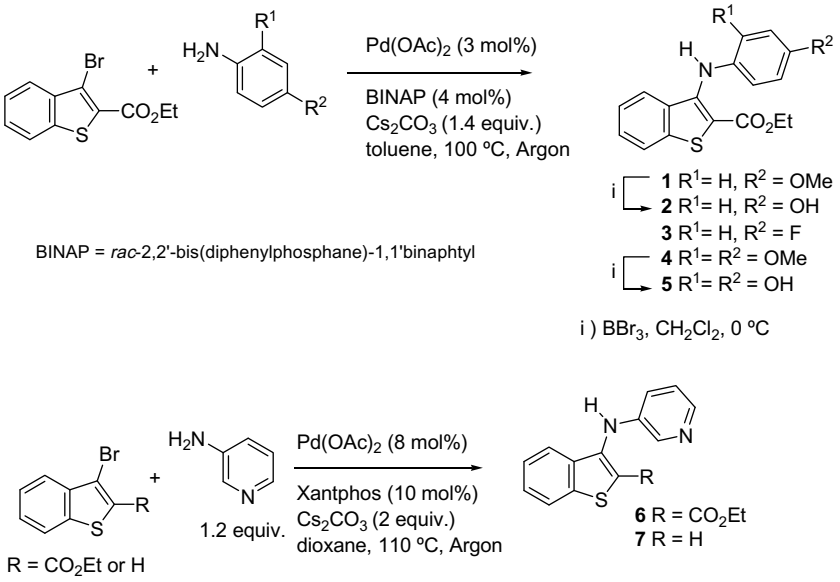
If compound **5** adds a moderate activity against the tested yeasts to its referred high activity against dermatophytes, compound **7** achieves the broadest spectrum of activity, including all tested pathogenic yeasts and moulds. It is, thus, active against fungi with decreased susceptibility to fluconazole, such as *C. krusei*, *C. glabrata*, and *Aspergillus* spp. The activity of these compounds was not affected by the fluconazole susceptibility profile of the tested strains.

2.3. Germ tube inhibition

Sub-inhibitory concentrations of compounds **5** and **7** strongly prevented germ tube formation, widely regarded as an important mechanism of pathogenicity in this yeast.[15] The highest concentration, half of the MIC, caused an almost complete inhibition in all tested strains (Fig. 1). *C. albicans* D5, the strain showing the lowest germ tube production in the controls, appears to be especially susceptible to this effect. In this strain, both compounds caused a virtually complete inhibition of the dimorphic transition even at concentrations four times lower than the respective MICs.

2.4. Flow cytometry studies

Regarding the flow cytometric results for *C. albicans* cells stained with FUN-1, the three highest test concentrations yielded over 90% of metabolically inactive cells, similarly to what happened to amphotericin B-treated cells (Fig. 2A). FUN-1 is a membrane-permeant fluorescent probe which is converted into orange/red cylindrical intra-vacuolar structures (CIVS) by metabolically active fungal cells, while remaining in the cytoplasm as a bright diffuse green/yellow stain in cells with impaired metabolism,[16] thus indicating the cells' metabolic vitality state. With the three test concentrations above 50 μg/mL resulting in over 90%



Scheme 1. Structures of the tested compounds **1**–**7**. Diarylamines **5** and **7** were synthesized and characterized in this work.

Table 1
Antifungal activity—MIC (MFC)—of seven tested compounds against yeasts and filamentous fungi (defined MIC values are shown in bold font)

Strains	Compounds/MIC (MFC) μg/mL (W/V)							Fluconazole	Amphotericin B
	1	**2**	**3**	**4**	**5**	**6**	**7**		
Yeasts									
ATCC									
Candida albicans ATCC 10231	>200 (>200)	>200 (>200)	>200 (>200)	>200 (>200)	**25–50** (>200)	>200 (>200)	**50** (**100**)	**1** (>128)	N.T.
C. krusei ATCC 6258	>200 (>200)	>200 (>200)	>200 (>200)	>200 (>200)	**25–50** (>50)	>200 (>200)	**100** (>100)	**64** (**64–128**)	N.T.
C. tropicalis ATCC 13803	>200 (>200)	>200 (>200)	>200 (>200)	>200 (>200)	**50** (>50)	>200 (>200)	**100** (>100)	**4** (>128)	N.T.
Clinical									
C. albicans D5	>200 (>200)	>200 (>200)	>200 (>200)	>200 (>200)	**25** (>50)	>200 (>200)	**50** (>50)	>128 (>128)	N.T.
C. glabrata D 10R	>200 (>200)	>200 (>200)	>200 (>200)	>200 (>200)	**25–50** (>50)	>200 (>200)	**50** (>50)	**32** (**32**)	N.T.
Filamentous fungi									
Dermatophytes									
Epidermophyton floccosum FF9	>200 (>200)	**3.13** (**12.5**)	>200 (>200)	>200 (>200)	**6.25–12.5** **(12.5)**	**6.25** (>50)	**12.5** (>**12.5**)	**16** (**16**)	N.T.
Trichophyton rubrum FF5	>200 (>200)	**6.25** (**6.25**)	>200 (>200)	>200 (>200)	**12.5–25** (**25**)	**3.13** (>50)	**3.13** (**12.5**)	**16–32** (**32**)	N.T.
T. mentagrophytes FF7	>200 (>200)	**6.25** (**6.25**)	>200 (>200)	>200 (>200)	**12.5** (**25**)	**3.13** (>50)	**12.5** (>**12.5**)	**16–32** (**32–64**)	N.T.
Microsporum canis FF1	>200 (>200)	**1.56** (**1.56**)	>200 (>200)	>200 (>200)	**12.5** (**12.5**)	**3.13** (>50)	**12.5** (>**12.5**)	**128** (**128**)	N.T.
M. gypseum FF3	>200 (>200)	**6.25** (**12.5**)	>200 (>200)	>200 (>200)	**12.5** (**25**)	**6.25** (>50)	**12.5** (>**12.5**)	>128 (>128)	N.T.
Aspergillus									
Aspergillus flavus F44	>200 (>200)	>200 (>200)	>200 (>200)	>200 (>200)	>100 (>100)	>200 (>200)	**50** (**100**)	N.T.	**2** (**8**)
A. fumigatus ATCC 46645	>200 (>200)	>200 (>200)	>200 (>200)	>200 (>200)	>100 (>100)	>200 (>200)	**25** (>50)	N.T.	**2** (**4**)
A. niger ATCC 16404	>200 (>200)	>200 (>200)	>200 (>200)	>200 (>200)	>100 (>100)	>200 (>200)	**50–100** (>100)	N.T.	**1–2** (**4**)

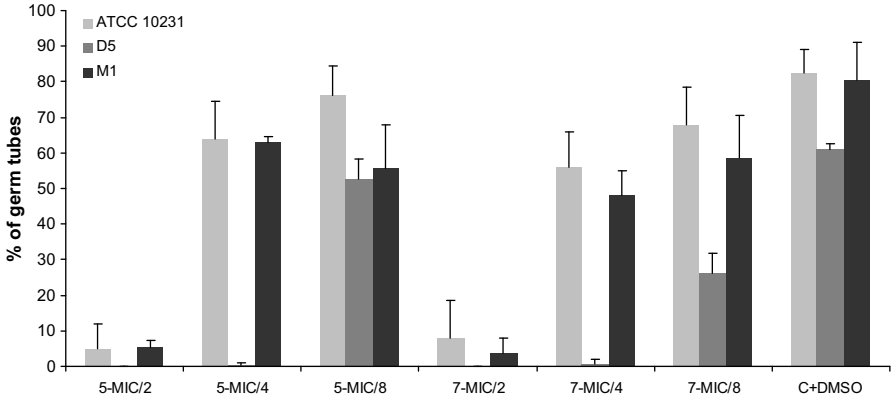


Figure 1. Percentage of positive germ tube bearing cells after treatment of *C. albicans* strains ATCC 10231, D5, and M1 with sub-inhibitory concentrations of compounds **5** and **7**.

of cells with impaired metabolism, manifested by a reduction in fluorescence in the FL-2 channel, these results show a strict correlation to the determined MIC. The concentration immediately below still yielded over 60% of cells with decreased CIVS formation in comparison to the control sample (Fig. 2A). Similar results were obtained for conidia of *A. fumigatus*, in which case a greater influence of lower tested concentrations (below the MIC) was found (Fig. 2A).

Concerning PI, after short incubation times of both *C. albicans* and *A. fumigatus* (a maximum of four hours), neither the test compound nor amphotericin B, in fungicidal concentrations, produced a shift of more than 40% of the cells to higher values of red fluorescence in comparison to the stained controls (data not shown). After 24 h, however, the two highest test concentrations of compound **7**, together with amphotericin B, led to the penetration of PI in over 80% of the *C. albicans* cells (Fig. 2B). Regarding *A. fumigatus* conidia,

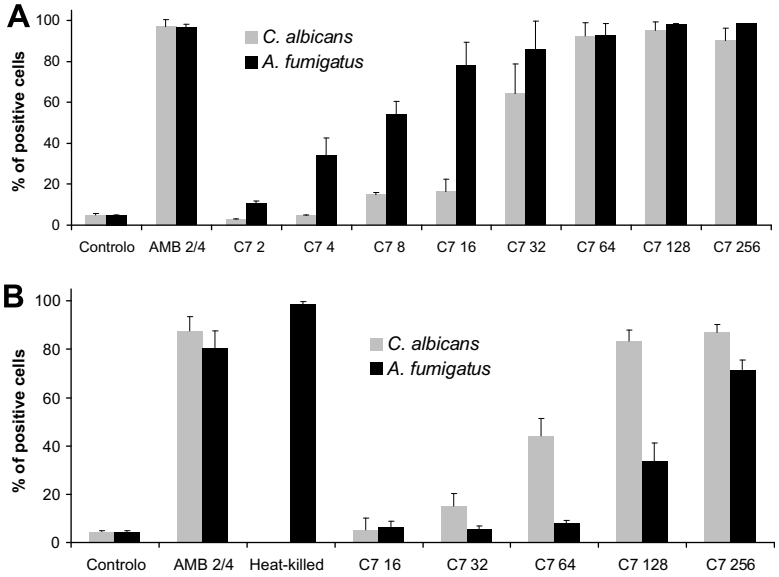


Figure 2. Flow cytometry results after treatment of *C. albicans* cells and *A. fumigatus* conidia with compound **7** and staining with the fluorescent probes FUN-1 (A) and PI (B).

similar results were obtained with the highest concentration of compound **7** and with the reference compound (Fig. 2B). A longer incubation time with both the test compound and amphotericin B was, this way, required to produce positivity. PI is a fluorescent marker that only penetrates cells with severe damage of the membrane, cells that are already dead, where it binds to nucleic acids and leads to increased red fluorescence.[17] Permeation to PI means that the compound was responsible for a fungicidal effect, resulting in extensive lesion to the plasmatic membrane, either from a direct effect or as a secondary result of metabolic impairment. Under our experimental conditions compound **7** showed similar effects to the reference fungicidal compound amphotericin B.

3. Conclusion

With the battery of di(hetero)arylamine derivatives of benzo[*b*]thiophenes tested it was possible to establish some structure–activity relationships. The presence of hydroxy groups in the aryl derivatives was found to be essential for the antifungal activity. The spectrum of activity in the pyridine derivatives was broadened by the absence of the ester group on position 2 of the benzo[*b*]thiophene system, with compound **7** inhibiting all tested fungal species.

The dermatophytes were found to be more sensitive to the active tested compounds than *Candida* and *Aspergillus* species, with four compounds (**2** and **5**–**7**) showing a considerably high activity. Among the presented set of compounds, compound **7** shows the highest potential. Although somewhat expressing its counterparts selectivity to dermatophytes, it inhibited all tested fungal strains, including fluconazole-resistant yeasts and *A. fumigatus*, especially important organisms from the clinical point of view. In fact, its activity was not affected by the fluconazole susceptibility profile of the tested strains. It clearly evidences a broad spectrum of activity that has been further confirmed by the prevention of germ tube production in *C. albicans* and the flow cytometry studies.

Given the results described, and as a future perspective, the structure–activity oriented preparation of a wider set of derivatives could, probably, allow the tuning of the antifungal activity of the compounds, particularly regarding the reduction of MICs for yeasts and *Aspergillus* species. Overall, these compounds show an interesting potential for development as antifungal agents and certainly deserve further investigation.

4. Experimental

4.1. Synthesis

General procedures: Melting points were determined on a Stuart SMP3 apparatus and are uncorrected. The ^{1}H NMR spectra were measured on a Varian Unity Plus at 300 MHz. Spin–spin decoupling was used to assign the signals. The ^{13}C NMR spectra were measured in the same instrument at 75.4 MHz (using DEPT θ 45°).

Elemental analyses were determined on a LECO CHNS 932 elemental analyzer. Mass spectra (EI) and HRMS were performed by the mass spectrometry service of University of Vigo-Spain.

Column chromatography was performed on Macherey–Nagel silica gel 230–400 mesh. Preparative layer chromatography (PLC) was performed on Macherey–Nagel 20 × 20 cm^2 silica plates, layer 2 mm SIL G-200 UV_{254}. Petroleum ether refers to the boiling range 40–60 °C. Ether refers to diethyl ether.

Xantphos corresponds to 4,5-bis(diphenylphosphino)-9,9-dimethylxanthene and was purchased from Strem.

Compounds **1**–**4** and **6**, as mentioned before, had been prepared and characterized by us in a previous work.[13]

4.1.1. Ethyl 3-(2,4-dihydroxyphenylamino)benzo[*b*]thiophene-2-carboxylate (5)

To a solution of diarylamine **4**[13] (100 mg, 0.280 mmol) in dry dichloromethane (10 mL), a solution of BBr_3 in dichloromethane 1 M (5 equiv) was added at 0 °C under argon and the mixture was stirred for 3 h. A saturated solution of $NaHCO_3$ (15 mL) was then slowly added and the extractions were done with dichloromethane (2× 20mL). The organic phases were collected, dried ($MgSO_4$), filtered, and removal of the solvent gave a yellow oil which was submitted to PLC (ether/petroleum ether 1:1) to give compound **5** as a yellow green solid (65 mg, 70%) mp 178–180 °C. ^{1}H NMR ($CDCl_3$): δ 1.44 (3H, t, J = 7.5 Hz, CH_2CH_3), 4.41 (2H, q, J = 7.5 Hz, CH_2CH_3), 4.87 (1H, s, OH), 6.28 (1H, s, OH), 6.32 (1H, dd, J = 8.4 and 2.7 Hz, 5′-H), 6.61 (1H, d, J = 2.7 Hz, 3′-H), 6.92 (1H, d, J = 8.4 Hz, 6′-H), 6.99–7.12 (2H, m, ArH), 7.34–7.42 (1H, m, ArH), 7.70–7.73 (1H, br d, *J* = 8.1 Hz, ArH), 8.45 (1H, br s, NH) ppm. ^{13}C NMR ($CDCl_3$): δ 14.42 (CH_2CH_3), 61.00 (CH_2CH_3), 102.45 (CH), 104.62 (C), 107.62 (CH), 121.31 (C), 123.14 (CH), 123.91 (CH), 124.68 (CH), 127.72 (CH), 128.50 (CH), 131.37 (C), 140.25 (C), 149.01 (C), 153.63 (C), 155.77 (C), 165.99 (C=O) ppm.

MS (EI) *m*/*z* (%): 331 (M^++2, 5), 330 (M^++1, 17), 329 (M^+, 81), 283 (100). HRMS M^+ calcd for $C_{17}H_{15}NO_4S$: 329.0722, found 329.0727.

4.1.2. *N*-(Benzo[*b*]thien-3-yl)pyridine-3-amine (7)

To a dried Schlenk tube dry dioxane (3 mL), 3-bromobenzo[*b*]thiophene (150 mg, 0.704 mmol), $Pd(OAc)_2$ (8 mol%), xantphos (10 mol%), Cs_2CO_3 (2 equiv), and 3-aminopyridine (80.0 mg, 0.845 mmol) were added under argon and the mixture was heated at 110 °C for 3 h:30 min. After cooling, the mixture was filtered and the filtrate was evaporated to give an oil. This was submitted to column chromatography using a gradient from 10% ethyl acetate/petroleum ether to 80% ethyl acetate/petroleum ether to give diarylamine **7** as an orange oil (128 mg, 80%). Crystallization from ethyl acetate/petroleum ether gave orange crystals mp114–116 °C. ^{1}H NMR ($CDCl_3$): δ 5.80 (1H, s, NH), 7.09 (1H, s, 2′-H), 7.16 (1H, dd, *J* = 8.3 and 4.7 Hz, 5-H), 7.24–7.28 (1H, m, 4-H), 7.37–7.44 (2H, m, ArH), 7.63–7.67 (1H, m, ArH), 7.85–7.89 (1H, m, ArH), 8.15 (1H, dd, *J* = 4.7 and 1.3 Hz, 6-H), 8.37 (1H, br d, J = 2.6 Hz, 2-H) ppm. ^{13}C NMR ($CDCl_3$): δ 111.33 (CH), 120.65 (CH), 121.82 (CH), 123.27 (CH), 123.78 (CH), 124.12 (CH), 125.05 (CH), 133.77 (C), 134.38 (C), 138.36 (CH), 138.90 (C), 140.97 (CH), 141.41 (C) ppm. MS (EI) *m*/*z* (%) 228 (M^++2, 5.75), 227 (M^++1, 17.30), 226 (M^+, 100) 225 (M^+-1, 50), 224 (M^+-2, 15). Calcd for $C_{13}H_{10}N_2S$: C 69.00, H 4.45, N 12.38, S 14.17%, found C 68.88, H 4.62, N 12.06, S 14.23%.

4.2. Antifungal evaluation

4.2.1. Antifungal drugs

Amphotericin B (Sigma) and fluconazole (Pfizer) were used as standard antifungal drugs. Amphotericin B was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma) at a starting concentration of 1600 μg/mL and fluconazole was dissolved in sterile distilled water at 12,800 μg/mL.

The test compounds **1**–**4** and **6** were prepared as previously reported[13] and compounds **5** and **7** (see chemical structures in Scheme 1) were prepared as described above. A 10 mg/mL stock solution of each of the test derivatives was prepared in 100% DMSO and then diluted in the test medium to achieve the required test concentrations. The final concentration of DMSO did not exceed 2%. According to previously performed tests (data not shown), DMSO at that concentration did not affect fungal growth.

4.2.2. Fungal organisms

The antifungal activity of di(hetero)arylamines **1**–**7** was evaluated against *Candida*, *Aspergillus*, and dermatophyte strains: two clinical strains of *Candida* isolated from recurrent cases of oral candidosis (*C. albicans* D5 and *C. glabrata* D 10R); three *Candida* reference strains (*C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803, and *C. krusei* ATCC 6258); three *Aspergillus* strains (*A. niger* ATCC 16404, *A. fumigatus* ATCC 46645, and *A. flavus* F44); and five clinical strains of dermatophytes isolated from nails and skin (*M. canis* FF1, *M. gypseum* FF3, *T. rubrum* FF5, *T. mentagrophytes* FF7, and *E. floccosum* FF9). *Candida parapsilosis* ATCC 90018 was used for quality control. *C. albicans* strains ATCC 10231 and D5, plus an additional clinical isolate, *C. albicans* M1, were used for the germ tube inhibition assay, while *C. albicans* ATCC 10231 and *A. fumigatus* ATCC 46645 were included in the flow cytometry studies. The clinical isolates were identified using standard microbiological methods. All strains were stored in Sabouraud dextrose broth (Becton–Dickinson) with 20% glycerol at −80 °C and passaged on Sabouraud dextrose agar (SDA; Becton–Dickinson) before use to ensure purity and viability.

4.2.3. Inoculum preparation

The isolates of yeasts, *Aspergillus* spp., and dermatophytes were grown for 1, 3, and 5 days, respectively, on SDA at 37 °C for *Candida*, and at 30 °C for filamentous fungi. The strains were then subcultured under the same conditions. Yeasts and conidial suspensions of these recent cultures were prepared in sterile 0.85% saline solution. Each suspension was diluted in RPMI 1640 medium, with L-glutamine and without sodium bicarbonate (Biochrom AG), buffered to pH 7.0 ± 0.2 with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma), and adjusted to $1–2 \times 10^3$ CFU/mL for yeasts and $1–2 \times 10^4$ CFU/mL for filamentous fungi. The concentration of each inoculum was confirmed by viable count on Sabouraud agar plates by plating 100 μL of serial dilutions onto the surface and incubating until visible growth.

4.2.4. Antifungal susceptibility testing

Broth macrodilution methods based on the CLSI (formerly NCCLS) reference documents M27-A2[18] and M38-A,[19] for yeasts and filamentous fungi, respectively, with minor modifications, were used to determine minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs). Since no reference method is available for dermatophytes, protocol M38-A[19] and previously published data on the subject[20] were taken into account. Briefly, serial twofold dilutions of each di(hetero)arylamine were prepared over the range of 200–0.8 μg/mL in RPMI 1640 medium immediately before testing. Each test tube containing a given concentration of the drug being tested was inoculated with the inoculum suspension and then incubated aerobically at 37 °C for 48 h (yeasts) or at 30 °C for 3/5 days (filamentous fungi/dermatophytes). The fungal growth was indicated by the turbidity and MICs were defined as the lowest drug concentration that reduced growth by 80% in comparison to the drug-free controls. To evaluate the MFCs, 20 μL aliquots were subcultured from each negative tube (optically clear tube) and the last positive tube, after MIC reading, onto Sabouraud dextrose agar plates. The plates were then incubated at 37 °C (yeasts) or 30 °C (*Aspergillus* species/dermatophytes) until growth was seen in the last positive tube subculture. Minimum fungicidal concentration was the lowest concentration of antifungal yielding subcultures without any visible fungal growth. In addition, a reference antifungal compound, fluconazole or amphotericin B, was used as the standard antifungal drug. Twofold serial dilutions, ranging from 128 to 0.25 μg/mL for fluconazole and 8 to 0.25 μg/mL for amphotericin B, were used. Quality control determinations of the MIC of fluconazole were ensured by testing *C. parapsilosis* ATCC 90018. The results obtained were within the recommended limits (data not shown). All results are from three independent and concordant experiments, performed in duplicate. A range of values are presented when different results were obtained. Two growth controls, using test medium alone and with 2.0% (v/v) DMSO, and a sterility control (drug-free medium only) were included in all assays.

4.2.5. Germ tube inhibition assay

Cell suspensions from overnight SDA cultures of *C. albicans* strains ATCC 10231, D5, and M1, at 37 °C, were prepared in NYP medium (*N*-acetylglucosamine [Sigma; 10^{-3} mol/L], Yeast Nitrogen Base [Difco; 3.35 g/L], proline [Fluka; 10^{-3} mol/L]) with NaCl (4.5 g/L, pH 6.7 ± 0.1)[21] and adjusted to obtain a density of $(1.0 \pm 0.2) \times 10^6$ CFU/mL. The compounds were dissolved and diluted in DMSO and added in a volume of 10 μL to 990 μL of the yeast suspensions (final DMSO concentration of 1%) to obtain appropriate sub-inhibitory concentrations (1/2, 1/4, and 1/8 of the MIC). After a 3-h incubation at 37 °C, 100 cells from each sample were counted, using a hemocytometer, and the percentage of germ tubes was determined. Germ tubes were considered positive when they were at least as long as the diameter of the blastospore. Protuberances showing a constriction at the point of connection to the mother cell, typical for pseudohyphae, were excluded. The re-

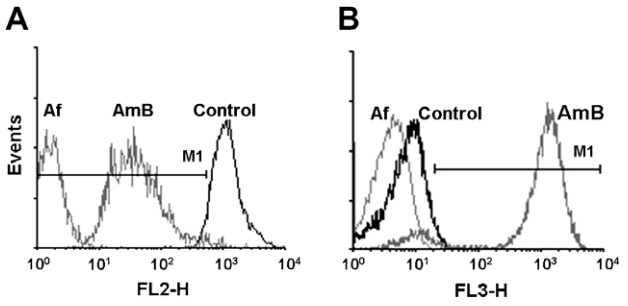


Figure 3. Monoparametric flow cytometry histograms showing overlaid peaks of different cell samples and the marker (M1), set to include a maximum of 5% of control cells and used to analyze drug-treated samples. (A) Samples of *A. fumigatus* conidia stained with FUN-1; (B) Samples of *C. albicans* cells stained with PI. (Af, auto-fluorescence of unstained cells; control, stained drug-free samples; AmB, cells treated with amphotericin B.)

sults are presented as averages ± standard deviations (SD) of three separate experiments.

4.2.6. Cell treatment for flow cytometry

Yeast and conidia suspensions in RPMI 1640 medium and sterile water with 0.01% Tween 80, respectively, were prepared from overnight SDA cultures of *C. albicans* ATCC 10231 and 4–7 days old SDA cultures of *A. fumigatus* ATCC 46645 at 35 °C and adjusted using a hemocytometer to a final density of $(2.0 \pm 0.2) \times 10^6$ CFU/mL. A suspension of heat-killed conidia (95 °C, 30 min) at the same density was also prepared. Serial twofold dilutions of compound **7** (256 to one μg/mL) and amphotericin B (two and four μg/mL) were then added to the cell suspensions and incubated at 35 °C in humid atmosphere without agitation for 2, 4, and 24 h for yeasts or in a water bath with moderate agitation for 3, 4, and 24 h for conidia, respectively. Drug-free control tubes were included in every experiment.

4.2.7. Cell staining and flow cytometry readings

After the incubations the cells were washed and resuspended in 500 μL of phosphate-buffered saline (PBS) with 2% D-glucose for FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide; Invitrogen, USA] staining and 0.125% sodium deoxycholate (Sigma) for PI (propidium iodide; Sigma) staining. Five microliters of the FUN-1 and PI solutions in DMSO and PBS, respectively, at appropriate concentrations (determined through preliminary tests, data not shown), were added to the cell suspensions in order to obtain final concentrations of 0.5 μM of FUN-1 and 1 μg/mL of PI. FUN-1 stained cells were then incubated at 35 °C, away from incident light, for 20 min, while PI stained samples were read after about 10 min at room temperature for yeast cells and after 1 h incubation at 35 °C, away from incident light, for *A. fumigatus* conidia. Unstained cell suspensions were always included, as auto-fluorescence controls. Flow cytometry was performed using a FACSCalibur® (Becton–Dickinson Biosciences, San Jose, CA) flow cytometer with a 15 mW blue argon laser emitting at 488-nm wavelength and the results were analyzed using CellQuest Pro Software (Becton–Dickinson). Intrinsic parameters (forward and side scatter, for cell size and complexity analysis, respectively) and fluorescence in the FL2 channel (log yellow/orange fluorescence, around 575 nm) for FUN-1 and the FL3 channel (log red fluorescence, above 605 nm) for PI were acquired and recorded, using logarithmic scales, for a minimum of 7500 events/sample. Markers (M1) were adjusted in monoparametric histograms of sample fluorescence intensity in order to include a maximum of 5% of the cells and then used in the analysis of the remaining samples to quantify the percentages of positive cells: cells showing altered fluorescence in comparison to the drug-free controls (see Fig. 3 for examples of such histograms). The results are presented as averages ± SD of at least three replicate experiments.

Acknowledgements

The authors thank the Foundation for the Science and Technology (Portugal) and FEDER for financial support through the research centres, project POCI/QUI/59407/2004 and for the post-doc grant attributed to Luís Vale-Silva (SFRH/BPD/29112/2006). The authors also thank the Socrates/Erasmus programme for financial support in Portugal to A.B. and J.-M.B. (Univ. Metz → Univ. Minho).

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