European Journal of Medicinal Chemistry 64 (2013) 357–364

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

2-Aminobenzothiazole derivatives: Search for new antifungal agents

Alessia Catalano [a], Alessia Carocci [a], Ivana Defrenza [a], Marilena Muraglia [a], Antonio Carrieri [a], Françoise Van Bambeke [b], Antonio Rosato [a], Filomena Corbo [a,*], Carlo Franchini [a]

[a] Dipartimento di Farmacia-Scienze del Farmaco, Università degli Studi di Bari "Aldo Moro", via E. Orabona n. 4, 70125 Bari, Italy
[b] Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium

ARTICLE INFO

Article history:
Received 23 October 2012
Received in revised form
27 March 2013
Accepted 28 March 2013
Available online 9 April 2013

Keywords:
Antifungal activity
2-Aminobenzothiazole derivatives
CA-CYP51
Docking
Cytotoxicity

ABSTRACT

A new series of 6-substituted 2-aminobenzothiazole derivatives were synthesized and screened *in vitro* as potential antimicrobials. Almost all the compounds showed antifungal activity. In particular, compounds **1n**,**o**, designed on the basis of molecular modeling studies, were the best of the series, showing MIC values of 4–8 μg/mL against *Candida albicans*, *Candida parapsilosis* and *Candida tropicalis*. None of the two compounds did show any cytotoxicity effect on human THP-1 cells.

1. Introduction

One of the major problems we are facing today in the context of infectious diseases is the relentless increase and spread of antimicrobial resistance. Thus, studies for the identification of novel targets and drugs for the treatment of infectious diseases are at the forefront. In this perspective, for example, we recently determined the X-ray crystallographic structure of *Enterococcus faecalis* thymidylate synthase, which should be a potential target for antibacterial therapy [1]. Many heterocyclic nuclei, such as 1,3,4-thiadiazole, benzimidazole, 1,3,5-triazine, and benzothiazole have been recently reviewed as antimicrobial agents [2,3]. Our attention was focused to the benzothiazole nucleus [4]. In fact, benzothiazole derivatives possess a wide spectrum of biological applications such as antitumor, schictosomicidal, anti-inflammatory, anticonvulsant, antidiabetic, antipsychotic, diuretic, and antimicrobial activities [5]. In the past, our research group was interested in the synthesis and microbiological screening of a series of 2-mercapto-1,3-benzothiazole derivatives, some of which showed antibacterial activity against Gram-positive and negative bacterial strains [6]. Results showed that the SH moiety at the 2 position of the heterocyclic nucleus led to a remarkable antibacterial activity. In a search for new leads toward potent antimicrobial agents, given the isosteric relationship existing between SH and NH_2 groups, following a previous work [7], we synthesized a series of 2-amino-1,3-benzothiazoles (Fig. 1) and tested their *in vitro* antimicrobial activity. It was reported, indeed, that several 2-aminobenzothiazole derivatives, variously substituted, showed antifungal activity, even though much lower than those of the reference antifungal agent used [8]. In particular, as it was already done for the series of 2-mercapto-1,3-benzothiazole derivatives [6], substitutions at position 6 of the aryl moiety was investigated. QSAR and docking studies gave valuable hints assessing lipophilicity and steric hindrance as main molecular determinants most likely affecting the newly synthesized benzothiazole derivatives in their antifungal activity.

2. Results and discussion

2.1. Chemistry

Compounds **1a** and **1j**–**m** were commercially available. Compounds **1b**–**i**,**n**,**o** (Table 1) were synthesized as depicted in Scheme 1. Alkyl and aryl alcohols **2e**–**h**,**n** were reacted with 4-nitrophenol under Mitsunobu conditions [9–12] to give their nitro derivatives

* Corresponding author. Tel.: +39 080 5442746; fax: +39 080 5442724.
E-mail address: filomena.corbo@uniba.it (F. Corbo).

0223-5234/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2013.03.064

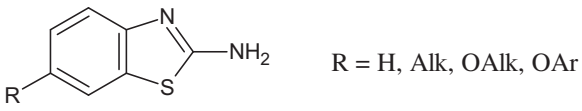


Fig. 1. Structures of 2-amino-1,3-benzothiazole derivatives (**1a**–**o**).

3e–**h**,**n**, which were reduced by catalytic hydrogenation to give anilines **6e**–**h**,**n**. Anilines **6b**–**d**,**o** were commercially available. Aniline **6i** was obtained by reducing nitro derivative **4** to aniline **5** and then by submitting the latter to a Mitsunobu reaction with phenol. 2-Aminobenzothiazole derivatives **1b**–**i**,**n**,**o** were prepared via thiocyanation of **6e**–**i**,**n**,**o** [13]. In this reaction ammonium thiocyanate and bromine were used to generate thiocyanogen in situ [14].

2.2. Antimicrobial studies

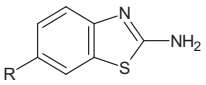
2.2.1. Antibacterial studies

According to the Clinical Laboratory Standards Institute (CLSI) guidelines [15] compounds **1a**–**o** (Table 1) were tested against Gram-positive and Gram-negative bacteria belonging to the ATCC collection (*Staphylococcus aureus* 29213, *E. faecalis* 29212, *Escherichia coli* 25922) using Norfloxacin (NRF) as reference drug. The results, expressed as MIC (μg/mL), are listed in Table 2. The antibacterial screening revealed that the compounds showed very low to no activity against all the bacterial strains tested, thus underlying that isosteric substitution SH/NH_2 brought to a loss of the antibacterial activity, as evidenced by comparing **1b**,**c**,**j**–**l** with their corresponding isosters previously reported [6].

2.2.2. Antifungal studies

Compounds **1a**–**o** (Table 1) were screened, according to CLSI guidelines [16], against a panel of fungi strains (*Candida albicans* 10231, *Candida parapsilosis* 22019, *Candida tropicalis* 750, *Candida krusei* 6258) belonging to the ATCC collection. Fluconazole was used as reference drug. The results, expressed as MIC (μg/mL), are listed in Table 3. All the 6-substituted 2-amino-1,3-mercapto benzothiazole derivatives show slight to high antifungal activity against all the *Candida* spp. tested, while unsubstituted **1a** was inactive. The electron-withdrawing effect at the 6-position of the benzothiazole moiety of halogens, such as a chlorine and fluorine atom (**1j**,**k**, Fig. 2) and a trifluoromethyl group (**1l**, Fig. 2) was first investigated. These three compounds were slightly active against all *Candida* spp. tested. In particular, **1l** was the most active of them, especially against *C. albicans*. Then, we introduced an alkoxy or aryloxy moiety (**1b**–**h**) in the same position. Results showed that antifungal activity enhanced with the increase of steric hindrance at position 6 of the heterocycle. In fact, compounds **1b** and **1c** were slightly active while phenoxy and benzyloxy derivatives (**1d** and **1e**, respectively) were much more potent. Compound **1d** was equipotent to **1c** against *C. albicans* but was much more active against *C. parapsilosis*, *C. tropicalis* and *C. krusei* (MIC values: 16, 32, 32 vs 128, 128, 256 μg/mL, respectively). The benzyloxy derivative **1e** was more active than the others against *C. albicans* and showed high activity also against *C. parapsilosis* and *C. tropicalis.* Thus, we investigated a possible homologation of the alkyl chain with one to three methylene moieties obtaining compounds **1f**, **1g** and **1h**, respectively. They were generally less active than compound **1e** evidencing that elongation of the alkyl chain was detrimental for activity. In particular, compound **1h** was nearly inactive against fungi. Compound **1i** was less active than its position isomer **1e**. This finding suggests that conceivably moving the oxygen atom is detrimental for activity. Among the series of alkoxy derivatives, we also tested the well-known drug riluzole (**1m**), the only FDA-approved drug to treat amyotrophic lateral sclerosis [17]. It was just slightly active against all the species of *Candida*. Finally, compounds **1n**,**o**, suggested by modeling studies (see below), were indeed the most active of the series against *C. albicans*, *C. parapsilosis* and *C. tropicalis.* It is noteworthy that compound **1n** was even as potent as the reference on *C. tropicalis*.

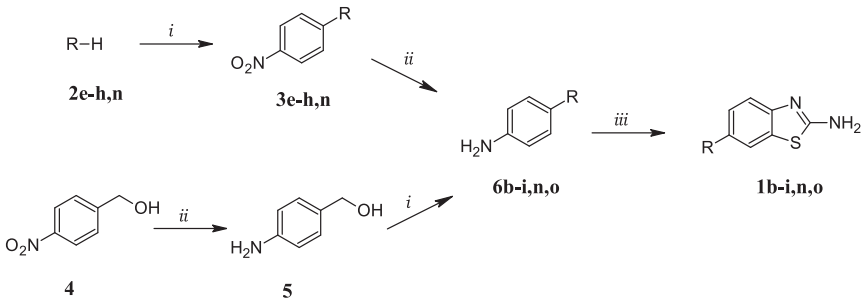
Table 1
Structures of compounds **1a**–**o**.



Compd	R
1a	H
1b	Me–O
1c	Et–O
1d	Ph–O
1e	Bn–O
1f	$Ph(CH_2)_2$–O
1g	$Ph(CH_2)_3$–O
1h	$Ph(CH_2)_4$–O
1i	$PhOCH_2$
1j	F
1k	Cl
1l	CF_3
1m	CF_3O
1n	4-Cl–Bn–O
1o	4-Cl–Ph–O

2.3. Molecular modeling studies

A molecular modeling study was carried out to better perceive and evaluate the biological profile within the series under study. In the first step we make use of classical 2D-QSAR to gain a sound regression model, showing the dependence of the antifungal activity on lipophilicity. In particular, as proposed by Hansch [18], pMIC data showed parabolic dependence by hydrophobic chemotype of benzothiazoles, as scored by the CLog*P* values (Fig. 2, Table 4). It has to be pointed out that model comprising compounds **1b**–**m** was indeed statistically significant ($r^2 = 0.624$), but a valuable increase of the explained variance ($r^2 = 0.750$) was achieved excluding from the regression model **1d**, the lone derivative bearing the phenyl ring directly branched to the oxygen at position 6 of the aryl moiety. Afterward, the antifungal activity was interpreted through docking experiments. Lanosterol 14α-demethylase (CYP51) is a member of the cytochrome P450 superfamily, which catalyzes the oxidative removal of the 14α-methyl group of lanosterol to give $\Delta^{14,15}$-desaturated intermediates in ergosterol biosynthesis [19]. The development of inhibitors of CYP51 in fungi has provided a rich source of drugs, such as clotrimazole, ketoconazole, fluconazole, and itraconazole [20], responsible for the cell growth inhibition due to ergosterol depletion. The same cytochrome is also targeted by drugs (i.e. benzothiazines and benzoxazines) [20–22] characterized by a molecular scaffold similar to the benzothiazoles here presented. Moreover, the importance of CYP51 as a plausible target in the treatment of *C. albicans* infections has been already and successfully reported [23–25]. On the basis of this evidence, compounds **1b**–**i** were therefore docked into the catalytic site of the homology based model of *C. albicans* CYP51 (CA-CYP51) [26,27] which has already been successfully used in a previous study on antifungal agents [7]. Docking binding poses highlighted some interesting features that might be in charge of the antifungal activity of the



Scheme 1. Reagents and conditions: (i) 4-nitrophenol (for compounds **3e**–**h**,**n**) or phenol (for compound **6i**), PPh_3, DIAD, anhyd THF, rt; (ii) $HCOONH_4$, 10% Pd/C, *i*-Pr2O/H2O/THF, rt (for compounds **6f**–**h**); H_2, 10% Pd/C, MeOH/abs EtOH, rt (for compounds **5** and **6e**), H_2, 10% Pd/C, abs EtOH, rt (for compounds **6n**,**o**); (iii) Br_2, NH_4SCN, $HCOOH/CH_3COOH$, 0 °C then rt.

compounds under study. All of them are actually capable to accommodate the active site mainly anchoring the heterocyclic system nearly perpendicular to the heme group of CA-CYP51, by mean of a coordination bond involving the iron and the nitrogen atoms, as it might be perceived from the docking of the most potent antifungal agent **1e** depicted in Fig. 3. As a figure of merit, the same binding topology has also been experimentally observed in the X-ray structures of other thiazoles in complex with cytochrome different from the CA-CYP51, but functionally related to our target [28]. At the same time, the amino group at position 2 reinforces the binding engaging Thr311 and Gly307 with hydrogen-bonding mediated polar interactions. This evidence then facilitates the positioning of the apolar and steric hindered substituents into a most likely hydrophobic dome placed above the heme group. It is very interesting to note that the benzyl moiety of the most potent antifungal agent **1e** make extensive π–π stackings with Phe228 and Tyr118. Other remarkable indications have to be ascribed to the reduced accessible surface of the active site lodge, shaped and delimited by bulky residues, namely Met508 and Val509. This evidence might justify, at least in part, the very low pMIC of derivatives bearing very large and long lipophilic groups hampering a proper docking (i.e. **1h**). On the other hand, compounds with a very small group, and lacking the phenyl ring (i.e. **1b**,**c**), might not be capable to establish significant interactions, resulting then with a lower antifungal potency. It may be noted that the need of a suitable functional group, in terms of bulkiness and/or lipophilicity, was already highlighted in the 2D-QSAR study previously mentioned. As long as this evidence was concerned, the synthesis of novel compounds was perceived and carried out according to the insights achieved by the modeling study. Indeed 2D-QSAR highlighted the affect of lipophilicity on antifungal activity, suggesting thereafter that an increase of this chemical cliché would have been at least mandatory to challenge the pMIC data. Moreover, the nature of the CA-CYP51 binding cleft was proved from dockings to be most likely characterized by aromatic, or at least very low polar, residues. This guided us in the design of **1n**,**o** as antifungals with improved activity. As expected these analogs were found to be the most potent of the series. The better antimicrobial profile might be ascribed not merely to a balancing in lipophilicity, but also to the additional and favorable van der Waals interactions occurring between the chlorine atom and the side chain of Val509 (see Fig. 3).

2.4. Cytotoxicity assay

The most active compounds of the series (**1n**,**o**) were tested for their cytotoxicity by the measurement of the release of the cytosolic enzyme lactate dehydrogenase (LDH) and by the uptake of trypan blue in damaged cells. The results obtained on human THP-1 cells indicate that these compounds show no cytotoxicity. Fig. 4 shows that **1n** caused a significant increase for both markers for concentrations ≥20 μg/mL (4× MIC) and that **1o** did not cause any LDH release and induced a small but significant increase in the number of trypan blue-stained cells when its concentration was ≥40 μg/mL (10× MIC).

Table 2
Antibacterial activity results of 2-aminobenzothiazole derivatives **1a**–**o** (MIC, μg/mL).

	Microorganism (MIC, μg/mL)		
	Gram-positive		Gram-negative
	S.a. 29213	*E.f.* 29212	*E.c.* 25922
1a	>512	>512	>512
1b	128	128	>512
1c	512	512	>512
1d	>512	>512	>512
1e	>512	>512	>512
1f	>512	128	>512
1g	512	128	>512
1h	512	256	>512
1i	256	128	>512
1j	>512	>512	>512
1k	>512	>512	>512
1l	256	256	>512
1m	256	512	512
1n	256	256	>512
1o	512	512	>512
NRF	0.5	4	0.03

Antibacterial activity was estimated by using CLSI assay [15]. Abbreviations: *S.a.*: *S. aureus*; *E.f.*: *E. faecalis*; *E.c.*: *E. coli*; NRF: norfloxacin.

Table 3
Antifungal activity results of 2-aminobenzothiazole derivatives **1a**–**o** (MIC, μg/mL).

	Microorganism (MIC, μg/mL)			
	C.a. 10231	*C.p.* 22019	*C.t.* 750	*C.k.* 6258
1a	>512	>512	>512	>512
1b	256	256	256	512
1c	128	128	128	256
1d	128	16	32	32
1e	8	8	8	64
1f	8	16	32	16
1g	32	16	16	16
1h	512	128	512	>512
1i	32	16	16	64
1j	256	128	256	256
1k	64	64	64	128
1l	16	64	64	64
1m	64	64	128	128
1n	4	8	4	64
1o	8	4	8	32
FCN	2	2	4	32

Antifungal activity was estimated by using CLSI assay [16]. Abbreviations: *C.a.*: *C. albicans*; *C.p.*: *C. parapsilosis*; *C.t.*: *C. tropicalis*; *C.k.*: *C. krusei*; FCN: fluconazole.

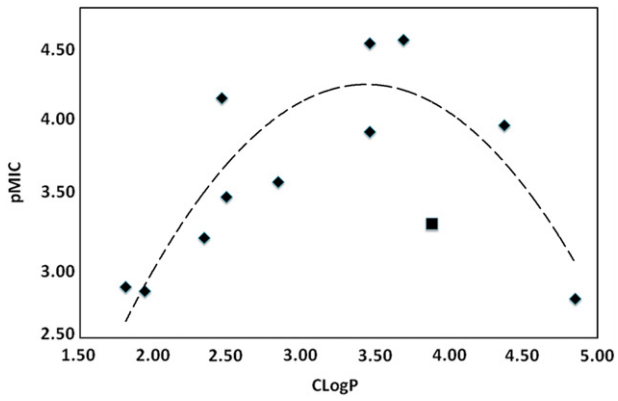


Fig. 2. Plot of pMIC vs CLog*P* data. Data not included in the regression model are represented by square.

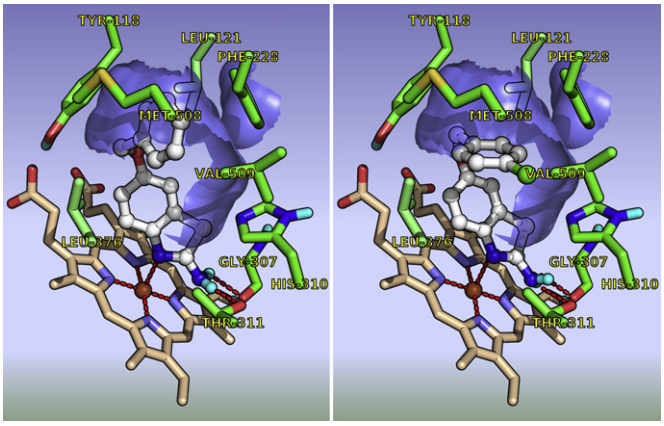


Fig. 3. Docking poses for **1e** (left) and **1n** (right) in the active site of CA-CYP51. Ligands and iron atom are displayed as ball and stick to help interpretation.

3. Conclusion

A new series of 2-amino-1,3-benzothiazole derivatives were synthesized, characterized by mass, ^{1}H NMR and IR spectra, and C, H, N, analyses. Comparison of these compounds with the series of 2-mercapto-1,3-benzothiazole derivatives previously reported [6] showed that the isosteric substitution SH/NH_2 at position 2 brought to the loss of activity against bacteria but to the appearance of an interesting activity toward fungi strains. This result confirms our previous hypothesis that the SH moiety at the 2 position of the heterocyclic nucleus is crucial for antibacterial activity. In this work, substitutions at the 6-position of the heterocyclic moiety were investigated. All the newly synthesized compounds (**1b**–**i**,**n**,**o**), together with commercial **1a**,**j**–**m**, were investigated for their *in vitro* antimicrobial activities. Among the screened samples, no compound showed interesting antibacterial activity, as expected. On the contrary, most of the compounds were interesting for their antifungal activity. In particular, compound **1n** showed the highest antifungal activity against *C. albicans* (MIC: 4 μg/mL), followed by compounds **1e**,**f**,**o** (MIC: 8 μg/mL). Compound **1n** was also very active against *C. tropicalis*, showing the same MIC value as the reference (4 μg/mL), followed by compounds **1n**,**o** (MIC: 8 μg/mL). Compound **1o** was the best of the series against *C. parapsilosis* (MIC: 4 μg/mL) followed by compounds **1e**,**n** (MIC: 8 μg/mL). It is worthy of note that both compounds **1f** and **1g** were indeed two-fold more potent than the reference compound fluconazole against *C. krusei*. In conclusion, the presence of the substitution at position 2 of the heterocyclic moiety is crucial for benzothiazole derivatives activity: in particular, 2-mercapto derivatives were generally more active against bacteria, while the 2-amino ones were more potent against fungi. Moreover, as also suggested by the molecular modeling studies, data suggests the importance of the substitution with bulky groups at the 6-position of the 2-aminobenzothiazole moiety for enhancing antifungal activity, being the 6-benzyloxy derivative **1e** the best of the series against most of the fungal strains used. Homologation of the alkyl chain brought to a reduction of antifungal activity, except for *C. krusei*, against which the most active of the series were compounds **1f** and **1g**. The best compounds of the series, **1n**,**o**, were also screened for their cytotoxicity and did not show any toxic effects for human THP-1 cells when concentrations are maintained below 4 (**1n**) or 10 (**1o**)× their MIC. The current study therefore suggests that 2-amino-1,3-benzothiazoles **1n**,**o** are promising scaffolds for the development of novel antifungal agents against *Candida* spp.

Table 4
Lipohilicity, binding energy and antifungal activity of derivatives **1b**–**o**.

	pMIC[a]	CLog*P*[b]	FEB (kcal/mol)[c]	Rank[d]	Population[e]
1b	2.85	1.81	−5.00	1	68
1c	3.18	2.34	−5.07	4	2
1d	3.28	3.88	−6.64	1	129
1e	4.51	3.46	−6.95	1	13
1f	4.53	3.69	−6.67	2	12
1g	3.95	4.37	−7.07	2	15
1h	2.77	4.85	nd[f]	–	–
1i	3.90	3.46	−7.16	1	53
1j	2.82	1.94	−4.87	1	88
1k	3.46	2.49	−5.42	1	171
1l	4.13	2.46	−5.08	1	110
1m	3.56	2.84	−4.59	2	31
1n	4.86	4.06	−6.49	1	70
1o	4.54	4.48	−6.19	5	3

[a] Expressed as Log = 1/MIC.
[b] Calculated using Advanced Chemistry Development (ACD) Software.
[c] Free Energy of Binding.
[d] Pose rank according to the FEB scoring.
[e] Number of similar docked conformations found in the same cluster.
[f] No docking poses (see text).

4. Experimental

4.1. Chemistry

Chemicals were purchased from Sigma–Aldrich or Lancaster. Yields refer to purified products and were not optimized. The structures of the compounds were confirmed by routine spectrometric and spectroscopic analyses. Only spectra for compounds not previously described are given. Alcohols **2e**–**h**,**n** and **4** and anilines **6b**–**d**,**o** were commercially available. Melting points were determined on a Gallenkamp apparatus in open glass capillary tubes and are uncorrected. Infrared spectra were recorded on a Perkin–Elmer (Norwalk, CT) Spectrum One FT spectrophotometer and band positions are given in reciprocal centimeters (cm^{-1}). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian VX Mercury spectrometer operating at 300 and 75 MHz for ^{1}H and ^{13}C, respectively, using $CDCl_3$ and DMSO-d_6 as solvents. Chemical shifts are reported in parts per million (ppm) relative to the residual non-deuterated solvent resonance: $CDCl_3$, δ 7.26 (^{1}H NMR) and δ 77.3 (^{13}C NMR); DMSO-d_6, δ 2.48 (^{1}H NMR) and δ 40.1 (^{13}C NMR). *J* values are given in Hz. Gas chromatography (GC)/mass spectroscopy (MS) was performed on a Hewlett–Packard 6890–5973 MSD at low resolution. Liquid chromatography (LC)/mass spectroscopy (MS) was performed on a spectrometer Agilent 1100 series LC-MSD Trap System VL. Elemental analyses were performed on a Eurovector Euro EA 3000 analyzer. Chromatographic separations were

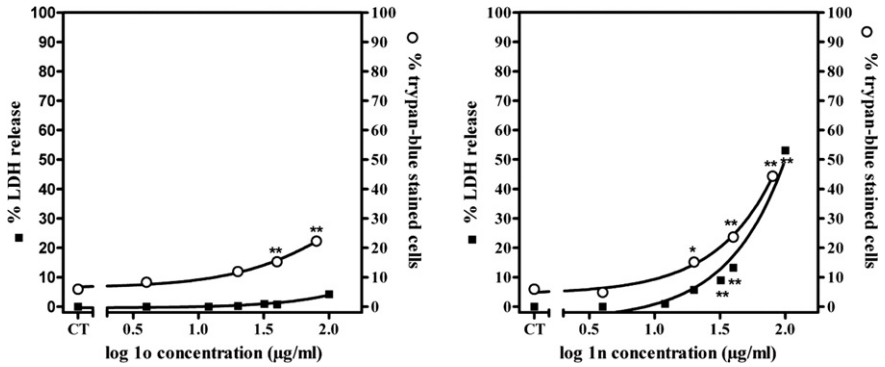


Fig. 4. Toxicity of **1o** (left) and **1n** (right) for THP-1 monocytes, as assessed by measuring LDH release (left axis) or trypan blue staining (right axis). Data are mean ± SD of 3 independent determinations (when non visible, SD are smaller than the symbol). Statistical analysis: one-way ANOVA with Dunnet's post-hoc test vs control: *: $p < 0.05$; **: $p < 0.01$.

performed on silica gel columns by column chromatography on silica gel (Kieselgel 60, 0.040–0.063 mm, Merck, Darmstadt, Germany) as described elsewhere [29–31]. TLC analyses were performed on precoated silica gel on aluminum sheets (Kieselgel 60 F_{254}, Merck).

4.2. General procedure for the synthesis of 4-substituted nitrobenzene derivatives (**3e**–**h**,**n**) and 4-(phenoxymethyl)aniline (**6i**)

The method adopted for the synthesis of 1-benzyloxy-4-nitrobenzene (**3e**) is described. A solution of diisopropyl azodicarboxylate (DIAD, 5.60 g, 27.8 mmol) in dry THF (60 mL) was added dropwise to a solution of benzyl alcohol (**2e**) (2.0 g, 18.5 mmol), 4-nitrophenol (3.86 g, 27.8 mmol), and triphenylphosphine (7.28 g, 27.8 mmol) in dry THF (100 mL) under N_2 atmosphere at room temperature. The reaction mixture was stirred overnight and then concentrated in vacuo. Et_2O was added to the residue and the solid filtered off. The filtrate was evaporated and the residue was purified by column chromatography on silica gel (EtOAc/petroleum ether 1:9) to give a white solid which was recrystallized from EtOAc/petroleum ether to give 3.33 g (79%) of white crystals: mp 107–108 °C; IR (KBr): 1509, 1347 (NO_2) cm^{-1}; GC/MS (70 eV) *m*/*z* (%) 229 (M^+, 3), 91 (100). Other spectroscopic data were in agreement with the literature [32].

4.2.1. 1-Nitro-4-(2-phenylethoxy)benzene (**3f**)

Prepared as reported above for **3e** starting from **2f** and 4-nitrophenol. Yield: 72%; white solid: mp 59–60 °C (EtOAc/petroleum ether); IR (KBr): 1510, 1338 (NO_2) cm^{-1}; GC/MS (70 eV) *m*/*z* (%) 243 (M^+, 14), 105 (100). Other spectroscopic data were in agreement with the literature [33].

4.2.2. 1-Nitro-4-(3-phenylpropoxy)benzene (**3g**)

Prepared as reported above for **3e** starting from **2g** and 4-nitrophenol. Yield: 25%; slightly yellowish crystals: mp 82–83 °C (EtOAc/hexane); IR (KBr): 1514, 1332 (NO_2) cm^{-1}; GC/MS (70 eV) *m*/*z* (%) 257 (M^+, 49), 91 (100); ^{1}H NMR ($CDCl_3$): δ 2.15 (quintet, $J = 6.9$ Hz, 2H, $CH_2CH_2CH_2$), 2.83 (t, $J = 7.4$ Hz, 2H, CH_2–Ar), 4.05 (t, $J = 6.3$ Hz, 2H, CH_2–OAr), 6.93 (d, $J = 9.3$ Hz, 2H, Ar), 7.15–7.35 (m, 5H, Ar), 8.19 (d, $J = 9.3$ Hz, 2H, Ar); ^{13}C NMR ($CDCl_3$): δ 30.7 (1C), 32.2 (1C), 67.9 (1C), 114.6 (2C), 126.1 (2C), 126.4 (1C), 128.7 (4C), 141.1 (1C), 142.0 (1C), 164.3 (1C).

4.2.3. 1-Nitro-4-(4-phenylbutoxy)benzene (**3h**)

Prepared as reported above for **3e** starting from **2h** and 4-nitrophenol. Yield: 78%; white crystals: mp 54–55 °C (EtOAc/petroleum ether); IR (KBr): 1498, 1331 (NO_2) cm^{-1}; GC/MS (70 eV) *m*/*z* (%) 271 (M^+, 13), 91 (100); ^{1}H NMR ($CDCl_3$): δ 1.75–1.95 (m, 4H, $CH_2CH_2CH_2CH_2$), 2.71 (t, $J = 6.9$ Hz, 2H, CH_2–Ar), 4.06 (t, $J = 5.6$ Hz, 2H, CH_2–OAr), 6.92 (d, $J = 9.1$ Hz, 2H, Ar), 7.15–7.35 (m, 5H, Ar), 8.19 (d, $J = 9.1$ Hz, 2H, Ar); ^{13}C NMR ($CDCl_3$): δ 27.9 (1C), 28.8 (1C), 35.7 (1C), 68.9 (1C), 114.6 (2C), 126.1 (2C), 126.2 (1C), 128.6 (4C), 141.6 (1C), 142.1 (1C), 164.4 (1C).

4.2.4. Synthesis of 1-chloro-4-[(4-nitrophenoxy)methyl]benzene (**3n**)

Prepared as reported above for **3e** starting from **2n** and 4-nitrophenol. Yield: 47%; off-white crystals: mp 114–115 °C (EtOAc/petroleum ether); IR (KBr): 1505, 1337 (NO_2) cm^{-1}; GC/MS (70 eV) *m*/*z* (%) 263 (M^+, 2), 125 (100); ^{1}H NMR ($CDCl_3$): δ 5.13 (br s, exch D_2O, 2H, NH_2), 7.02 (d, $J = 9.1$ Hz, 2H, Ar), 7.32–7.44 (m, 4H, Ar), 8.21 (d, $J = 9.3$ Hz, 2H, Ar); ^{13}C NMR ($CDCl_3$): δ 70.1 (1C), 115.1 (2C), 126.2 (2C), 129.0 (2C), 129.2 (2C), 134.2 (1C), 134.7 (1C), 142.1 (1C), 163.6 (1C).

4.2.5. 4-(Phenoxymethyl)aniline (**6i**)

Prepared as reported above for **3e** starting from **5** and phenol. Yield: 59%; gold crystals: mp 177–178 °C ($CHCl_3$/hexane); IR (KBr): 3398 (NH_2) cm^{-1}; GC/MS (70 eV) *m*/*z* (%) 199 (M^+, 3), 106 (100); ^{1}H NMR ($CDCl_3$): δ 3.70 (br s, exch D_2O, 2H, NH_2), 4.93 (s, 2H, CH_2), 6.68 (d, $J = 8.8$ Hz, 2H, Ar), 6.95–7.05 (m, 3H, Ar), 7.20–7.35 (m, 3H, Ar); ^{13}C NMR (DMSO-d_6): δ 60.4 (1C), 112.8 (2C), 115.4 (2C), 123.8 (1C), 127.4 (1C), 128.8 (2C), 130.1 (2C), 148.4 (1C), 171.0 (1C).

4.3. General procedure for the synthesis of 4-substituted anilines (**6f**–**h**) [34,35]

The method adopted for the synthesis of 4-(2-phenylethoxy) aniline (**6f**) is described. A stirring suspension of 10% Pd/C (0.17 g) in 2-propanol (10 mL) and water (1 mL) was brought to reflux. Next, nitro derivative **3f** (0.40 g, 1.64 mmol) dissolved in THF (4 mL) and solid ammonium formate (0.41 g, 6.58 mmol) were added. After 1.5 h the catalyst was removed by filtration and the solvent was evaporated in vacuo to give a crude residue that was taken up with EtOAc and washed with water. Then the solvent was removed in vacuo giving 0.28 g (80%) of a dark brown oil: GC/MS (70 eV) *m*/*z* (%) 213 (M^+, 100). Other spectroscopic data were in agreement with the literature [24].

4.3.1. 4-(3-Phenylpropoxy)aniline (**6g**)

Prepared as reported above for **6f** starting from **3g**. Yield: 72%; brown solid: mp 60–61 °C; IR (KBr): 3403 (NH_2) cm^{-1}; GC/MS (70 eV) *m*/*z* (%) 227 (M^+, 94), 109 (100); ^{1}H NMR ($CDCl_3$): δ 2.06 (quintet, $J = 7.0$ Hz, 2H, $CH_2CH_2CH_2$), 2.80 (t, $J = 7.6$ Hz, 2H, CH_2–Ar), 3.42 (br s, exch D_2O, 2H, NH_2), 3.89 (t, $J = 6.3$ Hz, 2H, CH_2–OAr), 6.64

(d, J = 8.5 Hz, 2H, Ar), 6.75 (d, J = 9.1 Hz, 2H, Ar), 7.15–7.35 (m, 5H, Ar); ^{13}C NMR ($CDCl_3$): δ 31.2 (1C), 32.4 (1C), 67.9 (1C), 116.0 (2C), 116.9 (2C), 126.1 (1C), 128.7 (4C), 139.8 (1C), 141.9 (1C), 152.6 (1C).

4.3.2. 4-(4-Phenylbutoxy)aniline (**6h**)

Prepared as reported above for **6f** starting from **3h**. Yield: 90%; brown oil; IR (neat): 3361 (NH_2) cm^{-1}; GC/MS (70 eV) m/z (%) 241 (M^+, 53), 109 (100); ^{1}H NMR ($CDCl_3$): δ 1.72–1.88 (m, 4H, $CH_2CH_2CH_2CH_2$), 2.62–2.75 (m, 2H, CH_2–Ar), 3.12 (br s, 2H, NH_2), 3.85–3.95 (m, 2H, CH_2–OAr), 6.60–6.68 (m, 2H, Ar), 6.70–6.78 (m, 2H, Ar), 7.15–7.35 (m, 5H, Ar); ^{13}C NMR ($CDCl_3$): δ 28.1 (1C), 29.3 (1C), 35.9 (1C), 68.7 (1C), 116.0 (2C), 116.7 (2C), 126.0 (1C), 128.5 (2C), 128.7 (2C), 140.0 (1C), 142.5 (1C), 152.6 (1C).

4.4. General procedure for the synthesis of 4-substituted anilines (**5** and **6e,n**)

The method adopted for the synthesis of 4-aminophenylmethanol (**5**) is described. Catalytic hydrogenation of **4** (1.50 g, 9.8 mmol) in 30 mL of a mixture of MeOH and absolute EtOH (2/1) was conducted at room temperature for 24 h in the presence of 10% palladium on carbon at 10 bar. The catalyst was removed by filtration and the residue taken up with EtOAc and washed with water. The solvent was removed to give 1.03 g (85%) of a yellow solid which was recrystallized from EtOAc/petroleum ether to give 0.65 g of yellowish crystals: mp 65–66 °C; GC/MS (70 eV) m/z (%) 123 (M^+, 100). Other spectroscopic data were in agreement with the literature [36].

4.4.1. 4-Benzyloxyaniline (**6e**)

Prepared as reported above for **5** starting from **3e**. Yield: 90%; dark green solid: mp 68–69 °C; IR (KBr): 3355 (NH_2) cm^{-1}; GC/MS (70 eV) m/z (%) 199 (M^+, 19), 108 (100); LC/MS m/z (%): 200 (M^+ + H). Other spectroscopic data were in agreement with the literature [24].

4.4.2. Synthesis of 4-[(4-chlorobenzyl)oxy]aniline (**6n**)

Prepared as reported above for **5** starting from **3n**. This reaction was carried out at room temperature for 20 min in the presence of 10% palladium on carbon at 3 bar. Yield: 49%; brown crystals: mp 108–109 °C (EtOAc/hexane); IR (KBr): 3366 (NH_2) cm^{-1}; GC/MS (70 eV) m/z (%) 233 (M^+, 14), 108 (100); ^{1}H NMR ($CDCl_3$): δ 3.44 (br s, exch D_2O, 2H, NH_2), 4.95 (s, 2H, CH_2), 6.58–6.67 (m, 2H, Ar), 6.75–6.85 (m, 2H, Ar), 7.34 (s, 4H, Ar).

4.5. General procedure for the synthesis of 6-substituted-2-amino-1,3-benzothiazoles (**1b–i**)

The synthesis of 6-ethoxy-1,3-benzothiazol-2-amine (**1c**), obtained following a general procedure for the preparation of aminobenzothiazoles described in the literature [13,14] is described. Aniline **6c** (1.0 g, 7.36 mmol) and NH_4SCN (1.6 g, 21.9 mmol) were dissolved in a 20% formic acid–glacial acetic acid mixture (100 mL) and cooled to −3 °C with stirring, under N_2. With the exclusion of light from the reaction mixture, bromine (0.30 mL dissolved in 20 mL of glacial acetic acid) was added dropwise, while the reaction temperature was kept between −3 °C and 0 °C. The light shield was removed and the mixture was allowed to warm to room temperature overnight. Sodium hydroxide pellets and ice were added with stirring until pH 11 was attained, and the mixture was extracted with EtOAc. The organic layer was separated and filtered through celite to remove polythiocyanogen $(SCN)_n$. The organic layer was then washed with water, saturated $NaHCO_3$ and brine; then, the solvent was evaporated in vacuo. The residue was purified by flash chromatography (EtOAc/petroleum ether 1:1) to give 0.93 g (65%) of an orange solid: mp 163–164 °C; IR (KBr): 3436 (NH_2) cm^{-1}; GC/MS (70 eV) m/z (%) 194 (M^+, 81), 165 (100); ^{1}H NMR (DMSO-d_6): δ 1.28 (t, J = 6.9 Hz, 3H, CH_3), 3.34 (br s, exch D_2O, 2H, NH_2), 3.96 (quartet, J = 7.0 Hz, 2H, CH_2), 6.64–6.80 (m, 1H, Ar), 7.16–7.28 (m, 2H, Ar); ^{13}C NMR (DMSO-d_6): δ 15.5 (1C), 64.1 (1C), 106.8 (1C), 114.0 (1C), 118.7 (1C), 132.5 (1C), 147.4 (1C), 154.1 (1C), 165.4 (1C). Anal. calcd. for $C_9H_{10}N_2OS\cdot0.17H_2O$ (197.25) %: C, 54.80; H 5.28; N 14.20. Found: C, 55.02; H 5.13; N 13.92.

4.5.1. 6-Methoxy-1,3-benzothiazol-2-amine (**1b**)

Prepared as reported above for **1c** starting from **6b**. Yield: 26%; beige crystals: mp: 162–163 °C ($CHCl_3$/hexane); IR (KBr): 3388 (NH_2) cm^{-1}; GC/MS (70 eV) m/z (%) 180 (M^+, 81), 165 (100); ^{1}H NMR (DMSO-d_6): δ 3.32 (br s, exch D_2O, 2H, NH_2), 3.71 (s, 3H, CH_3), 6.75–6.85 (m, 1H, Ar), 7.15–7.30 (m, 2H, Ar); ^{13}C NMR ($CDCl_3$): δ 56.1 (1C), 105.6 (1C), 113.9 (1C), 119.9 (1C), 132.9 (1C), 146.4 (1C), 155.9 (1C), 164.3 (1C). Anal. calcd. for $C_8H_8N_2OS\cdot0.50H_2O$ (189.24) %: C, 50.78; H 4.79; N 14.80. Found: C, 50.82; H 4.21; N 14.43.

4.5.2. 6-Phenoxy-1,3-benzothiazol-2-amine (**1d**)

Prepared as reported above for **1c** starting from **6d**. Yield: 65%; brown crystals: mp: 171–172 °C (EtOAc/petroleum ether); GC/MS (70 eV) m/z (%) 242 (M^+, 100). Anal. calcd. for $C_{13}H_{10}N_2OS\cdot0.20H_2O$ (245.90) %: C, 63.50; H 4.26; N 11.39. Found: C, 63.80; H 3.89; N 11.41. Other spectroscopic data were in agreement with the literature [13].

4.5.3. 6-(Benzyloxy)-1,3-benzothiazol-2-amine (**1e**)

Prepared as reported above for **1c** starting from **6e**. Yield: 20%; brown crystals: mp: 152–153 °C (EtOAc/petroleum ether); IR (KBr): 3436 (NH_2) cm^{-1}; GC/MS (70 eV) m/z (%) 256 (M^+, 15), 165 (100); ^{1}H NMR ($CDCl_3$): δ 5.09 (s, 2H, CH_2), 5.21 (br s, exch D_2O, 2H, NH_2), 6.98 (dd, J = 8.8, 2.5 Hz, 1H, Ar benzothiazole), 7.20 (d, J = 2.5 Hz, 1H, Ar benzothiazole), 7.28–7.48 (m, 6H, Ar); ^{13}C NMR ($CDCl_3$): δ 71.1 (1C), 106.9 (1C), 114.8 (1C), 120.0 (1C), 127.7 (2C), 128.2 (1C), 128.8 (2C), 132.9 (1C), 137.2 (1C), 146.6 (1C), 155.1 (1C), 164.2 (1C). Anal. calcd. for $C_{14}H_{12}N_2OS\cdot0.33H_2O$ (262.32) %: C, 64.10; H 4.87; N 10.68. Found: C, 64.27; H 4.75; N 10.32.

4.5.4. 6-(2-Phenylethoxy)-1,3-benzothiazol-2-amine (**1f**)

Prepared as reported above for **1c** starting from **6f**. Prepared as reported above for **1c** starting from **6f**. Yield: 59%; brown crystals: mp 116–117 °C (EtOAc/hexane); IR (KBr): 3432 (NH_2) cm^{-1}; GC/MS (70 eV) m/z (%) 270 (M^+, 100); ^{1}H NMR ($CDCl_3$): δ 3.10 (t, J = 7.1 Hz, 2H, CH_2–Ar), 4.18 (t, J = 7.0 Hz, 2H, CH_2–OAr), 5.19 (br s, exch D_2O, 2H, NH_2), 6.90 (dd, J = 8.8, 2.5 Hz, 1H, Ar), 7.12 (d, J = 2.5 Hz, 1H, Ar), 7.20–7.37 (m, 5H, Ar), 7.43 (d, J = 8.8 Hz, 1H, Ar); ^{13}C NMR ($CDCl_3$): δ 36.1 (1C), 69.8 (1C), 106.6 (1C), 114.6 (1C), 119.9 (1C), 126.7 (1C), 128.7 (2C), 129.2 (2C), 132.8 (1C), 138.4 (1C), 146.3 (1C), 155.1 (1C), 164.2 (1C). Anal. calcd. for $C_{15}H_{14}N_2OS$ (270.35) %: C, 66.64; H 5.22; N 10.36. Found: C, 66.27; H 5.22; N 10.26.

4.5.5. 6-(3-Phenylpropoxy)-1,3-benzothiazol-2-amine (**1g**)

Prepared as reported above for **1c** starting from **6g**. Yield: 41%; gold crystals: mp 126–127 °C (EtOAc/hexane); IR (KBr): 3419 (NH_2) cm^{-1}; GC/MS (70 eV) m/z (%) 284 (M^+, 81), 166 (100); ^{1}H NMR ($CDCl_3$): δ 2.05–2.20 (m, 2H, $CH_2CH_2CH_2$), 2.82 (t, J = 7.6 Hz, 2H, CH_2–Ar), 3.96 (t, J = 6.3 Hz, 2H, CH_2–OAr), 5.18 (br s, exch D_2O, 2H, NH_2), 6.91 (dd, J = 8.5, 2.6 Hz, 1H, Ar), 7.11 (d, J = 2.5 Hz, 1H, Ar), 7.15–7.35 (m, 5H, Ar), 7.44 (d, J = 8.5 Hz, 1H, Ar); ^{13}C NMR ($CDCl_3$): δ 31.1 (1C), 32.4 (1C), 67.9 (1C), 106.4 (1C), 114.6 (1C), 119.9 (1C), 126.2 (1C), 128.8 (4C), 132.8 (1C), 141.7 (1C), 146.3 (1C), 155.3 (1C), 164.1 (1C). Anal. calcd. for $C_{16}H_{16}N_2OS$ (284.38) %: C, 67.58; H 5.67; N 9.85. Found: C, 67.52; H 5.60; N 9.81.

*4.5.6. 6-(4-Phenylbutoxy)-1,3-benzothiazol-2-amine (**1h**)*

Prepared as reported above for **1c** starting from **6h**. Yield: 62%; beige solid: mp 98–99 °C; IR (KBr): 3428 (NH_2) cm^{-1}; GC/MS (70 eV) *m/z* (%) 298 (M^+, 28), 166 (100); ^1H NMR ($CDCl_3$): δ 1.70–1.90 (m, 4H, $CH_2CH_2CH_2CH_2$), 2.65–2.75 (m, 2H, CH_2–Ar), 3.90–4.05 (m, 2H, CH_2–OAr), 5.13 (br s, exch D_2O, 2H, NH_2), 6.89 (dd, *J* = 8.8, 2.5 Hz, 1H, Ar), 7.11 (d, *J* = 2.5 Hz, 1H, Ar), 7.14–7.34 (m, 5H, Ar), 7.43 (d, *J* = 8.8 Hz, 1H, Ar); ^{13}C NMR ($CDCl_3$): δ 28.1 (1C), 29.1 (1C), 35.8 (1C), 68.7 (1C), 106.3 (1C), 114.5 (1C), 119.9 (1C), 126.0 (1C), 128.6 (2C), 128.7 (2C), 132.9 (1C), 142.4 (1C), 146.3 (1C), 155.3 (1C), 164.1 (1C). Anal. calcd. for $C_{17}H_{18}N_2OS \cdot 0.2H_2O$ (302.00) %: C, 67.61; H 6.14; N 9.28. Found: C, 67.84; H 6.02; N 9.22.

*4.5.7. 6-(Phenoxymethyl)-1,3-benzothiazol-2-amine (**1i**)*

Prepared as reported above for **1c** starting from **6i**. Yield: 46%; yellow crystals: mp 168–169 °C (EtOAc/petroleum ether); IR (KBr): 3393 (NH_2) cm^{-1}; GC/MS (70 eV) *m/z* (%) 256 (M^+, 1), 163 (100); ^1H NMR ($CDCl_3$): δ 5.10 (s, 2H, CH_2), 5.27 (br s, exch D_2O, 2H, NH_2), 6.88–7.08 (m, 3H, Ar), 7.22–7.40 (m, 3H, Ar), 7.55 (d, *J* = 8.3 Hz, 1H, Ar), 7.69 (s, 1H, Ar); ^{13}C NMR ($CDCl_3$): δ 70.2 (1C), 115.1 (2C), 119.5 (1C), 120.4 (1C), 121.2 (1C), 126.0 (1C), 129.7 (2C), 131.6 (1C), 132.3 (1C), 152.2 (1C), 159.0 (1C), 166.2 (1C). Anal. calcd. for $C_{14}H_{12}N_2OS \cdot 0.5H_2O$ (265.32) %: C, 63.37; H 4.94; N 10.56. Found: C, 63.11; H 4.66; N 10.49.

*4.5.8. 6-[(4-Chlorobenzyl)oxy]-1,3-benzothiazol-2-amine (**1n**)*

Prepared as reported above for **1c** starting from **6n**. Yield: 20%; light pink crystals: mp 199–200 °C (EtOAc/petroleum ether); IR (KBr): 3439 (NH_2) cm^{-1}; GC/MS (70 eV) *m/z* (%) 290 (M^+, 18), 165 (100); ^1H NMR ($CDCl_3$): δ 5.03 (s, 2H, CH_2), 5.07 (br s, exch D_2O, 2H, NH_2), 6.95 (dd, *J* = 8.8, 2.7 Hz, 1H, Ar), 7.17 (d, *J* = 2.5 Hz, 1H, Ar), 7.36 (s, 4H, Ar), 7.45 (d, *J* = 8.8 Hz, 1H, Ar); ^{13}C NMR ($CDCl_3$): δ 69.7 (1C), 107.5 (1C), 114.4 (1C), 118.7 (1C), 129.1 (2C), 130.1 (2C), 132.5 (1C), 133.0 (1C), 137.0 (1C), 147.8 (1C), 153.7 (1C), 165.6 (1C). Anal. calcd. for $C_{14}H_{11}ClN_2OS$ (290.77) %: C, 57.83; H 3.81; N 9.63. Found: C, 58.16; H 3.77; N 9.70.

*4.5.9. 6-(4-Chlorophenoxy)-1,3-benzothiazol-2-amine (**1o**)*

Prepared as reported above for **1c** starting from **6o**. Yield: 87%; slightly green powder: mp 168–169 °C ($CHCl_3$/hexane); IR (KBr): 3377 (NH_2) cm^{-1}; GC/MS (70 eV) *m/z* (%) 276 (M^+, 100); ^1H NMR ($CDCl_3$): δ 5.27 (br s, exch D_2O, 2H, NH_2), 6.91 (d, *J* = 8.8 Hz, 2H, Ar), 7.0 (dd, *J* = 8.5, 2.5 Hz, 1H, Ar), 7.15–7.35 (m, 3H, Ar), 7.50 (d, *J* = 8.5 Hz, 1H, Ar); ^{13}C NMR ($CDCl_3$): δ 112.1 (1C), 118.5 (1C), 119.5 (2C), 120.0 (1C), 128.0 (1C), 129.9 (2C), 132.8 (1C), 148.8 (1C), 152.0 (1C), 157.1 (1C), 165.7 (1C). Anal. calcd. for $C_{13}H_9ClN_2OS$ (276.74) %: C, 56.42; H 3.28; N 10.12. Found: C, 56.25; H 3.38; N 9.85.

4.6. Antibacterial studies

The *in vitro* minimum inhibitory concentrations (MICs, μg/mL) were assessed by the broth microdilution method, using 96-well plates, according to CLSI guidelines [15]. Stock solutions of the tested compounds were obtained in DMSO. Stock solutions of lower concentrations were prepared for those substances which did not dissolve well. Then two-fold serial dilutions in the suitable test medium between 512 and 0.5 μg/mL were plated. To be sure that the solvent had no adverse effect on bacterial growth, a control test was carried out by using DMSO at its maximum concentration along with the medium. Bacteria strains available as freeze-dried discs, belonging to the ATCC collection, were used: Gram-positive strains such as *S. aureus* 29213, *E. faecalis* 29212, and Gram-negative one such as *E. coli* 25922. To preserve the purity of cultures and to allow the reproducibility, a series of criovials of all microbial strains in glycerolic medium were set up and stored at −80 °C. Pre-cultures of each bacterial strain were prepared in Cation Adjusted Mueller–Hinton broth (CAMHB) and incubated at 37 °C until the growth ceased. The turbidity of bacterial cell suspension was calibrated to 0.5 McFarland Standard by spectrophotometric method (625 nm, range 0.08–0.10), and further the standardized suspension was diluted 1:100 with CAMHB to have $1–2 \times 10^6$ CFU/mL. All wells were seeded with 100 μL of inoculum. A number of wells containing only inoculated broth as control growth were prepared. The plates were incubated at 37 °C for 24 h, and the MIC values were recorded as the last well containing no bacterial growth. The MICs were determined by using an antibacterial assay repeated twice in triplicate. Norfloxacin was used as reference drug.

4.7. Antifungal studies

Antifungal studies [16] were carried out against *C. albicans* 10231, *C. parapsilosis* 22019, *C. tropicalis* 750, *C. krusei* 6258, belonging to the ATCC collection. Preparation of stock solutions and purity of cultures preservation were obtained as above described for antibacterial studies. Pre-cultures of each yeast strain were prepared in Sabouraud broth 2% glucose (SAB), and incubated at 37 °C until the growth ceased. The turbidity of yeast stock suspension was calibrated to 0.5 McFarland Standard by spectrophotometric method (530 nm, range 0.12–0.15), and further the standardized suspension was diluted first 1:50 with SAB and then 1:20 in the same medium to have $1–5 \times 10^6$ CFU/mL. All wells were seeded with 100 μL of inoculum. A number of wells containing only inoculated broth as control growth were prepared. The plates were incubated at 37 °C for 24–48 h, and the MIC values were recorded as the last well containing no fungal growth. The MICs were determined by using an antifungal assay repeated twice in triplicates. Fluconazole was used as reference drug.

4.8. Molecular modeling studies

CLog*P* were calculated with ACD/ChemSketch v.12.01. Semi-rigid dockings were carried out by means of AutoDock ver. 4.2 [37] on the recently published homology models [26]. Blind dockings were initially carried out to explore the accessibility of the target, and also to prove the hypothesis of an inhibition of CA-CYP51 activity by the coordination of the iron atom elicited by the benzothiazole moiety of the studies compounds. Calculating the affinity maps on the entire protein surface the most favorable binding pose was, in all the instances, exactly inside the active site lodge surrounding the heme group. Afterward the cytochrome binding site was then defined as a $60 \times 60 \times 60$ cubic box, 0.374 Å spaced, centered on the iron atom of the heme group of CA-CYP51. Amber and AM1 charges were used for protein and ligands respectively. For each compound 200 runs were carried out, exploring the conformational space with the Lamarckian Genetic Algorithm (LGA). To increase the docking performance the population size and the number of energy evaluations were raised to 300 and 5,000,000 in that order, and for the whole docked molecules the best free energy of binding (FEB) poses were selected. To save computational time, the lone high active compound **1e** was submitted to flexible docking carried out exploring the side chains flexibility of the seven residues comprising the binding site proximity, namely Tyr118, Leu121, Phe228, His310, Thr311, Leu376 and Met508. The 200 samplings carried in this way gave results similar to the previous 200 rigid runs, with a RMSD between the selected poses of 0.619.

4.9. Toxicity for eukaryotic cells

Toxicity was assessed toward THP-1 cells (ATCC TIB-202), a human myelomonocytic cell line displaying macrophage-like activity [38]. These cells were maintained as a loose suspension in RPMI 1640

medium supplemented with 10% fetal calf serum (FCS) and 2 mmol L-glutamine/L in an atmosphere of 95% air/5% CO_2. Cells were subcultured every third day by gentle shaking, followed by pelleting and reseeding at a density of 3×10^3 cells/mL. The cultures were used after 2 days, when they reached a density of $0.1–5 \times 10^6$ cells/mL. Cells were incubated with compounds at increasing concentrations during 24 h and toxicity was evaluated using two assays in parallel, namely the release of lactate dehydrogenase (LDH), a cytoplasmic enzyme, and trypan blue exclusion assay [39]. This method represents a valid alternative to the MTT assay previously performed [11,40]. LDH release was measured with the Roche LDH kit (cytotoxicity detection kit [LDH], Roche, Basel, Switzerland) in accordance with the manufacturer's instructions. Percentage of LDH release was calculated relative to that of the control (cells incubated with 2% DMSO; 0% LDH release), and that of cells lysed with Triton X-100 (100% LDH release). For trypan blue assay, 90 μl of cell suspension incubated with compounds were mixed with 10 μL Trypan blue reagent (1:9 ratio) and incubated for 2–3 min at room temperature. 10 μL were transferred to a Burker counting chamber to determine the number of viable (transparent) and non-viable (blue) cells. At least 200 cells were counted per condition. Percentage mortality was determined as the ratio between the number of trypan blue labeled cells and the total cell number.

Acknowledgments

This work was accomplished thanks to the financial support of the Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR). The authors kindly acknowledge Prof. Antonio Macchiarulo for supplying the CA-CYP51 homology model.

References

[1] C. Pozzi, S. Ferrari, D. Cortesi, R. Lucini, R.M. Stroud, A. Catalano, M.P. Costi, S. Mangani, Acta Crystallogr. D68 (2012) 1232–1241.
[2] S.L. Khokra, K. Arora, H. Mehta, A. Aggarwal, M. Yadav, Int. J. Pharm. Sci. Res. 2 (2011) 1356–1377.
[3] R.V. Patel, P.K. Patel, P. Kumari, D.P. Rajani, K.H. Chikhalia, Eur. J. Med. Chem. 53 (2012) 41–51.
[4] A. Gupta, S. Rawat, J. Curr. Pharm. Res. 3 (2010) 13–23.
[5] G. Alang, R. Kaur, G. Kaur, A. Singh, P. Singla, Acta Pharm. Sci. 52 (2010) 213–218.
[6] C. Franchini, M. Muraglia, F. Corbo, M. Florio, A. Di Mola, A. Rosato, R. Matucci, M. Nesi, F. Bambeke, C. Vitali, Arch. Pharm. Chem. Life Sci. 342 (2009) 605–613.
[7] D. Armenise, M. Muraglia, M.A. Florio, N. De Laurentis, A. Rosato, A. Carrieri, F. Corbo, C. Franchini, Arch. Pharm. Chem. Life Sci. 345 (2012) 407–416.
[8] J.K. Malik, F.V. Manvin, Nanjade S. Singh, Drug Invent. Today 1 (2009) 32–34.
[9] A. Carocci, A. Catalano, F. Corbo, A. Duranti, R. Amoroso, C. Franchini, G. Lentini, V. Tortorella, Tetrahedron: Asymmetry 11 (2000) 3619–3634.
[10] R. Pascale, A. Carocci, A. Catalano, G. Lentini, A. Spagnoletta, M.M. Cavalluzzi, F. De Santis, A. De Palma, V. Scalera, C. Franchini, Bioorg. Med. Chem. 18 (2010) 5903–5914.
[11] A. Catalano, J.-F. Desaphy, G. Lentini, A. Carocci, A. Di Mola, C. Bruno, R. Carbonara, A. De Palma, R. Budriesi, C. Ghelardini, M.G. Perrone, N.A. Colabufo, D. Conte Camerino, C. Franchini, J. Med. Chem. 55 (2012) 1418–1422.
[12] A. Carocci, A. Catalano, A. Lovece, G. Lentini, A. Duranti, V. Lucini, M. Pannacci, F. Scaglione, C. Franchini, Bioorg. Med. Chem. 18 (2010) 6496–6511.
[13] J.B. Baell, P.J. Duggan, S.A. Forsyth, R.J. Lewis, Y.P. Lok, C.I. Schroeder, Bioorg. Med. Chem. 12 (2004) 4025–4037.
[14] K. Nagarajan, S.B. Hendi, A.N. Goud, H.G. Sen, B.N. Deb, Ind. J. Pharm. Sci. 48 (1991) 85–88.
[15] CLSI, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Approved Standard, eighth ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2009. M7–A8.
[16] CLSI, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast. Approved standard, third ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2008. M27–A3.
[17] J.E. Schuster, R. Fu, T. Siddique, C.J. Heckman, J. Neurophysiol. 107 (2012) 484–492.
[18] C. Hansch, T. Fujita, J. Am. Chem. Soc. 86 (1964) 1616–1626.
[19] H.V. Bossche, L. Koymans, Mycoses 41 (1998) 32–38.
[20] D.J. Sheehan, C.A. Hitchcock, C.M. Sibley, Clin. Microbiol. Rev. 12 (1999) 40–79.
[21] D. Lamb, D. Kelly, S. Kelly, Drug Res. Updat. 2 (1999) 390–402.
[22] G.I. Lepesheva, M.R. Waterman, Biochim. Biophys. Acta 1770 (2007) 467–477.
[23] H. Ji, W. Zhang, Y. Zhou, M. Zhang, J. Zhu, Y. Song, J. Lü, J. Zhu, J. Med. Chem. 43 (2000) 2493–2505.
[24] H. Ji, W. Zhang, M. Zhang, M. Kudo, Y. Aoyama, Y. Yoshida, C. Sheng, Y. Song, S. Yang, Y. Zhou, J. Lü, J. Zhu, J. Med. Chem. 46 (2003) 474–485.
[25] J. Zhu, J. Lu, Y. Zhou, Y. Li, J. Cheng, C. Zheng, Bioorg. Med. Chem. Lett. 16 (2006) 5285–5289.
[26] A. Macchiarulo, G. Costantino, D. Fringuelli, A. Vecchierelli, F. Schiaffella, R. Fringuelli, Bioorg. Med. Chem. 10 (2002) 3415–3423.
[27] F. Schiaffella, A. Macchiarulo, L. Milanese, A. Vecchierelli, G. Costantino, D. Pietrella, R. Fringuelli, J. Med. Chem. 48 (2005) 7658–7666.
[28] I.F. Sevrioukova, T.L. Poulos, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 18422–18427.
[29] M.M. Cavalluzzi, C. Bruno, G. Lentini, A. Lovece, A. Catalano, A. Carocci, C. Franchini, Tetrahedron: Asymmetry 20 (2009) 1984–1991.
[30] A. Catalano, A. Carocci, F. Corbo, C. Franchini, M. Muraglia, A. Scilimati, M. De Bellis, A. De Luca, D. Conte Camerino, M.S. Sinicropi, V. Tortorella, Eur. J. Med. Chem. 43 (2008) 2923–2925.
[31] C. Franchini, A. Carocci, A. Catalano, M.M. Cavalluzzi, F. Corbo, G. Lentini, A. Scilimati, P. Tortorella, D. Conte Camerino, A. De Luca, J. Med. Chem. 46 (2003) 5238–5248.
[32] U. Sharma, P.K. Verma, N. Kumar, V. Kumar, M. Bala, B. Singh, Chem. Eur. J. 17 (2011) 5903–5907.
[33] C.N. Carrigan, R.D. Bartlett, C.S. Esslinger, K.A. Cybulski, P. Tongcharoensirikul, R.J. Bridges, C.M. Thompson, J. Med. Chem. 45 (2002) 2260–2276.
[34] A. Catalano, A. Carocci, G. Lentini, I. Defrenza, M.M. Cavalluzzi, C. Franchini, Drug Metab. Lett. 6 (2012) 124–128.
[35] A. Catalano, A. Carocci, G. Lentini, I. Defrenza, C. Bruno, C. Franchini, Drug Metab. Lett. 6 (2012) 182–186.
[36] H. Yang, Y. Li, J. Wang, H. Fu, Chem. Eur. J. 17 (2011) 5652–5660.
[37] G.M. Morris, R. Huey, W. Lindstrom, M.F. Sanner, R.K. Belew, D.S. Goodsell, A.J. Olson, J. Comput. Chem. 16 (2009) 2785–2791.
[38] S. Tsuchiya, M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, K. Tada, Int. J. Cancer 26 (1980) 171–176.
[39] M.N. Berry, H.J. Halls, M.B. Grivell, Life Sci. 51 (1992) 1–16.
[40] A. Carocci, A. Catalano, C. Bruno, A. Lovece, M.G. Roselli, M.M. Cavalluzzi, F. De Santis, A. De Palma, M.R. Rusciano, M. Illario, C. Franchini, G. Lentini, Bioorg. Med. Chem. 21 (2013) 847–851.