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Bulky 1,4-benzoxazine derivatives with antifungal activity

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ABSTRACT

For many years the development of new azole antifungals has been quite empirically based. More recently, the publication of the crystal structure of CYP51 of *Mycobacterium tuberculosis* (MT-CYP51) provided new opportunities to rationalize the knowledge about antifungal action of this class of compounds. Recent studies reported that a 'channel 2 opened' conformation of the enzyme could better explain the interaction with ketoconazole (KTZ)-like drugs. Conformational changes were made on our model of *Candida albicans* CYP51 (CA-CYP51) previously reported and docking experiments were performed. The results allowed new KTZ analogues to be designed, by predicting that the 1,4-benzoxazine moiety could replace the KTZ aryl-piperazinyl chain. The synthesis of derivatives **12** and **13** was planned.
The in vitro antifungal activity was evaluated against different *Candida* species and low and high capsulated strains of *Cryptococcus neoformans*. Since the in vitro activity do not necessarily correlate with the in vivo antifungal activity the newly synthesized compounds were also tested in a murine model of systemic *C. albicans* infection. The therapeutic effect was evaluated in terms of animal survival and of fungal growth in the kidneys, the target organ in systemic candidiasis.

1. Introduction

In recent years, the incidence of fungal infections has markedly increased particularly in high risk units in which hospitalized patients are treated with immunosuppressive agents, anticancer, anti-AIDS drugs, or broad-spectrum antibiotics.[1,2] Amongst the different pathogens responsible for opportunistic fungal infections are yeasts belonging to the genera *Candida* and *Cryptococcus*. In particular, *Candida albicans* is a ubiquitous mucosal commensal in equilibrium with the microbial flora and the host immune system, but is also responsible for a wide range of infections in immunocompromised persons. A major obstacle in the treatment of *Candida* infections is the spread of antifungal drug resistance, particularly in patients subjected to prolonged antimycotic therapy.[3] Polyenic and azole antifungal agents are usually used to treat *Candida* infections, but despite the good antifungal activities observed in vitro, their therapeutic efficacy is variable, so that candidemia is still a major cause of death.[4] Moreover, antimycotic therapy is usually marred by drug solubility problems as well as by a number of adverse side effects.[5]

Therefore, new effective anti-*Candida* agents are needed to combat the drug-resistant strains and widespread diffusion of *C. albicans*.

For many years the development of new azole antifungals has been quite empirically based. More recently, the publication of the crystal structure of CYP51 of *Mycobacterium tuberculosis* (MT-CYP51, pdb code: 1ea1, 1e9x), both alone and co-crystallized with fluconazole (FLU),[6] provided new opportunities to rationalize the knowledge about antifungal action of this class of compounds.

In a program aimed at the design and synthesis of novel azole inhibitors of *C. albicans* CYP51 (CA-CYP51), a series of azole 1,4-benzothiazines (1,4-BTs) and 1,4-benzoxazines (1,4-BOs) were recently synthesized.[7–13] To better understand the SAR of these derivatives, and to hypothesize the modes of interactions between the synthesized compounds and the target enzyme, a 3D homology model of CYP51 of *C. albicans* was built on the basis of the homology relationship existing between the amino acidic sequences of CA-CYP51 and MT-CYP51 (40% similarity, 22% sequence identity).[11]

Docking studies were performed on the model using FLU and representative 1,4-BTs and 1,4-BOs chosen from those previously synthesized. The best docking solutions showed that FLU and the considered compounds adopt similar binding modes inside the catalytic site of CA-CYP51. For this purpose, the attention was focused on portions of the molecule that had not been investigated previously. To better characterize the putative interaction with the enzyme of bulky molecules similar to ketoconazole (KTZ), and

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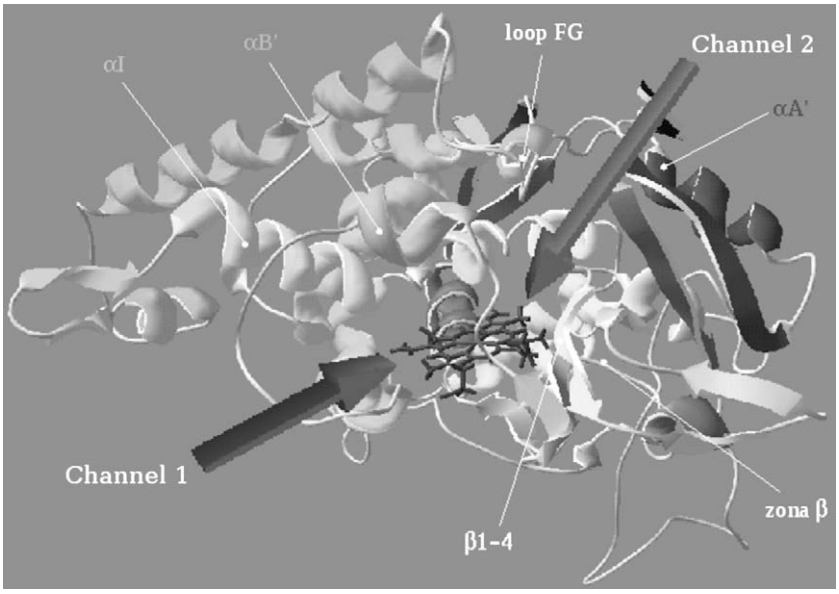


Figure 1. Homology model of CA-CYP51. The two channels controlling the access to the catalytic site are labeled and indicated with the arrows. Channel 1 runs parallel to the plane of the heme group whereas channel 2 is perpendicular to that plane. The principal secondary structure elements of the enzyme are also indicated.

compound **1** previously synthesized in our laboratory[14] further analyses on several CYP51 X-ray structures were carried out.[15,16] Results of these studies highlighted the presence of a second substrate access channel (channel 2), almost perpendicular to the heme, apparently closed in the MT-CYP51 published crystal.

Recent studies, carried on by Xiao and coworkers, reported that a 'channel 2 opened' conformation of the enzyme could better explain the interaction with KTZ-like drugs.[17] (Fig. 1)

According to this methodology, we have modified our model of CA-CYP51 in order to reproduce the 'channel 2 opened' conformation, and new docking experiments were thus performed on this new model. As a result, we show here that new KTZ analogues can be designed, by replacing the KTZ aryl-piperazinyl chain with 1,4-BO. Indeed, several structures were docked into the model, and those that showed the best binding energy values were 1,4-BO derivatives bound to the ketal moiety through the position C-6 of the nucleus-base.

The synthesis of derivatives **12** and **13** was so planned taking into account that the alkylation of the C-2 position was favorable in other series of compounds[12] (Fig. 2).

2. Docking experiments

According to the hypothesis of Xiao et al.,[17] the conformation of the side chain of two amino acids (Tyr118 and Met508) was manually changed in order to open channel 2 in our homology model of CA-CYP51.[11] In particular, this was accomplished by rotating of about 60° the dihedral angle defined by the atoms N, C_α, C_β, C_γ of Tyr118 and Met508. To evaluate the interaction energy between

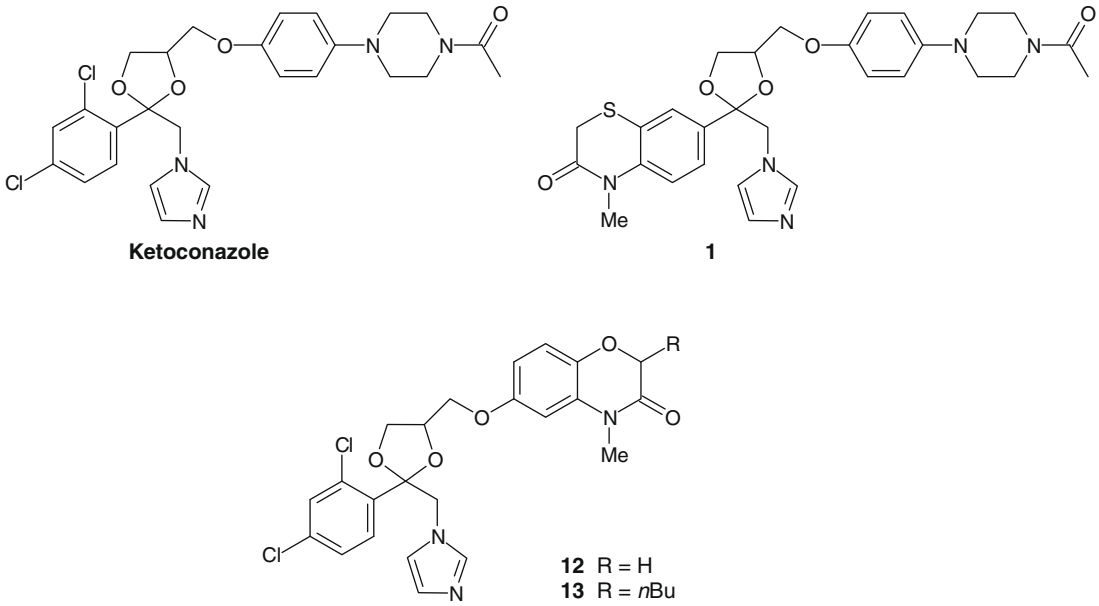


Figure 2.

Table 1
Binding energies and *Alog P* values of ketoconazole (KTZ) and 1,4-benzoxazine derivatives with best docking results

Compound	E_{bin} (kcal/mol)	Alog P
KTZ	−28.616	4.480
12	−35.000	3.660
(*S*)-**13**	−35.784	5.459
(*R*)-**13**	−7.846	5.459

the ligands and the enzyme, we performed automated docking experiments of the inhibitors as reported in Section 5.

Docking results presenting the binding pose of the inhibitors with the long side chain stretched out in the channel 2 were considered as 'productive' and stored for further analysis. The binding energies (E_{bin}, Table 1) were calculated using a knowledge-based strategy considering the protein as a rigid-body and removing all the constrains used during docking experiments.[11]

All compounds adopt a similar interaction pattern which is schematically represented in the following Figure 3.

As far as it concerns compound **13**, docking experiments reveal that the *S* isomer fits better the enzymatic site along channel 2 then the *R* isomer. The adopted binding pose of compounds **13*S*** and **12**, while in agreement with that of FLU, allows the benzoxazine moiety to form hydrogen bonding with the backbone of Tyr505 and Met508. Finally, the inspection of the *Alog* P values (Table 1) reveals a good balance of hydrophilic and hydrophobic properties of these compounds for crossing the fungi membrane.[11]

3. Chemistry

The synthesis and determination of the configuration of *O*-*p*-toluenesulfonyl (Ts) ketal precursors, *cis*- and *trans*-**8**, were performed as described by Rotstein and coworkers.[18] Since 1,4-BO of the 2-butyl serie did not react with the Ts-ketals with satisfactory yields, the corresponding methanesulfonyl (Ms) derivative **9** was also prepared. In this case, it was not possible to separate the enantiomeric couples by chromatography, so the successive reactions were performed on the whole mixture and the separation was carried out on the final products.

For the synthesis of the 1,4-BO moiety the key step was to obtain the 1,4-BO intermediates with a reactive function in the C-6 position: compound **6** was obtained by the selective 6-hydroxylation of 3,4-dihydro-2*H*-1,4-benzoxazin-3-one performed with

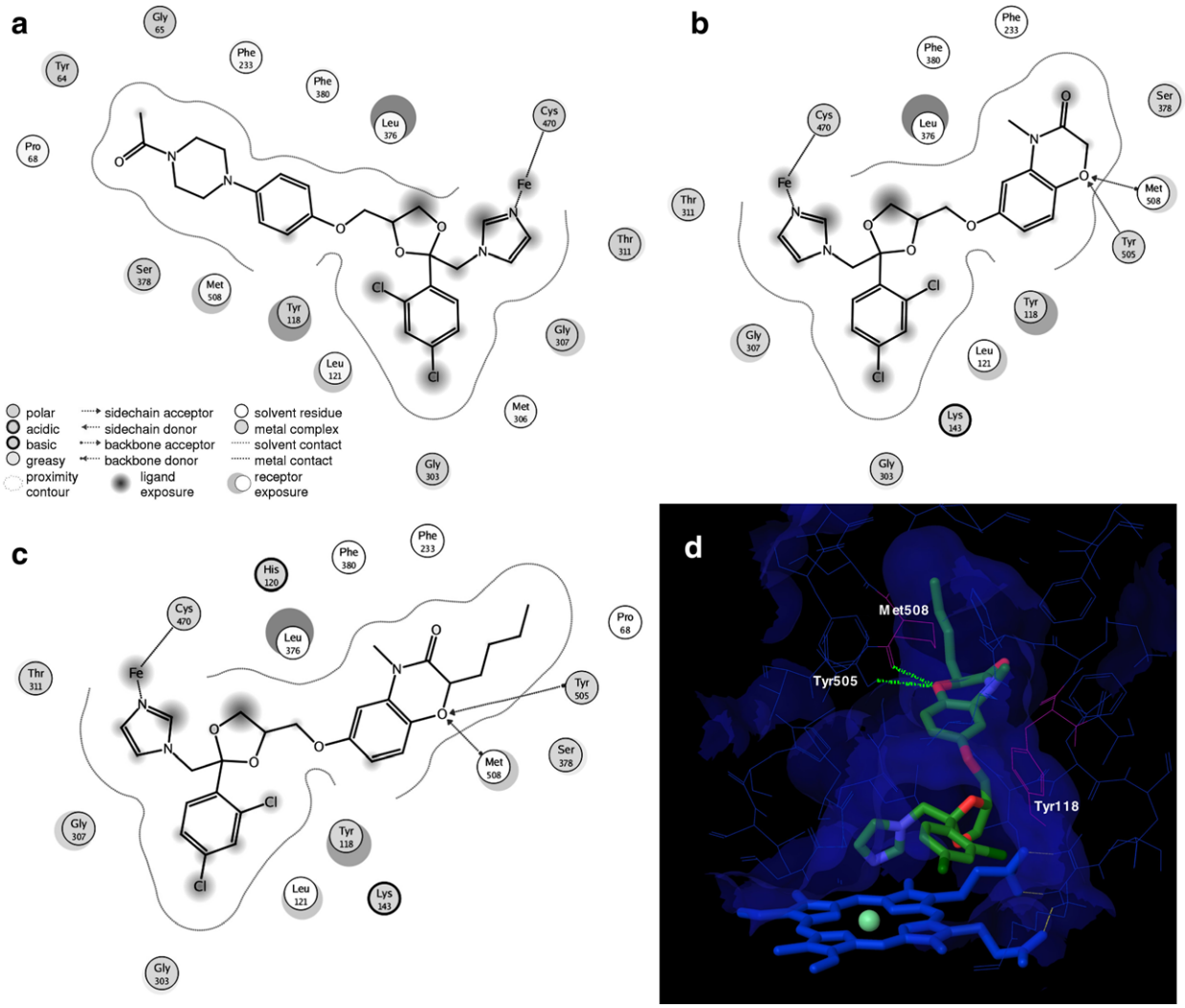
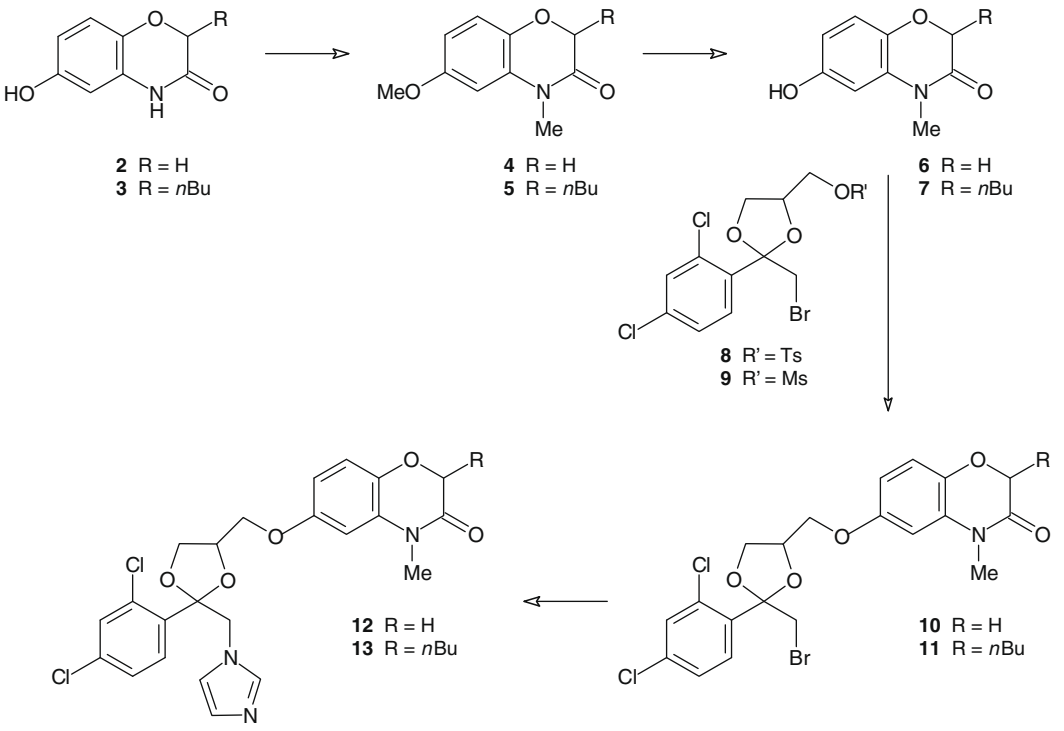


Figure 3. Best binding poses resulting from docking experiments of KTZ (a), NN compound (b) and MM compound (c, d). (a–c) were drawn using the MOE Ligand Interaction tool (Molecular Operating Environment, 2006.08 ed.; Chemical Computing Group, Inc. Montreal, Quebec, Canada.). (d) shows the binding pose of the compound MM (green sticks) highlighting the hydrogen bonds (green lines) with Tyr505 and Met508. In magenta are shown the 2 residues (Met508 and Tyr118) whose conformation was modified in order to open channel 2. In blue is shown the cavity of channel 2.



Scheme 1. Reagents: (a) *t*BuOK, MeI; (b) 48% HBr; (c) NaH; (d) K_2CO_3, 1*H*-imidazole.

[bis(trifluoroacetoxy)iodo]benzene (PIFA),[19] and the same reaction gave good yields of the 2-butyl derivative **7** starting from 2-butyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one.[20] The hydroxylated intermediates thus obtained were methylated (**4** and **5**) and the fenolic function was restored refluxing with 48% HBr. Compound **6** was then condensed with both *cis*- and *trans*-**8**, while derivative **7** reacted with the corresponding Ms-ketal **9**. Finally, imidazole was introduced thus obtaining the desired products **12** and **13** (Scheme 1).

As far as compound **13** concerns, since the ketal moiety employed in the synthesis was composed of all four diastereomers, and the 1,4-BO moiety introduces a third stereogenic center, our product was a mixture of eight isomers. This mixture was split into two fractions by flash chromatography. Examination of their ^{1}H NMR spectra, by analogy with data previously reported, has reasonably permitted to suppose that the less polar fraction is composed of stereoisomers with a *syn* configuration on the ketal moiety, *cis*-**13**, while the second fraction, *trans*-**13**, is the mixture of the four *anti* isomers.

4. Results and discussion

The antifungal activity of the synthesized compounds was evaluated against different *Candida* species and low and high capsulated strains of *Cryptococcus neoformans*. The results, reported in Table 2, indicate that all compounds exhibited good antifungal activity on *C. albicans* strain CA-6, with MIC equal to 0.49 μg/mL after 24 and 48 h incubation. The MIC of KTZ, used as reference, was <0.0076 μg/mL. Interestingly, only *cis*-**13** exhibited low (MIC 125 μg/mL) but appreciable activity against the low virulent *C. albicans* strain PCA-2 unable to undergo mycelial transition, all other compounds being totally ineffective. As regards *Candida krusei*, *Candida glabrata* and *Candida parapsilosis*, all compounds showed no or modest antifungal activity, with MIC ranging from 3.90 to >500 μg/mL.

When assayed on *C. neoformans*, the compounds showed a good antifungal activity, particularly against the high capsulated strain CAP-67, with MIC ranging from 0.0019 for *cis*-**12** to 3.90 μg/mL for *trans*-**13**.

Table 2
In vitro antifungal activity of different synthesized compounds

	MIC[a] (μg/mL)													
	C. albicans strain CA-6		*C. albicans* strain PCA-2		*C. krusei*		*C. glabrata*		*C. parapsilosis*		*C. neoformans* strain 6995		*C. neoformans* strain CAP67	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	48 h	72 h	48 h	72 h
cis-**12**	0.49	0.49	>500	>500	15.60	>500	7.80	>500	250	>500	0.24	0.24	0.0019	0.0019
trans-**12**	0.49	0.49	>500	>500	15.60	>500	15.60	250	>500	>500	0.97	1.95	0.03	0.03
cis-**13**	0.49	0.49	125	125	3.90	3.90	31.25	125	31.25	31.25	1.95	7.80	0.97	1.95
trans-**13**	0.49	0.49	>500	>500	3.90	7.80	62.50	>500	125	500	7.80	7.80	3.90	3.90
KTZ[b]	<0.0076	<0.0076	15.60	15.60	0.49	0.97	0.12	1.95	<0.00006	0.00095	0.00095	0.00095	0.00012	0.00012
FLU[c]	<0.0076	<0.0076	>500	>500	250	>500	125	250	7.80	15.60	1.95	3.90	0.49	0.49
Diluent[d]	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500

[a] MIC: minimum inhibitory concentration.
[b] KTZ: ketoconazole.
[c] FLU: fluconazole.
[d] Diluent: EtOH/H_2O 1:4.

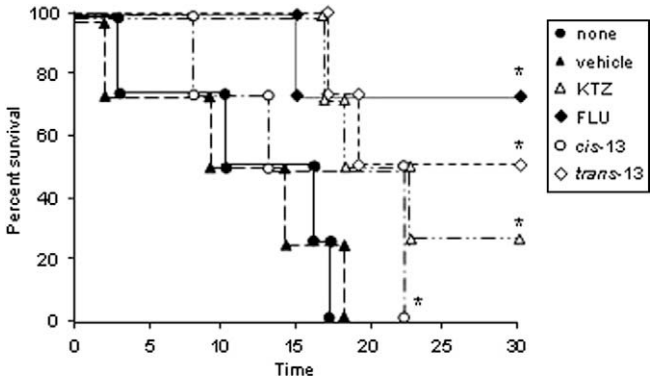


Figure 4. Percent survival of *C. albicans* infected mice. Mice were infected at day 0 with *C. albicans* CA-6 (7×10^5/mouse, i.v.) and treated 2 h before and once a day for 6 consecutive days after the infection with *cis*-**13**, *trans*-**13**, KTZ, FLU (all 10 mg/kg, i.p.) or vehicle (0.2 ml 2% EtOH in saline, i.p.). *: $p < 0.05$, treated versus untreated.

Collectively, the results demonstrate that synthesized compounds are active against *Candida* and *Cryptococcus* in vitro. Interestingly, data indicate that maximum activity is shown against *Candida* isolates capable of hypha transition like *C. albicans* CA6 and *C. krusei* rather than *C. albicans* PCA-2 and *C. glabrata*, both agerminative, suggesting that process leading to hypha formation could be targeted by the compounds.

Since the in vitro activity does not necessarily correlate with the in vivo antifungal activity[21–23] the newly synthesized compounds were also tested in a murine model of systemic *C. albicans* infection. To this purpose, mice were infected with *C. albicans* CA-6 and inoculated with the different compounds. Preliminary experiments indicated that treatment with *cis*-**12** and *trans*-**12** was ineffective (not shown), therefore they were not included in subsequent experiments. The therapeutic effect was evaluated in terms of animal survival and of fungal growth in the kidneys, the target organ in systemic candidiasis. Survival data, reported in Figure 4, indicate that treatment of infected mice with both *cis*-**13** and *trans*-**13** resulted in significant outcome improvement. In particular, treatment with *trans*-**13** resulted in 50% of mice surviving the full 30 days observation period, while treatment with *cis*-**13** resulted in significantly higher survival when compared to untreated controls (animals dying in 23 and 17 days, respectively). Noteworthy, treatment with *trans*-**13** resulted in improved survival rates when compared to KTZ treatment; of course (obviously), in the same experimental setting, FLU significantly improved outcome, resulting in 75% of animals surviving until the end of the observation period.

In parallel experiments, evaluation of fungal burden in the kidneys 7 days post infection, showed a significant reduction in CFU recovery in *cis*-**13** and *trans*-**13** treated mice. Remarkably, FLU treated mice almost completely cleared the infection (Fig. 5).

In the same experimental conditions, histological analysis of PAS-stained kidneys sections showed extensive fungal growth with multiple inflammatory foci in renal parenchima of *C. albicans* infected animals (Fig. 6A), while treatment with *cis*-**13**, *trans*-**13**, or FLU resulted in rare inflammatory foci with a small number of fungal cells (Fig. 6B–D).

To further assess the effect of synthesized compounds in murine systemic candidiasis, in selected experiments serum collected from mice 1 h after inoculation with *cis*-**13**, *trans*-**13**, FLU, or 2%

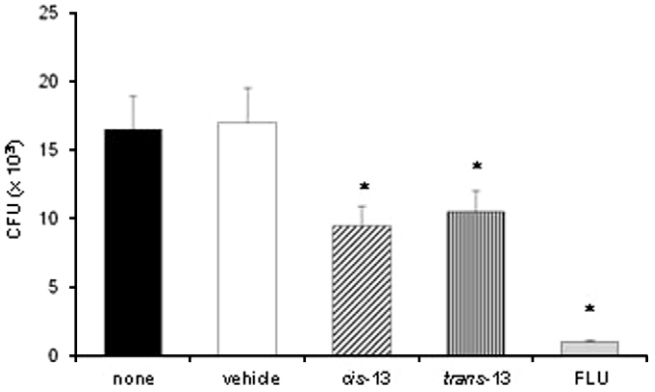


Figure 5. Fungal burden in the kidneys of *C. albicans* infected mice. Mice were infected at day 0 with *C. albicans* CA-6 (2.5×10^5/mouse, i.v.) and treated 2 h before and once a day for 6 consecutive days after the infection with *cis*-**13**, *trans*-**13**, FLU (all 10 mg/kg, i.p.) or vehicle (0.2 ml 2% EtOH in saline, i.p.). CFU recovery from kidneys was determined 7 days after infection. *: $p < 0.05$, treated versus untreated.

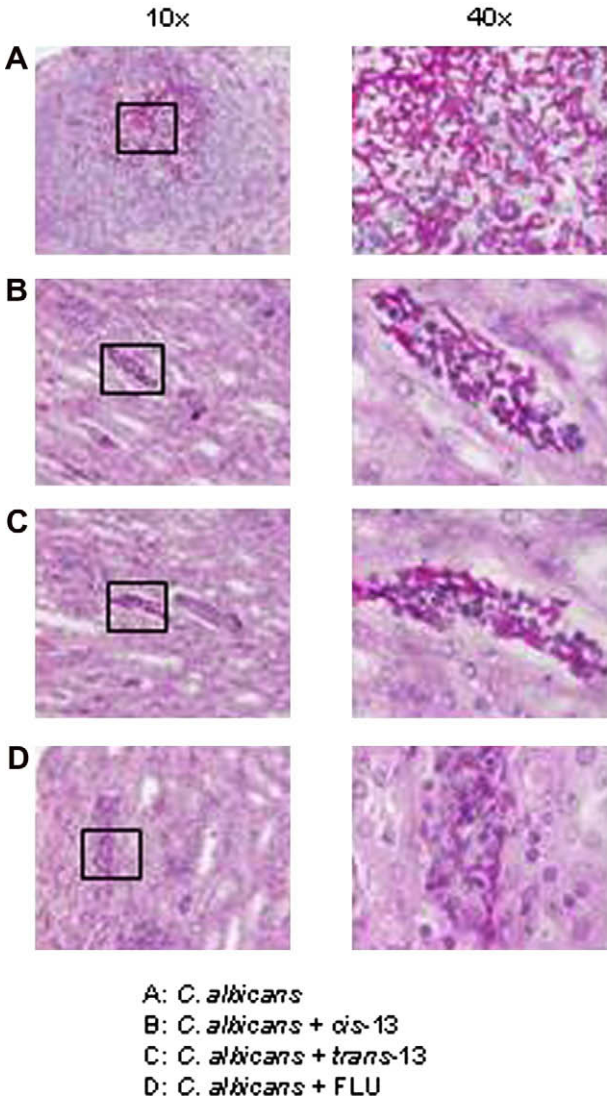


Figure 6. Histological analysis of kidneys from *C. albicans* infected mice. Mice were infected at day 0 with *C. albicans* CA-6 (7×10^5/mouse, i.v.) and treated 2 h before and once a day for 6 consecutive days after the infection with *cis*-**13**, *trans*-**13** or FLU (all 10 mg/kg). 7 days after infection, kidneys were excised and fixed in formalin. Sections of paraffin-embedded tissues were stained using the periodic acid-Schiff (PAS). Histologic evaluation of kidneys from mice infected with *C. albicans* shows extensive fungal growth, associated with the presence of numerous foci of inflammatory reaction throughout the parenchyma (A). Note the reduced fungal growth and few pathologic lesions in the kidneys of *C. albicans* infected mice treated with *cis*-**13** (B), *trans*-**13** (C), or FLU (D). Boxes indicate the areas magnified.

ethanol solution, was passively transferred into recipient mice subsequently challenged with *C. albicans* CA-6. Recovery of CFU in the kidneys, 7 days after challenge, showed that inoculation of serum from treated animals resulted in significantly lower fungal burden in kidneys (Fig. 7).

This result confirms that the synthesized compounds are indeed distributed by blood circulation to the different organs. Moreover, the high effect shown in Figure 7 could suggest that a sort of modification or activation of this compound would occur soon after inoculation in vivo.

We conclude that it may be feasible to modify the structure of KTZ to render more active the compound in vivo. Furthermore, we demonstrated that these conformational modifications lead to powerful antifungal activity in vivo, possibly due to shedding of potent antifungal molecules, as documented by protective effects of passive transfer of serum from *trans*-**13** treated mice into *C. albicans* infected animals.

5. Experimental

Melting points determined in capillary tubes (Electrothermal, model 9100, melting point apparatus) were uncorrected. Elemental analysis was performed on a Carlo Erba element analyzer 1106, and the data for C, H, and N are within ±0.4% of the theoretical values. ^{1}H NMR and ^{13}C NMR spectra were recorded at 200 MHz (Bruker AC-200 spectrometer) with Me_4Si as internal standard. Chemical shifts are given in ppm (δ), and the spectral data are consistent with the assigned structures. HPLC-MS was checked employing an Agilent 1100 binary HPLC system (Agilent Technologies, CA, USA) interfaced with an API 150EX single quadrupole MS spectrometer (Applied Biosystem/MDS Sciex, CA, USA) equipped with an Electrospray (ESI) Ion Source. Reagents and solvents were purchased from common commercial suppliers and used as received. Column chromatography separations were carried out on Merck Silica Gel 60 (mesh 230–400). Yields of purified products were not optimized. All starting materials were commercially available unless otherwise indicated.

5.1. 2-Butyl-6-hydroxy-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (3)

[Bis(trifluoroacetoxy)iodo]benzene (PIFA) (2.50 g, 5.81 mmol) in 10 mL of trifluoroacetic acid (TFA) was added to a refluxing solution of 2-butyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one[20] (1.00 g, 4.88 mmol) in TFA (10 mL). After refluxing for 5 min, the solution was concentrated at half of its volume, neutralized with a saturated solution of $NaHCO_3$, then extracted with EtOAc. The organic phase was dried, evaporated, and purified by chromatography eluting with cyclohexane/EtOAc 40:60 to give **3** (0.73 g, 67%) as a white solid, mp 191–193 °C, ^{1}H NMR (DMSO-d_6) δ: 0.88 (3H, t, J = 7.0 Hz, CH_2CH_3), 1.20–2.00 (6H, m, $CH_2CH_2CH_2CH_3$), 4.40 (1H, m, CHO), 6.15–6.85 (3H, m, aromatic H), 9.18 (H, br s, NH), 10.52 (H, br s, OH). Anal. Calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.03; H, 6.84; N, 6.31.

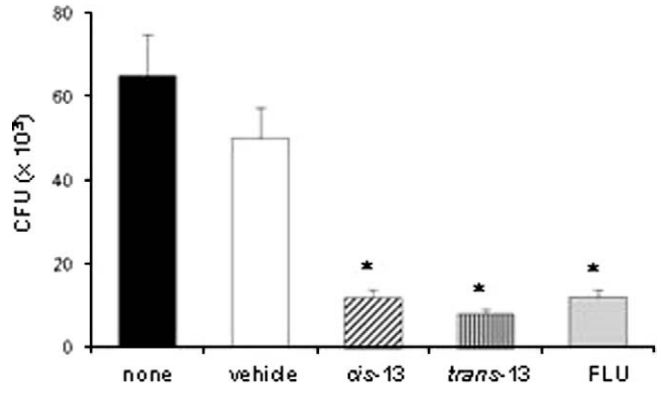


Figure 7. Fungal clearance after passive transfer of serum in *C. albicans* infected mice. Mice were infected with *C. albicans* CA-6 (7×10^5/mouse, i.v.) after intraperitoneal inoculation with 0.3 ml of serum recovered from mice 1 h after treatment with *cis*-**13**, *trans*-**13**, FLU (all 10 mg/kg, i.p.) or vehicle (0.2 ml 2% EtOH in saline, i.p.). CFU recovery from kidneys was determined 7 days after *C. albicans* infection. *: $p < 0.05$, treated versus untreated.

5.2. 6-Methoxy-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (4)

t-BuOK (1.68 g, 15.00 mmol) was added to a solution of 6-hydroxy-3,4-dihydro-2*H*-1,4-benzoxazin-3-one[19] (**2**) (1.00 g, 6.06 mmol) in dry DMF (30 mL). The mixture was stirred at room temperature for 15 min, and then MeI (2.13 g, 15.00 mmol) in dry DMF (10 mL) was added dropwise. After being stirred for 1 h, the mixture was poured into ice-chilled water and extracted with EtOAc. The residue was chromatographed, eluting with cyclohexane/EtOAc 65:35. Compound **4** (0.47 g, 40%) was obtained as a white solid, mp 70.6–71.8 °C, ^{1}H NMR ($CDCl_3$) δ 3.33 (3H, s, NCH_3), 3.80 (3H, s, OCH_3), 4.55 (2H, s, OCH_2), 6.45–6.60 (2H, m, H-5 and H-8), 6.90 (1H, dd, J = 8.0 and 2.5 Hz, H-7). Anal. Calcd for $C_{10}H_{11}NO_3$: C, 62.17; H, 5.74; N, 7.25. Found: C, 62.24; H, 5.74; N, 7.24.

5.3. 2-Butyl-6-methoxy-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (5)

It was synthesized starting from **3** using the same procedure described for **4**. Oil, 70% yield. ^{1}H NMR ($CDCl_3$) δ 0.93 (3H, t, J = 7.0 Hz, CH_2CH_3), 1.20–1.60 (4H, m, $CH_2CH_2CH_2CH_3$), 1.60–2.00 (2H, m, $CH_2CH_2CH_2CH_3$), 3.35 (3H, s, NCH_3), 3.81 (3H, s, OCH_3), 4.53 (1H, m, CHO), 6.40–7.00 (3H, m, aromatic H). Anal. Calcd for $C_{14}H_{19}NO_3$: C, 67.45; H, 7.68; N, 5.62. Found: C, 67.24; H, 7.70; N, 5.64.

5.4. 6-Hydroxy-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (6)

A solution of **4** (1.00 g, 5.18 mmol) in 48% HBr (15 mL) was refluxed for 2 h, then was poured into ice-chilled water and extracted with EtOAc. The organic phase was dried and evaporated in vacuo to give **6** (0.87 g, 93%) as a pale yellow solid, mp 207.7–208.5 °C, which was used without further purification. ^{1}H NMR ($CDCl_3$) δ 3.37 (3H, s, NCH_3), 4.60 (2H, s, OCH_2), 6.49 (1H, dd, J = 8.5 and 2.7 Hz, H-7), 6.57 (1H, d, J = 2.7 Hz, H-5), 6.89 (1H, d, J = 8.5 Hz, H-8), 8.13 (1H, s, OH). Anal. Calcd for $C_9H_9NO_3$: C, 60.33; H, 5.06; N, 7.82. Found: C, 60.33; H, 5.07; N, 7.79.

5.5. 2-Butyl-6-hydroxy-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (7)

It was synthesized starting from **5** using the same procedure described for **6**. White solid, mp 80–82 °C, 70% yield. ^{1}H NMR (acetone-d_6) δ 0.93 (3H, t, J = 7.0 Hz, CH_2CH_3), 1.25–1.65 (4H, m, $CH_2CH_2CH_2CH_3$), 1.65–1.90 (2H, m, $CH_2CH_2CH_2CH_3$), 3.31 (3H, s, NCH_3), 4.44 (1H, dd, J = 13.1 and 4.8 Hz, CHO), 6.51 (1H, dd, J = 8.6 and 2.6 Hz, H-7), 6.62 (1H, d, J = 2.6 Hz, H-5), 6.84 (1H, d, J = 8.6 Hz, H-8), 8.26 (1H, s, OH). Anal. Calcd for $C_{13}H_{17}NO_3$: C, 66.36; H, 7.28; N, 5.95. Found: C, 66.30; H, 7.30; N, 5.93.

5.6. *cis*-6-{[2-(Bromomethyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl]methoxy}-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (*cis*-10)

A solution of **6** (0.26 g, 1.44 mmol) in dry DMSO (3 mL) was added dropwise to a suspension of NaH (60% mineral oil disper-

sion, 0.063 g, 1.58 mmol) in dry DMSO (2 mL). After stirring at room temperature for 1 h, a solution of *cis*-[2-(bromomethyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl]methyl 4-methylbenzenesulfonate[18] (*cis*-**8**) (0.50 g, 1.01 mmol) in DMSO (3 mL) was added dropwise. The mixture was then heated to 80 °C for 4 h, poured into ice-chilled water and extracted with EtOAc. The residue was chromatographed, eluting with cyclohexane/EtOAc 80:20, thus giving *cis*-**10** (0.40 g, 79%), as a white solid that was crystallized from cyclohexane/EtOAc, mp 97.9–99.4 °C, ^{1}H NMR ($CDCl_3$) δ 3.39 (3H, s, NCH_3), 3.91 and 4.02 (each 1H, d, J = 11.4 Hz, CH_2Br), 4.02–4.32 (4H, m, CH_2CHCH_2OAr), 4.41–4.88 (1H, m, CH_2C*H*CH_2OAr), 4.61 (2H, s, OCH_2CO), 6.55–6.75 (2H, m, benzoxazine H-5 and H-7), 6.96 (1H, d, J = 8.5 Hz, benzoxazine H-8), 7.33 (1H, dd, J = 9.0 and 2.0 Hz, 2,4-dichlorophenyl H-5), 7.48 (1H, d, J = 2.0 Hz, 2,4-dichlorophenyl H-3), 7.70 (1H, d, J = 9.0 Hz, 2,4-dichlorophenyl H-6). Anal. Calcd for $C_{20}H_{18}BrCl_2NO_5$: C, 47.74; H, 3.61; N, 2.78. Found: C, 47.80; H, 3.60; N, 2.78.

5.7. *trans*-6-{[2-(Bromomethyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl]methoxy}-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (*trans*-10)

It was synthesized starting from **6** and ketal *trans*-[2-(bromomethyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl]methyl 4-methylbenzenesulfonate[18] (*trans*-**8**), using the same procedure described for *cis*-**10**. Amorphous solid, 59% yield, ^{1}H NMR ($CDCl_3$) δ 3.29 (3H, s, NCH_3), 3.85 and 4.00 (3H, m, C*H*HCHCH_2OAr), 3.89 (2H, s, CH_2Br), 4.46 (1H, dd, J = 8.2 and 6.5 Hz, CH*H*CHCH_2OAr), 4.55 (2H, s, OCH_2CO), 4.70–4.87 (1H, m, CH_2CHCH_2OAr), 6.30 (1H, d, J = 2.6 Hz, benzoxazine H-5), 6.37 (1H, dd, J = 8.5 and 2.6 Hz, benzoxazine H-7), 6.85 (1H, d, J = 8.5 Hz, benzoxazine H-8), 7.24 (1H, dd, J = 8.5 and 2.1 Hz, 2,4-dichlorophenyl H-5), 7.39 (1H, d, J = 2.1 Hz, 2,4-dichlorophenyl H-3), 7.68 (1H, d, J = 8.5 Hz, 2,4-dichlorophenyl H-6). Anal. Calcd for $C_{20}H_{18}BrCl_2NO_5$: C, 47.74; H, 3.61; N, 2.78. Found: C, 47.84; H, 3.61; N, 2.77.

5.8. 6-{[2-(Bromomethyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl]methoxy}-2-butyl-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (11)

It was synthesized using the same procedure described for *cis*-**10** starting from **7** and [2-(bromomethyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4yl]methymethanesulfonate[24] (**9**), the latter as the *cis*/*trans* diastereomeric mixture. The mixture of the eight isomers of **11** thus obtained (overall yield 48%) was used for the following condensation reaction. ^{1}H NMR according to diastereomeric mixture.

5.9. *cis*-6-{[2-(2,4-Dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}-4-methyl-3,4-dihydro-2H-1,4-benzoxazin-3-one (*cis*-12)

A mixture of *cis*-**10** (0.40 g, 0.79 mmol), 1*H*-imidazole (0.27 g, 3.98 mmol) and K_2CO_3 (0.55 g, 3.98 mmol) in DMA (5 mL) was refluxed for 6 h, then cooled, diluted with water, and extracted with EtOAc. The residue obtained after evaporation, was chromatographed eluting with $CHCl_3$ furnishing *cis*-**12** (0.25 g, 65%) as a white solid that was crystallized from cyclohexane/EtOAc, mp 160.2–160.8 °C, ^{1}H NMR ($CDCl_3$) δ 3.25 (1H, dd, J = 9.4 and 6.8 Hz, C*H*HCHCH2OAr), 3.41 (3H, s, NCH_3), 3.65–4.00 (3H, m, CH*H*CHCH_2OAr), 4.30–4.45 (1H, m, CH_2CHCH_2OAr), 4.46 and 4.58 (each 1H, d, J = 14.5 Hz, CH_2N), 4.60 (2H, s, OCH_2CO), 6.43 (1H, dd, J = 8.6 and 2.7 Hz, benzoxazine H-7), 6.55 (1H, d, J = 2.7 Hz, benzoxazine H-5), 6.93 (1H, d, J = 8.6 Hz, benzoxazine H-8), 7.06 (2H, br s, imidazolic H), 7.32 (1H, dd, J = 8.4 and 2.0 Hz, 2,4-dichlorophenyl H-5), 7.55 (1H, d, J = 2.0 Hz, 2,4-dichlorophenyl H-3), 7.60–7.70 (2H, m, 2,4-dichlorophenyl H-6 and imidazolic H); ^{13}C NMR ($CDCl_3$) δ: 28.37 (NCH_3), 51.30 (CH_2N), 67.00 (OCH_2CO), 67.52 (CH_2CHCH_2OAr), 67.58 (CH_2CHCH_2OAr), 75.00 (CH_2CHCH_2OAr), 104.70 (benzoxazine C-5), 107.94 (OCO), 111.40 (benzoxazine C-8), 111.85 (benzoxazine C-7), 121.10 (N*C*HCHN), 127.20 (2,4-dichorophenyl C-5), 128.50 (NCH*C*HN), 129.45 (2,4-dichorophenyl C-6), 130.20 (benzoxazine C-10), 131.30 (2,4-dichorophenyl C-3), 133.15 (2,4-dichorophenyl C-2), 134.48 (2,4-dichorophenyl C-1), 136.00 (2,4-dichorophenyl C-4), 138.60 (NCHN), 139,05 (benzoxazine C-9), 157.60 (benzoxazine C-6), 165.50 (OCH_2CO); HPLC-MS: 490.0 (M+). Anal. Calcd for $C_{23}H_{21}Cl_2N_3O_5$: C, 56.34; H, 4.32; N, 8.57. Found: C, 56.48; H, 4.31; N, 8.55.

5.10. *trans*-6-{[2-(2,4-Dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (*trans*-12)

It was synthesized starting from *trans*-**10**, using the same procedure described for *cis*-**12**. Amorphous solid, 68% yield. ^{1}H NMR ($CDCl_3$) δ 3.27 (3H, s, NCH_3), 3.65–3.97 (4H, m, CH_2CHCH_2OAr), 4.08–4.22 (1H, m, CH_2CHCH_2OAr), 4.44 (2H, s, CH_2N), 4.54 (2H, s, OCH_2CO), 6.26 (1H, d, J = 2.5 Hz benzoxazine H-5), 6.30 (1H, dd, J = 8.6 and 2.5 Hz, benzoxazine H-7), 6.83 (1H, d, J = 8.6 Hz, benzoxazine H-8), 7.01, 7.05 and 7.63 (each 1H, br s, imidazolic H), 7.20 (1H, dd, J = 8.4 and 2.0 Hz, 2,4-dichlorophenyl H-5), 7.42 (1H, d, J = 2.0 Hz, 2,4-dichlorophenyl H-3), 7.58 (1H, d, J = 8.4 Hz, 2,4-dichlorophenyl H-6); ^{13}C NMR ($CDCl_3$) δ: 28.37 (NCH_3), 51.70 (CH_2N), 67.00 (OCH_2CO), 67.55 (CH_2CHCH_2OAr), 75.49 (CH_2CHCH_2OAr), 104.70 (benzoxazine C-5), 108.10 (OCO), 111.42 (benzoxazine C-8), 111.85 (benzoxazine C-7), 121.10 (NCHCHN), 127.20 (2,4-dichorophenyl C-5), 128.50 (NCH*C*HN), 129.45 (2,4-dichorophenyl C-6), 130.20 (benzoxazine C-10), 131.30 (2,4-dichorophenyl C-3), 133.15 (2,4-dichorophenyl C-2), 134.45 (2,4-dichorophenyl C-1), 136.00 (2,4-dichorophenyl C-4), 138.50 (NCHN), 139.00 (benzoxazine C-9), 157.50 (benzoxazine C-6), 165.55 (OCH_2CO); HPLC-MS: 490.0 (M+). Anal. Calcd for $C_{23}H_{21}Cl_2N_3O_5$: C, 56.34; H, 4.32; N, 8.57. Found: C, 56.40; H, 4.30; N, 8.58.

5.11. 2-Butyl-6-{[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (13)

The mixture of the eight isomers of **11** was treated as described for the synthesis of *cis*-**12**. The residue obtained after the extraction was chromatographed eluting with EtOAc, thus obtaining *cis*-**13** (36% yield), and *trans*-**13** (25.3% yield), as amorphous solids.

cis-**13**: ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.8 Hz, CH_2CH_3), 1.15–1.65 (4H, m, $CH_2CH_2CH_2CH_3$), 1.65–1.95 (2H, m, $CH_2CH_2CH_2CH_3$), 3.10–3.25 (1H, m, C*H*HCHCH$_2$OAr), 3.33 (3H, s, NCH_3), 3.55–3.95 (3H, m, CH*H*CHCH_2OAr), 4.25–4.57 (4H, m, OCHCO, CH_2CHCH_2OAr and CH_2N), 6.35–6.50 (2H, m, benzoxazine H-5 and H-7), 6.86 (1H, d, J = 8.4 Hz, benzoxazine H-8), 6.98 (2H, br s, imidazolic H), 7.25 (1H, dd, J = 8.4 and 2.0 Hz, 2,4-dichlorophenyl H-5), 7.45 (1H, d, J = 2.0 Hz, 2,4-dichlorophenyl H-3), 7.54 (1H, br s, imidazolic H), 7.58 (1H, d, J = 8.4 Hz, 2,4-dichlorophenyl H-6); ^{13}C NMR ($CDCl_3$) δ: 14.10 ($CH_2CH_2CH_2CH_3$), 21.50 ($CH_2CH_2CH_2CH_3$), 26.00 ($CH_2CH_2CH_2CH_3$), 28.70 (NCH_3), 30.33 ($CH_2CH_2CH_2CH_3$), 51.40 (CH_2N), 67.48 (CH_2CHCH_2OAr), 67.70 (CH_2CHCH_2OAr), 75.00 (CH_2CHCH_2OAr), 81.38 (OCHCO), 104.40 (benzoxazine C-5), 108.00 (OCO), 111.00 (benzoxazine C-8), 111.40 (benzoxazine C-7), 121.00 (NCHCHN), 127.40 (2,4-dichorophenyl C-5), 128.00 (NCH*C*HN), 129.50 (2,4-dichorophenyl C-6), 129.75 (benzoxazine C-10), 131.05 (2,4-dichorophenyl C-3), 133.24 (2,4-dichorophenyl C-2), 133.60 (2,4-dichorophenyl C-1), 135.96 (2,4-dichorophenyl C-4), 138.63 (NCHN), 140.00 (benzoxazine C-9), 157.70 (benzoxazine C-6), 168.50 (OCH_2CO); HPLC-MS: 546.1 (M+). Anal. Calcd

for $C_{27}H_{29}Cl_2N_3O_5$: C, 59.35; H, 5.35; N, 7.69. Found: C, 59.38; H, 5.36; N, 7.71.

trans-**13**: ^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, J = 6.7 Hz, CH_2CH_3), 1.23–1.60 (4H, m, $CH_2CH_2CH_2CH_3$), 1.70–1.92 (2H, m, $CH_2CH_2CH_2CH_3$), 3.26 and 3.29 (1.92 and 1.08H, respectively, s, NCH_3), 3.60–3.95 (3H, m, $CHCHCH_2OAr$), 4.07–4.23 (1H, m, CH_2CHCH_2OAr), 4.37–4.55 (1H, m, OCHCO), 4.44 (2H, s, CH_2N), 6.21–6.38 (2H, m, benzoxazine H-5 and H-7), 6.78 and 6.83 (0.36 and 0.64H, respectively, d, J = 8.6 Hz, benzoxazine H-8), 7.01 and 7.04 (each 1H, br s, imidazolic H), 7.20 (1H, dd, J = 8.5 and 2.0 Hz, 2,4-dichlorophenyl H-5), 7.42 (1H, d, J = 2.0 Hz, 2,4-dichlorophenyl H-3), 7.60 (1H, d, J = 8.5 Hz, 2,4-dichlorophenyl H-6), 7.61 (1H, br s, imidazolic H); ^{13}C NMR ($CDCl_3$) δ: 14.10 ($CH_2CH_2CH_2CH_3$), 21.55 ($CH_2CH_2CH_2CH_3$), 26.00 ($CH_2CH_2CH_2CH_3$), 28.70 (NCH_3), 30.33 ($CH_2CH_2CH_2CH_3$), 51.84 (CH_2N), 67.48 (CH_2CHCH_2OAr), 75.50 (CH_2CHCH_2OAr), 81.38 (OCHCO), 104.40 (benzoxazine C-5), 108.20 (OCO), 111.05 (benzoxazine C-8), 111.40 (benzoxazine C-7), 120.90 (NCHCHN), 127.40 (2,4-dichorophenyl C-5), 128.00 (NCHCHN), 129.50 (2,4-dichorophenyl C-6), 129.70 (benzoxazine C-10), 131.05 (2,4-dichorophenyl C-3), 133.30 (2,4-dichorophenyl C-2), 133.50 (2,4-dichorophenyl C-1), 136.01 (2,4-dichorophenyl C-4), 138.55 (NCHN), 140.00 (benzoxazine C-9), 157.70 (benzoxazine C-6), 168.50 (OCH_2CO); HPLC-MS: 546.1 (M+). Anal. Calcd for $C_{27}H_{29}Cl_2N_3O_5$: C, 59.35; H, 5.35; N, 7.69. Found: C, 59.40; H, 5.35; N, 7.67.

6. Molecular modeling

The inhibitors were built using the sketch module of Cerius-2,[25] and minimized using the Universal force-field v.1.2[26] adopting the Smart Minimizer protocol of the Open Force Field module (OFF). Atomic charges were calculated using the semi-empirical Mopac/AM1 method. Docking experiments were carried out using the Ligand Fit module of Cerius-2. During these calculations, the atomic coordinates of the imidazole ring of all compounds were constrained to the relative coordinates of the imidazole ring as observed in the crystal structure of MT-CYP51 in complex with fluconazole (pdb code: 1ea1).

The Universal force-field 1.2 and the Smart Minimizer protocol of the Open Force Field module (OFF) were used to optimize the geometry of the complexes resulting from docking experiments. During the minimization protocol, the High Convergence option was used as criteria to stop the minimization cycles. After the optimization of the binding geometry, all compounds showed a conserved distance of 2.4 Å between the N-3 of the imidazole group and the iron of the heme which is in agreement with the relative distance (2.3 Å) observed in the crystal structure of MT-CYP51 bound to FLU.

7. Micology

7.1. Reagents

The liophylized synthetic compounds were dissolved in 20% ethanol to the concentration of 10 mg/ and stored at −20 °C until use. For in vitro and in vivo experiments, thawed compounds were further diluted to the desired concentration. FLU and KTZ were obtained from Pfizer (Pfizer Italia S.r.l., Latina, Italy).

7.2. Microorganisms

The different *Candida* species (*C. albicans*, *C. krusei*, *C. glabrata* and *C. parapsilosis*) were isolated from clinical specimens in the laboratories of the Microbiology Section, University of Perugia. For experimental systemic candidiasis, mice were inoculated with the highly virulent *C. albicans* strain CA-6.[27] The agerminative, low virulent echinocandin resistant strain PCA-2 was obtained from D. Kerridge, Department of Biochemistry, University of Cambridge. The low and high capsulated *C. neoformans* strains (6995 and CAP-67, respectively) were obtained from the The Centraal Bureau voor Schimmelcultures (Utrecht, The Netherlands). All fungal cultures were maintained by serial passages on Sabouraud agar (Bio-Mèrieux, Lyon, France). The cells were harvested by suspending a single colony in sterile saline, washed twice, counted in a hemocytometer and adjusted to the desired concentration.

7.3. Antifungal susceptibility testing

Broth microdilution testing was performed according to NCCLS document M27-A.[28] Briefly, serial twofold dilutions of synthetic compounds, FLU, or KTZ were done in RPMI 1640 with morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co., St. Louis, Mo.) buffered to pH 7.0. Dilutions were prepared in 96 well U-bottom microplates ranging from 1 mg to 0.00006 μg. Yeasts, suspended in RPMI 1640/MOPS, were added at the concentration of 5×10^4 CFU. The plates were incubated at 37 °C and the minimum inhibitory concentration (MIC), defined as the lowest concentration of the compound that completely inhibited the growth of the yeast, was determined by visual reading at 24 and 48 h.

7.4. Mice and Infection

Female, 8–10 weeks old, outbred CD1 mice were obtained from Harlan Nossan Laboratories (Milan, Italy) and housed at the Animal Facilities of the University of Perugia, Perugia, Italy. Procedures involving animals and their care were conducted in conformity with national and international laws and policies. For systemic infection, mice were infected intravenously on day 0 with 2.5×10^5 blastospores of the highly virulent *C. albicans* CA-6 strain and untreated or treated 2 h before challenge and once a day for 6 consecutive days after challenge with the synthetic compounds, KTZ or FLU (all at 10 mg/kg body weight, i.p.). 2% ethanol solution (0.2 mL, i.p.) was used as a negative control. In selected experiments mice were inoculated with serum (0.3 mL/mouse, i.p.) from mice untreated or treated for 1 h with *cis*-**13**, *trans*-**13**, FLU, or 2% ethanol solution, 1 h before *C. albicans* systemic challenge (7×10^5/mouse). Infected animals were monitored for survival and organ clearance. Quantification of fungal growth, 7 days after infection, was assessed by counting the number of colony forming units recovered after plating serial dilutions of kidney homogenates onto Sabouraud agar. The experiments were repeated three times by using 4 animals/experimental group.

7.5. Histological analysis

For histology, kidneys were excised 7 days after infection and immediately fixed in formalin. Sections (1.5 μm) of paraffin-embedded tissues were stained using the periodic acid-Schiff (PAS) procedure, which detects neutral and acid polysaccharides. On PAS stained sections, *C. albicans* blastospores and hyphae stain red.

7.6. Statistical analysis

Student's t test was used to determine the statistical significance of differences in organ clearance assays. Survival data were analyzed by the Mantel-Cox logrank test. A value of $p < 0.05$ was taken as significant.

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