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Novel ketoconazole analogues based on the replacement of 2,4-dichlorophenyl group with 1,4-benzothiazine moiety: Design, synthesis, and microbiological evaluation

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Abstract—As a part of a program to develop novel antifungal agents, new compounds which incorporate the 1,4-benzothiazine moiety into the structure of ketoconazole (KTZ) were prepared. These compounds were computationally investigated to assess whether the 1,4-benzothiazine moiety was a suitable bioisosteric replacement for the 2,4-dichlorophenyl group of KTZ in order to obtain a more potent inhibition of CYP51 enzyme of *Candida albicans*. Results of preliminary microbiological studies show that the racemic *cis*-**7** analogue has a good in vivo activity, comparable to that of KTZ, but the best activity was observed in the racemic *trans*-**7** analogue.
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1. Introduction

Ketoconazole (KTZ) was the first available compound for oral treatment of systemic fungal infections and up to the introduction of triazoles, it was indicated as the drug of choice in chronic mucocutaneous candidiasis[1] and as an effective alternative to amphotericin B in less severe (non-immunocompromised) cases of blastomycosis, histoplasmosis, and paracoccidioidomycosis.[2,3]

With time, a number of clinically relevant shortcomings of this compound became evident and its use has become limited due to adverse side effects and serious drug–drug interactions.[4]

The basis of the antifungal activity of KTZ and related azoles is the blockage of the conversion of lanosterol to ergosterol, which is necessary to maintain cell membrane integrity, by inhibiting the cytochrome P-450 lanosterol 14α-demethylase (CYP51), enzyme responsible for the oxidative removal of the C-14 methyl group of lanosterol.[5,6]

KTZ has two stereogenic centers. The drug is the racemic mixture of the two *cis* enantiomers (+)-(2*R*,4*S*) and (−)-(2*S*,4*R*). The diastereoisomeric *trans* pair of enantiomers is not contained in KTZ because it is a much weaker inhibitor than the *cis* pair.[7]

Different KTZ analogues have been reported in which the 1-piperazinyl substituent has been run in every conceivable type of alkyl, aryl, heteroaryl, aralkyl, and acyl group[8–13] or in which the 2,4-dichlorophenyl ring was replaced by the biphenyl portion of bifonazole[14] (Fig. 1).

In this context, as a part of a program to develop novel antifungal agents containing a 1,4-benzothiazine unit,[15–20] we decided to prepare new compounds which incorporate the 1,4-benzothiazine moiety, that in itself shows some antifungal activity,[21] into the KTZ structure (Fig. 1).

To explore whether 1,4-benzothiazine group is a suitable moiety to replace the 2,4-dichlorophenyl group of KTZ, we developed a computational strategy based on docking studies into the catalytic site of a homology model

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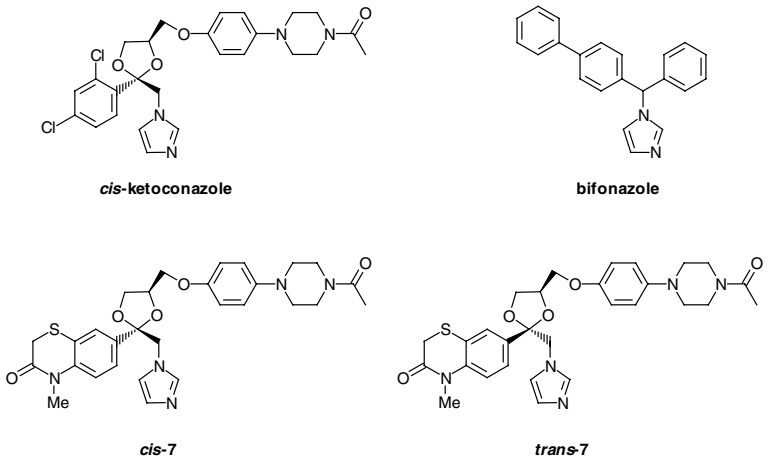


Figure 1.

of the CYP51 of *Candida albicans* previously reported by us.[19,20]

2. Chemistry

The synthesis of racemic **7**, starting from 7-(2-bromoacetyl)-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (**1**),[15] is outlined in Schemes 1 and 2. Ketalization with glycerol was performed in benzene with azeotropic removal of water in the presence of catalytic amount of *p*-toluenesulfonic acid (TsOH).

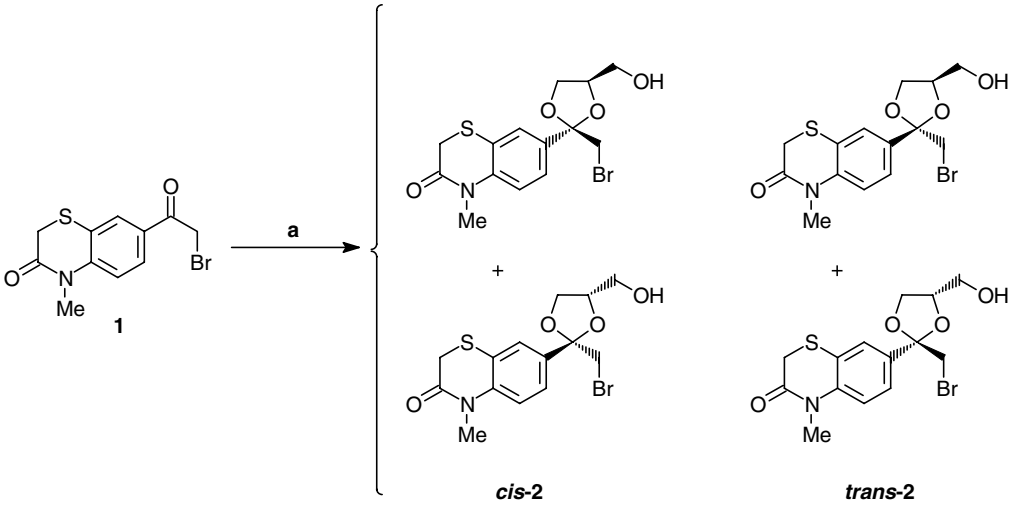
The *cis*/*trans* mixture (55:45) of bromo ketal **2** was resolved by flash chromatography into *cis* and *trans* diastereoisomers. The *cis* and *trans* nature of the isomers was deduced by examination of their ^{1}H NMR spectra and confirmed by Overhauser correlations measured by NOE and NOESY experiments. Proton H-4 of the dioxolane ring is shielded by the aromatic system of 0.4 ppm in compound *cis*-**2**. A similar shielding has been observed on CH_2OH protons in compound *trans*-**2**. A nuclear Overhauser correlation between H-4 of the dioxolane ring and aromatic protons and between OH and CH_2Br in compound *cis*-**2** confirms the *syn* relationship between the two groups. Furthermore, a dipolar correlation between OH and aromatic protons has been observed in compound *trans*-**2**.

Successive reactions were carried out separately on the *cis* and *trans* racemic mixtures.

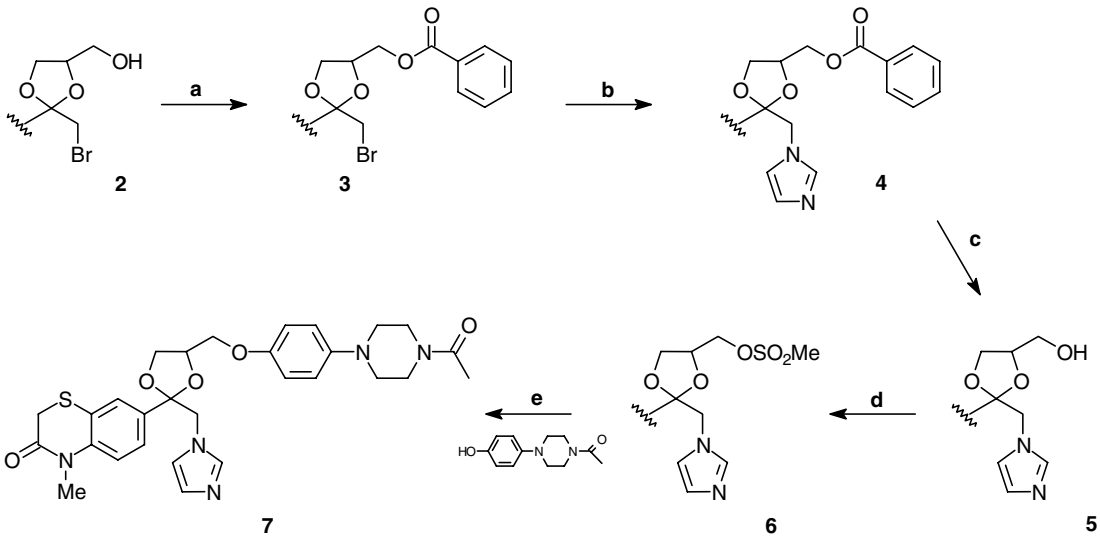
Benzoylation of **2** in CH_2Cl_2 afforded the ester **3** that was condensed with imidazole in dimethylacetamide (DMA) to give the imidazole derivative **4**. The ester was saponified at reflux with 6 N NaOH to the alcohol **5** which was converted to methanesulfonate **6** and finally coupled with 1-acetyl-4-(hydroxyphenyl)-piperazine[22] to furnish the final product **7**.[23]

3. Results and discussion

The concept of bioisosterism, introduced by Friedman,[24] describes functional groups or molecules that



Scheme 1. Reagents: (a) glycerine, benzene, TsOH.



Scheme 2. Reagents: (a) benzoyl chloride, CH_2Cl_2, Et_3N; (b) 1*H*-imidazole, DMA; (c) 6 N NaOH, dioxane; (d) CH_3SO_2Cl, Py; (e) NaH, DMSO.

are structurally different, but give similar intermolecular interactions. Bioisosteric replacements have been widely and successfully used in drug discovery processes to optimize lead compounds by removing side effects and improving absorption, metabolism, distribution, and elimination (ADME) profiles. Herein, we report the study of the bioisosteric relationship between the 2,4-dichlorophenyl moiety of KTZ and the 1,4-benzothiazine group.

To evaluate the degree of bioisosterism between the two moieties, the binding energies to CYP51 of *C. albicans* and other parameters were evaluated for each compound.

The binding energies were calculated on the basis of the previously reported scoring function[19,20] and the following considerations. Briefly, since our scoring function implies the approximation of considering the constrained conformational space of the protein, we assumed that the substructure common to both compound **7** and KTZ should contribute to the binding energy in a similar way. Thus, we neglected this portion of the molecule in our docking experiments in order to avoid the exploration of the conformational space of the enzyme around the common substructures of compound **7** and KTZ. Docking experiments of the above groups into the catalytic site of the cytochrome CYP51 showed that the two moieties have similar binding energies: −30.245 kcal/mol (2,4-dichlorophenyl-KTZ) and −31.217 kcal/mol (1,4-benzothiazine-KTZ).

In terms of docking pose, a slight root mean square displacement (rmsd) of 1.5 Å was observed between the two substructures (Fig. 2). This displacement could affect the induction of two different conformational adaptations of the amino acidic side chains of the enzyme upon ligand binding. This, in turn, would result in different contributions to the binding free energy of the commonly neglected substructures of compound **7** and KTZ.

The calculation of properties that affect the ADME profiles of compound **7** and KTZ is reported in Table 1.

The parameters that constitute Lipinski's rule-of-five were calculated.[25] Briefly, the rule-of-five got its name from the cutoff values for each of four useful parameters calculated to assess the drug-likeness of potential drug candidates in relation to their ADME profile. These parameters include the molecular weight (cutoff of violation >500), the number of hydrogen-bond acceptors (cutoff of violation >10) and donors (cutoff of violation >5), and the log *P* value (cutoff of violation >5; here calculated as A log *P*98).[26]

The analysis of the above parameters showed that all the compounds had acceptable ADME profiles and, thus, good drug-likeness. They only violated Lipinski's rule-of-five once with their molecular weight higher than 500.

The hydrophobic partition coefficient (expressed as A log *P*98) shows an increased polarity in compound **7** (A log *P*98 = 1.55) in which the 1,4-benzothiazine moiety replaced the 2,4-dichlorophenyl group of KTZ (A log *P*98 = 3.33).

In addition, the quantum mechanical parameters were calculated to gain more information about the bioisosteric relationship of 2,4-dichlorophenyl and 1,4-benzothiazine groups. Both configurations of compound **7** and KTZ had similar values for the highest occupied and lowest unoccupied molecular orbital energies (HOMO and LUMO). Interestingly, the magnitude of the dipole moment was greater in the *cis* than in the *trans* configuration of compound **7**; this means that the dipole property of *trans*-**7** is more closely related to the *cis* configuration of KTZ.

In vitro anti-*Candida* activity was determined according to the NCCLS guidelines.[27]

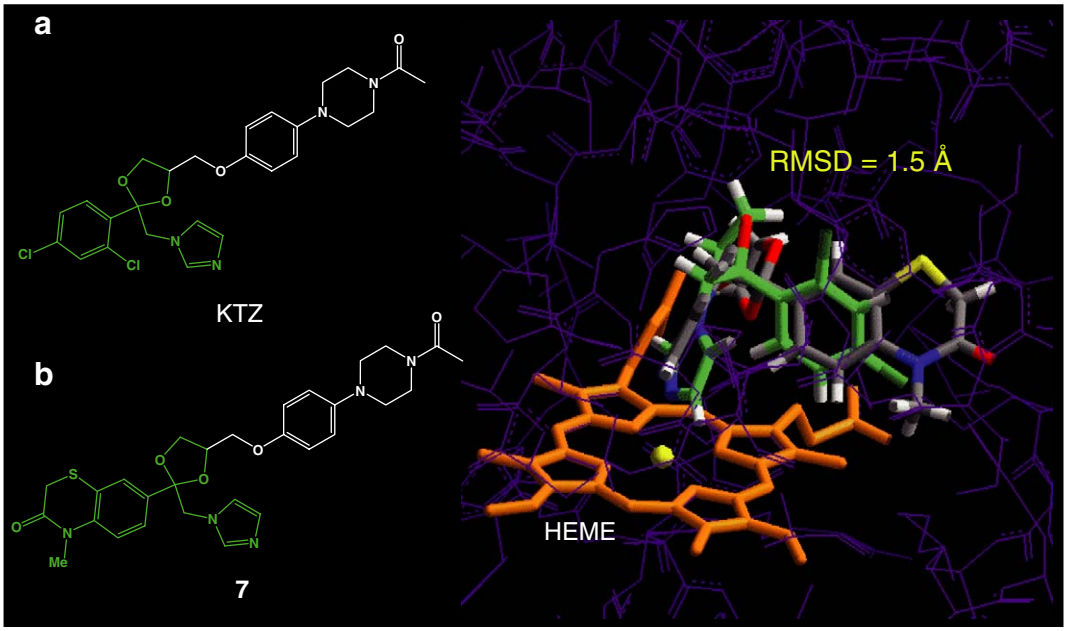


Figure 2. Docking experiments of KTZ (a) and **7** (b) substructures into the catalytic site of the cytochrome CYP51. Docked substructures are highlighted in green.

Table 1. Parameters for ADME profiling and quantum chemical parameters

Compound	MW	A log *P*98	H-bond donors	H-bond acceptors	Dipole moment	HOMO (kcal/mol)	LUMO (kcal/mol)
cis-**7**	547.671	1.55	0	6	6.613	−8.564	−0.422
trans-**7**	547.671	1.55	0	6	5.857	−8.500	−0.439
KTZ	515.438	3.33	0	6	5.379	−8.558	−0.405

Compounds were tested on *C. albicans* CA-6, clinically isolated, and identified according to taxonomical criteria. The results of preliminary studies are shown in Table 2.

In general, incorporation of the 1,4-benzothiazine moiety into the KTZ structure did not improve the in vitro activity. These data are explained by the docking experiments, which show the displacement between the positions of the longer chain-bearing carbons of compound **7** and KTZ in the catalytic site of cytochrome CYP51. This displacement implies an energetically costly rearrangement of the enzyme conformation in order to adapt to the KTZ core of **7**.

Table 2. In vitro and in vivo activity of different compounds against *Candida albicans*

Treatment[a]	MIC[b] (μg/mL)	MST[c]	*D*/*T*[d]
Diluent[e]	500	30.5	6/6
cis-**7**	62.5	25	4/6
trans-**7**	500	>60[*]	2/6
KTZ	0.45	20	4/6

[a] Diluent, KTZ, and indicated compounds were tested for in vitro activity (MIC values) or were given intraperitoneally at the dose of 10 mg/kg 2 h before intravenous challenge with *C. albicans* and once for eight consecutive days after challenge.
[b] Minimum inhibitory concentration.
[c] Median survival time.
[d] Dead mice at 60 days over total animals infected.
[e] EtOH/H_2O 1:4.
[*] $P < 0.01$ (compound-treatment vs vehicle-treatment).

Since the in vitro activity did not necessarily correlate with the in vivo activity,[28–30] newly synthesized compounds were also tested in a murine model of systemic *C. albicans* infection. To this end, mice were treated intraperitoneally with compounds 2 h before systemic challenge with *C. albicans* and once daily for eight consecutive days. The results are reported in Table 2. There was a good in vivo activity for racemic *cis*-**7** analogue (MST = 25, $D/T = 4/6$) comparable to that of KTZ (MST = 20, $D/T = 4/6$). Surprisingly, the racemic *trans*-**7** analogue had the best activity (MST > 60, $D/T = 2/6$).

This result was unexpected because the activity of diastereomeric pairs of enantiomers, in ketal-containing antifungal agents, is normally found in the *cis* pair.

It could be speculated that a role of the dipole moment is affecting the in vivo activity since the magnitude of *trans*-**7** is similar to that of *cis*-KTZ (Table 1).

In an attempt to explain this in vivo result, we tested whether the observed effect was ascribed, at least in part, to immunostimulating properties of our compounds. To evaluate this possibility, spleen cells from untreated mice

were stimulated with *cis*-**7** or *trans*-**7** analogue (1 μg/mL) for 24 h at 37 °C. The results showed that both compounds were able to produce a significant increase (50%) of killing activity of splenocytes against *C. albicans* with respect to untreated cells. Given that these data did not furnish an explanation for the better activity of *trans*-**7** with respect to *cis*-**7**, we analyzed another parameter that usually supports the in vivo results.

To this end, mice untreated or treated with both compounds was challenged intravenously with *C. albicans* (1×10^5 cells) and colony forming unit (CFU) from kidneys were recovered 15 days after the challenge. The results showed that the reduction of CFU obtained by using *cis*-**7** was 15% and 83% for *trans*-**7** with respect to CFU obtained from untreated mice. These results support the hypothesis of a direct activity of racemic *trans*-**7** analogue against *C. albicans*.

Previous results pinpointed that high log *P* values are required to cross the membrane of fungi and reach the enzyme.[19] Thus, on the basis of the biological results of these compounds, new chemical modifications will be carried out to increase the hydrophobic partition coefficient and thereby increase the activity.

4. Experimental

Melting points determined in capillary tubes (Electrothermal, Model 9100, melting point apparatus) are uncorrected. Elemental analysis was performed on a Carlo Erba element analyzer 1106, and the data for C, H, and N were within ± 0.4% of the theoretical values. ^{1}H NMR spectra were recorded at 200 MHz (Bruker AC-200 spectrometer) with Me_4Si as internal standard. Chemical shifts are given in parts per million (δ) and the spectral data are consistent with the assigned structures. 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 product were not optimized. Sodium sulfate was used when organic solutions are said to have been dried during workup. KTZ was purchased from Sigma (Milano, Italy). All starting materials were commercially available unless otherwise indicate.

5. Molecular modeling

Each compound was built and minimized using the Universal force-field v.1.2[31] and the smart minimizer protocol of the open force field module (OFF). Atomic charges were calculated using the semi-empirical Mopac/AM1 method.

Docking experiments and energetical scoring were performed using a knowledge-based strategy as previously reported.[19,20] Quantum mechanical descriptors (dipole moment, HOMO, and LUMO energies) and ADME profiling descriptors (molecular weight, number of hydrogen-bond acceptors and donors, and A log *P*98) were calculated using the Cerius-2 software package.[32]

6. Chemistry

6.1. 7-[2-(Bromomethyl)-4-(hydroxymethyl)-1,3-dioxolan-2-yl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (*cis*- and *trans*-*2*)

Glycerine (4.60 g; 10.00 mmol) and 7-(2-bromoacetyl)-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (**1**) (3.00 g; 10.00 mmol) in 300 mL of benzene were refluxed in the presence of TsOH for 24 h with azeotropic removal of water. The mixture was evaporated in vacuo, the residue was dissolved in EtOAc, washed with water, dried, and evaporated again in vacuo to give **2** as an oil that was chromatographed eluting with $CHCl_3$. Less polar *cis*-enantiomers *cis*-**2** (1.5 g) were then separated from *trans*-**2** (1.19 g), both as oils (total yield 72%). *cis*-**2**: ^{1}H NMR ($CDCl_3$) δ 2.25 (1H, br s, OH), 3.43 (2H, s, SCH_2), 3.45 (3H, s, NCH_3), 3.67 (2H, s, CH_2Br), 3.72 (1H, dd, J = 4.2 and 13 Hz, C*H*HOH), 3.85–4.00 (2H, m, CH*H*OH and C*H*HCH), 4.10 (1H, dd, J = 5.7 and 7.6 Hz, CH*H*CH), 4.15–4.25 (1H, m, CHHC*H*), 7.08 (1H, d, J = 8.5 Hz, H-5), 7.40 (1H, dd, J = 2.0 and 8.5 Hz, H-6), 7.52 (1H, d, J = 2.0 Hz, H-8). Anal. Calcd for $C_{14}H_{16}BrNO_4S$: C, 44.93; H, 4.31; N, 3.74. Found: C, 44.87; H, 4.28; N, 3.75.

trans-**2**: ^{1}H NMR ($CDCl_3$) δ 1.80 (1H, br s, OH), 3.43 (2H, s, SCH_2), 3.45 (3H, s, NCH_3), 3.55 (1H, dd, J = 4.7 and 12.0 Hz, C*H*HOH), 3.60–3.65 (2H, m, CH_2Br), 3.73 (1H, dd, 8.0 e 12.0 Hz, CH*H*OH), 3.80 (1H, dd, J = 7.9 and 8.0 Hz, CH*H*CH), 4.35 (1H, dd, J = 6.3 and 8.1 Hz, C*H*HCH), 4.50–4.65 (1H, m, CHHC*H*), 7.08 (1H, d, J = 8.5 Hz, H-5), 7.42 (1H, dd, J = 2.0 and 8.5 Hz, H-6), 7.53 (1H, d, J = 2.0 Hz, H-8). Anal. Calcd for $C_{14}H_{16}BrNO_4S$: C, 44.93; H, 4.31; N, 3.74. Found: C, 45.05; H, 4.32; N, 3.73.

6.2. 7-[2-(Bromomethyl)-4-(benzoyloxymethyl)-1,3-dioxolan-2-yl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (*cis*-3)

Benzoyl chloride (2.50 g; 6.68 mmol) was added dropwise to a solution of *cis*-**2** (2.50 g; 6.68 mmol) in 80 mL of dry CH_2Cl_2 and Et_3N (4.05 g; 40.02 mmol). The mixture was stirred at room temperature for 1 h, diluted with water, neutralized with a saturated solution of $NaHCO_3$, and evaporated in vacuo. The residue was chromatographed eluting with cyclohexane/EtOAc 80:20 yielding *cis*-**3**, (1.69 g; 53%), mp 116–119 °C. ^{1}H NMR ($CDCl_3$) δ 3.40 (2H, s, SCH_2), 3.42 (3H, s, NCH_3), 3.62 (2H, s, CH_2Br), 4.05 (1H, dd, J = 6.8 and 8.1 Hz, C*H*HCH), 4.12 (1H, dd, J = 5.6 and 8.1 Hz, CH*H*CH), 4.38–4.50 (1H, m, CHHC*H*), 4.55–4.60 (2H, m, CHCH_2OBz), 7.08 (1H, d, J = 8.5 Hz, H-5), 7.35–7.63 (5H, m, aromatic H), 8.00–8.10 (2H, m, aromatic H). Anal. Calcd for $C_{21}H_{20}BrNO_5S$: C, 52.73; H, 4.21; N, 2.93. Found: C, 54.78; H, 4.20; N, 2.92.

6.3. 7-[2-(Bromomethyl)-4-(benzoyloxymethyl)-1,3-dioxolan-2-yl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (*trans*-3)

It was obtained as an amorphous solid starting from *trans*-**2** by using the same procedure described for *cis*-**3**. Yield 55%. ^{1}H NMR ($CDCl_3$) δ 3.45 (2H, s, SCH_2), 3.48 (3H, s, NCH_3), 3.65–3.70 (2H, m, CH_2Br), 3.90–4.30 (2H, m, C*H*$_2$CH), 4.43–4.60 (2H, m, CHC*H*$_2$OBz), 4.75–4.83 (1H, m, CH_2C*H*), 6.95–8.14 (8H, m, aromatic H). Anal. Calcd for $C_{21}H_{20}BrNO_5S$: C, 52.73; H, 4.21; N, 2.93. Found: C, 53.82; H, 4.22; N, 2.93.

6.4. 7-[4-(Benzoyloxymethyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-2-yl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (*cis*-4)

A solution of *cis*-**3** (2.50 g; 5.23 mmol) in DMA (40 mL) was refluxed with 1*H*-imidazole (1.42 g; 20 mmol) for 7 days. The mixture was cooled, diluted with water, and extracted with EtOAc. The residue obtained after evaporation was chromatographed eluting with $CHCl_3$/MeOH 90:10 furnishing *cis*-**4** as an oil (0.97 g; 40%). ^{1}H NMR ($CDCl_3$) δ 3.47 (2H, s, SCH_2), 3.49 (3H, s, NCH_3), 3.76 (1H, dd, J = 5.7 and 8.4 Hz, C*H*HCH), 3.97 (1H, dd, J = 6.9 and 8.3 Hz, CH*H*CH), 4.20–4.25 (2H, m, CHC*H*$_2$OBz), 4.27–4.45 (3H, m, CHHC*H* and CH_2N), 7.00–8.10 (11H, m, aromatic and imidazolic H). Anal. Calcd for $C_{24}H_{23}N_3O_5S$: C, 61.92; H, 4.98; N, 9.03. Found: C, 62.12; H, 4.97; N, 9.05.

6.5. 7-[4-(Benzoyloxymethyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-2-yl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (*trans*-4)

It was obtained starting from *trans*-**3** and using the same procedure described for *cis*-**4**. Oil, yield 33%. ^{1}H NMR ($CDCl_3$) δ 3.39 (2H, s, SCH_2), 3.42 (3H, s, NCH_3), 3.48–3.52 (1H, m, C*H*HCH), 3.75–3.80 (1H, m, CH*H*CH), 4.00 (1H, dd, J = 5.9 and 8.2 Hz, CHC*H*HOBz), 4.10–4.30 (3H, m, CHCH*H*OBz and CH_2N), 4.45–4.55 (1H, m, C*H*CH$_2$OBz), 6.90–7.80 (11H, m, aromatic and imidazolic H). Anal. Calcd for $C_{24}H_{23}N_3O_5S$: C, 61.92; H, 4.98; N, 9.03. Found: C, 61.87; H, 4.99; N, 9.00.

6.6. 7-[4-(Hydroxymethyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-2-yl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (*cis*-5)

A solution of *cis*-**4** (2.70 g; 5.80 mmol) in 70 mL of dioxane was refluxed with 6 N NaOH (7 mL) for 40 min. The mixture was cooled, diluted with water, and extracted with $CHCl_3$. The organic phase was dried and evaporated in vacuo to give *cis*-**5** (1.11 g; 53%) as an oily residue which was used without further purification.^{1}H NMR ($CDCl_3$) δ 1.95 (1H, br s, OH), 3.25 (1H, dd, J = 5.2 and 11.8 Hz, C*H*HCH), 3.40–3.50 (1H, m, CH*H*CH), 3.43 (2H, s, SCH_2), 3.46 (3H, s, NCH_3), 3.67 (1H, dd, J = 5.2 and 8.0 Hz, CHC*H*HOH), 3.80 (1H, dd, J = 7.0 and 8.0 Hz, CHCH*H*OH), 4.10–4.20 (3H, m, CHHC*H* and CH_2N), 7.00–7.07 (3H, m, H-5 and imidazolic H), 7.37 (1H, dd, 2.1 and 8.5 Hz, H-6), 7.50–7.55 (2H, m, H-8 and imidazolic H). Anal. Calcd for $C_{17}H_{19}N_3O_4S$: C, 56.50; H, 5.30; N, 11.63. Found: C, 56.30; H, 5.28; N, 11.59.

6.7. 7-[4-(Hydroxymethyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-2-yl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (*trans*-5)

It was synthesized starting from *trans*-**4** and using the same procedure described for *cis*-**5**. Oil, yield 81%. ^{1}H NMR ($CDCl_3$) δ 2.10 (1H, br s, OH), 3.45 (2H, s, SCH_2), 3.50 (3H, s, NCH_3), 3.55–3.70 (2H, m, C*H*$_2$CH), 3.75–3.95 (2H, m, CHC*H*$_2$OH), 4.10–4.20 (3H, m, CH_2C*H* and CH_2N), 6.95–7.10 (3H, m, H-5 and imidazolic H), 7.35 (1H, dd, 2.1 and 8.5 Hz, H-6), 7.45–7.55 (2H, m, H-8 and imidazolic H). Anal. Calcd for $C_{17}H_{19}N_3O_4S$: C, 56.50; H, 5.30; N, 11.63. Found: C, 56.61; H, 5.31; N, 11.65.

6.8. 7-[2-(1*H*-Imidazol-1-ylmethyl)-4-(methanesulfonyloxymethyl)-1,3-dioxolan-2-yl]- 4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (*cis*-6)

Methanesulfonyl chloride (0.31 g; 2.77 mmol) was added in one portion to a solution of *cis*-**5** (1.00 g; 2.77 mmol) in dry pyridine (50 mL) while cooling on ice. The reaction mixture was stirred overnight, water was then added and extracted with EtOAc. The organic phase was dried, evaporated, and purified by chromatography eluting with $CHCl_3$/MeOH 90:10 to afford *cis*-**6** as an amorphous solid (0.73 g; 60%). ^{1}H NMR ($CDCl_3$) δ 3.03 (3H, s, SO_2CH_3), 3.42 (2H, s, SCH_2), 3.45 (3H, s, NCH_3), 3.60–3.90 (4H, m, C*H*$_2$CHC*H*$_2$OS), 4.17–4.20 (2H, m, CH_2N), 4.25–4.38 (1H, m, CH_2C*H*), 7.00–7.07 (2H, m, imidazolic H), 7.10 (1H, d, J = 8.5 Hz, H-5), 7.35 (1H, dd, J = 2.0 and 8.5 Hz, H-6), 7.45-7.55 (2H, m, H-8 and imidazolic H). Anal. Calcd for $C_{18}H_{21}N_3O_6S_2$: C, 49.19; H, 4.82; N, 9.56. Found: C, 49.30; H, 4.84; N, 9.58.

6.9. 7-[2-(1*H*-Imidazol-1-ylmethyl)-4-(methanesulfonyloxymethyl)-1,3-dioxolan-2-yl]-4- methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (*trans*-6)

It was synthesized starting from *trans*-**5** and using the same procedure described for *cis*-**6**. Amorphous solid, yield 58%. ^{1}H NMR ($CDCl_3$) δ 3.00 (3H, s, SO_2CH_3), 3.50 (2H, s, SCH_2), 3.52 (3H, s, NCH_3), 3.60–3.73 (1H, m, C*H*HCH), 3.85–4.00 (1H, m, CH*H*CH), 4.10–4.30 (5H, m, CH_2C*H*C*H*$_2$OS and CH_2N), 7.05–7.20 (3H, m, H-5 and imidazolic H), 7.40 (1H, dd, J = 1.85 and 8.50 Hz, H-6), 7.55 (1H, d, J = 1.85 Hz, H-8), 7.65 (1H, s, imidazolic H). Anal. Calcd for $C_{18}H_{21}N_3O_6S_2$: C, 49.19; H, 4.82; N, 9.56. Found: C, 49.17; H, 4.82; N, 9.54.

6.10. 7-[4-{[4-(4-Acetylpiperazin-1-yl)phenoxy]methyl}-2-(1*H*-imidazol-1-ylmethyl)1,3-dioxolan-2-yl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (*cis*-7)

1-Acetyl-4-(hydroxyphenyl)-piperazine (0.15 g; 0.68 mmol) in dry DMSO (4 mL) was added dropwise to a suspension of NaH (60% mineral oil dispersion, 0.03 g;

0.75 mmol) in dry DMSO (5 mL). After stirring at room temperature for 1 h, *cis*-**6** was added and stirring was continued for 5 h at 80 °C. The reaction mixture was cooled, water was added, and alkalized with 2.5 N NaOH. After extraction of the mixture with EtOAc, the organic layer was dried and evaporated to afford a residue which was purified by chromatography eluting with $CHCl_3$/MeOH 96:4. *cis*-**7** was obtained as amorphous solid (0.05 g; 20%). ^{1}H NMR ($CDCl_3$) δ 2.18 (3H, s, $COCH_3$), 3.00–3.15 (4H, m, piperazinic H), 3.30 (1H, dd, $J = 6.65$ and 9.44 Hz, C*H*HCH), 3.47 (2H, s, SCH_2), 3.50 (3H, s, NCH_3), 3.62–3.96 (7H, m, CH*H*CHCH_2OAr and piperazinic H), 4.20–4.27 (2H, m, CHCH_2OAr), 4.33-4.46 (1H, m, CH_2C*H*), 6.80 and 6.95 (each 2H, AA′BB′ system, aromatic H), 7.03, 7.06 and 7.61 (each 1H, s, imidazolic H), 7.12 (1H, d, $J = 8.5$ Hz, H-5), 7.40 (1H, dd, $J = 2.0$ and 8.5 Hz, H-6), 7.58 (1H, d, $J = 2.0$ Hz, H-8). Anal. Calcd for $C_{29}H_{33}N_5O_5S$: C, 61.79; H, 5.90; N, 12.42. Found: C, 61.93; H, 5.88; N, 12.38.

6.11. 7-[4-{[4-(4-Acetylpiperazin-1-yl)phenoxy]methyl}-2-(1*H*-imidazol-1-ylmethyl)1,3-dioxolan-2-yl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (*trans*-7)

It was obtained starting from *trans*-**6** and using the same procedure described for *cis*-**7**. Amorphous solid, yield 56%. ^{1}H NMR ($CDCl_3$) δ 2.20 (3H, s, $COCH_3$), 3.00–3.15 (4H, m, piperazinic H), 3.45 (2H, s, SCH_2), 3.50 (3H, s, NCH_3), 3.60–3.72 (2H, m, CH_2CH), 3.73–3.85 (4H, m, piperazinic H), 3.87–4.05 (2H, m, CHCH_2OAr), 4.12–4.28 (3H, m, CH_2C*H* and CH_2N), 6.85 and 6.92 (each 2H, AA′BB′ system, aromatic H), 7.00 (3H, m, H-5 and imidazolic H), 7.40 (1H, dd, $J = 1.9$ and 8.5 Hz, H-6), 7.52–7.62 (2H, m, H-8 and imidazolic H). Anal. Calcd for $C_{29}H_{33}N_5O_5S$: C, 61.79; H, 5.90; N, 12.42. Found: C, 61.85; H, 5.89; N, 12.45.

7. Mycology

7.1. Mice

Female CD1 mice (8–10 weeks old, weighing 25–30 g) were obtained from Charles River Breeding Laboratories (Calco, Lecco, Italy). *C. albicans* CA-6, used in this study, was isolated from a vaginal swab and identified according to the taxonomic criteria of van Uden and Buckley.[33] The yeasts were grown at 28 °C in Sabouraud's dextrose agar. Under these conditions the organisms grew as a pure yeast-phase population. Before use, yeast cells were harvested from a 24 h culture, suspended in pyrogen-free saline, washed twice, quantified by hemocytometry, and adjusted to the desired concentration.

7.2. Systemic candidiasis model

Mice were infected intravenously (iv) with 7×10^5 *C. albicans* blastoconidia via the lateral tail vein. Diluent (EtOH/H_2O 1:4), chemicals, and KTZ were administered intraperitoneally (ip) at a dose of 10 mg/kg of body weight 2 h before infection and then once daily for eight consecutive days. For survival studies, mice were observed for 60 days.

7.3. Quantification of *C. albicans* in the kidneys

The kidneys of mice were aseptically removed and homogenized with 3 mL of sterile distilled water. The number of CFU was determined by a plate dilution method. Colonies of *C. albicans* cells were counted after 48 h of incubation at room temperature and results were expressed as the number of CFU per organ.

7.4. In vitro activity of immune cells against *C. albicans*

Spleens from CD1 mice were recovered aseptically in RPMI. Splenocytes were harvested, counted and stimulated in the presence or absence of racemic *cis*-**7** or *trans*-**7** (1 μg/mL) for 24 h at 37 °C in RPMI containing 10% fetal calf serum.

After incubation, cells were washed three times in RPMI to remove the compound and then incubated with 5×10^4 cells of *C. albicans* for 4 h. After incubation, cultures were treated with 0.01% Triton X-100, diluted in sterile water, and plated on Sabouraud Agar Petri dishes. After 24 h, CFU were counted and the percentage of inhibition was calculated according to the formula: % of inhibition = 100 − (no. of CFU of samples/no. of CFU of control samples) × 100.

7.5. Susceptibility testing

Susceptibility testing was performed by the M27-A micro-dilution method of the National Committee for Clinical Laboratory Standards[27] in 0.165 M MOPS (morpholinepropanesulfonic acid)-buffered (pH 7) RPMI 1640 medium (Gibco BRL, Paisley, UK). The activity of compounds against *C. albicans* was tested using serial dilutions ranging from 0.9 to 500 μg/mL. The MIC was the lowest concentration of chemical that produced an 80% reduction in the turbidity compared to chemical-free normal subjects.

7.6. Statistical analysis

Differences in median survival time were determined by the Mann–Whitney *U*-test. The student *t* test was used to evaluate the significance of all other data. Each experiment was repeated three to five times.

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