European Journal of Medicinal Chemistry 47 (2012) 270–277

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

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

Original article

Antifungal activities of novel non-azole molecules against *S. cerevisiae* and *C. albicans*

Niina Tani [a,*], Minna Rahnasto-Rilla [a], Carsten Wittekindt [a,b], Kaisa A. Salminen [a], Anniina Ritvanen [a], Riina Ollakka [a], Jenna Koskiranta [a], Hannu Raunio [a], Risto O. Juvonen [a]

[a] School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, P.O. Box 1627, 70211 Kuopio, Finland
[b] COSMOlogic GmbH & Co. KG, Burscheider Str. 515, 51381 Leverkusen, Germany

ARTICLE INFO

Article history:
Received 25 August 2011
Received in revised form
24 October 2011
Accepted 28 October 2011
Available online 6 November 2011

Keywords:
S. cerevisiae
C. albicans
CYP enzyme
CYP51
Reactive group
Antifungal

ABSTRACT

Because of the increasing number of immunocompromised patients and due to problems with antifungal treatment, especially with the most widely used antifungals, azoles, there is an urgent need for new, potent and safe antifungals with fewer cytochrome P450 (CYP)-mediated interactions with other drugs. In the present study, 54 novel non-azole molecules were selected with the help of molecular modelling and virtual molecule database screening to identify new fungistatic or fungicidic compounds with functional groups that would produce reactive intermediates killing the yeast cells. Database screening and selection of tested compounds were based on the construction of two pharmacophores and docking hits to the active site of the CYP51 homology model. Inhibition potency of the compounds was tested against *Saccharomyces cerevisiae* and/or *Candida albicans*. Two new structured compounds, 2-({4-[(2-cyanoethyl)(methyl) amino] benzylidene} amino)-5-(3,4-dimethoxyphenyl)-4-methylthiophene-3-carbonitrile and 2-[([1,1′-biphenyl]-4-ylmethylene)amino]-5-(3,4-dimethoxyphenyl)-4-methylthiophene-3-carbonitrile were discovered to have promising antifungal properties based on bioassays. Inhibition screen of human hepatic CYP enzymes revealed that these two compounds did not inhibit potently five human recombinant CYP enzymes. The results of this study indicate that the functional groups of the two compounds may produce reactive intermediates when located at the active site of CYP51.

© 2011 Elsevier Masson SAS. All rights reserved.

1. Introduction

During recent decades, incidents of invasive fungal infections have increased significantly because of the rising number of patients at risk. These patients are mostly immunocompromised with diseases like AIDS or diabetes or patients who are receiving chemotherapy or undergone organ or bone marrow transplant or have an implanted catheter [1]. Invasive opportunistic fungal infections are associated with high morbidity and mortality [2,3] especially in seriously ill patients.

Candida species, mainly *Candida albicans*, is the source of most yeast infections in these patients [1]. Although *C. albicans* is one of the microbes normally in human gut flora, it can cause several different infections, depending on patient's immune system status. Even though systemic antifungals have been available for over 50 years and diagnostics and antifungal drugs have developed [2], there are still remarkable problems encountered in the successful treatment of yeast infections [3].

Four main classes of antifungal agents are presently available: polyenes, nucleoside analogues, echinocandins and azoles [4]. Today the use of the polyene amphotericin B is restricted because of its serious adverse effects, mainly renal toxicity [4,5]. The nucleoside analogue flucytosine is a prodrug deaminated to 5-fluorouracil (5-FU) by yeast cytosine deaminase enzyme [6,7]. The echinocandins comprise caspofungin, anidulafungin and micafungin. The azoles have activities against most yeasts and fungi and they are used for treatment of human mycosis and as fungicides in agriculture [8,9]. The first systemic azoles, ketoconazole and itraconazole appeared on the market in the 1980s and these were followed by several analogues, e.g. fluconazole, voriconazole, posaconazole, isavuconazole and ravuconazole. Recently, novel derivatives of azoles have been developed to improve solubility and optimize their pharmacokinetic profiles [10–12].

The mechanism of action of the azoles is reversible inhibition of lanosterol 14α-demethylase (cytochrome P450 51, CYP51, E.C. 1.14.13.70) that leads to the impairment of ergosterol synthesis in yeast and fungi. CYP51 is found in all biological kingdoms and it is the most widely distributed CYP gene family [13]. It was first found from *Saccharomyces cerevisiae* [14], the model organism in yeast

* Corresponding author. Tel.: +358 403552011; fax: +358 17162424.
E-mail address: niina.tani@uef.fi (N. Tani).

0223-5234/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2011.10.053

research [15–17]. Although azoles have revolutionized the treatment of mycosis there are still some challenges remaining. Azoles do not affect only fungi CYP51 but also some mammalian xenobiotic-metabolising CYP enzymes resulting in a wide range of interactions with other drugs. Another increasing problem is the emergence of resistant yeast strains due to extensive and prolonged use of antifungal agents [1,18,19]. Thus, there is a need for new, safer, more effective and new structured antifungals.

Several functional groups are known to be metabolised to reactive and toxic forms [20,21]. Most of these reactions are catalysed by CYP enzymes and therefore toxicity on cells is dependent on cell-specific expression of the activating CYPs. The mycotoxin aflatoxin B1 is an extremely potent hepatotoxin as it is oxidised to a reactive exo-epoxide by CYP3A4 and CYP1A2 in human liver [22] and CYP1A5 and CYP3A37 in turkey liver [23]. Aflatoxin B1 is lethal to yeasts at concentrations as low as 40 nM if the yeast cells express CYP enzymes catalysing the formation of the exo-epoxide [24].

In this study we searched for novel non-azole antifungal compounds using molecular modelling and virtual compound database screening. In analogy to the activation mechanism of aflatoxin B1, the compounds were designed to fit the active site of the yeast CYP51 enzyme and to contain functional groups that would produce reactive intermediates causing lethality to yeast cells. The antifungal activity of the hit compounds was tested against *S. cerevisiae* and *C. albicans*, and two novel potent non-azole antifungal compounds were identified.

2. Results

2.1. Design of pharmacophores and virtual screening

Designing of pharmacophores combined with virtual screening offers a means to systematically select compounds from large databases. Two pharmacophores of the CYP51 enzyme were designed (Fig. 1), and corresponding structures in the Maybridge databases were screened (Fig. 2). These pharmacophores were constructed by docking either the natural CYP51 substrate lanosterol (first pharmacophore) or ketoconazole/itraconazole (second pharmacophore) to the active site of a comparative model of CYP51 [25]. The dockings revealed that the CYP51 active site consisted of a large hydrophobic binding subsite above heme and two hydrophilic H-bond binding subsites with several polar positively charged amino acid residues (Lys and His). The core scaffold of lanosterol was located in the hydrophobic subsite above the porphyrin ring of heme. This information was used to define the location of the hydrophobic sites in the first pharmacophore (Fig. 1A), to filter the volume and shape of the screened compounds. An additional aim of this screen was to find compounds that could produce reactive metabolites. Therefore reactive functional groups causing irreversible binding to CYPs, such as nitrile, thiophene or furan above heme were included to the pharmacophore. The second pharmacophore was based on the docked positions of ketoconazole and itraconazole. It consisted also of two hydrophobic sites with one acceptor atom in the hydrophilic subsite and the azole ring above heme.

The lanosterol pharmacophore based screen yielded about 900 hits and the ketoconazole/itraconazole based screen about 300 hits. Since there were a large number of hit compounds in the first set, additional refinement was obtained by docking these hits into the active site of the CYP51 homology model [25]. After the docking procedure second hit compounds were obtained and subjected to testing of antifungal activity (Tables 1 and S1).

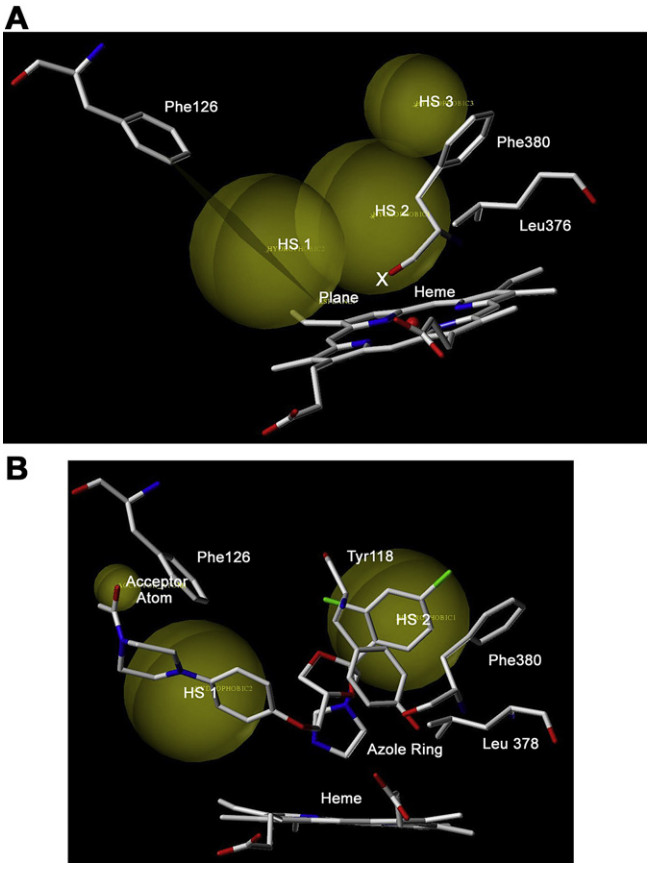


Fig. 1. Pharmacophores for virtual screening. Panel A illustrates the lanosterol based pharmacophore with three hydrophobic sites (HS1–3) and a reactive group (X; nitrile, thiophene or furan) in HS2. Panel B shows the ketoconazole/itraconazole based pharmacophore with two hydrophobic sites (HS1–2). Ketoconazole is the reference structure.

2.2. In vitro antifungal activities

These second hits contained three active antifungal compounds as identified in the *S. cerevisiae* spectrophotometric bioassay (Table 1). Compounds **1**, **2** and **3** were the most potent growth inhibitors with the MIC values of 118 μM, 120 μM and 100 μM, respectively, and MIC_{50} values of 2.7 μM, 35 μM and 10 μM, respectively (Fig. 3). Compounds **1** and **2**, but not compound **3**, inhibited the growth of *S. cerevisiae* also in the agar plate bioassay. This difference may be due to poor solubility of the compound **3** in agar. Table S1 (supplementary material) lists the compounds with weak or no inhibitory activity, including the five 4-benzylpyridine compounds (**45**–**49**) from our in-house hepatic CYP inhibitor repository which were also tested for growth inhibition of *S. cerevisiae* (Table S1, supplementary material). Compounds **48** and **49** were relatively potent with MIC values of 150 μM and 100 μM and MIC_{50} values of 18 μM and 14 μM, respectively. These compounds were not, however, pursued further in this study.

The positive control, ketoconazole (31.2 μM), inhibited yeast growth completely in all of the tests. In contrast, itraconazole (90.7 μM) did not inhibit the growth of *S. cerevisiae* completely after 24-h incubation when tested with both methods and after 48 h incubation the growth was approximately one-half of the growth in the negative control (2% DMSO).

The viability test showed that yeast cells treated with itraconazole (90.7 μM) and compound **1** (100 μM) did not proliferate after 24-h incubation in the spectrophotometric bioassay and in

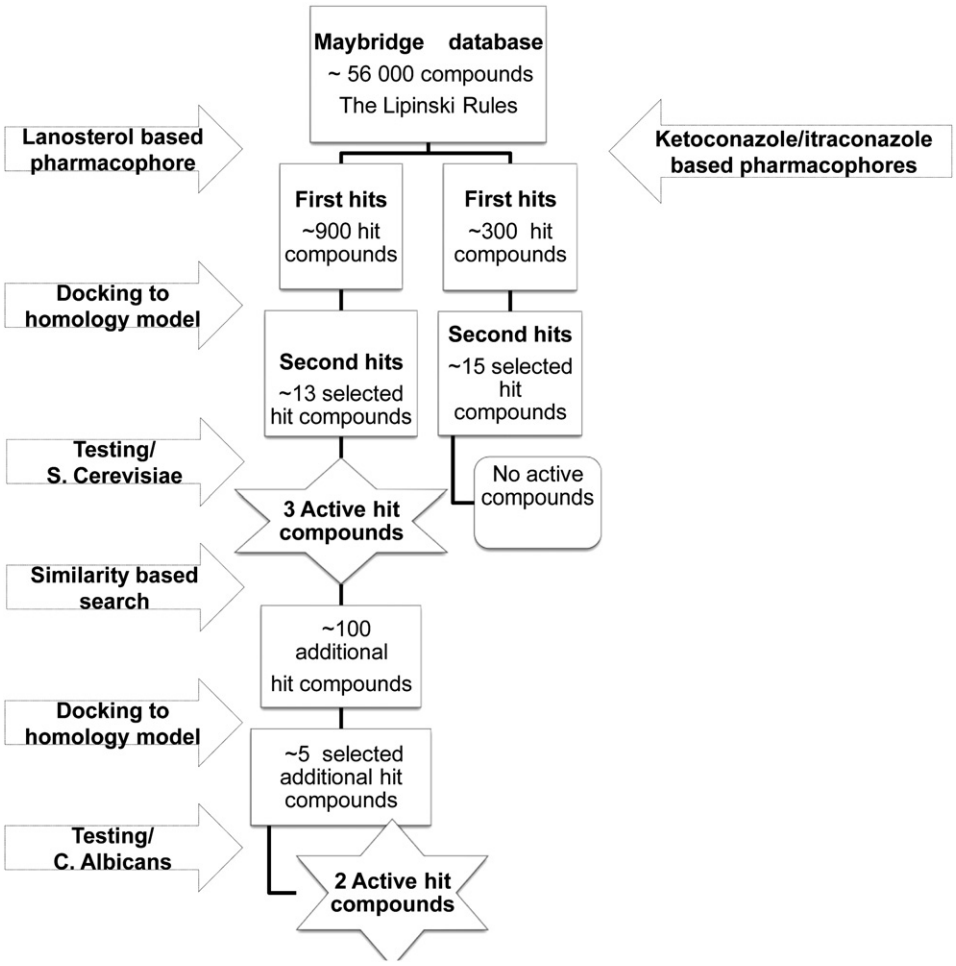


Fig. 2. Scheme of the overall process for identifying new antifungal compounds.

agar plate test the MIC value was 95%. After 48-h incubation, growth was also detected in the spectrophotometric bioassay. The yeast cells treated with ketoconazole and compounds **2** and **3** proliferated after 24-h and 48-h incubation in both bioassay types.

Since compound **1** had promising antifungal properties (MIC < 100 μM, $MIC_{50} < 5$ μM), it was tested with the pathogen *C. albicans* and selected as a basis for a new similarity based screening of the Maybridge database (Fig. 1). This screen produced about 100 hits and after docking them into the active site of CYP51, five compounds (**50**–**54**) were selected for antifungal testing with *C. albicans* (Table 1). Three of the tested compounds inhibited the growth of *C. albicans* markedly in the agar plate bioassay, i.e. compounds **1**, **50** and **51** with MIC values of 12 μM, 8 μM and 40 μM, respectively.

2.3. Binding model of the active compounds

To better understand the binding interaction between the active compounds and the active site of CYP51, their dockings were analyzed in more detail (Fig. 4A). The hydrophobic parts of compound **1** were located above heme porphyrin ring and interacted with the surrounding hydrophobic residues. This hydrophobic core formed van der Waals interaction with Tyr118, Thr122, Phe126, Met306, Leu376, Phe380, Met508 and Val509. Two reactive functional groups (thiophene or carbonitrile) in compound **1** were located above heme. Thiophene may form a charge-transfer complex with Tyr118. Another carbonitrile substitution in the phenyl ring was orientated towards a hydrophilic pocket constituted by Lys143, Lys144, and Lys147.

The van der Waals surfaces of the active site of CYP51 and compound **1** showed that a tight interaction was formed between the positive Lys147 residue and the negative carbonitrile group (Fig. 4B). Interestingly the absence of this phenyl ring substituted carbonitrile chain (compound **50**) increased the potency against *C. albicans*. Docking of compound **50** showed that its core structure was in an opposite orientation compared to compound **1**, as the methoxy group of compound **50** was located in this hydrophilic pocket near the Lys143, Lys144 and Lys147 residues (data not shown).

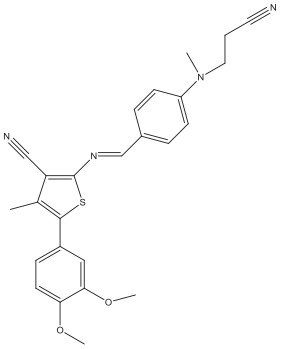
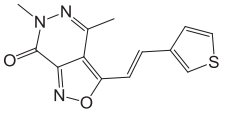
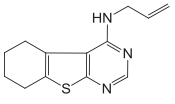
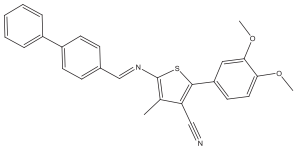
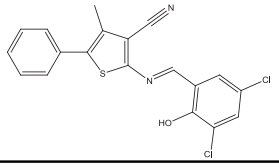
2.4. Hepatic CYP inhibition of the active compounds

Inhibition of human hepatic CYP enzymes 1A2, 2C9, 2C19, 2D6 and 3A4 was tested with compounds **1** and **50**. The compounds had only minor or no inhibitory effect on CYP1A2, CYP2D6, and CYP3A4 enzymes whereas they inhibited CYP2C9 and CYP2C19 enzymes with moderate potency (Table 2). Neither compound inhibited potently ($IC_{50} < 1$ μM) any of the tested human liver CYP enzymes.

3. Discussion

We identified novel non-azole inhibitors of yeast growth using molecular modelling and virtual screening approaches. A large compound database was searched for molecules having the

Table 1
Novel inhibitors of *S. cerevisiae* and *C. albicans*.

No.	Name; M_w (g/mol)	Structure	MIC (μM)	MIC_{50} (μM)	Yeast
1	2-({4-[(2-cyanoethyl)(methyl)amino]benzylidene}amino)-5-(3,4-dimethoxyphenyl)-4-methylthiophene-3-carbonitrile; 444.6		118; 12	2.7	*S. cerevisiae*; *C. albicans*
2	4,6-dimethyl-3-[2-(3-thienyl)vinyl]-6,7-dihydroisoxazolo [3,4-d]pyridazin-7-one; 273.3		120	35	*S. cerevisiae*
3	N-allyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4-amine; 245.3		100[a]	9.98[a]	*S. cerevisiae*
50	2-[([1,1′-biphenyl]-4-ylmethylene)amino]-5-(3,4-dimethoxyphenyl)-4-methylthiophene-3-carbonitrile; 438.54		8	n.d.	*C. albicans*
51	2-{[(3,5-dichloro-2-hydroxyphenyl)methylene]amino}-4-methyl-5-phenyl-3-thiophenecarbonitrile; 387.28		40	n.d.	*C. albicans*

[a] Inhibition detected only with spectrophotometric bioassay.

potential to serve as ligands for the CYP51 enzyme and possessing reactive substructures. Of the 54 hit compounds tested, two had potent inhibitory activity towards *S. cerevisiae* and *C. albicans*, comparable to that of itraconazole and ketoconazole. The MIC values were 12 μM and 8 μM for compounds **1** and **50**, respectively, against *C. albicans*, which are significantly less than MIC/MFC (Minimum Fungicidal Concentration) for the widely used antifungal drugs e.g. itraconazole in this study (MIC > 90.7 μM), fluconazole (104.5 μM), miconazole (76.9 μM), terbinafine (439.2 μM) and amorolfine (201.6 μM) [26]. The results indicate that these two molecules are potential candidates for further development as antifungal agents with less liability for drug interactions caused by inhibition of human liver CYP enzymes.

Two different methods were used to determine the inhibitory potency of the compounds. The agar plate bioassay and its variations are commonly used for testing antifungal properties [27–29]. However, it is not very accurate as the reading of the results is based on visible growth judged by the naked eye. There are also several other variables in the bioassay, such as inoculum size and time of incubation [27]. In contrast, in the spectrophotometric bioassay the results are numeric values (absorbance) and are therefore more objective. This method is also more sensitive than the agar plate bioassay and thus smaller amounts of test compounds are needed. The problem with the spectrophotometric bioassay is the variation between duplicate samples. A possible reason for this may be random aggregation of yeast cells due to e.g. inoculum size [27] or growth characteristics of the yeast isolates [28].

Although the azole group is important for inhibition of CYP51 activity and therefore for the antifungal activity of triazoles and imidazoles, it also causes several problems in practice [30–32]. Azoles do not bind only to fungal CYP51 but also to human CYP51 and other human CYP enzymes causing toxicity [9,30] and significant interactions with many drugs, especially via the inhibition of hepatic CYP3A4 [33]. The novel compounds **1** and **50** identified in this study showed inhibitory activity towards CYP2C9 and CYP2C19, thus resembling the CYP inhibitory profile of fluconazole [33]. Some fungi have become resistant to azole antifungals. One reason for azole resistance is that there have been changes in fungal CYP51, e.g. overexpression, alterations in the affinity, and cellular content. Due to these reasons, there is a clear need for more fungal specific, effective antifungal drugs with fewer interactions.

Previous studies have reported several new types of azole and triazole antifungal compounds [10,34] and a series of non-azole antifungal compounds containing benzopyran, aminotetralin

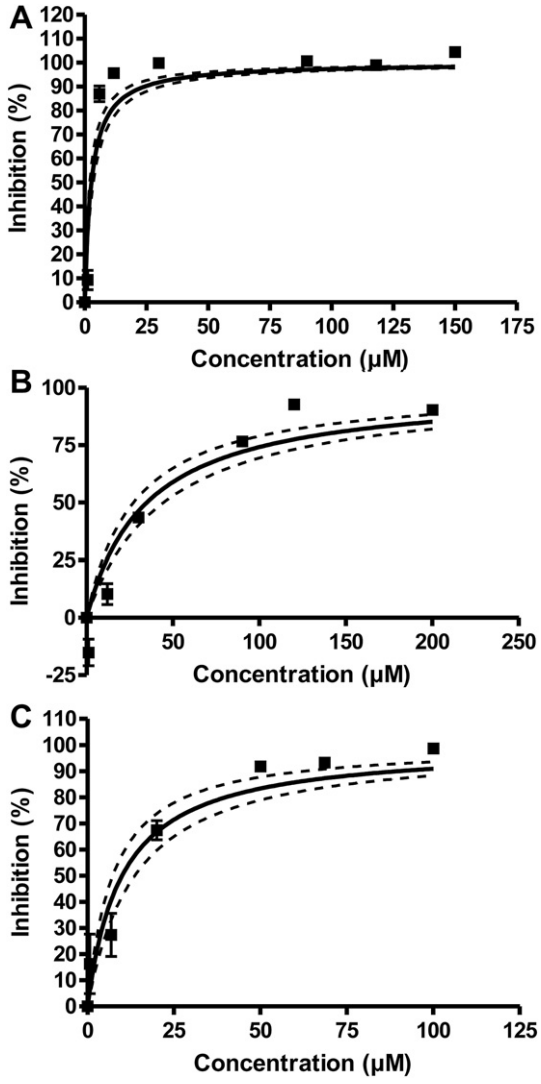


Fig. 3. Inhibition of *S. cerevisiae* growth by compound **1** (A), compound **2** (B), compound **3** (C).

[9,30] or thiophene/aminothiophene scaffolds [35,36]. In these studies thiophene and 2-aminothiophene derivatives have been observed to have antifungal activity against some fungal species, but they were not active against *C. albicans*. Stefancich and co-workers [37] reported antifungal activity of thiophene analogues of bifonazole against *C. albicans* but as azole derivatives they probably have same problems as other azoles. The potent inhibitor of ATP-ase concamycin A has also been described as having antifungal activity, but it is highly toxic, limiting its clinical use [38].

Azole derivatives are five-membered heterocyclic ring compounds containing at least one non-carbon atom such as nitrogen. Azoles bind to the CYP51 catalytic site so that the nitrogen atom of the azole ring coordinates with the iron atom of the CYP heme [9,39]. The previously reported [9,30] aminotetralin derivatives contained two hydrophobic parts consisting of a phenyl ring (hydrophobic part 1) and N-substituted alkyl side chains with 1–18 carbon atoms (hydrophobic part 2) (Fig. 5). These benzopyran or aminotetralin compounds probably interact with CYP51 without binding to heme. The novel antifungal compounds **1** and **50** reported here also consist of two similar hydrophobic parts with a functional group (thiophene or carbonitrile) between these hydrophobic units. Fig. 5 describes the structural similarity

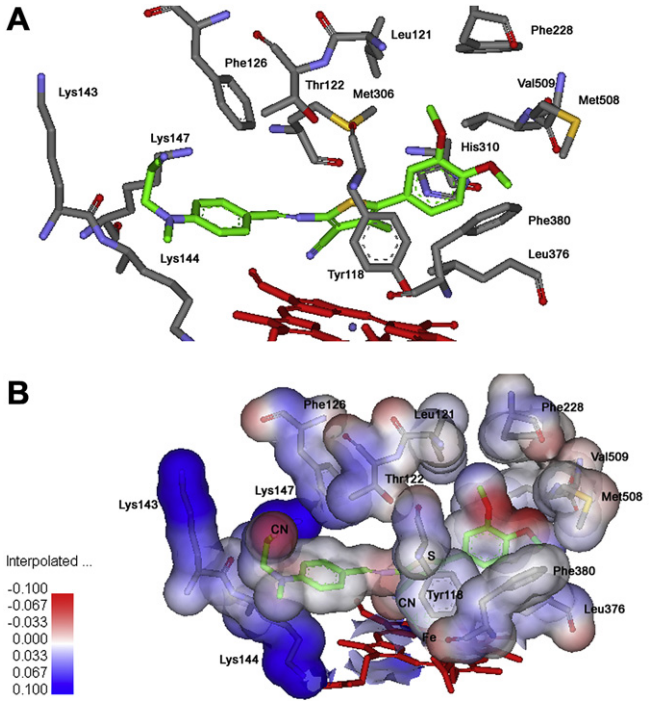


Fig. 4. The binding model of compound **1** (A) without van der Waals surface, (B) with van der Waals surface. Red denotes more negative regions, white neutral regions and blue more positive regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between aminotetralin derivatives and compound **50**. The scaffold of previously reported 2-aminothiophene derivatives [35] contained one hydrophobic moiety next to the functional group (thiophene), being more rigid than the one in our study.

In this study we employed virtual screening and biological testing to identify novel scaffolds that have antifungal activities against *C. albicans*. These non-azole scaffolds can be used as leads in the design of antifungal agents against *C. albicans* and other pathogenic fungi. The functional groups of compounds **1** and **50** have the potential that allow the yeast to produce reactive intermediates via its CYP51 enzyme. These reactive intermediates may attack either the CYP protein or other yeast proteins after their release from the enzyme. Our future studies will focus on elucidating the precise growth inhibitory characteristics of the novel compounds on *C. albicans* and especially on the interactions of the compounds with the CYP51 enzyme.

Table 2
Inhibition of human hepatic CYPs by compounds **1** and **50**.

CYP isoform	Compound **1**		Compound **50**	
	IC_{50} value, μM (category)	95% confidence intervals	Category (IC_{50} value, μM)	95% confidence intervals
1A2	11.9 (weak[a])	7.5–18.9	26.0 (weak[a])	16.9–39.9
2C9	1.2 (moderately potent[a])	0.80–1.72	1.4 (moderately potent[a])	0.89–2.28
2C19	1.1 (moderately potent[a])	0.78–1.63	2.3 (moderately potent[a])	1.18–4.66
2D6	23.3 (weak[a])	16.7–32.5	>100 (non-inhibitor[a])	–
3A4	33.2 (weak[a])	24.8–44.4	>100 (non-inhibitor[a])	–

[a] Classified as potent ($IC_{50} < 1$ μM), marginal/moderate potent (1 μM $< IC_{50} <$ 10 μM), or weak ($IC_{50} > 10$ μM) inhibitors, or non-inhibitor ($IC_{50} > 100$ μM).

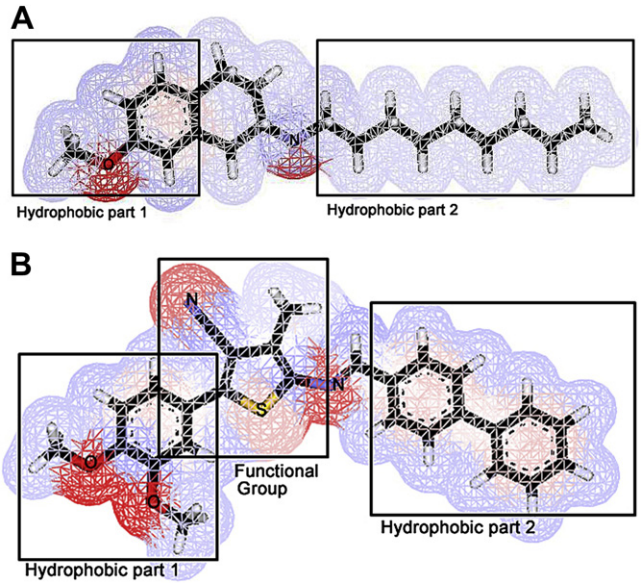


Fig. 5. Comparison of structures and van der Waals surfaces of an antifungal 2-aminotetralin derivative with compound **50**. Red and blue colours describe the electrostatic features of these compounds. Red denotes more negative regions, white neutral regions and blue more positive regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Experimental protocols

4.1. Chemicals

Analytical grade itraconazole and ketoconazole were purchased from Sigma-Aldrich, USA. The tested chemicals were purchased from Maybridge, UK. Stock solutions (5–20 mM) of all the chemicals were dissolved in dimethyl sulfoxide (DMSO, J.T. Baker, Netherlands). The stock solutions were further diluted in 100% DMSO and added to the incubation mixtures at a final DMSO concentration of 2% or less (a concentration of DMSO tested not to inhibit yeast growth).

4.2. Yeast strains and human recombinant enzymes

S. cerevisiae (wild-type, ATCC 24657) strain was purchased from ATCC, Sweden. *C. albicans* (DSM 3454, ATCC 32032) strain was purchased from DSMZ, Germany. Both yeasts were regenerated and cultured according to the manufacturers' instructions.

cDNA-expressed human wild-type CYPs (Supersomes™) were purchased from BD Biosciences Discovery Labware (Bedford, MA, USA).

4.3. Growth medium

The growth medium contained 1% of yeast extract (Bacto™ Yeast Extract, BD, USA), 2% of peptone (Bacto™ Peptone, BD, USA), 2% of glucose (D-(+)-glucose, Sigma, USA) and distilled water (Millipore Synergy water purification system, USA). The agar growth medium was prepared from the growth medium by adding 1% of agar (Agar bacterioloque type A, Biokar Diagnostics, France).

4.4. Methods

4.4.1. Molecular modelling and database search

4.4.1.1. Software used. The construction of the ligand structures was performed using the SYBYL (version 8.1) modelling package. The GOLD software package (version 3.2) was used for ligand docking, and the ChemScore or CScore programs were applied for ranking the docking results. The radius for docking was set at 20 Å around the porphyrin ring of the CYP heme. Virtual screening of the Maybridge database (56,000 compounds) was carried out with the UNITY module of SYBYL. The MOE similarity search tool was used to perform similarity search.

4.4.1.2. Virtual screening. Two different pharmacophores utilizing the docked ligands lanosterol and ketoconazole/itraconazole and the structure of the active site of CYP51 were utilized to screen the databases. The overall screening and filtering process is presented in Fig. 1. The Lipinski rule of five [40] was used as the filtration criterion for the screenings, limiting the range for molecular weight to ≤500 Da, calculated octanol–water partition coefficient to ≤5, the number of H-bond donor atoms (OH and NH) to ≤5, and H-bond acceptor atoms (N and O) to ≤10. Two different pharmacophores were applied for screening the database. Those pharmacophores were generated from the information given by the complex of the docked ligands lanosterol (first pharmacophore) and ketoconazole/itraconazole (second pharmacophore) in the active site of CYP51 [25]. The first hit compounds were docked to the catalytic site of the CYP51 homology model [25]. The selection of compounds for testing (second hits) was based on either the scoring values in docking or visual inspection for the desired properties. In the similarity search based virtual screening, the MOE similarity search tool was also used to find additional molecules similar to those active compounds identified in the pharmacophore based virtual screening.

4.4.2. Antifungal activity bioassay

Two kinds of bioassays were used to determine effects of the chemicals on yeast growth. The positive controls were ketoconazole (30 μM) and itraconazole (90 μM) throughout the study, and 2% DMSO was used as a negative control. In the agar plate bioassay, the plates were prepared according to the EUCAST document [29]. Chemical dilutions (final concentration in wells 1–100 μM) were mixed with 60 °C agar growth medium in sterile flat bottom 6-well plates (Nunclon™ DELTA-Surface). The plates were cooled and stored at +4 °C overnight. The yeasts were cultured in the growth medium overnight at 30 °C for *S. cerevisiae* and 37 °C for *C. albicans* in a shaking incubator. Yeast growth was then adjusted to absorbance of 0.2–0.4 at 600 nm using a spectrophotometer (Hitachi U-2000). This reading approximates to a concentration of 2×10^7 cells per ml [27]. Diluted yeast suspension (final cell count approximately 2×10^2 to 2×10^5) was then transferred to the surface of the agar wells [29] and incubated at +30 °C for *S. cerevisiae* and +37 °C for *C. albicans* in air. The colonies were observed and counted after 24 h and 48 h incubation.

The spectrophotometric bioassay was based on change of absorbance at 595 nm when the yeast was cultured in a medium in 48-plate wells [41]. This method was used only for *S. cerevisiae* due the regulations for handling *C. albicans*. The overnight pre-test incubation and dilutions of yeast were same as in agar plate bioassay. Then chemical dilutions were mixed with media and the solution was added to clear, sterile, flat bottom 48-well plate (Cellstar, Greiner Bio-one) and the yeast stock was added into this mixture (final volume 500 μl). Absorbance was determined prior to incubation and after 24 h and 48 h incubation at +30 °C in a spectrophotometer (ELX800UV, Bio-Tek Instruments) at 595 nm. Plates were incubated on a shaking incubator to avoid yeast cell aggregation.

The extent of growth inhibition was estimated via MIC and MIC_{50} values. The MIC (minimum inhibitory concentration) is the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after overnight incubation in the agar

plate bioassay. The MIC_{50} is the concentration that inhibits 50% of growth as determined by the spectrophotometric bioassay. MIC_{50} values were determined with Graph Pad Prism software (GraphPad Software, Inc.).

In order to determine whether the compounds killed the yeast cells or simply slowed down their growth, the viability of yeast cells was tested after 2-h exposure to the tested compounds. After exposure yeast suspension (*S. cerevisiae*) was centrifuged at 15,000 rpm for 15 min. The supernatant was discarded and the yeast cell pellet was washed with new medium. The washing step was repeated two times. The washed yeast cells were then cultured and the growth was determined as described above with the spectrophotometric assay and the agar plate bioassay.

4.4.3. Inhibition of human hepatic CYP activity

Human hepatic CYP inhibition assays for compounds **1** and **50** were performed essentially as described in detail by Salminen et al. [42] against human recombinant CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 enzymes. Both compounds were pre-screened using five concentrations ranging from 0.0068 to 68 μM according to their solubilities, and then tested for a second time with concentrations around the IC_{50} value detected in the pre-screen. Due to the high lipophilicity of the test compounds, they were dissolved in DMSO and then further diluted with water to achieve a final concentration of 1.3% DMSO in the incubation mixtures.

Acknowledgements

We thank Hannele Jaatinen for expert technical assistance, MSc. Maria Valkonen (National Institute for Health and Welfare) for help in *Candida Albicans* bioassays and Dr. Ewen MacDonald for revising the language of the manuscript. This work was supported by University of Eastern Finland.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.10.053.

References

[1] N.N. Mishra, T. Prasad, N. Sharma, A. Payasi, R. Prasad, D.K. Gupta, R. Singh, Pathogenicity and drug resistance in *Candida albicans* and other yeast species. A review, Acta Microbiol. Immunol. Hung. 54 (2007) 201–235.
[2] L. Ostrosky-Zeichner, A. Casadevall, J.N. Galgiani, F.C. Odds, J.H. Rex, An insight into the antifungal pipeline: selected new molecules and beyond, Nat. Rev. Drug Discov. 9 (2010) 719–727.
[3] C.C. Lai, C.K. Tan, Y.T. Huang, P.L. Shao, P.R. Hsueh, Current challenges in the management of invasive fungal infections, J. Infect. Chemother. 14 (2008) 77–85.
[4] R.P. Rapp, Changing strategies for the management of invasive fungal infections, Pharmacotherapy 24 (2004) 4–28.
[5] M. Klepser, The value of amphotericin B in the treatment of invasive fungal infections, J. Crit. Care 26 (2011) 1–10.
[6] T.D. Edlind, S.K. Katiyar, Mutational analysis of flucytosine resistance in *Candida glabrata*, Antimicrob. Agents Chemother. 54 (2010) 4733–4738.
[7] T.P. Ko, J.J. Lin, C.Y. Hu, Y.H. Hsu, A.H. Wang, S.H. Liaw, Crystal structure of yeast cytosine deaminase. Insights into enzyme mechanism and evolution, J. Biol. Chem. 278 (2003) 19111–19117.
[8] E. Giavini, E. Menegola, Are azole fungicides a teratogenic risk for human conceptus? Toxicol. Lett. 198 (2010) 106–111.
[9] H. Ji, W. Zhang, M. Zhang, M. Kudo, Y. Aoyama, Y. Yoshida, C. Sheng, Y. Song, S. Yang, Y. Zhou, J. Lu, J. Zhu, Structure-based de novo design, synthesis, and biological evaluation of non-azole inhibitors specific for lanosterol 14alpha-demethylase of fungi, J. Med. Chem. 46 (2003) 474–485.
[10] J. Xu, Y. Cao, J. Zhang, S. Yu, Y. Zou, X. Chai, Q. Wu, D. Zhang, Y. Jiang, Q. Sun, Design, synthesis and antifungal activities of novel 1,2,4-triazole derivatives, Eur. J. Med. Chem. 46 (2011) 3142–3148.
[11] C. Sheng, X. Che, W. Wang, S. Wang, Y. Cao, J. Yao, Z. Miao, W. Zhang, Structure-based design, synthesis, and antifungal activity of new triazole derivatives, Chem. Biol. Drug Des. 78 (2011) 309–313.
[12] X. Chai, J. Zhang, Y. Cao, Y. Zou, Q. Wu, D. Zhang, Y. Jiang, Q. Sun, Design, synthesis and molecular docking studies of novel triazole as antifungal agent, Eur. J. Med. Chem. 46 (2011) 3167–3176.
[13] G.I. Lepesheva, M.R. Waterman, CYP51 – the omnipotent P450, Mol. Cell. Endocrinol. 215 (2004) 165–170.
[14] Y. Aoyama, Y. Yoshida, R. Sato, Yeast cytochrome P-450 catalyzing lanosterol 14 alpha-demethylation. II. Lanosterol metabolism by purified P-450(14)DM and by intact microsomes, J. Biol. Chem. 259 (1984) 1661–1666.
[15] S.L. Kelly, S. Kenna, A. Arnoldi, D.E. Kelly, Studies on azole-induced cell death in *Saccharomyces cerevisiae*, FEMS Microbiol. Lett. 115 (1994) 219–222.
[16] C.J. Jackson, D.C. Lamb, N.J. Manning, D.E. Kelly, S.L. Kelly, Mutations in *Saccharomyces cerevisiae* sterol C5-desaturase conferring resistance to the CYP51 inhibitor fluconazole, Biochem. Biophys. Res. Commun. 309 (2003) 999–1004.
[17] K.S. Barker, M.M. Pearson, P.D. Rogers, Identification of genes differentially expressed in association with reduced azole susceptibility in *Saccharomyces cerevisiae*, J. Antimicrob. Chemother. 51 (2003) 1131–1140.
[18] M.M. Balkis, S.D. Leidich, P.K. Mukherjee, M.A. Ghannoum, Mechanisms of fungal resistance: an overview, Drugs 62 (2002) 1025–1040.
[19] M.A. Ghannoum, L.B. Rice, Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance, Clin. Microbiol. Rev. 12 (1999) 501–517.
[20] A.S. Kalgutkar, I. Gardner, R.S. Obach, C.L. Shaffer, E. Callegari, K.R. Henne, A.E. Mutlib, D.K. Dalvie, J.S. Lee, Y. Nakai, J.P. O'Donnell, J. Boer, S.P. Harriman, A comprehensive listing of bioactivation pathways of organic functional groups, Curr. Drug Metab. 6 (2005) 161–225.
[21] E. Fontana, P.M. Dansette, S.M. Poli, Cytochrome p450 enzymes mechanism based inhibitors: common sub-structures and reactivity, Curr. Drug Metab. 6 (2005) 413–454.
[22] Y.F. Ueng, T. Shimada, H. Yamazaki, F.P. Guengerich, Oxidation of aflatoxin B1 by bacterial recombinant human cytochrome P450 enzymes, Chem. Res. Toxicol. 8 (1995) 218–225.
[23] S. Rawal, S.S. Yip, R.A. Coulombe Jr., Cloning, expression and functional characterization of cytochrome P450 3A37 from turkey liver with high aflatoxin B1 epoxidation activity, Chem. Res. Toxicol. 23 (2010) 1322–1329.
[24] P. Pelkonen, M.A. Lang, M. Negishi, C.P. Wild, R.O. Juvonen, Interaction of aflatoxin B1 with cytochrome P450 2A5 and its mutants: correlation with metabolic activation and toxicity, Chem. Res. Toxicol. 10 (1997) 85–90.
[25] B. Rupp, S. Raub, C. Marian, H.D. Holtje, Molecular design of two sterol 14alpha-demethylase homology models and their interactions with the azole antifungals ketoconazole and bifonazole, J. Comput. Aided Mol. Des. 19 (2005) 149–163.
[26] S. Hawser, K. Islam, Comparisons of the effects of fungicidal and fungistatic antifungal agents on the morphogenetic transformation of *Candida albicans*, J. Antimicrob. Chemother. 43 (1999) 411–413.
[27] C. Brass, J.Z. Shainhouse, D.A. Stevens, Variability of agar dilution-replicator method of yeast susceptibility testing, Antimicrob. Agents Chemother. 15 (1979) 763–768.
[28] D.R. Hospenthal, C.K. Murray, M.G. Rinaldi, The role of antifungal susceptibility testing in the therapy of candidiasis, Diagn. Microbiol. Infect. Dis. 48 (2004) 153–160.
[29] EUCAST Definitive Document E.DEF 3.1, June 2000: Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution, Clin. Microbiol. Infect. 6 (2000) 509–515.
[30] B. Yao, H. Ji, Y. Cao, Y. Zhou, J. Zhu, J. Lu, Y. Li, J. Chen, C. Zheng, Y. Jiang, R. Liang, H. Tang, Synthesis and antifungal activities of novel 2-aminotetralin derivatives, J. Med. Chem. 50 (2007) 5293–5300.
[31] Z. Lv, C. Sheng, Y. Zhang, T. Wang, J. Feng, H. Sun, H. Zhong, M. Zhang, H. Chen, K. Li, Synthesis and in vitro antifungal activities of new 3-substituted benzopyrone derivatives, Bioorg. Med. Chem. Lett. 20 (2010) 7106–7109.
[32] G.I. Lepesheva, M.R. Waterman, Sterol 14alpha-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms, Biochim. Biophys. Acta 1770 (2007) 467–477.
[33] O. Pelkonen, M. Turpeinen, J. Hakkola, P. Honkakoski, J. Hukkanen, H. Raunio, Inhibition and induction of human cytochrome P450 enzymes: current status, Arch. Toxicol. 82 (2008) 667–715.
[34] C. Sheng, X. Che, W. Wang, S. Wang, Y. Cao, J. Yao, Z. Miao, W. Zhang, Design and synthesis of antifungal benzoheterocyclic derivatives by scaffold hopping, Eur. J. Med. Chem. 46 (2011) 1706–1712.
[35] P.S. Fogue, P.K. Lunga, E.S. Fondjo, J. De Dieu Tamokou, B. Thaddee, J. Tsemeugne, A.T. Tchapi, J.R. Kuiate, Substituted 2-aminothiophenes: antifungal activities and effect on *Microsporum gypseum* protein profile, Mycoses (2011).
[36] N. Fokialakis, C.L. Cantrell, S.O. Duke, A.L. Skaltsounis, D.E. Wedge, Antifungal activity of thiophenes from *Echinops ritro*, J. Agric. Food Chem. 54 (2006) 1651–1655.
[37] G. Stefancich, M. Artico, G. Ortar, R. Silvestri, G. Simonetti, G. Apuzzo, Antibacterial and antifungal agents. XV. Synthesis and antifungal activity of structural analogues of bifonazole and ketoconazole, Arch. Pharm. (Weinheim) 325 (1992) 687–694.
[38] I. Okoli, J.J. Coleman, E. Tampakakis, W.F. An, E. Holson, F. Wagner, A.L. Conery, J. Larkins-Ford, G. Wu, A. Stern, F.M. Ausubel, E. Mylonakis, Identification of antifungal compounds active against *Candida albicans* using

an improved high-throughput *Caenorhabditis elegans* assay, PLoS ONE 4 (2009) e7025.
[39] N. Strushkevich, S.A. Usanov, H.W. Park, Structural basis of human CYP51 inhibition by antifungal azoles, J. Mol. Biol. 397 (2010) 1067–1078.
[40] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Adv. Drug Deliv. Rev. 46 (2001) 3–26.
[41] T. Patton, J. Barrett, J. Brennan, N. Moran, Use of a spectrophotometric bioassay for determination of microbial sensitivity to manuka honey, J. Microbiol. Methods 64 (2006) 84–95.
[42] K.A. Salminen, A. Meyer, L. Jerabkova, L.E. Korhonen, M. Rahnasto, R.O. Juvonen, P. Imming, H. Raunio, Inhibition of human drug metabolizing cytochrome P450 enzymes by plant isoquinoline alkaloids, Phytomedicine 18 (2011) 533–538.