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Identification and mode of action of a plant natural product targeting human fungal pathogens

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Abstract

Candida albicans is a major cause of fungal diseases in humans and its resistance to available drugs is of concern. In an attempt to identify novel antifungal agents, we initiated a small scale screening of a 199 natural plant compounds (NPs) library. *In vitro* susceptibility profiling experiments identified 33 NPs with activity against *C. albicans* (MIC_{50} ≤ 32 µg/ml). Among the selected NPs, the sterol alkaloid tomatidine was further investigated. Tomatidine originates from *Solanum lycopersicum* (tomato) and exhibited high fungistatic activity against *Candida* species (MIC_{50} ≤ 1 µg/ml) but no cytotoxicity against mammalian cells. Genome-wide transcriptional analysis of *C. albicans* tomatidine-treated cells revealed a major alteration (upregulation) of ergosterol genes suggesting that the ergosterol pathway was targeted by this NP. Consistent with this transcriptional response, sterol content analysis of tomatidine-treated cells showed not only inhibition of Erg6 (C-24 sterol methyltransferase) but also of Erg4 (C-24 sterol reductase) activity. A forward genetic approach in *Saccharomyces cerevisiae* coupled with whole genome sequencing identified 2 non-synonymous mutations in *ERG6* (amino acids: D249G and G132D) responsible for tomatidine resistance. Our results therefore identified unambiguously Erg6, a sterol C-24 methyltransferase absent in mammals, as the main direct target of tomatidine. We tested the *in vivo* efficacy of tomatidine in a mouse model of *C. albicans* systemic infection. Treatment with a nano-crystal pharmacological formulation successfully decreased the fungal burden in infected kidneys as compared to placebo and thus confirmed the potential of tomatidine as a therapeutic agent.

Introduction

Invasive fungal infections are an increasing threat to human health. In the developed countries, these infections predominantly occur in the context of increasingly aggressive immunosuppressive therapies. The overall mortality for invasive diseases caused by *Candida* and *Aspergillus* spp. is 30–50%, despite the advent of new diagnostic and therapeutic strategies (1). The fight against *C. albicans* infections necessitates the use of antifungal agents and continued efforts are required to improve the therapeutic outcomes associated with fungal infections.

Antifungal drugs that are currently available in the treatment of *Candida* infections belong to four different chemical classes and include polyenes, azoles, pyrimidine analogues and echinocandins (2). While polyenes and azoles target sterols and their biosynthesis, pyrimidine analogues perturb nucleic acid biosynthesis and echinocandins interfere with cell wall biosynthesis. The activity against common fungal pathogens and their detailed mode of action is summarised in several available reviews (3, 4). The repeated or long-term use of antifungal agents in medicine has facilitated the development of resistance in clinically relevant species (5). When occurring, antifungal resistance can be a serious clinical problem due to the limited number of available agents. In general, the incidence of antifungal resistance among human fungal pathogens is low to moderate, especially when compared to the incidence of antibiotic resistance among bacterial pathogens. Antifungal resistance occurrence has to be considered independently for each antifungal class and for each fungal species. Moreover, epidemiological data regarding incidence of resistance among fungal species is not identically distributed worldwide (6, 7). Taken together, the small number of available antifungal agents and the occurrence of resistance reveal the urgent need for novel active compounds.

Natural products (NPs) have already provided a vast resource for active ingredients in medicines. The reason for this success can be explained by the high chemical diversity of NPs and the effects of evolutionary pressure to create biologically active molecules and/or the structural similarity of protein targets across many species (8). In the field of antimicrobials, NPs met important successes. Starting with the discovery of penicillin, the pharmaceutical industry has relied on this source extensively for antibiotic development. Nowadays, 80% of

all available clinically used antibiotics are directly (or indirectly) derived from NPs (9). Some antifungals including polyenes and echinocandins derive directly from NPs.

The discovery of structurally novel NPs with suitable pharmacological properties as antibiotic leads has weakly progressed in the recent decades (10). Innovative strategies provided comprehensive antifungal profilings of given NPs and an understanding of their mode of action for target identification and validation (11).

In a precedent study, we reported a strategy to identify antifungal NPs from plant crude extracts (12). This strategy relied on the use of a *C. albicans* isolate highly susceptible to growth inhibitors and in which traces of inhibitory NPs could be detected. NPs were identified with a bioassay enabling a rapid detection tool of antifungal activity. With the determination of chemical structures of the identified NPs, novel compounds could be readily processed for further evaluation with *in vivo* approaches (13).

In this study, we report a small scale screening of selected NPs with an in-depth characterization of their biological properties. The compounds were tested on the basis of activity against different pathogenic and non-pathogenic yeasts and of their toxicity for mammalian cells. One of promising compounds (tomatidine) showing high activity against *C. albicans* was further investigated. Tomatidine mode of action was in-depth characterized for the first time and its activity was confirmed *in vivo*.

Results

Screening of small scale library of plant NPs for antifungal activities

A library of 199 natural products (NPs) with potential antifungal activity was built. Compounds were selected either according to previously reported activities or by structural analogy to scaffolds that were known to be active. These compounds were obtained in two ways, either by targeted isolation from plant extracts (29 different plants were investigated making 53% of the investigated NPs) or by commercial acquisition after selection based on structural similarity with documented antifungals (see Material and Methods; compounds are listed in Supplementary Table S1). The 199 NPs were subjected to standard *in vitro* microdilution susceptibility assays (EUCAST method) with *C. albicans* under acidic and neutral conditions (pH of 4.6 and 7.0). These different values were chosen to reflect pH changes in the different host niches of *C. albicans*. Results are summarized in Table 1. Considering a threshold for antifungal activity of 32 µg/ml, our analysis identified 33 NPs exhibiting antifungal activities. The activity threshold (32 µg/ml) was selected since we estimated that setting a high threshold for a MIC value obtained *in vitro* would be problematic when testing activities *in vivo* and to reach therapeutic concentration ranges in animals. With this threshold, while 2 compounds were active at neutral pH and 18 at low pH only, 13 were active at both pH conditions (Table 1).

In order to further characterize their antifungal properties, active NPs were profiled for their activity spectrum against several other clinically relevant *Candida* strains (*C. glabrata, C. tropicalis, C. parapsilosis* and *C. krusei)* as well as another related non-pathogenic yeast (*Saccharomyces cerevisiae*). As shown in Fig. 1, a variety of activity profiles was observed with compounds active on all strains, while others only on a small species subset, suggesting a diversity in their mode of action and in the target cell response. *C. glabrata* strains (azole sensitive (AS) and azole resistant (AR)) exhibited the most resistant phenotypes. To identify compounds with high antifungal potential, two major clinical antifungal agents (fluconazole and caspofungin) were added to the selected NPs and all clustered according to their activity profile. Four NPs (pyridoxatin, glc-3-medicagenic acid, medicagenic acid, plumbagin) were grouped together with caspofungin (Fig. 1, purple highlighted) and exhibited a strong inhibitory activity against all species tested. The nearest neighbouring cluster was containing

fluconazole and included three other NPs (formosanin C, tomatidine and taxodion; Fig. 1, red highlighted). This cluster was characterized by strong overall activities but with reduced susceptibility of specific yeast such as C. *glabrata* (AR) and *C. krusei* for fluconazole and *C. glabrata* strains for tomatidine.

Biofilms are defined as complex cell populations with intrinsic resistance to many antifungal drugs as compared to planktonic cells (14). We tested the selected NPs on *C. albicans* mature biofilms formed *in vitro* using an activity cut-off value higher than the one used for planktonic cells (MIC ≤ 50 µg/ml). Most of the compounds (25 out of 33) were active against biofilms and exhibited a MIC against biofilm higher than the MICs measured with planktonic cells, except for NPs originating from *Waltheria indica*. Anti-biofilm activity of these NPs was previously reported (15).

We next tested the activity of the selected NPs (33) on a Gram-negative bacterium (*E. coli*). All but one compound (pyridoxatin, Table 1) were inactive against this bacterial species (MIC >64 µg/ml), arguing for a fungal-specific inhibitory activity.

The last step in the screening process was to determine the potential acute toxicity of selected NPs on mammalian cells. The 24 most active antifungal NPs (MIC ≤ 16 µg/ml) were therefore tested with standard cytotoxicity assays on Hela cells and the lethal dose (LD_{50}) was determined. Our results indicated that only 3 NPs including tomatidine, medicagenic acid and medicagenic acid 3-0-glucopyranoside, showed a selectivity index (SI) with an acceptable range (>100). All the antifungal profiling assays are summarized in Table 1. Since the antifungal activity of the two medicagenic acid-based NPs were already reported (16), we focused our efforts in understanding the antifungal properties and mode of action of tomatidine.

Antifungal activity of tomatidine

Tomatidine (Fig. 2A) is a sterol alkaloid produced by tomato (*Solanum lycopersicum*). It is the precursor of the sterol glycol-alkaloids α-tomatine, which is a well-known antifungal saponin against phytopathogens (17). Antifungal and antiparasitic activities of tomatidine have been reported against *S. cerevisiae* and some parasites such as *Leishmania amazonensis* and

Phytomonas serpens (18-20). These studies revealed that tomatidine exposure induces a perturbation of ergosterol biosynthesis and suggested the 24-sterol methyl transferase (24-SMT-Erg6) as potential target within the sterol biosynthetic pathway. Erg6 is responsible for the key structural difference between cholesterol and ergosterol (20, 21). This is consistent with the fact that *ERG6* gene is present in fungi but absent in higher eukaryotes such as mammals. Erg6 therefore represents an attractive target for the development of antifungal agents.

Tomatidine susceptibility assays on several clinically-relevant *Candida* species are shown on Fig. 2B. All strains, except *C. glabrata*, were highly susceptible to tomatidine. Similar results were obtained with standard microdilution susceptibility assays with *C. krusei* and *C. albicans* showing the highest sensitivity (Table 2, MIC = 0.625 µM). We used a larger panel of *C. albicans* isolates (n=9) and found that the tomatidine MIC50 (concentration needed to inhibit at least 50% of the tested population) was at 0.3125 µM. MIC50 values for *C. krusei* (n=8) and *C. tropicalis* (n=9) were both at 1.25 µM (Table S4). Tomatidine was not active against *C. albicans* mature biofilms (Table 1), but the ability of *C. albicans* to form hyphae in filamentation-inducing *in vitro* conditions was severely compromised in presence of tomatidine. As shown in Fig. 2C, the addition of tomatidine to *C. albicans* cultures under hyphal inducing conditions resulted in the formation of pseudohyphae and/or unseparated budding yeast cells. Tomatidine exhibited fungistatic activity in *C. albicans* as revealed by time-kill assays at different drug concentrations (Fig. 2D). Only weak fungicidal activity of tomatidine (10 µM) was observed in *C. krusei* with CFU counts decreased by about 80% as compared to the starting inoculum after 24 h of incubation (Fig. 2D).

Global transcriptional analysis of tomatidine-treated *C. albicans* cells

Genome-wide transcriptional analysis in the presence of given drugs has been often used as a mean to highlight their modes of action and to propose possible cellular targets (22). The effect of tomatidine treatments on *C. albicans* transcriptome was investigated *in vitro* using two different exposure times (1- and 3 h) at a fixed drug concentration (2.5 µM). This drug concentration was corresponding to the MIC obtained in YEPD (see Table 2) and led to a 30 % and 50 % growth inhibition after 1 h and 3 h incubation, respectively (data not shown).

Total RNAs was recovered from treated and untreated cells and genome-wide transcriptional analysis was performed using RNA seq (see Materials and Methods). After 1- and 3 h of tomatidine exposure, 129 and 1149 genes (2% and 21 % of the genes expressed) were identified as differentially expressed between treated and untreated cells (false discovery rate (FDR) ≤ 0.05 and fold-change ≥ 2), respectively. Hundred twelve (112) genes were found to be affected at both 1- and 3 h exposure time. Seventy seven (77) genes were upregulated, 32 genes downregulated and 3 genes were inversely regulated as compared to untreated conditions (Fig. 3A, supplementary File S1). GO term analysis on the 77 commonly upregulated genes revealed a clear alteration of the ergosterol biosynthetic pathway (corrected p-value < 0.0004) which included 6 genes (*ERG2, ERG3, ERG4, ERG6, ERG11, ERG25*) (Fig. 3B). Interestingly, other components of the ergosterol pathway were found to be upregulated by tomatidine at 1 h exposure time only including *ERG1, ERG5, ERG24* and *UPC2* (the master sterol transcriptional regulator) and at 3 h exposure time only including *ERG9* and *ERG28* (supplementary File S1). Interestingly, among all the *ERG* genes differentially regulated by tomatidine treatment, the most affected was *ERG6* with 7.4- and 11.8 fold-change of expression after 1- and 3 h treatment, respectively. This important fold-change was confirmed by qPCR analysis (up to 40-fold increase at 3 h treatment) (Fig. 3C).

Response to steroid hormone stimulus was another overrepresented GO term among upregulated genes (corrected p-value < 0.002). The three corresponding genes were part of the *TAC1* regulon and included the major drug efflux system in *C. albicans* with the two ABC transporters (*CDR1* and *CDR2*) and the transcriptional regulator itself (*TAC1*) (Fig. 3B). qPCR analysis on *ERG4, ERG6, ERG11, CDR1* and *CDR2* expression on the same RNA samples validated the RNA-seq data analysis (Fig. 3C, supplementary Fig. S1).

GO analysis identified only one enriched GO term (ion transport; corrected p-value < 0.003) in commonly down-regulated gene (32 genes) with 2 genes involved in iron or calcium transport (*FET34*, *FTR1*).

Exposing *C. albicans* cells to tomatidine for 3 h leads to large transcriptional changes (total of 1149 genes) with 1037 genes specifically affected after that exposure time. GO term analysis identified processes that are characteristics of growth inhibition/arrest or drug-related stress

such as glucose transport (in upregulated genes) and the transcriptional and translational machinery in downregulated genes (see supplementary File S1).

In order to better characterize the response to tomatidine, we used a Gene Set Enrichment Analysis (GSEA) to identify transcriptional signatures from other drug-induced transcriptional studies overlapping with the present tomatidine transcriptional data (Supplementary File S2). As shown in Fig. 3D, tomatidine up- and downregulated genes (red and blue, respectively) shared the highest number of regulated genes with the fluconazole_UP/DOWN gene sets, followed by another azole-related gene set (ketoconazole_UP/DOWN) and gene set from cells lacking the master regulator of ergosterol genes *UPC2*. Using another published set of transcriptional data that includes several *in vitro* growth stress conditions (such as hypoxia, (23), tomatidine gene set was sharing regulated genes with cells under hypoxic conditions, which adaptive response mainly is mediated by *UPC2* (supplementary Fig. S2) (24). Taken together, these analyses revealed that tomatidine had a transcriptional signature closely related to azole drugs and strongly suggests that the ergosterol biosynthetic pathway is also targeted by this NP.

Tomatidine as an inhibitor of the sterol biosynthetic pathway

As suggested by our transcriptional analysis, tomatidine is likely to interfere with ergosterol biosynthesis given that the upregulation of *ERG* genes upon treatment has been shown to be a cell response mechanism to drugs targeting the ergosterol pathway (25). In order to detect more precisely the affected step(s) of this pathway, we performed an in-depth sterol content analysis of *C. albicans*, *C. krusei* and *S. cerevisiae* cells treated with tomatidine. Total sterols were extracted from yeast cells and next subjected to gas liquid chromatography coupled with mass spectrometry (GC-MS) analysis.

As shown in Fig. 4A, untreated *C. albicans* cells contained, as expected, ergosterol (blue color) as the most abundant sterol (>96% of total sterol). Zymosterol (the product known to accumulate in the mutant for *ERG6*, a 24-C methyl transferase) (red color) was the major sterol (84% of total sterols) in tomatidine-treated cells, thus suggesting a strong inhibition of this enzymatic step by tomatidine. Furthermore, careful analysis of sterol content allowed the

identification of small amount of two other sterol intermediates including cholesta-trienol (6 %) and ergosta-5-7-24-(28)-tetraenol (8%). Enzymes involved in the late stage of ergosterol biosynthesis are usually acting independently of each other which enables the pathway to proceed even if one step is inhibited by a drug or is genetically suppressed leading to the synthesis of alternative sterols. One of those is cholesta-trienol (green color) which was detected in tomatidine-inhibited cells, where successive actions of Erg2, Erg3 and Erg5 occurred in absence of previous Erg6 activity. However, Erg4, the last enzyme involved in the sterol pathway, requires first Erg6 action to produce its substrate. More specifically, the methylene group added by Erg6 at position C-24 of zymosterol is the substrate for the C-24 reductase activity of Erg4. Therefore the detection of the Erg4 substrate (ergosta-5-7-24-(28)-tetraenol (purple color)), was unanticipated and suggested that (i) tomatidine was also inhibiting Erg4 and that (ii) Erg6 was not completely inhibited by tomatidine at the concentration tested.

In order to be able to assess more accurately the inhibition of Erg4 activity, we next performed a progressive decrease of Erg6 inhibition by titrating down tomatidine concentration. As shown in Fig. 4A, decreasing tomatidine concentration from 2 µM to 0.25 µM resulted in the release of Erg6 inhibition (zymosterol decreased from 84 % to 7 % of total sterol) and the accumulation of ergosta-5-7-24-(28)-tetraenol (up to 72% of total sterol at 0.25 µM tomatidine), thus confirming the ability of tomatidine to inhibit Erg4. As the concentration of tomatidine required to accumulate zymosterol and ergosta-5-7-24-(28)-tetraenol up to 84 % and 72 % of total sterols is approximatively 2 µM and 0.25 µM, respectively, one can estimate that tomatidine is about 10 times more effective as an inhibitor of Erg4 as compared to Erg6. Interestingly, this Erg4/Erg6 dual inhibition was also found in *C. krusei* but not in *S. cerevisiae* in which only Erg6 inhibition could be detected (supplementary Fig. S3).

Following these analysis, a *C. albicans ERG6* deletion strain was engineered in order to validate that Erg6 is a target of tomatidine. We reasoned that, if the Erg6 target is absent, then tomatidine will not be active against this mutant. As already described, the deletion of *ERG6* results in increased susceptibility to a large variety of drugs, with the exception of azoles and polyenes (26). The *erg6*Δ/Δ mutant was indeed resistant to fluconazole with a 128-fold higher MIC than the wild type (Fig. 4B). Interestingly, the *erg6*Δ/Δ mutant constructed here was as susceptible to tomatidine as the parental strain (Fig. 4B). This indicates a pleiotropic

susceptibility of the strain or potential secondary target(s) of tomatidine in addition to the ergosterol pathway. Similar results were obtained using a *S. cerevisiae ERG6* mutant (supplementary Fig. S4).

Forward genetic approach to identify targets of tomatidine

A forward genetic screen in *S. cerevisiae* was finally undertaken as an unbiased way to discover the target(s) of tomatidine. The strain chosen in this approach lacked *PDR5* and *MSH2* to avoid multidrug transporter-dependent resistance mechanisms and to increase the rates at which resistance mutation may occur, respectively (27, 28). A *pdr5*Δ *msh2*Δ strain (P1) was plated on YEPD media containing 10 µM tomatidine and, after a screening of more than 2 x 10^8 cells, one resistant mutant (R1) was isolated. To obtain additional resistant mutants, a second strategy inspired by Ojini *et al.* (28) was developed and consisted of submitting cells to two drug-exposure periods in liquid media (72- and 48 h) interspersed by a period of drug-free growth (48 h). Cultures with a robust growth at the end of the experiment were further plated on solid YEPD media containing 10 µM tomatidine which resulted in the isolation of four resistant mutants (R2, R3.1, R3.2 and R3.4) from two different cultures. Tomatidine resistance of the five obtained mutants was confirmed by a serial dilution assay (Fig. 5A). MIC results showed a 4-fold decrease in susceptibility for R1, R3.1 and R3.2 as compared to the parental strain P1. Isolates R3.3 and R2 exhibited only a slight decrease of susceptibility. Tomatidine resistance was specific since susceptibility assays with different drug classes (fluconazole, caspofungin and amphotericin B) were in the range of strain P1 (Table 3). Simplified sterol composition analysis using the unique spectrophotometric absorbance signature of sterols confirmed that the mutants had normal sterol composition with no detectable differences compared to the parental strain (supplementary Fig. S5A).

The five mutant genomes were sequenced with that of parental strain P1. Alignment of parental and mutant genomes to the reference genome (S288C) was followed by the

identification of non-synonymous polymorphisms uniquely present in the coding regions of the resistant mutants (see Materials and Methods). All the non-synonymous mutations inventoried in the five resistant mutants (between 26 to 60 according to the strain) are listed in supplementary File S3. Interestingly, four out of the five resistant mutants contained a missense mutation in *ERG6* with the following amino acid substitutions: D249G for R1, R3.1, R3.2 and G132D for R2. These amino acid residues might play an important role in Erg6 function as they are conserved among fungi, plants or protozoa (29) (Fig. 5B), but seemed dispensable for normal ergosterol synthesis. To determine if the two mutations were sufficient to confer tomatidine resistance, the G132D and D249G substitutions were inserted in a wild type *S. cerevisiae* strain (IMX585) using site-directed CRISPR-Cas9 genome editing technology (30). Both strains (G132D and D249G) were 4-fold more resistant to tomatidine than the wild type (Table 3), thus recapitulating (or increasing for G132D) the tomatidine resistant phenotype of the original resistant mutants. Sterol profiles analysis showed that the G132D strain, in contrast to the corresponding original resistant mutant and the D249G strain, exhibited an altered sterol composition with intermediate profiles between an *ERG6* deletion and a wild-type strain (supplementary Fig. S5B). This might explain the observed increase fluconazole susceptibility of this strain (Table 3).

While the last resistant mutant (R3.3) retained a wild-type allele of *ERG6*, it exhibited a frameshift mutation in *ACE2.* This gene encodes for a transcription factor required for septum degradation after cytokinesis (31). Strain R3.3 exhibited a multicellular clumping phenotype, which is identical to an *ACE2* deletion strain (supplementary Fig. S6). A decrease in tomatidine susceptibility was observed in the *ACE2* deletion strain and thus recapitulated the resistance phenotype of the R3.3 initial strain (Table 3).

Tomatidine is targeted by efflux pumps

The forward genetic approach was designed to exclude multidrug transporter-dependent resistance mechanisms by using a *S. cerevisiae* strain lacking *PDR5*. However, the activation of drug efflux in yeast is a common cell defence mechanism against toxic drugs, as observed in our *C. albicans* genome-wide transcriptional analysis, in which the genes of the *TAC1* regulon (including *CDR1, CDR2*) were among the tomatidine-upregulated genes (Fig. 3B). To

address whether tomatidine was a target of the efflux pump system in *C. albicans*, a set of deletion mutants lacking each *CDR1*, *CDR2* or *MDR1* (another import efflux pump belonging to the major facilitator superfamily of transporters) were used to evaluate their tomatidine susceptibility. The *cdr1*Δ/Δ strain was the only one to exhibit increased susceptibility to tomatidine in contrast to *CDR2* and *MDR1* mutants and the wild-type strain (Fig. 6A). This data clearly indicated that, as many other drugs such as fluconazole (32), tomatidine is targeted by the *CDR1* efflux pump.

A major problem arising during clinical treatment of candidiasis is the emergence of resistant isolates. One important underlying mechanism consists of an upregulation of efflux pumps by the acquisition of a hyperactive alleles of their regulator (33). To evaluate the potential cross-resistance of hyperactive *TAC1* strains between fluconazole and tomatidine, we measured the tomatidine susceptibility of two pairs of matched azole-susceptible (AS) and azole-resistant (AR) *C. albicans* clinical isolates. The AR isolates carried *TAC1* hyperactive alleles. While the difference in fluconazole MIC between DSY732 (AR) and DSY731 (AS) was of 64-fold in magnitude, the tomatidine MIC between the two strains diverged only by 4-fold (Fig. 6B left panel, Table 4). Similar results were obtained using *C. albicans* clinical isolates DSY1843 (AR) and DSY1841 (AS). Azole and tomatidine MICs increased by only 4- and 2-fold, respectively (Fig. 6B right panel, Table 4). Taken together, these results suggest that the common resistance mechanism against azole triggered by *TAC1* hyperactivity seems to have limited effect on tomatidine susceptibility.

As *C. albicans* exhibited limited cross-resistance between fluconazole and tomatidine, we next investigated the potential synergistic effect of their combination. Classical checkerboard combination assays were performed and Fractional Inhibitory Concentration (FIC) values determined (as described in Materials and Methods). As expected for drug targeting the same pathway, fluconazole and tomatidine exhibited a strong additive effect in both *C. albicans* and *C. krusei* with cell growth inhibition of more than 50% in the zone of additivity (Fig. 6C). We then tested if the combination of the two drugs changed their fungistatic properties. Interestingly, time-kill assay showed that fluconazole-tomatidine combination leads to a fungicidal activity in *C. krusei* (>100-fold decrease in cells counts after 24 hours), but not in *C. albicans* (Fig. 6D).

In vivo activity of tomatidine

To confirm the high potential of tomatidine as a therapeutic agent, the *in vivo* efficacy of the compound was tested in a mouse model of *C. albicans* systemic infection. Due to its hydrophobic nature, a nanoparticle-based formulation of tomatidine was developed to allow its administration and to potentially enhance its bioavailability. Mice were infected through the tail vein with *C. albicans* inoculum and were treated intraperitoneally (i.p.) with tomatidine (50 mg/kg) or placebo at 6 hours, 24 hours and 31 hours post-infection (pi). Colony forming units (CFU) were then determined in the kidneys 48h pi. As illustrated in Fig. 7, mice treated with tomatidine exhibited statistically-significant reduced CFU compared to controls (Mann-Whitney test, p-value = 0.031), thus highlighting the *in vivo* activity of tomatidine and a therapeutic potential.

Discussion

NPs as source of antifungal agents

The aim of our study was to identify promising antifungal natural compounds starting with a small scale screening of carefully selected compounds, proceeding with an extensive *in vitro* characterisation of their antifungal properties and cytotoxicity, and then extending to the identification of their cellular targets and the validation of their *in vivo* therapeutic potential.

A library of 199 natural products was built using both direct isolation from extracts of plants with documented antifungal properties (29 different plants were investigated allowing to the isolation of 53% of the investigated NPs) and commercial acquisition after selection based on structural similarities with known antifungals. The efficiency of the preselection process explains the high positive hit rate (17%; 33/199) of NPs with activity against human pathogens using a cut-off MIC value (≤32 µg/ml). From extended bioactivity profiling procedure including assays on different fungal strains, a bacterial strain and cytotoxicity assays for therapeutic index evaluation, only three interesting leads were identified. Tomatidine stood out as being a novel anti-*Candida* drug with a putative promising target.

Tomatidine is a sterol alkaloid from tomato plants with a cholesterol derived hydrophobic 27-carbon skeleton and serves as precursor intermediate in the synthesis of a plant defence metabolite, the glycol sterol alkaloid α-tomatine (34). The latter has been characterized as an antifungal agent against a large variety of phytopathogens and possesses membrane disruption properties (unspecific toxicity) caused by its ability to form complexes with cholesterol and ergosterol (17, 35). Alpha-tomatine is present in most green parts of tomato plants where it is subjected to degradation during maturation. In contrast, tomatidine, its aglycone, has a different mode of action as it does not show any sterol binding activity (35). It is poorly active on phytopathogens, it exhibits no toxicity *in vivo* and is present only in traces in the tomato plant (17, 18, 36). An antifungal and antiparasitic activity of tomatidine has been reported in *S. cerevisiae* and *Leishmania amazonensis* (18, 19) and a putative target, the 24-sterol methyl transferase (24-SMT-Erg6) was proposed. In the present study, the antifungal activity of tomatidine was in-depth characterized for the first time against important fungal pathogens. All *Candida* spp. except *C. glabrata* were susceptible to tomatidine (MIC = 0.25 to 10 µM, Table 2).

Tomatidine mode of action

Multi-level investigations were then conducted in order to determine its mode of action and to identify its molecular target. A first genomic strategy characterized tomatidine-specific transcriptional signature in *C. albicans* and identified an important upregulation of the ergosterol biosynthetic genes including *ERG6* as the most affected gene (Fig. 3). These results confirmed studies published in *S. cerevisiae* (18) and pointed out the ergosterol pathway as the target of tomatidine. Interestingly, the GSEA analysis identified fluconazole as mediating the closest-related transcriptional signature to tomatidine (Fig. 3), which is consistent with their inhibitory activity directed to the same pathway. Furthermore, this convergence of azole and tomatidine activity was also showed by microscopic analysis of the cytological effect of the drugs on the ultrastructure of *C. albicans* cells (supplementary Figure S7).

Considering the above findings, detailed sterol analysis using GC-MS was achieved in *C. albicans* and *C. krusei* resulting in the indirect identification of (i) Erg6 inhibition through a strong accumulation of the Erg6 substrate zymosterol in cells treated with high concentration of tomatidine and (ii) Erg4 inhibition through a strong accumulation of ergosta-5-7-24-(28)-tetraenol, its substrate, in cells treated with low concentration of tomatidine (Fig. 4). As Erg4 needs Erg6 activity to obtain its substrate, the identification of this dual Erg4-Erg6 inhibition was made possible by the likely higher affinity of tomatidine for Erg4 as compared to Erg6. Both enzymes are targeting the C-24 position in the ergosterol precursor and thereby probably exhibit similar binding pocket conformation even if their protein sequence identity is low (15 %). A dual effect on Erg4 and Erg6 had already been reported in *S. cerevisiae* for azasterol, a sterol carrying a nitrogen in the side chain (37) and thus reinforces the idea of structural similarities between the targets. Tomatidine dual inhibition was not detected in *S. cerevisiae*, where no ergosta-5-7-24-(28)-tetraenol was identified even at low concentration. This suggests that either Erg4 in *S. cerevisiae* is not inhibited by tomatidine or it has a lower affinity for tomatidine than does Erg6. These intra-species differences could be attributed to Erg4 independent genetic evolution as single point mutations in *ERG6* can affect sensitivity to tomatidine.

It has been shown that deleting the target of fluconazole (*ERG11)* in *C. albicans* caused a marked increase in its resistance to the drug (38). We applied the same paradigm to tomatidine and tested the susceptibility of an *ERG6* deletion strain. Late-acting ergosterol genes are not essential for cell viability and ergosterol is substituted by altered sterols in the membrane of deletion strains (39). However, these substitutions impact the regulation of membrane permeability and fluidity and are associated with diverse phenotypic alterations. Yeast *ERG6* deletion mutant showed pleiotropic hypersensitivity to a broad range of antifungal compounds and metabolic inhibitors reflecting an increased membrane permeability and passive diffusion to small molecules (hydrophobic mostly) (26, 39, 40). The fact that tomatidine susceptibility of *C. albicans ERG6* deletion strain was similar to the wild type strain was intriguing. It is known that *ERG6* deletion can alter cell membrane permeability to different drugs and this phenotype has been utilized by others in order to improve the effect of different drugs (41). Given that *ERG6* deletion results in a slight increased susceptibility to tomatidine as compared to wild type, it suggests that the compound could still target other cellular components. Consistent with this hypothesis, we identified *ACE2* as another putative target of tomatidine (see below). It is interesting to note that the *C. albicans ERG6* deletion strain was resistant to fluconazole (Fig. 4B), which indicates drug-dependent susceptibility phenotypes and thus excludes thereby an unspecific hypersensitivity response to a given stressor.

Our forward genetic approach in *S. cerevisiae* was aimed to identify alternative targets of tomatidine by avoiding the interference of efflux pump hyperactivity mechanisms. However, our screen revealed Erg6 as major target and several explanations can be proposed. First, our set of obtained resistant mutants was relatively small (5), thus limiting the discovery of additional targets. Second, an alternative target could be accessible or present only in the background of an *ERG6* deletion background, in which altered sterols are present with compromised cell membrane functions. The hypothesis of a pleiotropic effect of the *ERG6* deletion on the susceptibility to small hydrophobic molecules was tested using a sterol alkaloid (solasodine) which possesses highly similar chemical structure to tomatidine (only the planar configuration of the piperidine ring and an unsaturated alpha ring bond differs). While this molecule was inactive against a *C. albicans* wild type strain (MIC >40 µM), the deletion of *ERG6* increased drug susceptibility (MIC = 20 µM) and thus argued for an

unspecific drug hypersensitivity of this strain. Furthermore, *in vitro* filamentation studies with *C. albicans* (Fig. 2D) attested of the similar pseudohyphae and/or unseparated budding yeast cell phenotype between tomatidine-treated wild type cells and *ERG6* depleted cells arguing for tomatidine has the major target. The forward genetic approach identified *ACE2* as a possible tomatidine target. Interestingly, *ACE2* deletion in *C. albicans* results in upregulation of several *ERG* genes of involved in sterol biosynthesis (42) and thus we reasoned that *ERG6* could be upregulated in the resistant mutant R3.3, thus resulting in tomatidine resistance. *ERG6* expression was however not significantly altered in R3.3 as compared to parent and thus the basis of resistance by *ACE2* still remains unresolved.

In comparison to previous reports in *S. cerevisiae* that suggest Erg6 as a tomatidine target (18), our forward genetic approach evidenced a direct interaction between Erg6 and tomatidine, with a single substitution of well conserved amino acids (G132D or D249G) sufficient to confer resistance. As a loss of function in Erg6 results in an increased susceptibility to tomatidine, the two mutations did not affect drastically Erg6 function (ergosterol was detected and amphotericin B susceptibility remained unaffected). In the absence of X-ray-structure for sterol methyltransferase (Erg6), previous functional analysis had identified functionally important residues in three regions using bioinformatics analysis, mechanism-based inactivation and site-directed mutagenesis experiments (Fig. 5B) (29). The two residues (G132D and D249G) were not included in these analyses and their effect on the kinetic of C-methylation reaction remains to be determined. To address the mechanism of Erg6 inhibition by tomatidine, studies of a known Erg6 inhibitor, 25-azalanosterol, have suggested a non-competitive binding to a different site than the sterol binding site in the active centre which leads to conformational changes deleterious to the catalytic reaction (29, 43). The two identified residues could mediate direct interactions to tomatidine and non-conserved changes in these positions may result in a decreased affinity to the drug.

An important issue concerning the potential of tomatidine as an antifungal drug regard is its resilience to efflux pump-mediated mechanisms, which are commonly acquired by azole exposure. We showed here that tomatidine is the substrate of Cdr1 in *C. albicans*. By extrapolation, we can hypothesize that the absence of activity against *C. glabrata*, which is known to possess intrinsic resistance to antifungals, is related to its potent efflux machinery. Nevertheless, we showed using matched azole-sensitive and azole-resistant clinical isolates

that the common resistance mechanism against azole through *TAC1* hyperactivity had limited effect on tomatidine susceptibility. This reduced cross-resistance and the increased fluconazole susceptibility of tomatidine-resistant strain (via G132D) raised the question about the potential therapeutic advantage of the combined use of the two drugs. Our analysis revealed an additive effect of their combination in a wild-type strain of *C. albicans*. In a recent study (44), a screen for synergistic molecules in combination with fluconazole on a *C. albicans* azole-resistant strain (with *ERG11* and *TAC1* mutations) identified a synergistic interaction with tomatidine, thus confirming the high potential of tomatidine in drug combination therapies.

In vivo activity of tomatidine

Our initial choice to screen a library of NPs was guided by the intrinsic properties of these natural compounds as compared to synthetic products, namely their immense chemical diversity, target specificity and intrinsic cell permeability. The critical step in developing new antifungal agent is to validate the promising *in vitro* characteristics *in vivo* and, by choosing NPs, we were hoping to bypass this bottle-neck. *In vivo* studies have validated that the target of tomatidine (Erg6) is required for virulence in both mice and insect mini-host model of *C. albicans* systemic infection ((45) and data not shown). Tomatidine is a highly hydrophobic sterol-like molecule difficult to dissolve using common aqueous solvents. First attempts to demonstrate *in vivo* activity of tomatidine in an animal model of *C. albicans* systemic infection using (co)solvents (DMSO, ethanol) or cyclodextrin complexation (hydroxypropyl betacyclodextrin) or surfactant (polyoxyl 35 castor oil) were not successful. We next reasoned that other types of drug formulations could be more successful. Several studies have used nanoparticles preparations to increase drug efficacy *in vivo*. For example, Moazeni et al (46) have reverted *in vitro* azole resistance of *Candida* spp. with solid lipid nanoparticles prepared with fluconazole. Inspired by this work, we prepared a nanoparticle-based tomatidine formulation. This formulation, which did not affect the *in vitro* activity of tomatidine and did not modify its chemical structure, was injected IP and decreased significantly fungal burden in the kidney as compared to placebo. Further studies would be necessary to understand

tomatidine bioavailability and bio-distribution in mice, however our work clearly raised tomatidine as a novel potential therapeutic antifungal agent.

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Materials and Methods

Yeast strains culture and growth media

Yeast and bacterial strains used in the study are listed in Table S1. NPs were dissolved in DMSO to 10 µg/ml. Tomatidine (Tomatidine-HCL) was purchased from Phytolab (GmbH), dissolved in DMSO at 2 mM concentration, heated for 20 min at 50°C for solubilization and stored at -20°C upon usage.

Antimicrobial susceptibility testing

(i) Antifungal broth microdilution assays.

Antifungal susceptibility testing was carried out on the basis of EUCAST protocols with slight modifications (47). Briefly, yeast strains were cultivated overnight at 30°C under constant agitation in YEPD (1% yeast extract, 2% peptone, 2% glucose). Cultures were diluted to a density of 2 x 10^5 cells per ml in RPMI (R8755- SIGMA) or Yeast Nitrogen Base (YNB) (MPbio) buffered to adequate pH with HCL or NaOH and with a final concentration of 2% glucose. Compounds from the NP library were dissolved in DMSO to 10 mg/ml as final concentration. The final DMSO concentration was 1%. Two-fold serial dilutions were prepared from 32 to 1 µg/ml. Plates were incubated at 35°C for 24 h and then MICs were read with a spectrophotometer plate reader set at 450 nm. The minimal inhibitory concentration (MIC) was defined as the drug concentration at which the optical density was equal or decreased more than 50% from that of the drug-free culture. For *S. cerevisiae* MICs, the media (YNB) was complemented with Complete Supplement Mixture (CSM; Mpbio, Santa Ana, California, USA) according to the supplier instructions. Assays were performed in triplicates.

For the detection of combinatory effect of tomatidine with fluconazole, a checkerboard assay was set up in YNB pH 7 with 2-fold dilutions of fluconazole (0.008 to 0.5 µg/ml, 1 to 64 µg/ml) and tomatidine (0.08 to 40 µM, 0.02 to 10 µM). Combinations of the different drug concentrations enable to determine a Fractional Inhibitory Concentration Index (FIC index) as described in (48). Drug combinations that give rise to a growth reduction of more than 50% are then identified and the associate FIC index determined the properties of their

combination. A FIC index between 2 and 0.5 indicate an additive effect, while FIC < 0.5, a synergetic effect. Average values are shown from three replicates.

(ii) Antifungal serial dilution susceptibility assay.

Drug susceptibility testing was also performed on solid YEPD agar plates containing specific drug concentrations or vehicle (1% DMS0). Ten-fold serial dilutions of cells were spotted starting with cell concentration of 10^6 cells/ml. Assays were performed at least in duplicates.

(iii) Antifungal biofilm susceptibility assay.

Antifungal susceptibility tests on *C. albicans* biofilms were conducted according to a published protocol (49) with 48 h of biofilm formation and 48 h of antifungal treatment. Briefly, an aliquot of a 100 µl cell suspension (10^6 cells/ml) per well prepared in RPMI medium 0.2 % glucose (pH 7) was deposited in each well of a 96-well plate and incubated at 37°C for 48 h to allow biofilm formation. Wells were then washed twice with phosphate-buffered saline (PBS). Two-fold serial dilutions of the compounds were prepared from 50 to 1.56 µg/ml and added to the wells containing the biofilms. Plates were incubated again for 48 h at 37°C and then washed twice with PBS. A measurement of the metabolic activity of the sessile cells was performed using a colorimetric assay with 2H-tetrazolium,2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-hydroxide salt (XTT) (X4626, Sigma Aldrich). Plates were read with a spectrophotometer plate reader at 492 nm. The MIC was defined as the drug concentration at which the optical density value was equal or less than 50% of the one of the drug-free biofilm. Assays were performed in duplicates.

(iv) Antifungal drug time-kill assay.

Time-kill assay was performed as follows. Cells were cultured overnight in YEPD at 30°C, adjusted to 2 x 10^5 cells/ml in YEPD and submitted to corresponding concentration of the drugs, their combination or solvent. After 0-, 4-, 8- and 24 h incubation with the drug at 30°C under agitation in a 3 ml liquid volume, cell viability was determined by plating cells on YEPD

agar plate for 16 h at 34°C and counting of colonies (colony forming unit, CFU). Fungicidal effect was determined when at least a 2-fold log decrease of CFU/ml from the initial cell density was measured.

(v) Antibacterial susceptibility assay.

Antibacterial susceptibility testing was carried out on the basis of CLSI Approved Standard M7-A7 using microdilution method with CAMH (Cation-adjusted Müller-Hinton) broth. Briefly, overnight *Escherichia coli* (ATCC® 25922) cell cultures were adjusted to McFarland 0.5 (10^8 Cells/ml) with NaCl. Final cell concentration was 3 x 10^5 CFU/ml. Drug two-fold serial dilutions were prepared from 64 µg/ml to 2 µg/ml. Microplates were incubated at 37°C for 24 h and then MICs were read with a spectrophotometer plate reader at 450 nm. The MIC was defined as the drug concentration at which the optical density was equal or decreased more than 50% from that of the drug-free culture. Assays were performed in duplicates. Drugs were tested at the pH at which an antifungal activity was detected during the microdilution susceptibility screen (pH 7 if active at both pH values).

Cell cytotoxicity assay

Cytotoxicity assay were performed according to standard procedure with sulforhodamine B (SRB) as a reporter (50). HeLa cells (ATCC CCL-2, Manassas, Virginia, USA) were cultured in DMEM + 10% FBS, at 37°C with 5% CO2. Ninety six-well plates were filled with a seeding density of 10^4 cells/well. After 24 h of growth (day 1), cells were washed twice with PBS and 2-fold serial dilutions (starting at 100 µg/ml) of the compounds were added to the cells and incubated for 48 h. The starting amount of cells was monitored by fixing the cells at day 1. At day 3, cells were all washed twice with PBS and then fixed and labelled as described in the standard procedure. Optical density was measured at 492 nm and the percentage of killed cells could be determined using the following formula: $100-(OD_{day3}/OD_{day1}) \times 100$. LD50 (lethal dose 50) corresponded to the concentrations at which at least 50% of cells were killed. Assays were performed in duplicates. The selectivity index (SI) was then calculated by dividing the LD50 by the MIC against *C. albicans*.

625

626 **Hierarchical clustering of activity profiles**

627 MIC of the 40 active NPs against the 7 yeast strains was used to generate the heatmap and
628 pH of activity was indicated by a color scheme. NPs that showed activity at both pH were
629 labelled as active at neutral pH. Cluster analyses were performed by calculating the distance
630 matrix using the Euclidean method followed by Ward (Ward.D) hierarchal clustering using the
631 gplots package in R version 3.3.2.

632

633 **Calcofluor white staining**

634 Yeast cells were grown overnight and washed twice with PBS. Cells (10^5) were resuspended
635 in 200 µl RPMI 0.2% glucose in 96-well plate and incubated for 3 h at 37°C. Ten µl of calcofluor
636 white stain (Sigma) was added to the well. After 10 min at room temperature, 4 µl of cell
637 suspension was mixed with 2 µl of Mowiol (Sigma) and directly thereafter, fluorescence
638 microscopy was performed with a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany).

639

640 **Sterol content analysis**

641 (i) Total sterol extraction

642 About 2 x 10^5/ml cells were cultured for 16 h at 30°C under agitation in 15 ml YEPD
643 supplemented with the indicated tomatidine concentrations (with 1% DMSO final
644 concentration). Cells were treated with tri-chloro-acetic acid (TCA) to a final concentration of
645 5% to stop metabolism and the cells incubated for 10 minutes on ice. Harvested cells were
646 then washed twice, first in 5% TCA (distilled water) and then in distilled water to remove
647 traces of the YEPD medium. Cells were resuspended in 3 ml of distilled water and 10^9 cells
648 were used to perform total sterol extraction as described in (51) and to determine the
649 amounts of total cellular sterols (esterified and non-esterified). Cells were resuspended in 1
650 ml 60% KOH to which 1 ml of 0.5% pyrogallol-containing methanol and 1 ml of methanol were
651 added in a screw cap glass tube. Tubes were heated at 85°C for 2 h and returned to room
652 temperature. Sterols were extracted three times with 2 ml of petroleum ether (high boiling

point). The combined petroleum ether phases were dried under N_2 flow, resuspended in 1/1 methanol/chloroform (v/v) and sonicated for 5 min for further analysis by GC–MS.

(ii) GC-MS analysis

Sterols were analysed by gas liquid chromatography-mass spectrometry (GC- MS) as described in (51). *ERG4* and *ERG6* deletion mutant extracts were used to determine the positions of the known sterols.

Tomatidine formulation

The nanosuspension was obtained by adding 6 mg of tomatidine, 300 µL 2% (w/V) D-α-tocopherol polyethylene glycol 1000 succinate (TPGS, Sigma-Aldrich) as stabilizer and 700 µL of purified water as non-solvent in a 2 ml tube (tomatidine/TPGS (50/50)). A wet milling with 579 mg of zirconium bead (BeadBug TM, Sigma-Aldrich, prefilled tubes of 2.0 mL with 0.5 mm Zirconium beads, triple-pure, high impact) was performed for 70 hours on a vortex Genie 2. Then, the nanosuspensions were frozen by dipping the tubes in liquid nitrogen. Subsequently, the nanosuspensions were lyophilized for 48 hours using a Christ Alpha 2-4 LD Plus freeze-dryer. The particle size distribution was determined by dynamic light scattering (DLS) using a Zetasizer 3000HSa. The samples were dispersed in 9/10 volume of filtered purified water and stirred for 20 min with a vortex to ensure a uniform dispersion free of aggregates. 1/10 volume of PBS 10X was added prior injection.

Transmission electron microscopy (TEM)

The *C. albicans* strain CAF2-1 was grown in YNB liquid cultures for 2 h at 37°C (15 ml plastic tubes). Next, miconazole (1 mg/ml in DMSO) was added at a concentration of 10 µg/ml and cultures were grown during 18 h to evaluate the cytotoxic effect of this commercial product

on the yeast strain. The cytotoxic effect of tomatidine was evaluated according to the same experiment, except that this compound (1 mg/ml in DMSO) was added at a concentration of 20 µM and cultures were grown during 18 h. Cell preparation was performed as described in (12). Thin sections were observed with a transmission electron microscope (Philips CM10) with a Mega View II camera. Control cells were obtained in the same way without drug treatment.

Selection of NPs

A smart chemical library containing 199 natural products (NPs) with potential antifungal activity was constructed. Among these compounds, 53% were previously isolated from crude plant extracts that presented an antifungal activity in our laboratory. The bioguided isolation process of these compounds were performed by bioautography using wild and genetic modified strains of *C. albicans* (12). In parallel, NPs with i) structures closely related to NPs possessing antifungal activity from published sources and ii) with unknown antifungal activity, were selected and acquired in commercial catalogues (compounds are listed in Supplementary Table S1). The identity and the purity of the commercial compounds obtained were systematically performed by nuclear magnetic resonance (NMR) and high resolution mass spectrometry (HRMS) analysis.

Genome-wide transcriptional analysis

(i) RNA extraction and processing

Overnight YEPD culture of *C. albicans* SC5314 strain was diluted 1:200 in 5 ml YEPD media and incubated under agitation at 30° C until early exponential growth phase (OD_{540} = 0.3). Fifty µl of solvent (DMSO) or 250 µM tomatidine (diluted in DMSO) were added to the culture to reach a concentration 1% DMSO and 2.5 µM tomatidine. Total RNA was extracted after 1- or 3 h tomatidine/solvent exposure by mechanical disruption of the cells with glass beads as previously described (52). Experiments were carried out in triplicates with 12 samples. Total RNA extracts were treated with DNase using the DNA-free kit (Ambion-Life Technologies, Zug,

Switzerland) and RNA quality and integrity was verified with Fragment Analyzer™ Automated CE System (Advanced Analytical). One µg of RNA was used to create sequencing libraries through standard Illumina TruSeq stranded mRNA protocol. Each library (sample) received a different index enabling several libraries to be multiplexed. Before RNA sequencing, libraries were analyzed with a fragment analyzer to assess quality and fragment size and with a Qubit fluorometer (Invitrogen) to determine cDNA concentration. Libraries were kept at -20°C until sequencing.

(ii) RNA sequencing

The 12 libraries were run on Illumina HiSeq platform (HiSeq2500). Sequencing data were processed using Illumina Pipeline software. Reads were filtered, trimmed, and counts align to the SC5314 C*. albicans* reference genome using CLC workbench pipeline. The numbers of read counts per gene locus was extracted. All reads were deposited at GEO under accession number GSE96965.

(ii) RNA seq data analysis

Data normalization and gene expression analysis were performed in R (v3.2.3), using Bioconductor packages (as described in (23)). The read count data were normalized using TMM (trimmed mean of M-values) method available in the R package edgeR (53) and transformed into log2 counts per million by Voom method from R package Limma (54). This package was then used to apply a linear model with one factor per condition (4 conditions: untreated 1h, tomatidine-treated 1h, untreated 3h, tomatidine-treated 3h (all in triplicates)) to the transformed data. Two contrasts representing the difference between tomatidine-treated and untreated cells at each drug exposure time (1- and 3 h) were extracted from the linear model to result in a moderated t statistic for all genes expressed.

qPCR analysis

Total RNA (same RNA samples as those used for RNA-seq experiments: 12 samples representing 4 conditions in 3 biological replicates) were treated with DNase and 1 µg of treated-RNA was used as template for cDNA synthesis using the high-fidelity cDNA synthesis kit (Roche Diagnostics, Switzerland). Real-time quantitative PCR (qPCR) were performed to determine relative gene expression of using primers and TaqMan probes (FAM-TAMRA modified) with the iTaq Supermix with ROX (Bio-Rad AG, Switzerland) in a StepOnePlus real-time PCR system (Applied Biosystems-Life Technologies, Switzerland). Each reaction was run in duplicate. Primers and probes are listed in supplementary Table S3. Relative transcript quantities were assessed using the 2^(-ΔΔCT) method (55) to determine a normalized expression ratio with *ACT1* as reference gene.

C. albicans ERG6 deletion strain constructions

To delete the first allele of *ERG6*, two fragments of 571 bp and 569 bp of flanking 5'- and 3'-UTR regions, respectively, were PCR amplified on SC5314 DNA with the following primer pairs (ERG6_5For_KPN1 and ERG6_5Rev_Xho1; ERG6_3For_SacII and ERG6_3Rev_SacI). These primers contained restriction sites in order to insert the two amplicons sequentially in pSFS2A (56). The plasmid obtained (pSD1) was then digested with ScaI and transformed in *C. albicans* SC5314. Yeasts were transformed by a lithium-acetate procedure previously described (57).

Transformants were positively selected on YEPD plate containing 200 µg/ml nourseothricin (Nour) (Werner Bioagents, Germany). The Nour selective cassette was then removed by growing cells in YEPD media containing 2% maltose. Nour susceptible cells were used to deleted the second allele. The same strategy was repeated for the second allele but, in order to achieve it, a different 3'-end *ERG6* homologous region (at the end of the CDS and upstream of the first allele, 343 bp) was amplified with the primers ERG6_3CDSFor_SacII, ERG6_3CDSRev_SacI. The resulting construct was named pSD4.

Nour-resistant transformants were phenotypically screened using a simplified ergosterol extraction and detection method (58). An alteration of the expected UV spectrophotometric sterol profiles was detected in some transformants with an additional peak of absorbance at 230 nm indicating a perturbation in the ergosterol pathway and thus suggesting the deletion

of the second *ERG6* allele. GC-MS analysis confirmed the loss of *ERG6* function with zymosterol being most abundant sterol (data not shown).

S. cerevisiae forward genetic screen

(i) Deletion of *MSH2* in *S. cerevisiae* DSY4743

The *MSH2 PDR5* deletion strain was constructed from a *PDR5* deletion strain (DSY4743) using a PCR-based gene deletion approach as described previously [61]. Primers For_msh2_Sc and Rev_msh2_Sc were used to amplify the *HIS3* selection marker. The *HIS3*-containing amplicons was purified using the Nucleospin Gel and PCR Clean-Up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions and used to transform *S. cerevisiae* *pdr5*Δ (DSY4743) using the standard lithium acetate protocol.

(ii) Selection of resistant mutants

Overnight cultures of *msh*2Δ *prd5*Δ strain (P1) cells were plated on solid media containing several tomatidine concentration and incubated for two to seven days. One pop-out (R1) was identified and resistance to tomatidine was confirmed using MIC broth dilution method. The other tomatidine-resistant strains were identified as described previously by Ojini and Gammie (28) with few modifications. The *msh*2Δ *prd5*Δ strain (P1) was grown to saturation in 5 ml of YEPD medium at 30°C. Overnight cultures were diluted 1:200 in YEPD medium containing 50 µg/ml of ampicillin and grown in the presence of 10 µM of tomatidine in 96-well microtiter plates (Costar) in a shaking incubator at 30°C for 72 h. Cells were diluted 1:200 in the respective media and distributed into new 96-well plates and grown for 48 h at 30°C in absence of the drug. The cultures were then diluted 1:200 in media containing tomatidine in a new 96-well plate and optical density at 540 nm was recorded at six different time points (T0, T16, T20, T24, T38 and T42) over a 42 h period. Several wells were selected based on their growth profiles in presence of drug with high optical density after 40 h, and plated onto YEPD containing 7.5 µM of tomatidine and incubated for four days at 30°C. Resistant pop-outs were cultured overnight and resistance was retested with broth dilution method. Four

792 resistant strains arising from 2 different cultures (R2 and R3.1, R3.2, R3.3) were identified and
793 together with R1 submitted to whole genome sequencing.

794

795 (iii) Whole-genome sequencing

796 The five tomatidine-resistant cells and the parental strain were grown overnight in YEPD
797 medium at 30°C under constant agitation. Genomic DNA was extracted from yeast using the
798 Gentra Puregene Yeast/Bact. Kit (Qiagen, Hilden, Germany) with RNase treatment. DNA
799 concentration was verified by Qubit 2.0 Fluorometer (Thermo Fisher) and adjusted to 10
800 ng/ml for whole-genome sequencing. DNA quality was verified with Fragment Analyzer
801 (Advanced Analytical Technologies, Ankeny, IA, USA). Whole-genome sequencing was
802 performed at Fasteris SA (Plan-les-Ouates, Switzerland) using TrueSeq Nano DNA library
803 preparation and Illumina MiSeq. Paired-end read of 250 bp were performed, giving an
804 average of 118x coverage of each genome. The sequencing data was analysed using the CLC
805 Genomics Workbench (v.9.5.2) (Qiagen). The sequence reads were mapped to the reference
806 genome S288C. The average percentage of mapped reads was 93%. Mutations were
807 identified using the Variant Detector option by comparing P1 and tomatidine-resistant strains
808 to the reference genome. Functional consequences option was used to identify amino acid
809 changes. To identify mutations specific to the resistant strains, the variants of tomatidine-
810 resistant strains were compared to the P1 strain using the Compare Variants option. Only
811 non-synonymous mutations were taken into account with a frequency of ≥ 50% of reads for
812 insertions and deletions (INDELS) and of ≥ 90% of reads for single nucleotide variants (SNV).
813 Genome data are deposited at NCBI under BioProject PRJNA380059.

814

815 (iv) Construction of *S. cerevisiae ERG6* point mutated strains

816 To introduce specific point mutations in *ERG6* in *S. cerevisiae*, the clustered regularly
817 interspaced short palindromic repeat (CRISPR)-Cas9 genome editing system was used as
818 described previously (30). All primers are listed in Supplementary Table S3. A 20-nucleotide
819 guide sequence was selected using an online tool named CHOPCHOP
820 (http://chopchop.cbu.uib.no/). PAM 11 (position 251 849) and PAM 19 (position 252 596)

were chosen for the D249G (position 252 245) and G132D mutation (position 252 596), respectively. The two repair fragments were constructed as described previously (59). All fragments were purified using Nucleospin Gel and PCR Clean-up Kit (Macherey-Nagel).

Genome editing was performed by co-transformation of the guide, pMEL10 and repair fragments into S*. cerevisiae* IMX581 and selection was carried out in YNB agar lacking uracil. Verification of introduced mutations was performed by PCR amplification of *ERG6* (Erg6_verif_for and Erg6_verif_rev, see supplementary Table S3) and by sequence analysis as described above. The gRNA plasmid pMEL10 were removed by counter-selection pressure with 5-fluoroorotic acid (5-FOA, Toronto Research Chemicals, TRC) and effect of mutations on tomatidine susceptibility was verified by broth dilution method.

Mice experiments and ethics statement

All animal experiments were performed at the University Hospital Center of Lausanne with approval through the Institutional Animal Use Committee, Affaires Vétérinaires du Canton de Vaud, Switzerland (authorization n∘ 1734.3), according to decree 18 of the federal law on animal protection. Female BALB/c mice (8 weeks-old; Charles River France) were housed in ventilated cages with free access to food and water. SC5314 strain was grown overnight under agitation at 30°C in YEPD medium, subsequently diluted 100-fold in YEPD medium and grown again overnight under agitation at 30°C. Overnight cultures were washed twice with PBS and resuspended in 5 ml PBS. The concentration of each culture was measured through optical density, and each strain was diluted in PBS to the desired concentration (4 x 10^5 CFU/ml). Mice were injected through the lateral tail vein with 250 µl of a cell suspension containing 1.6 × 10^6 cells/ml. At 7-, 24- and 31 h post-infection, tomatidine formulation or placebo was administered through intraperitoneal (IP) injection in a volume of 200 µl. At 48 h post-infection, the kidneys were recovered, weighted and the CFU were determined as previously described (60). The ratio CFU/g of kidney was determined. Outliers analysis was first performed in GraphPad Prism using default parameters (ROUT, Q= 1%) and final number of individuals per group were 10 for placebo and 9 for tomatidine-treated mice. Statistical analyses of the differences between CFU values were performed using the Mann–Whitney test. The weight and temperature of the animals were monitored daily.

851

852 **Figures Legends**

853

854 **Figure 1:** Heatmap and cluster analysis of the activity profiles for 33 active NPs.

855 MIC values of the NPs against seven yeast species are ordered by hierarchical clustering using
856 Euclidean distance method and represented with a heatmap intensity color code (Heatmap
857 legend, MIC (µg/ml): dark blue = 0.125, light blue = 32, white >32 (inactive)). The pH at which
858 activity was detected is indicated by a color code in the first column (orange: pH 4.6, green:
859 pH 7). Fluconazole and caspofungin were added to the list of compounds and their clusters
860 highlighted in red and purple colors, respectively.

861

862 **Figure 2**: Tomatidine susceptibility assays on *Candida* spp.

863 (A) Structure of tomatidine.

864 (B) Spotting susceptibility assays of *Candida spp.* in YEPD agar plates. Ten-fold serial dilutions
865 of indicated strains were spotted onto agar plates containing increasing tomatidine
866 concentrations (DMSO, 1, 5, 10 µM) and were incubated for 1 day at 34°C. Azole-resistant
867 strains are indicated with "AR".

868 (C) Calcofluor white staining of *C. albicans* cells exposed to tomatidine (magnification: 100X).
869 Cells were exposed to 2.5 µM tomatidine for 3 h or untreated (DMSO 1%) in RPMI (0.2 %
870 glucose) at 37°C and then labelled with calcofluor white to stain chitin. Depicted cells are
871 representative of the vast majority of cells from three independent experiments.

872 (D) Time-kill assay for tomatidine treated-cells. 2 x10^5 *C. albicans* and *C. krusei* cells were
873 treated with different supra-MIC concentrations of tomatidine in YEPD and the CFU/ml were
874 determined after 4-, 8- and 24 h of exposure. Relative expressions to initial (T0) CFU/ml values
875 (100%) were calculated. Experiments were performed in duplicates with average and SEM
876 graphically represented. Y-axis is on log10 scale.

877

878 **Figure 3**: Genome-wide transcriptional analysis of tomatidine-treated cells.

(A) Venn diagram showing the number of genes differentially regulated by tomatidine (2.5 µM) as compared to control cells after 1- and 3 h exposure (FDR >0.05, fold-change ≥ 2).

(B) GO term analysis of the commonly up- and down- regulated genes upon 1h and 3h tomatidine treatment. GO term and their FDR value are indicated and the expression of corresponding genes represented with a heat-map for both drug exposure durations. The scaled expression of each gene, denoted as the row Z-score, is plotted in red-green colour scale with red indicating high expression and blue indicating low expression. A Z-score of 0 (black) correspond to the mean expression level of a particular gene and Z-score scale indicates the numbers of standard deviation (positive or negative). Genes within each GO term group are listed in an increasing adjusted p-value manner.

(C) qPCR analysis of *ERG11*, *ERG6* and *ERG4* expression in tomatidine-treated cells. Fold-change increase in expression compared to untreated cells is represented in a barplot. T-test analysis were performed between treated and untreated group for each gene relative expression and significant differences detected (all p-value < 0.05).

(D) Gene set enrichment analysis (GSEA) of *C. albicans* genes regulated by tomatidine. The list of drug-regulated genes was generated from published transcriptional data (see supplementary File S2, Candida_drug_treatment.gmt). Tomatidine regulated genes (3h) were ranked according to their fold-change. The list was then imported into the GSEA software. Analysis parameters were as follows: norm, meandiv; scoring_scheme, weighted; set_min, 15; nperm, 1000; set_max, 500. GSEA results were uploaded into Cytoscape 3.0 with the following parameters: p-value cut-off, 0.01; FDR q-value, 0.05. Red nodes represent enriched gene lists in upregulated genes from the GSEA. Blue nodes represent enriched gene lists in downregulated genes from the GSEA. Nodes are connected by edges when overlaps exist between nodes. The size of nodes reflects the total number of genes that are connected by edges to neighbouring nodes. The labels of the list (corresponding to list of genes up- and down-regulated by drug treatments, gene deletions or stress conditions) are indicated next to the nodes (details in supplementary File S2). XSA, XSB: oxidative shock, HU6H: hydroxyurea treatment.

Figure 4: Total sterol analysis in tomatidine-treated cells with GC-MS.

(A) Sterol composition of *C. albicans* cells treated with increasing amount of tomatidine. GC-MS analysis of cells exposed to tomatidine with the percentage of the different sterols are shown in a bar plot. Bar colors correspond to sterol molecules illustrated on the left panel where the last steps in the ergosterol biosynthetic pathway and an alternative pathway following *ERG6* inhibition are shown. Experiment was repeated (duplicates) and gave similar results.

(B) Susceptibility assay of the *C. albicans ERG6* deletion strain. Wild type (*ERG6*/*ERG6*), heterozygous (*ERG6*/*erg6*Δ) or homozygous *ERG6* (*erg6*Δ/*erg6*Δ) deletion strains were subjected to serial dilution susceptibility assay on YEPD plate containing indicated concentration of tomatidine or fluconazole (right panel) and to standard MIC determination assay in YNB pH 7 media (left panel).

Figure 5: Forward genetic approach in *S. cerevisiae.*

(A) Tomatidine susceptibility of resistant mutants. Fungal cells were spotted on YEPD agar containing different concentrations of tomatidine (and a drug-free control) as indicated. Plates were incubated at 30°C for 48 h.

(B) Alignment of the sterol methyltransferase protein sequences. Sequence alignment of sterol methyltransferase (SMT) from distant eukaryote species (Fungi: *S. cerevisiae*, *C. albicans*, *Pneumocystis carinii* (*P. carinii*), *Giberella zeae* (*G. zeae*); Green plants: *Glycine max* (*G. max*), *Arabidopsis thaliana* (*A. thaliana*); Euglenozoa: *Trypanosoma brucei* (*T. brucei*). Conserved residues are highlighted in grey. Black boxes indicated highly conserved region that have a role in substrate binding and enzymatic activity (29). The two mutated residues found in sterol C-24 methyltransferase (Erg6) of tomatidine-resistant strains (G132D and D249G) are represented by red boxes and are conserved across all aligned sequences. Strains carrying the *ERG6* mutation are indicated below the alignment. Protein sequences were retrieved from the following Genbank sources: *S. cerevisiae* KZV08836, *C. albicans* AOW28252, *P. carinii* KTW25893, *G. zeae* ESU10532, *G. max* NP_001238391, *A. thaliana* NP_173458, *T. brucei*

AAZ40214. A multiple alignment was performed using MUSCLE in the Geneious software (version 9.1.4, default parameters).

Figure 6: Tomatidine targets efflux pumps and induces only partial cross-resistance with fluconazole

(A) Serial dilution susceptibility assays of *C. albicans* strains carrying deletion in efflux pumps (genotype are indicated) on YEPD agar plates. Ten-fold serial dilutions of indicated strains were spotted onto agar plates containing increasing tomatidine concentrations (Ø, 0.5, 1, 5 µM) and were incubated for 1 day at 34°C.

(B) Tomatidine MICs of two matched pairs of azole-susceptible (AS) and azole-resistant (AR) strains. MICs were performed in YNB media for both tomatidine and fluconazole and are indicated by the arrows. The "Δ" symbol indicates the MIC fold-change differences between AS and AR matched strains.

(C) Two heatmaps representing color plots of checkerboard MIC tests. Each box corresponds to the relative growth (compared to drug-free control) resulting from a specific combination of tomatidine and fluconazole (RPMI, pH 7). Individual MICs of each drug are underlined by black lines. Black zones correspond to additive interactions (FIC index between 2 and 0.5). Yellow zones indicate a synergetic effect (FIC < 0.5).

(D) Time-kill assay of *C. ablicans* and *C. krusei* cells exposed to a combination of tomatidine and fluconazole. Cells were treated with indicated amount of drugs, individually or in combination, in YEPD and CFU/ml determined after 4-, 8- and 24 h of exposure and relative expression to initial (T0) CFU values (100%) calculated. Experiments were performed in duplicates with average and SEM graphically represented. Y-axis is on log10 scale. A dashed line was plotted on *C. krusei* graph at Y = 1 to delimit the fungicidal threshold (2-times log10 decrease).

Figure 7: *In vivo* efficacy of tomatidine in a mice model of systemic infection

(A) Schematic representation of the infection and treatment protocol. Mice were infected with 4 x 10^5 CFU through the tail vein. Tomatidine treatment (50 mg/kg) (or placebo) was administered intraperitoneally (IP) at 7-, 24- and 31 h post-infection (pi). At 48 h pi, animals were sacrificed and kidneys collected for CFU determination.

(B) Fungal burden in tomatidine-treated mice. CFU per g of kidney are plotted for placebo and tomatidine-treated mice. Significant differences in CFU distribution were assessed using Mann Whitney test (n=9,10; p-value = 0.031).

973 **Table 1:** List of the 33 NPs with their biological activity profiles

Name	N°	Source	CAS number	*C. albicans* MIC (µg/ml)		*C. albicans* biofilm MIC (µg/ml)	*E. coli* MIC (µg/ml)	Cytotoxicity assay LD_{50}	Selectivity Index LD_{50}/MIC *C. albicans*
				pH 7	pH 4.6				
morindone	1	*Morinda tomentosa*	478-29-5	32[a)]	>32	6.25	>64	ND [c)]	ND
lucidine ω-methyl ether	2	*Morinda tomentosa*	NA[b)]	>32	32	NA	>64	ND	ND
morindoquinone	3	*Morinda tomentosa*	New NP	>32	16	25	>64	>100	6.25
avocadene	4	*Persea americana*	24607-08-7	16	8	25	>64	50	6.25
plumbagin	5	Sigma	481-42-5	2	4	50	>64	100	50
alpha-hederin	6	*Schefflera systila*	27013-91-8	16	4	50	>64	100	6.25
Glc-3 medicagenic acid	7	*Dolichos kilimandscharicus*	49792-23-6	>32	2	>50	>64	>100	≥100
2-Propen-1-one, 1-(2,4-dihydroxy-6-methoxy-3,5-dimethylphenyl)-3-phenyl	8	*Myrica serrata*	65349-31-7	>32	8	25	>64	25	3.125
O-methyllawsone	9	*Swertia calycina*	2348-82-5	16	8	12.5	>64	25	1.5625
dihydrochelerythrine	10	*Fagara zanthoxyloides*	6880-91-7	8	>32	25	>64	25	3.125

simplexene D	11	*Swartzia simplex*	New NP	>32	16	25	>64	>100	≥12.5
waltherione G	12	*Waltheria indica*	1632043-42-5	>32	32	25	>64	ND	ND
waltherione F	13	*Waltheria indica*	1632043-41-4	>32	8	12.5	>64	50	6.25
8-deoxoantidesmone	14	*Waltheria indica*	NA	>32	16	25	>64	>100	12.5
waltherione E	15	*Waltheria indica*	954367-81-8	>32	4	12.5	>64	>100	≥50
pterostilbene	16	Sigma	537-42-8	32	32	50	>64	ND	ND
(5*S*,10*S*)-11,15(*S*)-dihydroxy-12-methoxyswartziarboreol G	17	*Swartzia simplex*	1830306-56-3	32	16	50	>64	50	3.125
pulsatilla saponin D	18	*Odondatenia puncticulosa*	68027-15-6	>32	16	>50	>64	100	6.25
3β-O-[β-D-xylopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-[β-D- glucopyranosyl-(1→4)]-α-L-arabinopyranosyl] hederagenin	19	*Odondatenia puncticulosa*	NA	>32	8	>50	>64	50	6.25
garcinone C	20	Phytolab	76996-27-5	8	32	>50	>64	50	6.25

pennogenin tetraglycoside	21	Phytolab	68124-04-9	4	8	>50	>64	6.25	1.5625
tomatidine hydrochloride	22	Phytolab	6192-62-7	< 1	16	>50	>64	>100	≥ 200
formosanin C	23	Phytolab	50773-42-7	1	4	25	>64	12.5	12.5
medicagenic acid	24	Phytolab	599-07-5	>32	2	>50	>64	>100	≥100
pyridoxatin	25	Sigma	135529-30-5	4	32	25	16	100	25
Isograndifoliol	26	*Perovskia atriplicifolia*	1445475-53-5	16	32	>50	>64	100	25
taxodion	27	*Salvia leriifolia*	19026-31-4	16	8	12.5	>64	50	3.125
waltherione N	28	*Waltheria indica*	New NP	>32	32	12.5	>64	ND	ND
5(*R*)-vanessine	29	*Waltheria indica*	New NP	>32	32	12.5	>64	ND	ND
waltherione Q	30	*Waltheria indica*	New NP	>32	32	25	>64	ND	ND
antidesmone	31	*Waltheria indica*	222629-77-8	>32	32	12.5	>64	ND	ND
waltherione I	32	*Waltheria indica*	1632043-44-7	>32	32	12.5	>64	ND	ND
waltherione J	33	*Waltheria indica*	1632043-46-9	>32	16	12.5	>64	50	3.125

[a)]: numbers in bold face indicate that the value met threshold requirements.

[b)] : NA: not available

[c)] : ND: not determined

978 **Table 2:** Tomatidine and fluconazole MICs for *Candida* spp.

Strain	RPMI pH 7		YNB pH 7		YPED pH 6.5	
	Tomatidine (µM)	Fluconazole (µg/ml)	Tomatidine (µM)	Fluconazole (µg/ml)	Tomatidine (µM)	Fluconazole (µg/ml)
C. albicans (SC5314)	0.625	0.125	0.625	0.5	1.25-2.5	0.5
C. krusei (DSY471) (AR)[a)]	0.625	32	0.625	32	0.3125	>128
C. tropicalis (DSY472)	1.25	0.25 - 0.5	5	>128	2.5	32
C. parapsilosis (DSY473)	10	2	2.5	8	5	16
C. glabrata (DSY562)	>40	2	>40	32	>40	64
C. glabrata (DSY562) (AR)	>40	128	>40	>128	>40	>128

979 [a)]: (AR): azole-resistant strains

980

Table 3: MICs of *S. cerevisiae* strains to different antifungals

Strain	MIC			
	Tomatidine (µM)	Fluconazole (µg/ml)	Caspofungin (µg/ml)	Amphotericin B (µg/ml)
P1	1.25	4	0.25	2
R2	1.25	4	0.25	2
R3.1	5	4	0.25	2
R3.2	5	4	0.25	2
R3.3	2.5	4	0.25	2
R1	5	4	0.25	2
IMX581	5	16	0.125	2
D249G	20	16	0.125	2
G132D	20	4	0.125	2

Table 4: Susceptibility of *C. albicans* clinical isolates to tomatidine and fluconazole

Strain	MIC (YNB pH 7)	
	Tomatidine (µM)	Fluconazole (µg/ml)
DSY731 (AS)[a)]	0.625	1
DSY732 (AR[a)]	2.5	64
DSY1841 (AS)	2.5	8
DSY1843 (AR)	5	32

[a)] (AR): azole-resistant strains, (AS): azole-susceptible strains

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