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Antifungal Activity of Fused Mannich Ketones Triggers an Oxidative Stress Response and Is Cap1-Dependent in Candida albicans

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Abstract

We investigated the antifungal activity of fused Mannich ketone (FMK) congeners and two of their aminoalcohol derivatives. In particular, FMKs with five-membered saturated rings were shown to have minimum inhibitory concentration (MIC50) ranging from 0.8 to 6 μg/mL toward C. albicans and the closely related C. parapsilosis and C. krusei while having reduced efficacy toward C. glabrata and almost no efficacy against Aspergillus sp. Transcript profiling of C. albicans cells exposed for 30 or 60 min to 2-(morpholinomethyl)-1-indanone, a representative FMK with a five-membered saturated ring, revealed a transcriptional response typical of oxidative stress and similar to that of a C. albicans Cap1 transcriptional activator. Consistently, C. albicans lacking the CAP1 gene was hypersensitive to this FMK, while C. albicans strains overexpressing CAP1 had decreased sensitivity to 2-(morpholinomethyl)-1-indanone. Quantitative structure–activity relationship studies revealed a correlation of antifungal potency and the energy of the lowest unoccupied molecular orbital of FMKs and unsaturated Mannich ketones thereby implicating redox cycling-mediated oxidative stress as a mechanism of action. This conclusion was further supported by the loss of antifungal activity upon conversion of representative FMKs to aminoalcohols that were unable to participate in redox cycles.

Introduction

The number of invasive fungal infections has dramatically increased over the recent years causing morbidity and mortality of immunocompromised patients [1]. In particular, patients after transplantation, under corticosteroid therapy, burn patients, drug abuser and neonates are in a high risk group for systemic fungal infection. Current treatment of invasive fungal infections relies mainly on three families of antifungal compounds: azoles that target membrane ergosterol biosynthesis, echinocandins that target membrane ergosterol biosynthesis, echinocandins that target cell wall beta-1,3-glucan biosynthesis and polyenes that target membrane ergosterol biosynthesis, echinocandins that target membrane ergosterol biosynthesis, echinocandins that target cell wall beta-1,3-glucan biosynthesis and polyenes that target membrane ergosterol biosynthesis [2]. Nevertheless, high mortality rates for invasive candidiasis (30–50%) and invasive aspergillosis (50–90%) are observed despite this antifungal arsenal [1]. Therefore, the discovery and development of new, efficient antifungal agents are important endeavors [2].

Mannich ketones (MKs) are versatile starting materials for synthetic organic and medicinal chemistry [3]. Their decomposition in an elimination reaction yields very reactive vinyl ketones as possible intermediates for ring closure in the synthesis of heterocycles. MKs can also be used as prodrugs [4,5]. Furthermore, unsaturated MKs are efficient alkylating agents for thiol enzymes [6,7] and their water solubility, especially in the case of the aminoketones, makes them good model compounds for antimicrobial investigations.

Previously, we have reported the synthesis and structure verification of two families of MKs: a set of 23 unsaturated MKs [8] and another one represented by 21 fused MKs (FMKs) [9]. Their reduction yielded 11 compounds as the corresponding aminoalcohols [10]. Interestingly, compounds of these three families showed antibacterial activity [8–10]. This activity could be correlated, to some extent, with the ability of the unsaturated and fused MKs to deplete thiols. However, several compounds with efficient antibacterial activity did not cause thiol depletion, suggesting that there were other chemical determinants of the antibacterial efficacy of MKs beside thiol depletion [11,12]. The antifungal activity of MKs was also investigated. In particular,
sixteen compounds among the unsaturated MKs and three compounds from the family of aminoalcohols showed antifungal activity towards reference Candida strains and some clinical isolates [13]. However, the antifungal mode-of-action of these compounds has remained unknown.

Here we report the characterization of the antifungal activity of a family of FMKs (Fig. 1) and their two aminoalcohol congeners (Fig. 2), along with a preliminary investigation of their mode of action in Candida albicans using transcript profiling, analysis of C. albicans mutants and quantitative structure–activity relationship (QSAR) analysis. Our combined approach strongly suggests that FMKs trigger oxidative damage in C. albicans, which contributes to their antifungal activity.

**Results**

Fused Mannich Ketones (FMK) show Antifungal Activity Towards several pathogenic yeasts

In a previous study [9], we have reported the synthesis of 21 FMKs (Fig. 1) and their aminoalcohol congeners (Fig. 2). Here, we focus on their antifungal activity against several fungal pathogens of humans; namely Candida albicans, Candida krusei and Candida parapsilosis. These fungi belong to the CTG clade of hemiascomycetous yeasts, Candida glabrata (a hemiascomycetous yeast closely related to Saccharomyces cerevisiae) and the filamentous ascomycete Aspergillus fumigatus. We also evaluated the antifungal activity of these compounds towards a Saccharomyces sp. clinical isolate.

MIC$_{90}$s for each of the FMKs are given in Table 1. Noticeably, most compounds, except compound 2 (2-(morpholinomethyl)-1-indanone), had MIC$_{90}$s above 50 mg/mL, when assayed towards Aspergillus sp. Moreover, compound 5, 7 and the alkoxy-substituted six-membered compounds 9–18 and the two amino alcohols 22–23 showed weak potency with MIC$_{90}$s from 6.25 mg/mL to 200 mg/mL (Table 1) across all species tested. In contrast, compounds 1–4, 6, 8 and 19–21 displayed appreciable antifungal activity against the different yeast species (MIC$_{90}$ ranging from 0.8 to 12.5 µg/mL). However, MIC$_{90}$s were significantly higher for C. glabrata than for other yeast species which was in agreement with previous observations showing decreased sensitivity of C. glabrata to antifungals such as azoles and the antifungal peptide ApoEdpL-W [1,14,15,16,17]. Compound 6 and compounds with seven-membered saturated rings (19 and 20) showed reduced activity towards C. parapsilosis. Overall, MKs with a five-membered saturated ring (compounds 1–4) exerted the most potent antifungal effect against all yeast strains tested, with compound 2 being the only one exerting activity toward Aspergillus sp. Taken together, these results indicated that a subset of the tested FMKs have antifungal activity against pathogenic yeasts, with the best compounds being FMKs 1–4 that contain a five-membered saturated ring.

We have previously shown that, according to the Hodge and Sterner Scale [18], compounds 1, 3 and 4 have acceptable toxicity to HeLa cells (IC$_{50}$ around 15, 12 and 14 µg/mL, respectively), while compound 2 showed a higher toxicity (IC$_{50}$ = 2.5 µg/mL) [9]. As compound 2 has a broader spectrum of antifungal activity (i.e., it is the only one among the tested FMKs with activity against Aspergillus sp.), it was selected for further in vivo investigation. Compound 2 was given to BALB/c mice intraperitoneally and the LD$_{50}$ value was calculated from the dose–effect curve using the Lichfield–Wilcoxon graphic method [19,20]. An LD$_{50}$ of 450 mg i.p./body weight kg (1.68 mM i.p./body weight kg) was obtained, indicating moderate toxicity according to the Hodge and Sterner Scale [18]. Practically this value is at the borderline between moderate and weak toxicity.

Quantitative Structure–activity Relationships

We performed a QSAR study to correlate the potency to inhibit the growth of the various yeast strains (C. albicans, C. glabrata, C. Figure 1. Chemical structures of the fused Mannich ketones investigated.

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Krusei, C. parapsilosis and Saccharomyces sp.) with the molecular properties of FMKs (Table 1), unsaturated cyclic MKs [13] and aminoalcohols [13]. The best three- and two-parameter equations that were obtained from this analysis are listed in Table 2. As shown by the representative example in Fig. 3, data for the unsaturated cyclic MKs and the FMKs could be merged to give common QSARs. The universal descriptor found to correlate with pMIC was the energy of the lowest unoccupied molecular orbital (LUMO, eV). In addition, a shape index (first-order basic k-type, SI<sub>k1</sub>) was represented for all Candida strains. Solvent accessible surface area (SASE, Å<sup>2</sup>) and dielectric energy (E<sub>De</sub>, kcal/mol) were also common descriptors among the best QSARs considering two and three descriptors. Ionization potential (IP, eV), steric energy (E<sub>St</sub>, kcal/mol), the energy of the highest occupied molecular orbital (HOMO, eV), the valence connectivity index (zero-order, standard VC<sub>0</sub>) and molar refractivity (MR) were represented in single equations. The additional descriptors (SI<sub>k1</sub>, SASE, IP, E<sub>St</sub>, HOMO, VC<sub>0</sub> and MR) may indicate the role of specific mechanisms (e.g., binding to protein targets yet to be identified) not directly related to or complementing the antifungal activity of unsaturated MKs and FMKs.

**Gene Expression Profiling of Candida albicans SC5314 Exposed to Fused Mannich Ketones**

To investigate the antifungal mode of action of FMKs, we performed transcriptional profiling of C. albicans SC5314 cells exposed to compound 2—the only compound having broad antifungal activity in our set of FMKs. Compound 2 showed an MIC<sub>90</sub> of 6.25 µg/mL towards C. albicans strain SC5314 grown in SD medium, similar to the MIC<sub>90</sub> defined for strain ATCC90028 (Table 3). Hence, exponentially growing C. albicans SC5314 cells were exposed to compound 2 at 6.25 µg/mL for 30–60 min and

**Table 1. MIC90s of fused Mannich ketones and aminoalcohols towards Candida, Saccharomyces and Aspergillus species.**

| Comp. | n | R | Config. |
|-------|---|---|---------|
| 22    | 1 | 4-Morpholiny | trans |
| 23    | 2 | 1-Pyrrolidinyl | trans |

![Figure 2. Chemical structure of the aminoalcohols investigated.](http://example.com/figure2.png)

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Table 1. MIC<sub>90</sub>s of fused Mannich ketones and aminoalcohols towards Candida, Saccharomyces and Aspergillus species.

| Comp. | MIC<sub>90</sub> (µg/mL) |
|-------|------------------|
|       | Candida albicans | Candida glabrata | Candida krusei | Candida parapsilosis | Saccharomyces sp. | Aspergillus sp. |
| 1     | 3.125            | 6.25            | 3.125          | 1.56              | 0.8              | 100            |
| 2     | 6.25             | 6.25            | 3.125          | 3.125             | 1.56             | 6.25           |
| 3     | 0.8              | 6.25            | 3.125          | 3.125             | 1.56             | 50             |
| 4     | 12.5             | 100             | 12.5           | 12.5              | 12.5             | 12.5           |
| 5     | 6.25             | 25              | 12.5           | 12.5              | 12.5             | 100            |
| 6     | 12.5             | 50              | 25             | 12.5              | 25               | 100            |
| 7     | 6.25             | 12.5            | 3.125          | 6.25              | 6.25             | 50             |
| 8     | 12.5             | 200             | 25             | 25                | 100              | 50             |
| 9     | 50               | 200             | 100            | 100               | 50               | 200            |
| 10    | 25               | 100             | 50             | 50                | 25               | 100            |
| 11    | 100              | 200             | 200            | 200               | 200              | >200           |
| 12    | 25               | 200             | 25             | 25                | 12.5             | 200            |
| 13    | 25               | 100             | 25             | 25                | 25               | 50             |
| 14    | 25               | 50              | 25             | 25                | 25               | 50             |
| 15    | 25               | 50              | 25             | 25                | 6.25             | 25             |
| 16    | 25               | 50              | 50             | 50                | 25               | 100            |
| 17    | 6.25             | 50              | 12.5           | 25                | 50               | 100            |
| 18    | 12.5             | 50              | 50             | 50                | 25               | 200            |
| 19    | 6.25             | 12.5            | 6.25           | 25                | 6.25             | 50             |
| 20    | 12.5             | 12.5            | 1.56           | 25                | 3.125            | 100            |
| 21    | 1.56             | 6.25            | 0.8            | 0.8               | 1.56             | 50             |
| 22    | >200             | >200            | >200           | >200              | >200             | >200           |
| 23    | >200             | >200            | >200           | >200              | >200             | >200           |
| A<sup>a</sup> | 0.4       | 0.4            | 0.4            | 0.4               | 0.8              | 3.125           |

<sup>a</sup>Amphotericin B was used as a standard.
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the levels of expression of 6852 putative ORFs in the treated and untreated cells were compared using microarrays (see Materials and Methods for details). Following 30 min of exposure, we identified 138 genes up-regulated in response to compound 2 while 40 genes were down-regulated (Supplemental Table S1). After 60 min, 111 genes were identified as up-regulated and 84 as down-regulated (Supplemental Table S1). Overall, 56 up-regulated genes and 8 down-regulated genes were shared by the 30- and 60-min transcript profiles.

In order to get further insights on the transcriptional responses of cells exposed to compound 2, up-regulated and down-regulated genes were queried according to the gene ontology of cells exposed to compound 2. 60-min transcript profiles. 111 genes were identified as up-regulated and 84 as down-regulated (Supplemental Table S1). Overall, 56 up-regulated genes and 8 down-regulated genes were shared by the 30- and 60-min transcript profiles. In order to get further insights on the transcriptional responses of cells exposed to compound 2, up-regulated and down-regulated genes were queried according to the C. albicans gene ontology [21,22]. Noticeably, a significant enrichment for genes associated with oxidative stress was observed among the genes up-regulated at 30 min (P-value = 3.23 × 10⁻⁴) or 60 min (P-value = 7.59 × 10⁻⁵). Furthermore, the set of genes down-regulated at 30 min was enriched for genes involved in fatty acid biosynthesis (P-value = 1.2 × 10⁻³), whereas the set of genes down-regulated at 60 min was enriched for genes involved in monocarboxylic acid metabolism (P-value = 3.97 × 10⁻⁶), including genes involved in the glyoxylate cycle and fatty acid metabolism. This suggested an oxidative stress signal and a down regulation of fatty acid metabolic pathways in response to compound 2.

Additionally, we noted that a large number of the genes up-regulated at both time-points had been previously annotated as “Cap1 regulated” (Table 4; [23]). Cap1 is a transcription factor central to C. albicans oxidative stress response [24]. Wang et al. [25] have identified C. albicans genes that respond to oxidative stress (H₂O₂) and defined those regulated in a Cap1-dependent manner. Genes induced by compound 2 overlapped with the H₂O₂-induced genes defined by Wang et al. (18 out of 56 genes; [25]), and the majority of the overlapping genes were Cap1 regulated (Table 4). Strikingly, 35 of the 56 genes induced by compound 2 have been characterized as being bound by Cap1 in their promoter region (Table 4; [26]). Moreover, 50 of the 193 genes up-regulated in response to compound 2 at any one of the two time points were part of the 89 Cap1 targets identified by Znaidi et al. [26]. These results suggested that compound 2 triggered oxidative damage and activation of the Cap1 regulon in C. albicans.

Another interesting feature of the response to compound 2 was the induction of several multidrug transporter genes; namely MDR1, SNQ2 and CDR1. MDR1 showed particularly strong up-regulation (up to 43-fold at 60 min) and is known to be induced in response to oxidative stress [27], as well as by a hyperactive CAP1 allele [24] consistent, again, with compound 2 triggering oxidative stress in C. albicans. However, it cannot be excluded that up-regulation of these multidrug transporter genes in response to compound 2 reflects a general drug response.

A Candida albicans cap1 Mutant Defective for Oxidative Stress Responses shows Hypersensitivity to Fused Mannich Ketones

Inactivation of the CAP1 gene in C. albicans results in hypersensitivity to oxidative stress [24]. Hence, we compared the sensitivity of a wild-type strain and a cap1Δ/Δ mutant to compound 2. The MIC₉₀ of the cap1Δ/Δ strain was 4-fold lower than that of the wild-type strain (data not shown). Growth of the cap1Δ/Δ strain in microtitre plate was completely abolished at

![Figure 3. QSAR analysis of Mannich ketones for antifungal activity towards Candida albicans.](https://plosone.org/figure/10.1371/journal.pone.0062142.g003)
6.25 μg/mL of compound 2 while the wild type strain showed a delayed but efficient growth compared to untreated cells (Fig. 4A). Moreover, the cap1Δ/Δ strain was more sensitive to lower concentrations of compound 2, as indicated by a strong growth delay (Fig. 4A). In contrast, over-expression of CAP1 resulted in an increase of the growth rate, compared to control and cap1Δ/Δ strains, when exposed to compound 2 under inducible conditions (Fig. 4B). Taken together, these results indicated that a functional Cap1 regulon contributed to C. albicans survival upon exposure to FMKs.

Candida albicans Mutants Defective for Non-oxidative Stress Responses do not show Hypersensitivity to Fused Mannich Ketones

In order to assess whether compound 2 triggered additional stress responses, we tested the sensitivity to this FMK of several C. albicans strains with null mutations in genes encoding signaling components involved in the response to a variety of stresses: HOG1 encoding a MAP kinase involved in the response to osmotic stress and, to some extent, oxidative stress [28,29]; MLN1 encoding a transcription factor involved in the response to weak acid stress [30]; and CTA4 encoding a transcription factor involved in the response to nitrosative stress [31]. Results presented in Fig. 4C showed that, while growth of the cap1Δ/Δ strain is inhibited at 6.25μg/mL compound 2, this was not the case for the hog1Δ/Δ, mnl1Δ/Δ and cta4Δ/Δ mutants. Similar results were obtained when the mutant strains were exposed to H2O2–with the mnl1Δ/Δ and cta4Δ/Δ/Δ mutants behaving like the wild-type strain, the cap1Δ/Δ strain showing high sensitivity to H2O2 and the hog1Δ/Δ strain showing moderate sensitivity to H2O2 (data not shown). In contrast, only the hog1Δ/Δ mutant showed sensitivity to osmotic stress (data not shown). Again, these results indicated that compound 2 elicited a specific oxidative stress in C. albicans and that survival of C. albicans after exposure to compound 2 was specifically dependent upon a functional Cap1 regulon.

Discussion

We have shown that a subset of fused Mannich ketones have antifungal activity against pathogenic yeasts. In particular, FMKs with a five-membered saturated ring (compounds 1–4) showed the best potency toward C. albicans and the closely related C. parapsilosis and C. krusei while having reduced potency toward C. glabrata. Among them, only compound 2 was effective against Aspergillus sp. Interestingly, these four FMKs (1–4) showed moderate toxicity to mammalian cells, and our results with compound 2 indicated that it also had moderate toxicity in mice. In this study, we have additionally investigated the effect of one of these FMKs, compound 2, on C. albicans by transcript profiling and shown that it elicited a strong oxidative stress-like transcriptional response. Notably, many of the genes whose transcription was increased upon compound 2 treatment were regulated by the CAP1 gene increased the sensitivity of C. albicans cells to compound 2. Taken together, these data have suggested that generation of oxidative stress is an important component of the antifungal activity of compound 2 and, more generally, FMKs.

Whether FMKs directly or indirectly generate an intracellular oxidative stress has not been determined. Dimmock et al. [32] have proposed that Mannich bases of unsaturated ketones can be considered alkylating agents reacting with low molecular-weight and protein-associated thiols through Michael addition. In particular, they have investigated compound NC1173, a conjugated styryl ketone exhibiting a wide-spectrum activity against pathogenic fungi, and suggested that its activity is probably based on the inhibition of H+-ATPase-mediated proton pumping [33]. Alternatively, it has been proposed that Mannich ketone-induced thiol depletion could cause oxidative stress leading to oxidative damage of the cell and production of reactive oxygen species [34]. The Mannich ketones that have been investigated in the present study can be classified as fused cyclic Mannich ketones. While the unsaturated Mannich ketones contain two alkylation sites for the
Table 4. *C. albicans* genes showing increased expression upon 30 min and 60 min exposure to compound 2.

| Systematic name | Gene name | Ratio 30 min | P-value 30 min | Ratio 60 min | P-value 60 min | Benomyl$^1$ | Cap1-dependent$^2$ | H$_2$O$_2$ | Benomyl$^4$ | Cap1-dependent$^5$ | Cap1-regulated$^6$ | Short description |
|-----------------|-----------|--------------|----------------|--------------|----------------|-------------|------------------|-------------|-------------|-----------------|------------------|------------------|
| orf19.113       | CP1       | 215.4        | 1.07E-03       | 95.99        | 6.54E-06       |             |                  |             |             |                 |                  | Possible oxidoreductase |
| orf19.3131      | OYE32     | 61.7         | 9.09E-04       | 30.13        | 2.87E-06       |             |                  |             |             |                 |                  | NAD(P)H oxidoreductase family protein |
| orf19.5604      | MDR1      | 24.98        | 2.01E-03       | 42.8         | 1.16E-06       |             |                  |             |             |                 |                  | Plasma membrane multidrug efflux pump |
| orf19.3139      | 23.76     | 1.36E-04     | 3.842          | 5.47E-08     |             |             |                  |             |             |                 |                  | Hap43p-repressed gene |
| orf19.2285      | 17.63     | 4.76E-03     | 14.65          | 3.35E-05     |             |             |                  |             |             |                 |                  | Increased transcription is observed upon benomyl treatment |
| orf19.2351      | 16.43     | 4.27E-08     | 2.994          | 9.48E-06     |             |             |                  |             |             |                 |                  | Thi/Ppl protein |
| orf19.5285      | PST3      | 15.56        | 9.92E-03       | 6.958        | 1.14E-07      |             |                  |             |             |                 |                  | Putative flavodoxin |
| orf19.3443      | OYE2      | 11.7         | 8.98E-04       | 6.748        | 4.21E-07      |             |                  |             |             |                 |                  | Putative NAPDH dehydrogenase |
| orf19.1149      | MRF1      | 11.42        | 4.92E-03       | 6.223        | 1.56E-07      |             |                  |             |             |                 |                  | Putative mitochondrial respiratory protein |
| orf19.2262      | 11.28     | 2.38E-03     | 6.234          | 5.68E-10     |             |             |                  |             |             |                 |                  | Protein similar to quinone oxidoreductases |
| orf19.6898      | 9.273     | 3.03E-03     | 9.889          | 7.11E-10     |             |             |                  |             |             |                 |                  | Predicted ORF |
| orf19.1321      | GST1      | 9.049        | 1.81E-02       | 1.588        | 4.03E-02      |             |                  |             |             |                 |                  | Putative glutathione S-transferase |
| orf19.7042      | 8.096     | 2.48E-03     | 7.62           | 1.99E-03     |             |             |                  |             |             |                 |                  | Increased transcription is observed upon benomyl treatment |
| orf19.1763      | IFR1      | 6.616        | 2.78E-02       | 3.001        | 1.08E-04      |             |                  |             |             |                 |                  | Predicted ORF |
| orf19.5286      | YCP4      | 6.288        | 6.19E-03       | 3.602        | 4.10E-06      |             |                  |             |             |                 |                  | Putative flavodoxin |
| orf19.2396      | IFR2      | 5.712        | 1.06E-04       | 3.128        | 2.11E-07      |             |                  |             |             |                 |                  | Zinc-binding dehydrogenase |
| orf19.5517      | 5.633     | 1.67E-04     | 1.952          | 4.42E-06     |             |             |                  |             |             |                 |                  | Similar to alcohol dehydrogenases |
| orf19.7306      | 5.539     | 3.30E-02     | 3.896          | 1.62E-06     |             |             |                  |             |             |                 |                  | Aldo-keto reductase family protein |
| orf19.847       | YIM1      | 5.318        | 5.94E-04       | 2.895        | 3.70E-05      |             |                  |             |             |                 |                  | Protein similar to protease of mitochondrial inner membrane |
| orf19.1340      | 5.312     | 4.93E-05     | 1.582          | 1.38E-03     |             |             |                  |             |             |                 |                  | Putative aldose reductase |
| orf19.1237      | ARO9      | 5.082        | 2.22E-02       | 2.141        | 1.98E-04      |             |                  |             |             |                 |                  | Aromatic transaminase of the Ehrlich fusel oil pathway of aromatic alcohol biosynthesis |
| orf19.3150      | GRE2      | 5.012        | 4.43E-03       | 3.405        | 3.33E-06      |             |                  |             |             |                 |                  | Putative reductase; benomyl- induced |
| orf19.7531      | 4.633     | 1.87E-02     | 2.131          | 1.26E-06     |             |             |                  |             |             |                 |                  | Putative protein of unknown function |
| orf19.2693      | GST2      | 4.453        | 1.16E-03       | 3.151        | 8.04E-06      |             |                  |             |             |                 |                  | Putative glutathione S transferase |
| orf19.1048      | IFD6      | 3.835        | 7.52E-03       | 2.548        | 1.73E-02      |             |                  |             |             |                 |                  | Aldo-keto reductase family member |
| orf19.1868      | RNR22     | 3.83        | 4.09E-02       | 1.825        | 3.42E-02      |             |                  |             |             |                 |                  | Putative ribonucleoside diphosphate reductase |
| orf19.3244      | OYE2      | 3.777        | 2.30E-02       | 1.713        | 7.88E-04      |             |                  |             |             |                 |                  | Putative NADPH dehydrogenase |
| orf19.3122      | ARR3      | 3.609        | 1.75E-06       | 2.951        | 1.19E-08      |             |                  |             |             |                 |                  | Protein not essential for viability |
| orf19.4309      | GRP2      | 3.59        | 4.39E-03       | 1.756        | 6.74E-07      |             |                  |             |             |                 |                  | Methylglyoxal reductase |
| orf19.1167      | 3.29      | 4.06E-02     | 2.175          | 8.90E-03     |             |             |                  |             |             |                 |                  | *S. cerevisiae* ortholog Jlp1p |
| orf19.1162      | 3.22      | 1.09E-02     | 2.398          | 8.66E-07     |             |             |                  |             |             |                 |                  | Predicted ORF |
| Systematic name | Gene name | Ratio 30 min | P-value 30 min | Ratio 60 min | P-value 60 min | Benomyl¹ | Cap1-dependent² | H₂O₂³ | Benomyl⁴ | Cap1-dependent⁵ | Cap1-regulated⁶ | Short description |
|-----------------|-----------|--------------|----------------|--------------|----------------|---------|----------------|--------|---------|----------------|----------------|------------------|
| orf19.7611      | TRX1      | 3.179        | 2.38E-03       | 1.543        | 5.88E-05       |         |                |        |         |                |                | Thioredoxin, involved in response to reactive oxygen species |
| orf19.6586      |           | 3.138        | 3.50E-02       | 2.88         | 3.02E-05       |         |                |        |         |                |                |                  |
| orf19.2500      |           | 2.998        | 2.26E-03       | 1.625        | 3.08E-04       |         |                |        |         |                |                |                  |
| orf19.6059      | TTR1      | 2.908        | 1.00E-03       | 2.024        | 1.51E-04       |         |                |        |         |                |                |                  |
| orf19.2461      | PRN4      | 2.8          | 4.61E-03       | 2.263        | 5.67E-06       |         |                |        |         |                |                | Putative glutaredoxin |
| orf19.4449      |           | 2.766        | 4.93E-03       | 1.655        | 3.31E-03       |         |                |        |         |                |                |                  |
| orf19.1027      | PDR16     | 2.456        | 4.71E-02       | 2.278        | 2.58E-05       |         |                |        |         |                |                | Phosphatidylinositol transfer protein |
| orf19.2369.1    | ATX1      | 2.389        | 3.37E-03       | 1.608        | 1.63E-04       |         |                |        |         |                |                | Putative cytosolic copper metallochaperone |
| orf19.2825      |           | 2.356        | 7.77E-05       | 1.822        | 4.46E-05       |         |                |        |         |                |                | Putative cytosolic Fe-S protein assembly protein |
| orf19.2862      | RIB1      | 2.216        | 1.16E-04       | 1.653        | 1.22E-04       |         |                |        |         |                |                | Putative GTP cyclohydrolase II; enzyme of riboflavin biosynthesis |
| orf19.2558      |           | 2.156        | 4.63E-03       | 1.681        | 1.05E-04       |         |                |        |         |                |                | Predicted ORF |
| orf19.1623      | CAP1      | 2.11         | 6.74E-04       | 1.738        | 2.30E-04       |         |                |        |         |                |                | Transcription factor, AP-1 bZIP family; role in oxidative stress response |
| orf19.2022      |           | 2.093        | 4.55E-02       | 1.508        | 2.51E-02       |         |                |        |         |                |                | Predicted ORF |
| orf19.2463      | PRN2      | 1.993        | 6.57E-03       | 1.851        | 1.93E-04       |         |                |        |         |                |                | Protein similar to pirin; Hap43p-repressed gene |
| orf19.5800      |           | 1.984        | 4.95E-02       | 1.792        | 9.01E-04       |         |                |        |         |                |                | Predicted ORF |
| orf19.784       | AMO1      | 1.911        | 1.16E-02       | 1.699        | 3.29E-05       |         |                |        |         |                |                | Putative peroxisomal copper amine oxidase |
| orf19.344       |           | 1.885        | 1.46E-04       | 1.803        | 1.80E-02       |         |                |        |         |                |                | Predicted ORF |
| orf19.1724      |           | 1.878        | 2.09E-03       | 1.975        | 9.36E-06       |         |                |        |         |                |                | Protein of unknown function |
| orf19.6748      | YCF1      | 1.861        | 3.96E-03       | 1.648        | 8.07E-05       |         |                |        |         |                |                | Putative glutathione S-conjugate transporter |
| orf19.3448      |           | 1.812        | 1.08E-02       | 1.528        | 2.59E-04       |         |                |        |         |                |                | Predicted ORF |
| orf19.330       |           | 1.762        | 4.42E-03       | 1.883        | 1.18E-05       |         |                |        |         |                |                | Predicted ORF |
| orf19.282       |           | 1.73         | 4.96E-04       | 1.527        | 8.33E-03       |         |                |        |         |                |                | Hap43p-repressed gene |
| orf19.3462      | PRN3      | 1.555        | 2.49E-02       | 1.972        | 4.43E-05       |         |                |        |         |                |                | Protein similar to pirin |
| orf19.3395      |           | 1.544        | 6.52E-03       | 1.615        | 2.89E-02       |         |                |        |         |                |                | Predicted membrane transporter |
| orf19.4757      | NAR1      | 1.518        | 3.72E-03       | 1.851        | 1.02E-03       |         |                |        |         |                |                | Putative cytosolic iron-sulfur (FeS) protein |

¹ Annotated as benomyl regulated in CGD database.
² Annotated as Cap1 dependent regulation in CGD database.
³ Induced by H₂O₂ according to Wang et al. [23].
⁴ Induced by benomyl according to Znaidi et al. [26].
⁵ Induced by Benomyl according to Znaidi et al. [26].
⁶ Direct target of Cap1 according to Znaidi et al. [26].

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potential thiol nucleophiles (i.e., the C = C bond and a latent alkylation site), FMKs have only one latent alkylation site. Indeed, the amine group can be considered a latent alkylation site, since its 1,2-elimination under physiological conditions affords a very reactive vinyl ketone that can react with thiols according to Dimmock et al. [32]. The observation that aminoalcohols 22 and 23 devoid of this latent alkylation site did not elicit antifungal activity suggests that FMKs could directly trigger oxidative stress. The implication of oxidative stress into the prevailing mechanism of action of FMKs is further supported by the strong correlation between FMKs’ antifungal potency and their LUMO energies in QSAR studies. Specifically, the formation of radical anions from FMKs initiating ROS formation through redox cycling would be facilitated by the low LUMO energies of these compounds. A similar mode of action has been proposed for 7-chlorotetrazolo[5,1-c]benzo[1,2,4]triazine (CTBT), a compound with chemosensitizing activity in yeasts [35]. Specifically, transcript profiling of S. cerevisiae exposed to CTBT revealed the induction of oxidant- and stress-response defense genes as well as nuclear translocation of Vap1p, a transcriptional regulator with functions similar to C. albicans Cap1p [35].

While QSAR results suggested that FMKs antifungal activity is a direct consequence of their ability to trigger oxidative stress, it should be taken into consideration that other antifungals with defined modes of action have been shown to elicit oxidative stress, in possibly indirect ways. This is the case for benomyl that targets microtubules. Benomyl triggers transcriptional responses similar to those associated with oxidative stress inducers such as H2O2 in C. albicans [25,36,37] and oxidative stress responses in S. cerevisiae [38]. In this respect, we have observed a striking overlap between the 56 genes identified in our study as up-regulated in response to compound 2 and genes identified as up-regulated in response to benomyl by Karababa et al. [37] and Znaidi et al. (Table 4, [26]). However, the mechanisms by which benomyl elicits oxidative stress are not known. Polyenes, azoles and echinocandins have also been shown to elicit an oxidative stress response, although to an apparently much lower extent than compound 2.

While amphothericin B has been known to bring forth oxidative damage [39,40], the amphothericin B-dependent transcriptional and translational responses of oxidative stress response genes are considerably weaker than those we observed upon treatment with compound 2 [41,42]. Moreover, most of the oxidative stress-response genes induced upon amphothericin B treatment are not Cap1 target genes [26], and no change in CAP1 expression or levels of the Cap1 protein are associated with amphothericin B treatment. In S. cerevisiae, YAPI does not influence the susceptibility to amphothericin

Figure 4. Growth kinetics in 96-well microtiter plates of C. albicans strains exposed to various concentrations of compound 2. A. Representative growth kinetics of BWP17 and cap1Δ/Δ strains exposed to decreasing concentrations of compound 2 in SD minimal medium. B. Representative growth kinetics of BWP17, cap1Δ/Δ and CAP1 overexpression strains in inducible medium (YNB-casa) exposed to 12.5 μg/mL compound 2. C. Representative growth kinetics of several transcription factor mutants and parent strains in SD minimal medium exposed to 6.25 μg/mL compound 2.

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B [43]. Nuclear translocation of Cap1 in response to the echinocandin caspofungin has been reported along with the induction of two Cap1-regulated genes, GLR1 and SOD2 [44]. However, transcript profiling of caspofungin-treated C. albicans demonstrated repression of CAPI and no up-regulation of oxidative stress genes [42], suggesting that very high concentrations of caspofungin, as those used by Kelly et al. [44], are required to observe oxidative stress. Finally, exposure to ketoconazole does not trigger overexpression of stress genes [42] while fluconazole can induce 2 Cap1-dependent oxidative stress genes, TRR1 and GRE2 [45]. Thus, massive Cap1-dependent oxidative stress responses, as those observed upon treatment of C. albicans by compound 2, do not appear to be a generic trait of most antifungal agents, providing support for the hypothesis that FMKs directly trigger oxidative stress that explains their antifungal activity. Nevertheless, the precise mode of antifungal action of FMKs and whether their triggering of oxidative damage is direct or indirect will require further investigation.

Finally, our results outline a possible chemosensitizing potential for Mannich ketones, as QSAR and transcript profiling against C. albicans have been very similar to those observed with the chemosensitizing agent CTBT on S. cerevisiae [35]. Chemosensitizing approaches are particularly promising to enhance the efficacy of clinically-relevant antifungals, allowing dose and side effects reduction. Compounds with redox potential and targeting the oxidative stress response, such as compound 2, have been described as chemosensitizing agents and proven to enhance antifungal efficacy of azole and amphotericin B against pathogenic yeasts including C. albicans [43,46]. Thus, the chemosensitizing potential of Mannich ketones should be explored in the future.

Materials and Methods

Strains, Media and Antifungal Agents

Strains are listed in Table 3. To construct the CAPI overexpressing strain (CEC3490), the CAPI gene was cloned in the Clp10-PCK1p-GTW-TAPtag vector and the resulting plasmids were introduced at the C. albicans RPS1 locus as described in Cabral et al. [47]. This construction placed the CAPI gene under the casaminoacids inducible PCK1 promoter. International standard strains, C. albicans strains, Saccharomyces sp. and Aspergillus sp. strains from clinical samples were cultivated in Sabouraud medium (OXOID Ltd., England), RPMI-1640 medium (Sigma-Aldrich, Hungary) buffered to pH 7.0 with 0.165 M MOPS (Sigma-Aldrich, Hungary) or SD minimal medium (0.67% yeast nitrogen base without amino acids [Difco] plus 2% glucose) supplemented with uridine (40 mg/l), arginine (20 mg/l), or histidine (20 mg/l), when needed. C. albicans overexpression strains were cultivated in SD minimal medium (uninduced condition) or YNB-casa (0.67% yeast nitrogen base without amino acids [Difco] plus 2% casaminoacids; induced condition). Amphotericin B (Fungizone, Bristol-Myers Squibb, Epernon, France) was used as an antifungal standard. Fused Mannich ketones (compounds 1–21) and the corresponding aminoalcohols (compounds 22–23) were synthesized as described previously [8,9]. Some have been known compounds prepared according to the literature [48,49,50,51,52,53,54,55,56,57,58,59,60]. All data of the test compounds were in accordance with the compounds published in the references above. Minimum inhibitory concentration (MIC) values of standard and synthesized agents were determined using the macro-tube dilution method [13]. Briefly, the test compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted in RPMI. In the first tube there was 100 μg/mL test material in 5% (v/v) DMSO. From this tube we made double dilution series. The inoculum concentration was 10^4 cells/mL. The tubes were incubated for 48 h at 30°C. They were checked by naked eye and subcultures were made from the tubes on Sabouraud medium agar. The colony forming unit (CFU) values were determined after incubation for 48 hours at 30°C. MIC_{50} stands for the drug concentration that yielded a 10-fold reduction in CFUs relative to an untreated control. MIC_{90} were determined three times for each compound and strain. Other technical details have been given in a previous publication [13].

In vivo Toxicity Testing

Cytotoxicity tests in HeLa cells were performed as described previously [9]. Animal experiments were approved by the Animal Ethics Committee of Pécs University (Permission Number: BA/02/2000-1/2007). The work regulation of this Committee is based on Good Laboratory Practice (GLP) and harmonized with Directive 2010/63/EU on the protection of animals. For the determination of median lethal dose (LD_{50}), inbred male and female BALB/c mice, weighing 18–22 g each, were provided with normal mice chow and water ad libitum. All animals were allowed to acclimate for at least 5 days prior to the first treatment. The 160 mice were randomly divided into 8 groups. Compound 2 was resuspended in physiologic saline solution at the final concentration of 1, 2, 4, 8, 10, 12, 16 and 20 mg/mL and 1 mL of each solution was injected intraperitoneally into 20 mice each. The animals were under control for 7 days and the death rate was determined. The LD_{50} value was determined from the dose–effect curve by Lichfield-Wilcoxon graphic methods [19,20].

QSAR Calculations

QSAR analysis was done using the BioMedCACHe 6.1 program for Windows (Fujitsu, Beaverton, OR). Structures were preoptimized using augmented MM3 parameters followed by semiempirical PM3 optimization. A total of 32 descriptors were calculated for each compound. The empirical MICs were expressed in molality and converted to pMIC (negative logarithm of MIC) values. Only compounds with an MIC <200 mg/l were used in the QSAR analysis. The best QSAR equations with three and two descriptors were selected by multiple linear regressions via the Project Leader module of BioMedCACHe. Model validations were done by randomization of pMIC and leaving out 10% of the compounds.

Microarray Experiments

Gene expression analysis of the C. albicans sequenced laboratory strain SC5314 was performed by comparing planktonic cells with and without exposure to compound 2. An exponentially growing C. albicans culture in SD medium at 30°C was split into two flasks, one exposed to MIC_{90} concentration of compound 2 (6.25 μg/mL in water), the other to the same volume of water. Samples were collected after 30 and 60 min for transcript profiling. Total RNA was isolated using an RNeasy minikit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. The concentration, purity, and integrity of the isolated RNA were evaluated using a Nanodrop spectrophotometer (Thermo Fisher, Illkirch, France) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). cDNA synthesis, labeling and hybridization on C. albicans oligonucleotide microarrays (Eurogentec, Liege, Belgium) were performed as described in Rossignol et al. [14]. Sample comparisons at 30 and 60 min were performed on two biological replicates, and each biological replicate was subjected to technical replicates with dye swap.
Microarray Analysis

Microarray scans were performed with a GenePix 4000 A scanner using GenePix 5 software and analyses were performed using GeneSpring GX software (Agilent Technologies, Massy, France). Data normalization was performed with the LOWESS method, and the statistical analysis with the t-test from GeneSpring. We used the September 2011 annotation from the Candida Genome Database [23]. Some oligonucleotides on the microarrays did not match a gene in the current version of CGD as some genes have been removed from CGD or coordinates have been refined. Data for these oligonucleotides were not analyzed further. Genes regulated by at least 1.5-fold with P<0.05 were considered significant. Microarray data have been deposited at ArrayExpress under accession number E-MEXP-3334. Normalized data are available in Table S1 in the supplemental material. Gene ontology analyses were performed using tools available at the Candida Genome Database, with p-values calculated by GO Term Finder [22]. GO TermFinder calculates a P-value using the hypergeometric distribution:

\[
P = \frac{1}{P} \sum_{i=0}^{k} \binom{M}{i} \frac{(N-M)}{n-i} \frac{n}{N}
\]

where N is the total number of genes in the background distribution, M is the number of genes within this distribution that are annotated to the node of interest, n is the list size of the distribution, and k is the number of genes within this list annotated to the node.

Growth Kinetics

Strains were inoculated at a final OD₆₀₀ = 0.05 in 100 μL and were grown in 96-well plates at 30°C for 24 h. Growth was monitored every 20 minutes using a microplate reader (TECAN Sunrise). For growth kinetics comparison of wild type and cap1 deleted strains, growth curves were performed in triplicate in independent experiments in SD minimal medium complemented with various concentrations of compound 2 ranging from 25 μg/mL to 0.0975 μg/mL. For CAP1 overexpression experiments, growth curves were performed in duplicates in independent experiments in SD minimal medium (non-induction condition) and in YNB-casa (induction condition) with various concentrations of compound 2 ranging from 25 μg/mL to 0.0975 μg/mL. For comparison between transcription factor mutants and parent strains experiments, growth curves were obtained in duplicates from independent experiments in SD minimal medium with various concentrations of compound 2 ranging from 25 μg/mL to 0.0975 μg/mL, or H₂O₂ ranging from 4 mM to 15.62 μM. For all these experiments, OD₆₀₀ readings at final point were also recorded in two independent experiments for validation.

Supporting Information

Table S1 Microarray Gene expression data. Normalized expression data for all ORFs with fold change ratios and p-values at 30 min and 60 min as described in materials and methods section. (XLS)

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Author Contributions

Conceived and designed the experiments: TL TR BK CE. Performed the experiments: TR BK OB BK AN PBJ KR. Analyzed the data: TR LP TL CE. Contributed reagents/materials/analysis tools: FK LP CE TL. Wrote the paper: TR BK LP CE TL.

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