Chemical Genetic Profiling and Characterization of Small-molecule Compounds That Affect the Biosynthesis of Unsaturated Fatty Acids in Candida albicans*§

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The balance between saturated and unsaturated fatty acids plays a crucial role in determining the membrane fluidity. In the diploid fungal pathogen Candida albicans, the gene for fatty acid Δ9 desaturase, OLE1, is essential for viability. Using a reverse genetic approach, termed the fitness test, we identified a group of structurally related synthetic compounds that induce specific hypersensitivity of the OLE1+/− strain. Genetic repression of OLE1 and chemical inhibition by two selected compounds, ECC145 and ECC188, resulted in a marked decrease in the total unsaturated fatty acids and impaired hyphal development. The resulting auxotroph of both was suppressed by the exogenous monounsaturated fatty acids (16:1Δ9 and 18:1Δ9). These correlations suggest that both compounds affect the level of unsaturated fatty acids, likely by impairing Ole1p directly or indirectly. However, the residual levels of monounsaturated fatty acids (MUFAs) resulted from chemical inhibition were significantly higher than OLE1 repression, indicating even partial inhibition of MUFAs is sufficient to stop cellular proliferation. Although the essentiality of OLE1 was suppressed by MUFAs in vitro, we demonstrated that it was required for virulence in a murine model of systemic candidiasis even when the animals were supplemented with a high fat diet. Thus, the fungal fatty acid desaturase is an attractive antifungal drug target. Taking advantage of the inhibitors and the relevant conditional shut-off strains, we validated several chemical genetic interactions observed in the fitness test profiles that reveal novel genetic interactions between OLE1/unsaturated fatty acids and other cellular processes.

Candida albicans is a commensal Hemiascomycete of the normal microflora of the healthy humans. However, it can cause mycoses ranging from superficial mucosal to hematogenously disseminated infections. In fact, C. albicans persists as the single most significant fungal pathogen leading to life-threatening infections in terms of the total number of cases and the mortality rate in the hospital setting (1). Our understandings of its pathobiology as well as our efforts to identify therapeutic agents have been greatly facilitated by studying another Hemiascomycete, Saccharomyces cerevisiae. Genomic studies indicate that Hemiascomycetes represent a phylogenetically homogenous group of eukaryotes (2). However, they are highly diverse at the physiological and ecological levels. Unlike the model yeast, C. albicans is a diploid without a sexual life cycle based on meiosis and sporulation. The application of the conventional forward genetic approaches is restricted. Its unique pathobiology, however, urges that biological studies be performed directly in the pathogen. To this end, large-scale construction of mutants has been undertaken in this diploid organism (3).

One of the genetic strategies took advantage of the diploidy of C. albicans to create a library of heterozygous mutants in which each strain contains an insertional disruption of the Tn7 transposon. A screen of that library yielded a set of genes that was defective in the yeast-to-hypha transition when one allele was disrupted (4). Haploinsufficiency (reduction in growth by a loss-of-function mutation of one allele in a diploid organism; e.g. phenotypic defects associated with heterozygous deletion strains) and conditional haploinsufficiency, in particular, provide the genetic basis to identify genes whose dosages become susceptible to a defined condition and, thus, are functionally involved in the relevant biological processes. As the genome of C. albicans has been determined and annotated (5, 6), a reverse genetic approach can be used to explore haploinsufficiency at the genome level. The resulting assay, the C. albicans fitness test (CaFT)2, adapted from an analogous strategy first developed in S. cerevisiae (7, 8), consists of ~2900 heterozygous deletion strains (~45% genome coverage) in a single pool that

2 The abbreviations used are: CaFT, C. albicans fitness test; MOA, mechanism of action; SFA, saturated fatty acid; UFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; IC, inhibitory concentration; GRACE, gene replacement and conditional expression; pTET, tetracycline-repressible promoter; FAS, fatty acid synthase; DEX, dextrose; ER, endoplasmic reticulum; YNB, yeast nitrogen base and dextrose; CSR, complete supplement mixture; ORF, open reading frame; MYC, myristic acid; 14:0, palmitic acid; 16:0, palmitoleic acid; 16:1 (16:1Δ9), palmitoleic acid; 18:0, stearic acid; 18:1 (18:1Δ9), oleic acid; 18:2 (18:2Δ9Δ12), linoleic acid; 18:3 (18:3Δ9Δ12Δ15), linolenic acid.

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can be screened for phenotypic variations in parallel. Because each strain was double-bar-coded at the deleted locus, the relative variations in growth (hence, the term of fitness test) of all the strains in response to chemical perturbations could be determined en masse by DNA microarrays (9).

It has been demonstrated in both fungi that specific and significant responses (hypersensitivity and resistance) elicited by antiproliferative compounds are restricted to only small sets of heterozygous deletion strains (7–9). Usually, they contain strains corresponding to the targets and other factors that are directly associated with or involved in the targets or aspects of the mechanisms of action (MOAs). For example, fluconazole, the therapeutic drug used to treat fungal infections, induces specific hypersensitivity of heterozygotes for ERG11, NCP1, and CDR1, corresponding to the target, the co-factor of Erg11p, and the efflux pump for the drug, respectively (9). In most cases the fitness test profiles are highly indicative of the biological activities of the compounds tested, as the hypersensitive and resistant strains reveal aspects of MOAs of antiproliferative compounds that are susceptible to a 50% loss of gene dosage. Indeed, CaFT profiling was used to determine the MOAs of novel antifungal synthetic compounds (9, 10) and natural products (11–13). In this report we present the identification of structurally related small-molecule inhibitors that elicited significant hypersensitivity of the OLE1+/− strain in the fitness test and demonstrate that the two selected compounds reduce the level of unsaturated fatty acids and block hyphal growth.

The balance between saturated (SFA) and unsaturated (UFA) fatty acids is a critical determining factor of membrane fluidity important for the function and integrity of the membrane systems of the cell. In the yeast S. cerevisiae, the fatty acid Δ9 desaturase (ScOle1p) is located at the endoplasmic reticulum (ER) surface and converts coenzyme A-bound SFAs (palmitic (16:0) and stearic (18:0) acids) to monounsaturated fatty acids (MUFAs, palmitoleic (16:1) and oleic (18:1) acids) by introducing double-bonds at carbon 9 in the carbon chains (14, 15). The C. albicans Ole1p was shown to play the same role (16). However, although 16:1 is the predominant UFA in S. cerevisiae, the pathogen produces, in addition, polyunsaturated fatty acids (PUFAs, linoleic (18:2) and linolenic (18:3) acids) through the sequential formation of extra double bonds in 18:1 via Δ12 and Δ15 desaturases (16, 17).

In yeast, the level of unsaturated fatty acids is elaborately controlled. For example, multiple transcription regulatory elements have been identified in the promoter of ScOLE1. Its transcription is repressed by UFAs (which also regulate the stability of ScOLE1 mRNA) but activated by hypoxia and low temperature via the ER-bound transcription factors ScSpt23p and ScMga2p (18). Both factors are, in turn, activated by ubiquitin/proteasome-dependent ER-associated degradation, a process that is also involved in regulating the stability of ScOle1p (19). Moreover, the UFA/SFA balance is important for other cellular processes. For example, a temperature-sensitive mutant of ScOLE1, identified as mdm2, resulted in a 2.5-fold decrease of monounsaturated fatty acids at the nonpermissive temperatures and caused the fragmentation of reticular network of mitochondria and accumulation of defective mitochondria in the mother cell during division (20). Similarly, low levels of unsaturated fatty acids affect the nuclear envelope structure and, to a less extent, vesicular traffic (21). In C. albicans, diminished levels of OLE1 block hyphal development and formation of chlamydomospores under the normoxic conditions (16).

Our results show that the effects of chemical inhibition by two selected compounds correlate with genetic repression of OLE1 (in a conditional shut-off strain), suggesting that Ole1p be the target. However, it is possible that their affects on Ole1p is indirect, as neither compound was tested for direct inhibition of the Δ9 desaturase activity in vitro. Nonetheless, we provide evidence that partial depletion of monounsaturated fatty acids is sufficient to be antiproliferative and demonstrate the essentiality of OLE1 in a murine model of systemic candidiasis.

EXPERIMENTAL PROCEDURES

C. albicans Fitness Test—The CaFT was described in Xu et al. (9). Briefly, it relies on chemically induced responses (hypersensitivity and resistance) of heterozygous deletion strains, each of which is double-bar-coded with two specific DNA sequences flanking the deleted allele (replaced with HIS3). A pool of 2868 heterozygous deletion strains with approximately equal representation was subjected to treatments of a given antifungal compound at multiple sublethal inhibitory concentrations (ICs) and mock treatment. The relative abundance of each strain in the compound-treated cultures was determined by DNA microarrays that identify the two corresponding DNA barcodes. An error model was used to account for technological and biological variability. The relative response of each strain was indicated by the normalized z-scores of two barcodes, with a positive value indicating hypersensitivity and negative resistance. The z-scores were not converted to p values to avoid over-interpretation (9). For each strain, the z-score with higher absolute value is selected to compile the final list for comparison (supplemental Table S1). Multiple experiments were analyzed with Cluster 3.0 (hierarchical clustering by centroid linkage (22)), displayed in TreeView.

Antifungal Compounds and Reagents—Reference compounds cerulenin and fluconazole were from Sigma-Aldrich and Toronto Research Chemicals Inc. (Toronto, ON, Canada). ECC145, ECC188, and other compounds in Fig. 1, A and B, were from Chembridge Corp. (San Diego, CA). Other chemical reagents (palmitic, stearic, palmitoleic, and oleic acids, tergitol, etc) were from Sigma-Aldrich.

C. albicans Heterozygous Deletion Strains, Conditional Shut-off Strains, and Growth Inhibition Assays—The C. albicans strain SC5314 was used to construct all the strains, heterozygous deletion, and conditional shut-off (pTET, also known as gene replacement and conditional expression (GRACE) strains), employed in this study as described in Roemer et al. (23). Growth inhibition assays were performed in liquid medium (RPMI, as used in the CaFT), with the starting A600 of 0.01 for each strain. Compounds were added at the indicated concentrations with DMSO (1% final). The mock treatment consisted of 1% DMSO. The A600 was determined after 16 h of growth at 30 °C and normalized as percentage by the growth of the same strain under the identical conditions without antifungal compound (mock). For spot tests, solid medium of YNBD
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FIGURE 1. Chemical structures and C. albicans fitness test profiles of putative OLE1 compounds. A and B, two groups of synthetic compounds tested in the CaFT. C, the fitness test profiles. For each compound (with the exception of ECC120 and ECC146), two independent CaFT experiments are selected for overall comparison. Cerulenin is included as a control. In the two additional experiments with ECC145 (145/MUFA), monounsaturated fatty acids (16:1 and 18:1) were added at the final concentration of 0.5 mM each (see “Results” for detail).

The strains were selected with the absolute value of z-scores > 3.5 in at least two experiments. They (57 in total) were grouped by hierarchical clustering using centroid linkage method (22) and displayed inTreeView. The experimental conditions (concentrations and the resulting ICs) are shown on the top, and scale of heat map is shown above the gene annotation (black arrows indicate genes of which the conditional shut-off strains were tested in Fig. 2; other functionally related genes are those highlighted by red boxes, and genes involved in fatty acid metabolism are in red). The four subunits of the putative signal recognition particle and one of the receptors are boxed in black. Other functionally related genes are those involved in transcription and regulation (§), ubiquitin-dependent protein degradation (●), and cell wall structure or biosynthesis ($). Black arrows indicate genes of which the conditional shut-off strains were tested in Fig. 7. D, spot tests of selected heterozygous deletion strains against ECC145 and ECC188 (1-day growth on solid YNBd+CSM medium at 30 °C).

supplemented with CSM was used with 2% agar. The suppression of lethality of the pTET strains under the repression conditions was achieved by adding fatty acid(s) (dissolved in tergitol at 100× of the final concentration) to the media, with 1% tergitol as the mock treatment. Cultures in exponential growth were diluted to A<sub>600</sub> 1.0 (Fig. 2) or 0.2 (see Figs. 1D and 7) followed by 1/5 serial dilutions with 2-μl aliquots spotted. The growth was photographed after 1 or 2 days of growth at 30 °C.

Analysis of Fatty Acids—A gas chromatograph was used to determine the ratio of unsaturated and saturated fatty acids, as described in Stukey et al. (14). To determine the effect of OLE1 repression on fatty acids, the pTET-OLE1 strain was grown in liquid medium in the presence of 100 μg/ml tetracycline for 15 h at 30 °C. The culture was harvested, and the cell pellet was used to extract fatty acids. Cells from a culture of the pTET-OLE1 strain under the non-repressing conditions were used as the control. To examine the effect of ECC145 and ECC188, cultures of the pTET-HIS3 strain were grown in the presence of ECC145 (5 μg/ml) and ECC188 (0.5 μg/ml) for 15 h at 30 °C and harvested for extraction of fatty acids.

Cells of the same strain grown under the same conditions without drug treatment were used as the control.

Hyphal Growth—Cultures of indicated strains were grown in yeast extract/peptone/dextrose (YPD) for 6 h and diluted with RPMI to A<sub>600</sub> 0.05. Aliquots of 75 μl were dispensed in the 96-well microtiter plate. Aliquots of equal volume of RPMI medium containing tetracycline or ECC145 or ECC188 at 2× final concentration were mixed with diluted culture. The mixtures were kept at 30 °C for ≥15 h and examined microscopically.

Virulence Test in a Marine Model of Systemic Candidiasis—The conditional shut-off strains for OLE1, FAS1, and FAS2 were tested in a murine model of systemic candidiasis for virulence as described in Roemer et al. (23). All the animal experiments were performed according to the National Institutes of Health guidelines for the ethical treatment of animals.

RESULTS

Identification of OLE1 Compounds by Chemical Genetic Profiling—From a group of synthetic compounds with whole-cell antifungal activities, tested in the CaFT, we identified eight that share a core structure of 1,2,4-triazolidine-3-thione and
yielded highly comparable profiles consisting of significant and consistent hypersensitivity of the OLE1+/− (orf19.5177) strain (with z-scores > 5) (Fig. 1C and supplemental Table S1). Dubbed as putative OLE1 compounds, they could be divided into two subclasses, A and B, based on chemical structures (Fig. 1, A and B). Their profiles are readily differentiated from that of cerulenin, a known inhibitor of fatty acid synthase that elicits prominent hypersensitivity of the FAS1+/− strain (Ref. 9, Fig. 1C). Additional hypersensitive heterozygous strains specific to the ScSRP68, a known inhibitor of fatty acid synthase that elicits prominent hypersensitivity of the FAS1+/− strain (Ref. 9, Fig. 1C). Additional hypersensitive heterozygous strains specific to the

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** Suppression of conditional shut-off strains for genes involved in fatty acid biosynthesis by exogenous fatty acids. A, schematics of the OLE1 loci in the conditional shut-off (pTET) strain. In this strain the first allele was deleted and replaced with HIS3, and the native promoter of the remaining allele was replaced by pTET, which is repressed in the presence of tetracycline in the media (23). B, suppression of transcriptional repression of FAS1 and FAS2 by palmitic acid (16:0, 1.25 mM) partially reverses the suppression by 16:0. C, suppression of transcriptional repression of OLE1 by palmitoleic (16:1, 0.5 mM) and oleic (18:1, 0.5 mM) acids. The pTET-OLE1 strain was partially suppressed by 16:1, which was enhanced by 18:1 in mixture (in both B and C, all solid YNBD-CSM media contained 1% ethanol and 1% tergitol; the non-repressing conditions are indicated by no tet; top panels), and the repression conditions are indicated 100 μg/ml tet; bottom panels). The solid YNBD + CSM medium was used in the spot tests (2 days at 30 °C). D, a proposed model of the fatty acid biosynthesis pathway in C. albicans based on results described. Gray arrows indicate steps that are not described in this study. Dashed lines indicate possible feedback regulation at the level of gene expression.

| Compounds | ECC145 | ECC188 | Cerulenin |
|-----------|--------|--------|-----------|
| H3S       | +/−    | pTET   | +/−       | pTET   |
| OLE1      | 0.22 ± 0.06 | 0.02 ± 0.01 | 0.013 ± 0.007 | 0.002 ± 0.002 |
| FAS1      | 0.42 ± 0.03 | 0.23 ± 0.10 | 0.036 ± 0.009 | 0.038 ± 0.017 |

TABLE 1 Susceptibilities (average IC50 μg/ml ± S.D. from three experiments) of heterozygous deletion (+/−) and conditional shut-off (pTET, under the non-repressing conditions) strains to ECC145, ECC188, and cerulenin
under the repressing conditions. This suppression was largely antagonized by 18:0 in mixture (Fig. 2B). As reported (16), OLE1, but not OLE2, was essential for growth (Fig. 2B). Exogenous palmitoleic acid (16:1, 0.5 mM) suppressed partially OLE1 repression, which was enhanced by oleic acid (18:1, 0.5 mM) in mixture. However, the latter was not sufficient to suppress by itself (Fig. 2C). These results suggest that 1) the C. albicans FAS is responsible for the synthesis of 16:0, which is converted to 18:0 by another enzyme(s), 2) both 16:0 and 18:0 are converted to 16:1 and 18:1 by Ole1p, and 3) 16:1 can be converted to 18:1 by another enzyme(s), although much less efficiently (Fig. 2D).

It is also evident that the fatty acid biosynthesis in C. albicans is regulated by analogous feedback mechanisms in yeast (e.g. Ref. 25) such that replacement of the native promoters with a heterologous one may escape feedback and other regulations and result in deleterious effects on growth. Consistently, both pTET-FAS1 and pTET-OLE1 strains were partially impaired in growth under the non-repressing conditions (Fig. 2B, mock). Moreover, the former was severely impaired by exogenous 18:0 and 16:1 and to a less extent by 18:1 (Fig. 2, B and C). Similarly, the pTET-FAS2 strain was moderately impaired in the presence of 16:1 and 18:1 (Fig. 2C). In S. cerevisiae, the expression of ScOLE1 is repressed in response to the exogenous MUFAs (26), which also reduces the half-life of the ScOLE1 transcript (27). The growth defects of the pTET-OLE1 strain in the presence of 18:0 (Fig. 2B) and 18:1 (Fig. 2C) might result from the failure of regulating OLE1 expression in response to fatty acids present in the medium.

We noted that the level of the OLE1 transcript expressed from pTET was significantly lower than that from the native promoter.3 Indeed, the pTET-OLE1 strain was intrinsically highly susceptible to both ECC145 and ECC188 (Fig. 3, A and B), with ~20- and ~30-fold shifts in IC_{50} (versus the pTET-HIS3 strain), respectively, compared with 2- or 3-fold shifts of the heterozygous deletion strain (Table 1). However, the under-expression of OLE1 conferred resistance to cerulenin (Fig. 3C). The altered susceptibility of the pTET-OLE1 strain was restricted to ECC145, ECC188, and cerulenin (data not shown). The pTET-FAS1 strain, on the other hand, displayed only modest changes in susceptibility to cerulenin, ECC145, and ECC188 (Fig. 3, Table 1). These results suggest that in C. albicans regulation of expression of OLE1 is fine-tuned in response to free fatty acids. But in the cases of FAS1, promoter replacement does not change the intrinsic susceptibility to cerulenin under the non-repressing conditions.

**Effects of ECC145 and ECC188 on Unsaturated Fatty Acids and Hyphal Growth**—The genetic results (Figs. 1D and 3; Table 1) suggest that these two compounds affect the activity of Ole1p in the fungus. To characterize mechanism of action, we compared the metabolic defects and terminal phenotypes caused by chemical inhibition of EC145 and ECC188 with genetic repression of OLE1. Because the putative OLE1 compounds were from a high-throughput screen chemical library and limited in quantity, we decided to compare ECC145 and ECC188 side-by-side with the pTET-OLE1 strain. The effects of OLE1 repression on fatty acids were determined using the pTET strain grown in the absence or presence of tetracycline. Under the non-repressing conditions, unsaturated fatty acids constituted ~70% of the total fatty acids, with ~20% monounsaturated fatty acids (mostly 16:1 + 18:1), ~50% polyunsaturated fatty acids (18:2 + 18:3), and ~25% saturated fatty acids (mostly 16:0 + 18:0) (Table 2). After growth under the repressing conditions for 15 h (at which point the culture had ceased to grow), the level of MUFAs was markedly declined to 3% (~6-fold decrease) and PUFAs to 34% (~1.5-fold decrease). Saturated fatty acids increased concomitantly, with a significant drop in the UFA/PUFA ratio.3 D. Xu and T. Roemer, unpublished observations.
The overall distribution of saturated fatty acids and unsaturated fatty acids in the latter (Table 2). This observation is consistent with the lower level of monounsaturated fatty acids was slightly higher than what was observed in the pTET-OLE1 strain under the same conditions. The difference was largely because of a much lower level of monounsaturated fatty acids in the latter (Table 2). This observation is consistent with the lower level of OLE1 expression in the conditional shut-off strain. The overall distribution of saturated fatty acids and unsaturated fatty acids (in the pTET-HIS3 strain) is in agreement with results of other studies with quantitative differences, likely because of different strains and growth conditions employed (16, 17, 28). The treatment with ECC145 (at ~5× minimal inhibitory concentration for 15 h) resulted in ~1.5- and ~2.5-fold decreases in MUFAs and PUFAs, respectively, and ~3-fold increase in SFAs, with the overall UFA/SFA ratio dropping from 3.60 to 0.59 (Table 2). Similar results were obtained with ECC188 (Table 2). Although the UFA/SFA ratios were comparable between genetic repression of OLE1 and chemical inhibition of both compounds, the relative changes in mono- and polyunsaturated fatty acids were different in the two strains with different treatments (Table 2, see “Discussion” for details).

It was noted previously that when expression of OLE1 from the promoter of MET3 was repressed, C. albicans failed to undergo hyphal growth (16). This phenotype provided an additional criterion to examine the mechanism of action of ECC145 and ECC188. The control pTET-HIS3 strain developed true hyphae when grown in the RPMI medium under the non-repressing and the repressing conditions at 30 °C for ≥15 h (Fig. 4, A and B). However, even under the non-repressing conditions, the pTET-OLE1 strain was compromised in its ability to undergo full hyphal growth. Only yeast cells, pseudohyphae, elongated germ tubes, and limited hyphae were observed (Fig. 4D). When the expression of OLE1 was repressed (by tetracycline at 25 μg/ml or higher), hyphal development was completely blocked. Most cells remained in the yeast form and enlarged, whereas pseudohyphae and germ tubes were occasionally observed (Fig. 4E). These defects were largely reproduced in the pTET-HIS3 strain treated with ECC145 at concentrations ≥1IC50 (Fig. 4C) and ECC188 (data not shown). The overall correlations between phenotypes associated with genetic repression of OLE1 and chemical inhibition of ECC145 and ECC188 support the conclusion that both compounds inhibit the unsaturated fatty acid biosynthesis.

Genetic Characterization of ECC145 and ECC188—If these two compounds indeed inhibit the unsaturated fatty acids by affecting the activity of Ole1p directly or indirectly (see “Discussion”), their antiproliferative activities should be suppressed by the exogenous monounsaturated fatty acids, as in the case of OLE1 repression (Fig. 2C). When tested in the RMPI medium containing 1% tergitol, used to dissolve fatty acids, the potential of ECC145 and ECC188 were markedly reduced, with minimal inhibitory concentrations shifted to ~25 (~25-fold increase) and ~100 (>1000-fold) μg/ml, respectively (Fig. 5, A

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**FIGURE 4. Impairment of hyphal growth by repression of OLE1 and chemical inhibition of ECC145.** As a control, the pTET-HIS3 strain was grown in the absence (A) or presence (B) of tetracycline. The effect of ECC145 was tested with the pTET-HIS3 strain under the non-repressing conditions (C). Morphologies of the pTET-OLE1 strain under the non-repressing and the pressing conditions are shown in D and E, respectively.
and B). Nevertheless, the hypersensitivity of the OLE1+/− strain to both compounds was preserved. The exogenous MUFAs (16:1 and 18:1, 0.5 mM each) suppressed the antiproliferative activities of ECC145 (at concentrations ≤50 µg/ml) and ECC188 (at ≤100 µg/ml) against the HIS3+/−, and OLE1+/− strains (Fig. 5, A and B). On the other hand, tergitol moderately reduced the potency of cerulenin. As expected, the exogenous palmitic acid ablated almost fully the antiproliferative activity of cerulenin (Fig. 5, C).

As ECC145 was partially suppressed by MUFAs, it is likely that its inhibitory effect is not restricted to Ole1p (see “Discussion”). When it was tested in the CaFT at concentrations of ≥15 µg/ml (i.e. >IC20) in the presence of MUFAs (16:1 + 18:1, 0.5 mM each), almost all of the ECC145-responsive strains were suppressed (Fig. 1C), indicating that the fitness test profile of ECC145 is directly related to the inhibition of MUFAs. With the exceptions of the haploinsufficient STR2 (orf19.1033) and orf19.5291, ECC145 did not generate an informative fitness test profile in the presence of MUFAs (Fig. 1, C and D).

As noted earlier, the CaFT profiles of the OLE1 compounds share a common secondary profile of hypersensitive and resistant heterozygous strains for genes involved in a number of cellular processes (Fig. 1C). We sought to confirm these results independently. From the profiles, representative genes were selected based on their non-essentiality (23); PHO88 (orf19.7327-ScPho88p is a membrane protein involved in inor...
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In this study we applied chemical genetic profiling in *C. albicans* to identify a series of structurally related synthetic compounds that induced significant and specific hypersensitivity of the heterozygous deletion strain for OLE1, encoding the fungal fatty acid Δ9 desaturase (Fig. 1). The fitness test results suggest that these compounds affect unsaturated fatty acids, likely by impairing Ole1p. Two representative compounds were selected for characterization of mechanism of action. The conditional shut-off strains provided a genetic means to examine the level of cellular fatty acids (Table 2), and the effect on hyphal growth (Fig. 4) when OLE1 expression was repressed. The overall correlations between genetic repression and chemical inhibition (Table 2 and Fig. 4), and suppression of both by the exogenous monounsaturated fatty acids (Figs. 2 and 5) supported the MOA hypothesis. The significant reduction in total unsaturated fatty acids and the concomitant increase in total saturated fatty acids (Table 2) are clear indications that both ECC145 and ECC188 inhibit UFA biosynthesis. The shared chemical structures and the CaFT profiles (Fig. 1) suggest that other OLE1 compounds act in a similar manner. They provide another example that illustrates the predicative power of the fitness test in studying mechanisms of action of unknown compounds; thus, its application in antifungal drug discovery (9–13). However, the question remains of whether the effects of ECC145 and ECC188 are because of direct inhibition of Ole1p, as neither was tested for direct inhibition of the Δ9 desaturase activity *in vitro*.

**Fatty Acid Biosynthesis in C. albicans**—Fatty acids are a fundamental constituent of the cellular membrane systems that are involved in diverse biological processes. Their biosynthesis is energy-consuming and, thus, subject to elaborate regulation in

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**DISCUSSION**

The essentiality of *FAS1, FAS2*, and *OLE1* for viability was suppressed by the exogenous fatty acids (Fig. 2). This raises the question about their requirement in *C. albicans* pathogenesis. The virulence of these three *pTET* strains was tested in a murine model of systemic candidiasis (23). In the first group (sugar) of animals fed orally with sugar; *i.e.* under the non-repressing conditions, all three strains were virulent via lateral tail vein injection (*A, B, and C*, respectively, Fig. 8). The other two groups were fed with doxycycline starting either 3 days before (dox(−3d)) or 2 days post (dox(+2d)) infection and kept on doxycycline for 18 or 20 days, after which two mice from each group were sacrificed, and kidney fungal burden was determined. The surviving mice, dox(-3d) and dox(+2d) treatments (Fig. 8, A and B), with kidney fungal burden (18 days post-infection) <5.0 x 10⁸ and <10³ CFU/g (*i.e.* below detection), respectively. When the *pTET-OLE1* strain was tested, one mouse in the dox(+2d) group died, whereas the rest from both groups survived till the end of the experiments (Fig. 8C). The fungal burdens were below detection (<10³ colony-forming units/g of kidney) 20 and 34 days post-infection. When the experiments were repeated for the *pTET-OLE1* strain, all the animals from the dox(-3d) and the dox(+2d) groups survived (Fig. 8D), with fungal burdens below detection.

Because the mouse diet in these experiments consisted of 1% unsaturated fatty acids (from linoleic safflower, ~5× higher in linoleic acid than monounsaturated fatty acids), virulence studies were repeated for the *pTET-OLE1* strains with animals fed on 5 and 21% corn oil, which contains more MUFAs than safflower. Unexpectedly, the strain was found avirulent in both groups of dox(-3d) and dox(+2d) under both conditions with supplemented MUFAs in diet (Fig. 8, E and F), with the resulting fungal burdens below detection. We concluded that the three genes involved in fatty acid biosynthesis, although conditionally essential *in vitro*, are essential for establishing and maintaining a lethal systemic candidiasis in mice. Moreover, the addition of dietary MUFAs was not sufficient to suppress the failure of OLE1 repression to establish systemic infection. These results validate Ole1p as a new antifungal drug target.

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**FIGURE 7.** Hypersensitivity of conditional shut-off strains to ECC145 and ECC188 under the repressing conditions. The strains were selected based on the hypersensitivity of the corresponding heterozygous deletion strains in the CaFT shown in Fig. 1C. They were grown under the non-repressing (1% EtOH, top) and the pressing (100 µg/ml tetracycline, bottom) conditions in the absence (mock) or the presence of ECC145, ECC188, cerulenin (CRL), or fluconazole (FLC) at the concentrations indicated for 1 day at 30 °C. For orf19 designations, see Fig. 1C.

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**DISCUSSION**

In this study we applied chemical genetic profiling in *C. albicans* to identify a series of structurally related synthetic compounds that induced significant and specific hypersensitivity of the heterozygous deletion strain for OLE1, encoding the fungal fatty acid Δ9 desaturase (Fig. 1). The fitness test results suggest that these compounds affect unsaturated fatty acids, likely by impairing Ole1p. Two representative compounds were selected for characterization of mechanism of action. The conditional shut-off strains provided a genetic means to examine the level of cellular fatty acids (Table 2), and the effect on hyphal growth (Fig. 4) when OLE1 expression was repressed. The overall correlations between genetic repression and chemical inhibition (Table 2 and Fig. 4), and suppression of both by the exogenous monounsaturated fatty acids (Figs. 2 and 5) supported the MOA hypothesis. The significant reduction in total unsaturated fatty acids and the concomitant increase in total saturated fatty acids (Table 2) are clear indications that both ECC145 and ECC188 inhibit UFA biosynthesis. The shared chemical structures and the CaFT profiles (Fig. 1) suggest that other OLE1 compounds act in a similar manner. They provide another example that illustrates the predicative power of the fitness test in studying mechanisms of action of unknown compounds; thus, its application in antifungal drug discovery (9–13). However, the question remains of whether the effects of ECC145 and ECC188 are because of direct inhibition of Ole1p, as neither was tested for direct inhibition of the Δ9 desaturase activity *in vitro*.

**Fatty Acid Biosynthesis in C. albicans**—Fatty acids are a fundamental constituent of the cellular membrane systems that are involved in diverse biological processes. Their biosynthesis is energy-consuming and, thus, subject to elaborate regulation in
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(9) our results provide further evidence for the conservation of such regulatory scheme.

OLE1 repression was partially suppressed by 16:1 and further enhanced by 18:1, the latter of which did not suppress by itself but impaired severely the \( pTET-OLE1 \) strain under the non-repressing conditions (Fig. 2C). 18:0 had a similar but less severe deleterious effect on this strain (Fig. 2B). Our analyses indicated that the heterologous \( pTET \) drove a lower level of \( OLE1 \) expression than the native promoter. This was further confirmed by two additional lines of evidence. 1) The level of monounsaturated fatty acids in this strain (MUFA/SFA ratio = 0.75) was significantly lower than that in the control strain (MUFA/SFA = 1.40); a less significant difference was observed with polyunsaturated fatty acids (Table 2). 2) The \( pTET-OLE1 \) strain was intrinsically more susceptible to both ECC145 and ECC188 than the \( OLE1^{+/−} \) counterpart (Table 1, Fig. 3). In \( S. cerevisiae \), transcription of \( ScOLE1 \) is repressed by unsaturated fatty acids, which also destabilizes the mRNA via its 5′-untranslated region. Saturated fatty acids, on the other hand, activate transcription of \( ScOLE1 \) without affecting the RNA stability (18). Our results suggest that the ~250-base pair region immediately upstream of the \( OLE1 \) ORF is important for its regulation in response to both exogenous 18:0 and 18:1. The interplay between saturated and unsaturated fatty acids was further demonstrated by partial suppression of cerulin, an inhibitor of fatty acid synthase, by low concentrations of ECC145 and ECC188 (Fig. 6), suggesting relative growth advantage of partially restored UFA/SFA balance even when the overall fatty acid syntheses are impaired.

Mechanisms of Action of ECC145 and ECC188—Consistent with the MOA hypothesis generated in the fitness test, our results corroborate the chemical inhibition of both compounds with the genetic repression of \( OLE1 \) (Table 2, Figs. 4 and 5). However, neither compound was tested for in vitro inhibition of the \( \Delta9 \) desaturase activity (although the microsomal \( ScOle1p \) activity was tested in \( S. cerevisiae \) (26), none has been reported for \( C. albicans \)). We cannot, therefore, rule out the possibility that \( Ole1p \) is not the direct molecular target of these compounds and that their effects on unsaturated fatty acids are indirect.

In \( S. cerevisiae \), expression of \( ScOLE1 \) is exquisitely regulated by two homologous transcription factors, \( ScSpt23p \) and \( ScSpt21p \).
ScMga2p (18). The only ortholog, SPT23/orf19.1751, in C. albicans has been shown to play the same role. Its repression leads to reduction in monounsaturated fatty acids, and the resulting lethality is rescued by exogenous UFAs (28). The heterozygous deletion for SPT23 was not constructed for the CaFT; neither was the pTET strain. Nevertheless, the chemical inhibition of ECC145 and ECC188 is also in agreement with the terminal phenotypes of SPT23 repression as reported (28). It is, thus, possible that these compounds inhibit Spt23p, which in turn stalls the OLE1 expression. The chemical genetic interactions characterized here reflect the exclusive role the transcription factor in regulating OLE1.

On the other hand, the partial suppression of ECC145 (and likely ECC188) by exogenous MUFAs (Fig. 5) suggests that the antiproliferative activities are not restricted to inhibition of monounsaturated fatty acids. Although genetic repression of OLE1 predominantly reduced the level of monounsaturated fatty acids (MUFA/SFA = 0.06), both MUFAs and PUFAs were affected by the two compounds (MUFA/SFA ~ PUFA/SFA ~ 0.3, Table 2). The genes for the two polydesaturases, FAD2 (orf19.118) and FAD3 (orf19.4933), are not essential (30). The two heterozygous deletion strains displayed no significant growth variations against any of the OLE1 compounds or ECC145 in the presence of MUFAs in the fitness test (supplementary Table S1). We cannot, however, exclude a possible synergistic effect of impairing both MUFAs and PUFAs by ECC145 or ECC188 (particularly at high concentrations).

The structural variability of the putative OLE1 compounds (Fig. 1, A and B) suggests the possibility that they could be metabolized in C. albicans, which in turn generates a common product that causes the observed effects. If so, the fitness test did not ostensibly reveal this aspect of the mechanism of action. As only ~45% of the genome was represented in the current version of the fitness test (9), another possibility exists that these compounds affect another cellular process(es) which becomes synthetically lethal when chemically perturbed, with a genetic impaired unsaturated fatty acid biosynthesis (as in the case of OLE1+/- strain). If so, the potential target of these compounds could be related to the so-called secondary profiles in the fitness test (Fig. 1C and see below). We noted that the fatty acid profile of the parental strain (CAI-4), used to construct all the pTET strains, is different (Table 2). In particular, the level of monounsaturated fatty acids is significantly higher than both HIS3 (the control) and OLE1 conditional shut-off strains. It remains to be determined if genetic manipulations in CAI-4 and/or the pTET strains are responsible for changes in the biosynthesis of unsaturated fatty acids and if such changes affect the susceptibility to these compounds.

**OLE1 as an Antifungal Target**—Regardless of the exact molecular target of these compounds, our results and others (16, 28) suggest that an incomplete depletion of monounsaturated fatty acids is sufficient to block cellular proliferation. Although the results in Table 2 were not replicated biologically and, thus, must be interpreted with cautions, the fatty acid profiles resulted from ECC145 and ECC188 inhibition are in general agreement with each other. We note that the residual level of monounsaturated fatty acids was significantly higher in cells treated with either compound (at ~5× minimal inhibitory concentration for 15 h, with ~5-fold reduction in the MUFA/SFA ratio from ~1.5 to ~0.3) than in those depleted of OLE1 expression (~15-fold decrease from ~0.75 to ~0.06) (Table 2). Using a similar promoter replacement strategy but with the MET3 promoter, Krishnamurthy et al. (16) demonstrated that repression of OLE1 decreased the MUFA/SFA ratio from 0.73 to 0.34. Although differences in the MUFA/SFA ratio may reflect the promoters used for genetic repression and/or different experimental designs, the 2-fold reduction was enough to stop proliferation and block hyphal growth (16). In another study, repression of STP23 (from the MAL2 promoter) led to marginal, but statistically significant changes in the level of MUFAs and growth inhibition (28). Consistent with these results, the MUFA/SFA ratio of the pTET-OLE1 strain (under the non-repressing conditions) was ~50% that of the control strain (Table 2), its growth was impaired (Fig. 2B), and the ability to develop true hyphae was compromised (Fig. 4C). This shows the importance of balancing saturated and unsaturated fatty acids for proliferation, hyphal growth, and other cellular processes.

Although the OLE1 function can be effectively bypassed by the exogenous MUFAs in vitro (Fig. 2C), it is nonetheless essential in a murine model of systemic candidiasis (Fig. 8, C and D) even when the animals were fed with diet containing high MUFAs (Fig. 8, E and F). The OLE1 ortholog in Aspergillus fumigatus is also essential for growth. Indeed, three genes involved in fatty acid biosynthesis, FAS1, FAS2 (Fig. 8, A and B), and OLE1, all appear to be attractive antifungal targets. The fungal fatty acid desaturases contain a cytochrome b6 domain fused to the carboxyl terminus, which is absent in the mammalian homologs (18). However, the desaturase domain is highly conserved (but sufficiently diverged) in eukaryotes. If the hypothesis is true that incomplete depletion of MUFAs (e.g. partial inhibition of Ole1p) is sufficient to prevent cellular proliferation, potential inhibitors of Ole1p need not be enzymatically potent enough to exert antiproliferative activity. The apparent gap between enzymatic and antiproliferative potencies may provide much-needed space for medicinal chemical optimization of Ole1p inhibitors for selectivity (fungi versus humans) and broad antifungal specificity. Moreover, the balance between saturated and unsaturated fatty acids is critical to hyphal development (Ref. 16, Fig. 4). The combination of antiproliferative and anti-hyphal properties makes Ole1p/UFAs inhibitors ideal antifungal compounds, as the ability of C. albicans to opt for hyphal growth in vivo is one of the virulence factors (31).

**Chemical Genetics in C. albicans**—The natural diploidy and lack of a meiosis-based sexual life cycle has hindered the direct application of conventional genetic approaches in the fungal pathogen. Alternative strategies have been successfully developed in C. albicans (3). Although it is possible to study genetic interactions between genes (for example, see Refs. 4 and 32), forward genetics is cumbersome in the pathogen. Target-specific antiproliferative compounds may circumvent this difficulty and address genetic interactions by chemical genetic means. As demonstrated with characterized compounds, fitness test profiling reveals not only the targets and/or aspects of mechanisms of action but also biology of the targets and their
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interactions with other processes (9). In this study the fitness test profiles of the OLE1 compounds (Fig. 1C) contain additional genes whose genetic interactions with ECC145 and ECC188 were validated using the conditional shut-off strains (Fig. 7). Their interactions with ECC145 are directly related to inhibition of monounsaturated fatty acids, as all the hypersensitive heterozygous deletion strains were suppressed by the exogenous MUFAs (Fig. 1C, 145/MUFAs). These chemical genetic interactions reflect aspects of the fatty acid desaturase that are known and reveal additional biological processes involved in or connected to the Ole1p and/or Spt23p function and the unsaturated fatty acid biosynthesis.

The hypersensitivity of heterozygous deletion strains for the four subunits of the SRP and one of its receptors (Fig. 1C) suggests the SRP is involved in translocating the nascent Ole1p peptide to the lumen of the ER, where the enzyme normally resides, and/or Spt23p, which is likely ER-bound and activated by ubiquitin-dependent ER-associated degradation (18). The hypersensitivity of repression of UBP1 (encoding an ubiquitin-specific protease) to ECC145 and ECC188 (Fig. 7) is an indication of the role of ubiquitin involved in activation of Spt23p and/or the unsaturated fatty acid biosynthesis in the pathogen. The hypersensitivity and resistance of heterozygous deletion strains for genes involved in gene expression (Fig. 1C) may shed light on other aspects of the transcriptional regulation of OLE1. Similarly, the hypersensitivity of repression of SM11 may reflect the functional connection between fatty acids and cell wall organization and/or biogenesis. The link between fatty acid synthesis and fat storage is suggested by the hypersensitivity of repression of orf19.5291, as its orthologs have been demonstrated (33). On the other hand, the genetic interaction between OLE1 and PHO88 remains to be elucidated. However, one interesting possibility is the connection between Ole1p and the mannosyltransferase complex, as ScOle1p and ScPho88p are constituents of the complex in S. cerevisiae.

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