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Squalene is lipotoxic to yeast cells defective in lipid droplet biogenesis

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ABSTRACT

The toxic effect of overloaded lipids on cell physiology and viability was described in various organisms. In this study we focused on the potential lipotoxicity of squalene, a linear triterpene synthesized in eukaryotic cells as an intermediate in sterol biosynthesis. Squalene toxicity was studied in the yeast *Saccharomyces cerevisiae*, a model unicellular eukaryote established in lipotoxicity studies. Squalene levels in yeast are typically low but its accumulation can be induced under specific conditions, e.g. by inhibition of squalene monooxygenase with the antimycotic terbinafine. At higher levels squalene is stored in lipid droplets. We demonstrated that low doses of terbinafine caused severe impairment of growth and loss of viability of the yeast mutant *dga1Δ lro1Δ are1Δ are2Δ* unable to form lipid droplets and that these defects were linked to squalene accumulation. The hypersensitivity of the lipid droplet-less mutant to terbinafine was alleviated by decreasing squalene accumulation with low doses of squalene synthase inhibitor zaragozic acid. Our results proved that accumulated squalene is lipotoxic to yeast cells if it cannot be efficiently sequestered in lipid droplets. This supports the hypothesis about the role of squalene in the fungicidal activity of terbinafine. Squalene toxicity may represent also a limiting factor for production of this high-value lipid in yeast.

1. Introduction

Lipotoxicity is generally defined as the deleterious effect of lipid overload on cellular functions and viability. Such effects have been described for free fatty acids (FA) [1], cholesterol [2], sphingolipids and their derivatives [3] or diacylglycerols [4]. The interest in lipotoxicity was stimulated by the involvement of this phenomenon in the pathogenesis of several human morbidities, e.g. type 2 diabetes, cardiovascular diseases, non-alcoholic hepatosteatosis, cardiomyopathy and metabolic syndrome [5]. The mechanisms of lipotoxicity include mitochondrial damage and production of reactive oxygen species [6,7], impaired ER functions [8,9] or disturbed intracellular signaling [10]. The final outcome of severe lipotoxicity in humans is usually cellular death resulting in organ dysfunction [11].

Toxic effects of accumulated lipids are manifested also in the unicellular eukaryote *Saccharomyces cerevisiae*. Mechanisms of lipid homeostasis are very similar in *S. cerevisiae* and humans and therefore it is not surprising that *S. cerevisiae* was utilized as a model to elucidate lipotoxicity on molecular and cellular levels [12]. Lipotoxicity studies in yeast were focused mostly on the toxic effect of free FA. Excessive FA are efficiently sequestered in lipid droplets (LD) in the form of triacylglycerols (TAG) and steryl esters (SE) in *S. cerevisiae*. The *dga1Δ lro1Δ are1Δ are2Δ* quadruple mutant deficient in four major acyltransferases involved in the synthesis of TAG (Dga1p, Lro1p) and SE (Are1p, Are2p) is viable but unable to form lipid droplets (LDs) [13]. Several studies showed that this LD-less mutant is sensitive to exogenous unsaturated FA [14–17]. Similarly to human cells, the effects induced by exogenous FA in LD-less yeast include production of reactive oxygen species [14], induction of unfolded protein response in the ER [14,15], and induction apoptotic cell death [14].

In this report we investigated possible toxicity of squalene in the yeast *S. cerevisiae*. Squalene is an important lipidic metabolite with many medical and industrial applications. In eukaryotic organisms it is synthesized from acyl-CoA in the mevalonate pathway as the first precursor dedicated to sterol synthesis [18]. Intracellular levels of squalene are usually low due to its rapid conversion to oxidosqualene in an oxygen-dependent reaction. In multicellular organisms, high levels of squalene were observed in some tissues where it fulfills specific functions, e.g. human skin [19] or liver of

Abbreviations: CFU, colony forming units; ER, endoplasmic reticulum; FA, fatty acids; IC_{50}, concentration causing 50% inhibition of growth; LD, lipid droplet; SE, steryl esters; TAG, triacylglycerols; YPD, yeast extract/peptone/dextrose.

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deep-sea sharks [20]. In yeast, increased squalene was found in hypoxic or hem-deficient cells [21–24]. In addition, high amounts of squalene are accumulating in fungi treated with the antimycotic terbinafine, a specific inhibitor of squalene monooxygenase. Ryder [25] suggested that accumulation of squalene may contribute to the fungicidal effect of terbinafine, however, this hypothesis was not experimentally verified. Since squalene is widely used as food supplement, in cosmetics and in pharmacology (reviewed in Ref. [18]), its toxicity may have significant implications also for human subjects. In addition, yeast is a perspective source for industrial production of squalene which makes squalene toxicity a biotechnologically relevant issue.

2. Materials and methods

2.1. Reagents

Terbinafine, zaragozic acid, ergosterol and squalene were obtained from Sigma–Aldrich (Germany). Yeast extract, peptone and agar were from Becton Dickinson (USA). Organic solvents (HPLC grade) were from Merck (Germany). Other chemicals of the highest purity available were purchased from various suppliers.

2.2. Strains and growth conditions

S. cerevisiae strain W303-1B (*α ade2-1 ura3-52 trp1-1 leu2-3,112 his3-11,15 can1-100*) and its LD-less derivative mutant (*α ade2-1 ura3-52 trp1-1 leu2-3,112 his3-11,15 can1-100 dga1Δ lro1Δ are1Δ are2Δ*) [13] were obtained from Scan Bi Ltd. (Alnarp, Sweden). Strains were grown aerobically in liquid YPD media (1% yeast extract, 2% peptone, 2% glucose) on rotatory shaker at 28 °C. Where indicated, media were supplemented with inhibitors terbinafine (stock solution 2 mg/mL in DMSO) and zaragozic acid (stock solution 5 mg/mL in water). Culture growth was determined by counting in Bürker chamber and expressed as the number of generations (population doublings) according to the formula:

$$\text{number of generations} = [\log(N_{24}/N_0]/\log(2)$$

where N_0 is the initial cell count (time = 0 h) and N_{24} is the final cell count (time = 24 h).

2.3. Inhibitor susceptibility assays

Sensitivity to inhibitors (terbinafine and/or zaragozic acid) on agar plates was determined by serial dilution spot assay. Cells grown overnight at 28 °C were counted in Bürker chamber, washed and the cell density was adjusted to 2×10^6 cells/mL. Drops of 10-fold serial dilutions (corresponding to 10^4-10^3-10^2-10^1 cells/drop) were spotted on YPD plates containing indicated concentrations of inhibitors. Growth was evaluated after 3–5 days of incubation at 28 °C.

Growth sensitivity in liquid media was estimated as the decrease of the number of generations after 24 h of cultivation in YPD media with indicated concentrations of inhibitors. IC_{50} was determined as the concentration of terbinafine reducing the number of generations to 50% of the untreated control.

Viability was estimated as colony forming units (CFU) in cultures cultivated for 24 h in YPD media with corresponding inhibitors. Cells were washed in sterile water and counted in Bürker chamber. Washed cells were diluted in sterile water to the density of 1000 cells/mL and 100 and 200 cell aliquots were plated on YPD agar plates. Number of colonies was determined after 3 days of incubation at 28 °C.

2.4. Quantitative analysis of sterols and squalene as non-saponifiable lipids

Cells cultivated in liquid media for 24 h were washed and disrupted in FastPrep 24 instrument (MP Biomedicals) (glass beads diameter 0.4 mm, disruption 2 × 45 s at 6 m/s speed with 5 min cooling on ice between the runs). Cell homogenate was incubated in 3 mL of 60% KOH (w/v) – 50% methanol (v/v) for 2 h at 70 °C. Non-saponifiable lipids were extracted twice with 3 mL of n-hexane, combined extracts were dried under nitrogen. Lipid residue was dissolved in acetone and sterols were analyzed by HPLC on Agilent 1100 instrument equipped with Eclipse XDB-C8 column, diode array detector (Agilent Technologies, USA), and Corona Charged Aerosol Detector (ESA Inc., USA). Sterols were eluted with 95% methanol (flow rate of 1 mL/min, temperature 30 °C). Individual peaks were identified by retention times of ergosterol and squalene standards and verified by their UV spectra. The quantity of sterols was calculated from the output of the Corona CAD detector using calibration curves constructed for ergosterol and squalene standards.

3. Results

3.1. Yeast mutant lacking lipid droplets is hypersensitive to terbinafine

S. cerevisiae quadruple mutant strain carrying deletions of genes encoding four acyltransferases (*dga1Δ lro1Δ are1Δ are2Δ*) is unable to form lipid droplets (LDs) [13]. This LD-less strain cannot sequester excess FA in TAG or SE and is thus sensitive to exogenous unsaturated FA [14–17]. LDs are also the site of squalene storage in human adipose cells [26] or yeast *hem1* mutants [22,23]. The relation between squalene and LDs is indicated also by increased cellular content of LDs in terbinafine-treated yeast cells accumulating high levels of squalene [22,27,28]. Interestingly, *dga1Δ lro1Δ are1Δ are2Δ* strain lacking LDs showed increased sensitivity to terbinafine, an inhibitor of squalene monooxygenase [29]. This terbinafine hypersensitivity might reflect lipotoxicity of accumulating squalene in cells with compromised lipid storage. We therefore analyzed the effect of terbinafine on the LD-less strain in more detail.

The serial dilution spot assay demostrated terbinafine hypersensitivity of the LD-less strain (Fig. 1A) which was confirmed also in liquid media (Fig. 1B, Supplementary Fig. S1). Wild type cells responded to terbinafine treatment by a gradual decline in the number of generations in 24 h with 4 generations at 15 μg/mL terbinafine compared to 8 generations in control culture. On the other hand LD-less strain showed an abrupt loss of the growth ability at terbinafine concentrations over 1 μg/mL with only one generation in 24 h at 3 μg/mL of terbinafine. There was about tenfold difference between both strains in the IC_{50} of terbinafine calculated from the number of generations (15 μg/mL and 1.3 μg/mL for wild-type and LD-less strain, respectively). The growth curves (Fig. S1) revealed no significant lag phase in control and terbinafine-treated cultures. Interestingly, terbinafine treatment did not affect the growth rate of wild-type or LD-less cells during the initial 3–4 h and the concentration-dependent inhibitory effect of terbinafine was evident in both strains only after 4 h of growth (Supplementary Table I). This indicates that terbinafine-induced defects accumulated gradually in cells treated with subinhibitory concentrations of terbinafine.

The viability estimated as CFU (Fig. 1C) corresponded well with the growth ability in both strains. Wild type strain started to lose viability at 10 μg/mL of terbinafine and significant viability (about 34%) was retained even at the highest terbinafine concentration

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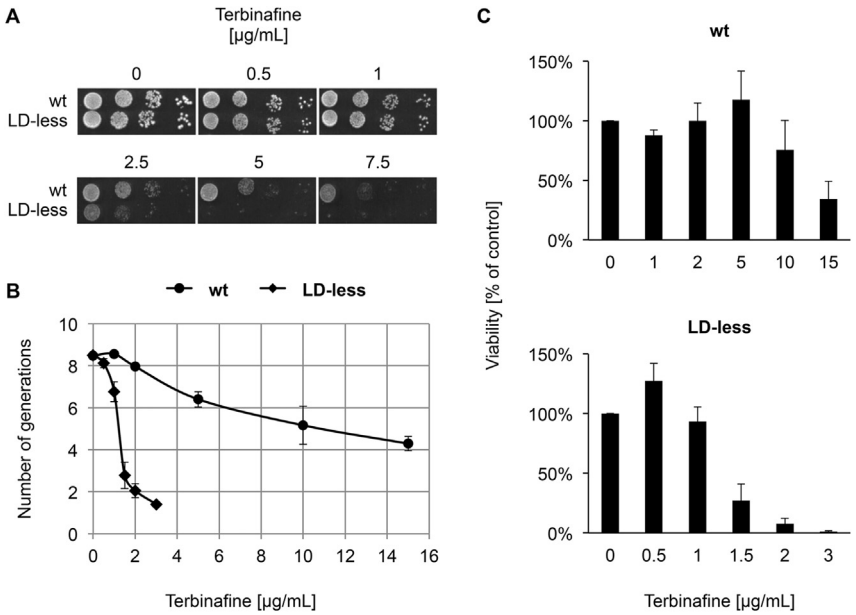

Fig. 1. Hypersensitivity of the LD-less *dga1Δ lro1Δ are1Δ are1* strain to terbinafine. A – Terbinafine sensitivity in serial dilution spot assay. W303-1B strain and its LD-less derivative were diluted and spotted on terbinafine-containing YPD agar plates as described in Material and Methods. Growth was evaluated after 3 days of incubation at 28 °C. B – Terbinafine sensitivity in liquid YPD medium. The number of generations after 24 h growth in liquid YPD media containing indicated concentrations of terbinafine was evaluated as described in Material and Methods. Data points represent the mean ± s.e.m. of 3 independent experiments. Symbols: ● – wild type strain; ◆ – LD-less strain. C – Viability of terbinafine-treated cells. Viability of cells grown for 24 h in YPD media containing indicated concentrations of terbinafine was estimated as colony-forming units (CFU) according to Materials and Methods. CFU in variants treated by terbinafine was normalized to the values of untreated controls. Values represent the mean ± s.e.m. of 3 independent experiments.

tested (15 μg/mL). The LD-less strain showed a rapid loss of viability at terbinafine concentrations over 1 μg/mL with negligible viability (1.3%) at 3 μg/mL.

3.2. Squalene accumulation is causing terbinafine hypersensitivity of LD-less mutant

Terbinafine is a specific inhibitor of ergosterol biosynthesis at the squalene epoxidation step and its antifungal activity may include ergosterol depletion and accumulation of squalene [25]. To distinguish between these two effects we analyzed ergosterol and squalene content in terbinafine-treated cells. As shown in Fig. 2, terbinafine in the tested concentration ranges did not affect significantly ergosterol levels. Reduced ergosterol levels observed in terbinafine-treated wild type strain reflects the consumption of ergosteryl esters in cells with partial inhibition of squalene monooxygenase activity [22]. Ergosterol levels in LD-less cells at 2 and 3 μg/mL terbinafine (where cell growth and viability were severely affected, see Fig. 1) were comparable to those in untreated control. This result excludes ergosterol depletion as the cause of terbinafine hypersensitivity in LD-less cells. Accumulation of squalene with increasing terbinafine concentrations was observed in both strains. Wild type cells able to store squalene in LDs tolerated high squalene levels (up to 400 μg/10^9 cells at 10 μg/mL of terbinafine) without major effects on growth and viability. On the other hand, LD-less cells showed severe growth defects and significant loss of viability starting at 40–50 μg of squalene per 10^9 cells. This strongly indicates that accumulated squalene is toxic to yeast cells if it cannot be efficiently sequestered in LDs.

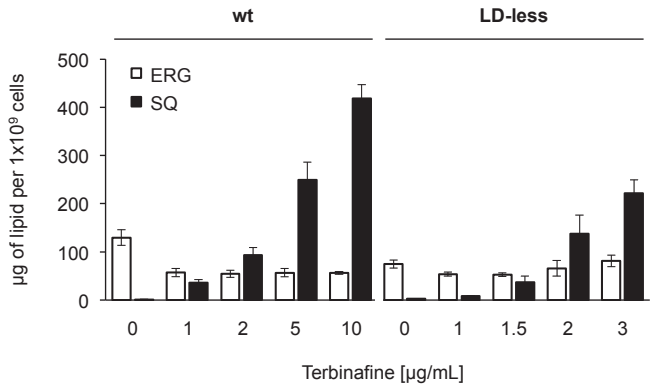

Fig. 2. The response of cellular ergosterol and squalene levels to terbinafine treatment. Wild type and LD-less strains were grown for 24 h in YPD with indicated concentrations of terbinafine. Non-saponifiable lipids were extracted and ergosterol and squalene were quantified by HPLC as described in Material and Methods. Values represent the mean ± s.e.m. of 3 independent experiments. Empty bars – ergosterol, black bars – squalene.

3.3. Co-treatment with zaragozic acid rescues terbinafine hypersensitivity of LD-less mutant

To verify the lipotoxicity of high squalene in LD-less *dga1Δ lro1Δ are1Δ are2Δ* strain we attempted to manipulate squalene levels by reducing squalene production in the mevalonate pathway. We expected that partial inhibition of squalene synthase activity with low concentrations of the specific inhibitor zaragozic acid [30] would counteract squalene accumulation in terbinafine-treated cells. We tested therefore the effect of zaragozic acid on terbinafine sensitivity of the LD-less cells in serial dilution spot assay on agar plates (Fig. 3A). We used terbinafine concentrations significantly inhibiting the growth of LD-less strain (2, 2.5 and 3 μg/mL) and zaragozic acid concentrations showing no effect on growth in both wild type and LD-less strain (0.5 and 2 μg/mL). In agreement with our hypothesis, zaragozic acid at 2 μg/mL improved the ability of LD-less cells to form colonies at all tested terbinafine concentrations. This rescuing effect was particularly evident at 3 μg/mL of terbinafine causing complete growth arrest in the absence of zaragozic acid. In

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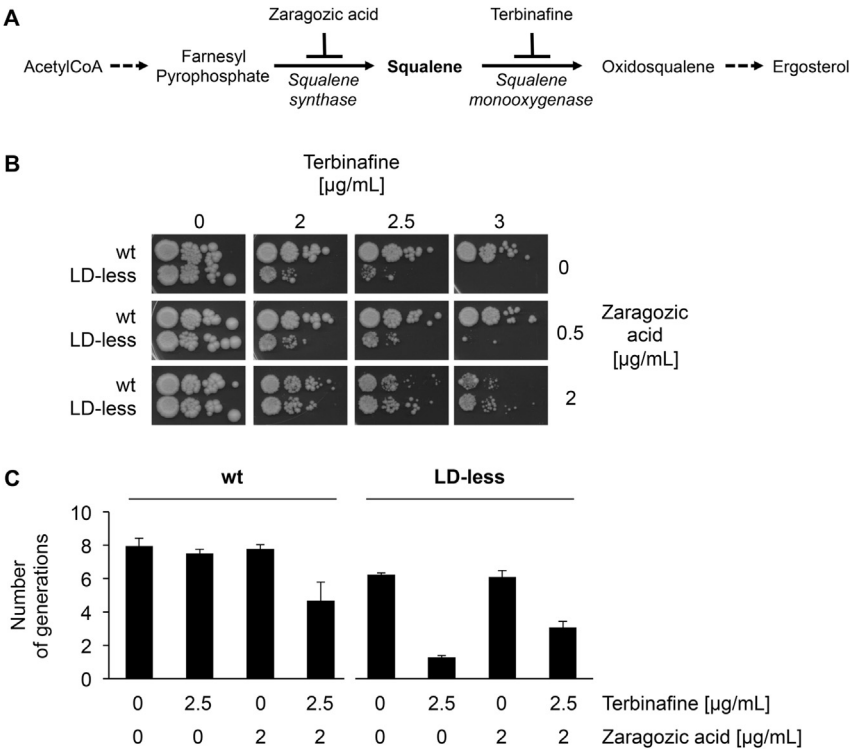

Fig. 3. Subinhibitory concentrations of zaragozic acid rescued the growth of terbinafine-treated LD-less cells. A – Sites of ergosterol biosynthesis inhibition by zaragozic acid and terbinafine. B – Growth sensitivity to zaragozic acid and terbinafine in serial dilution spot assay. W303-1B strain and its LD-less derivate were serially diluted and spotted on the YPD agar plates containing indicated concentrations of inhibitors as described in Material and Methods. Growth was evaluated after 5 days of incubation at 28 °C. C – Growth sensitivity to zaragozic acid and terbinafine in liquid YPD medium. The growth of both strains in liquid YPD media containing indicated concentrations of inhibitors was evaluated after 24 h as described in Material and Methods. Values represent the mean ± s.e.m. of 3 independent experiments.

contrast to the effect on the LD-less cells, the combination of subinhibitory concentrations 2.5 or 3 μg/mL of terbinafine and 2 μg/mL of zaragozic acid reduced the growth of wild type cells indicating a synergic effect after simultaneous inhibition of ergosterol biosynthesis at two sites.

The result of the serial dilution spot assay were confirmed in growth experiments in liquid media (Fig. 3B, Supplementary Fig. S2 and Table II). The growth of wild-type and LD-less cells was not affected by single treatment with 2 μg/mL of zaragozic acid. Terbinafine at 2.5 μg/mL reduced the duplication rate in the period 4–12 h (supplemetary Table II), but the final number of generations after 24 h was similar as in the control cells (Fig. 3C). In LD-less cells, this concentration of terbinafine showed a very strong inhibitory effect both on the growth kinetics (Supplementary material Fig. S2B and Table II) and on the number of generations (Fig. 3C). Both strains responded differently to the inhibition of squalene synthase in terbinafine-treated cells. Zaragozic acid aggravated the growth defects in terbinafine-treated wild-type cells which was demonstrated as reduced number of generations after 24 h. On the other hand, severe growth inhibition in LD-less mutant treated by terbinafine was partially rescued by zaragozic acid both with respect to final number of generations (Fig. 3C) and the growth kinetics (Supplementary Fig. S2B and Table II).

3.4. The rescuing effect of zaragozic acid on terbinafine hypersensitivity is mediated by reduced squalene in LD-less mutant

The mechanism of the rescuing effect of zaragozic acid in terbinafine-treated *dga1Δ lro1Δ are1Δ are2Δ* strain was analyzed by quantification of ergosterol and squalene levels (Fig. 4). Treatment by 2.5 μg/mL of terbinafine increased squalene levels to about 200 μg/10^9 cells both in wild-type and LD-less strains. Partial inhibition of squalene synthase by 2 μg/mL of zaragozic acid reduced squalene levels in terbinafine-treated cells by 33% in wild type strain and by 70% in LD-less strain. Squalene levels in the LD-less cells treated by the combination of zaragozic acid and terbinafine (64 ± 5 μg/10^9 cells) were compatible with partial growth observed under these conditions (about 3 generations, Fig. 3C). These results thus reveal a direct relationship between squalene accumulation and defects in growth and viability in yeast cells deficient in the formation of LDs.

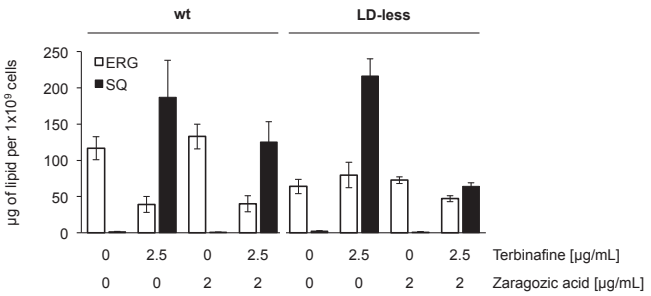

Fig. 4. Subinhibitory concentrations of zaragozic acid reduced squalene levels in terbinafine-treated cells. Wild type and LD-less strains were grown for 24 h in YPD with indicated concentrations of inhibitors. Non-saponifiable lipids were extracted and the ergosterol and squalene were quantified by HPLC as described in Material and Methods. Values represent the mean ± s.e.m. of 3 independent experiments. Empty bars – ergosterol, black bars – squalene.

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4. Discussion

Toxic effect of lipid overload has been described for many lipids, however, the possible lipotoxic effect of squalene escaped the attention so far. The strongest indication of a detrimental effect of squalene was its relation to the fungicidal effect of the antimycotic terbinafine in the dermatophyte *Trichophyton* suggested by Ryder [25]. Under physiological conditions eukaryotic cells rapidly metabolize squalene to sterols. Its levels in human tissues are thus generally very low [19]. However, increase in cellular squalene concentration was described under specific pathological conditions. For example, squalene accumulation in arthritogenic cells residing in lymph nodes was observed after transdermal injection of squalene as adjuvant [31]. Moreover, squalene accumulation was suggested as the cause of encephaloneuropathy in rats after tellurium administration [32].

Our idea to test possible lipotoxicity of squalene in yeast was based on three observations: 1) high accumulation of squalene can be induced in yeast by treatment with squalene monooxygenase inhibitor terbinafine [22,25], 2) yeast cells store accumulated squalene in LDs [23], and 3) *dga1Δ lro1Δ are1Δ are2Δ* mutant unable to form LDs shows increased sensitivity to terbinafine [29]. In this report we showed that LD-less cells have severely impaired growth and viability at 10-fold lower terbinafine concentrations than the wild-type cells. Lipid analyses indicated that this detrimental effect of terbinafine was linked to squalene accumulation and not to ergosterol depletion of LD-less cells. The proof of squalene lipotoxicity in LD-less cells was provided by the effect of low doses of squalene synthase inhibitor zaragozic acid. Partial inhibition of squalene synthesis by zaragozic acid resulted in a similar reduction of squalene levels in terbinafine-treated wild type and LD-less cells, however, the growth response of these two strains to zaragozic acid was strikingly different. The number of generations reached after 24 h cultivation of wild-type cells treated simultaneously by zaragozic acid and terbinafine was reduced by almost 50% compared to single-drug treatments. On the contrary, inhibition of squalene synthesis partially rescued the growth of terbinafine-treated LD-less cells. This indicates that squalene levels were reduced by zaragozic acid treatment below the level critical for lipid storage deficient cells.

The intracellular target of squalene lipotoxicity is not clear yet. Spanova et al. [23,33] reported that squalene accumulating in LD-less *hem1Δ dga1Δ lro1Δ are1Δ are2Δ* strain is deposited in ER, mitochondria and plasma membrane. LD-less cells show altered lipid composition, *e.g.* increased content of saturated FA in LD-less cells [13,17,33] or reduced ergosterol level in the plasma membrane [23,29,33] which can make these membranes more susceptible to squalene. LD-less *hem1Δ* mutants showed normal growth in these experiments, however, squalene accumulation caused by hem deficiency is relatively low and toxic levels of squalene might not be reached here [22].

Our results showed that accumulated squalene is toxic to yeast mutant deficient in LD biogenesis. Extreme concentrations of squalene may be, however, detrimental also in cells with functional LDs. We have recently demonstrated that wild type yeast accumulated squalene up to a limiting value of about 700 μg/10^9 cells when treated with high terbinafine concentrations (30–50 μg/mL) severely disturbing cell growth [22]. These results indicated that the storage capacity of LDs has been exceeded and further accumulation of squalene impaired the viability of yeast cells. Similarly, yeast strains engineered to accumulate high levels of squalene by overexpression of HMG-CoA reductase showed poor growth which might be related to toxicity of accumulated squalene [34,35].

In conclusion, we showed here that high squalene is toxic to yeast cells if it cannot be efficiently stored in lipid droplets. This lipotoxicity was demonstrated as compromised growth and loss of viability of LD-less mutant treated with low concentrations of squalene monooxygenase inhibitor terbinafine. Hypersensitivity of LD-less mutant to terbinafine was alleviated by sub-inhibitory doses of squalene synthase inhibitor zaragozic acid that counteracted squalene accumulation induced by terbinafine. Our results support the idea that squalene accumulation is an important factor in the fungicidal activity of terbinafine. Toxicity of squalene at levels exceeding the storage capacity of LDs may also limit the maximum levels of squalene that can be reached during biotechnological production in yeast. Further studies of the effects of squalene accumulation on cellular functions in LD-less strain are in progress in our laboratory.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.12.050.

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