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LETTERS

Remodeling of membrane lipid homeostasis in azole resistant isolates of *Candida albicans*

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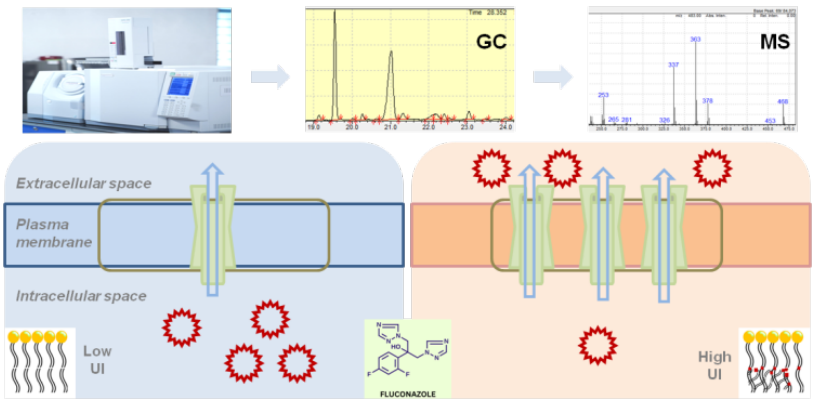
Research Article

ABSTRACT

Azole resistance mechanisms in *Candida albicans* infections majorly focus around the alteration of target enzymes, overexpression of efflux pump proteins, and changes in lipid metabolism. Our earlier lipidomic studies have linked changes in cellular lipid compositions to drug susceptibilities and phenotypic defects. This study investigates the relationship between whole cell and membrane lipid profiles in isogenic drug-susceptible and resistant isolates of *C. albicans*. We have examined the fatty acid and sterol snapshot lipidomics in whole cells, plasma membrane, and lipid rafts. Correlations were discovered between these lipid compositions and the observed drug resistance in *C. albicans*. Although the correlations drawn from cellular and plasma membrane data corroborate, understanding plasma membrane and suborganellar (rafts in this case) lipid changes may provide better insights into their roles in efflux pump activities and localization, and drug susceptibilities.



Keywords: Candida albicans, lipids, azole resistance, gas chromatography, mass spectrometry

INTRODUCTION

Candida infections pose a serious threat to human population in clinics, and have found their place in WHO fungal priority pathogens list (WHO FPPL).[1] *Candida* infections range from superficial, oro/oeso -pharyngeal, gastrointestinal and vulvovaginal.[1] Majority of these fungal infections are secondary in nature and prevalent in patients with pulmonary disorders, immunodeficiency, chemotherapy, burns, diabetes, etc.[2] Invasive candidiasis patients show mortality rates as high as 50%.[1] The annual global recurrence of vulvovaginal candidiasis alone is over 138 million with an estimated annual loss of US $14 billion by 2030.[3] In India, over 5.7 million patients suffer from a severe fungal illness, where *Candida* driven infections show a high prevalence of relapse.[4] Among *Candida* spp., *C. albicans* is the major cause of observed candidiasis in clinics. The available treatment options like azoles and echinocandins are becoming largely ineffective due to rise in resistance to these antifungals.[5]

Antifungal resistance poses a major threat towards the treatment of *C. albicans* infections. The main mechanisms attributed to drug resistance in *C. albicans* include: (i) alteration of target enzyme (14-α-demethylase, ERG11), preventing the binding of azoles; (ii) overexpression of MFS (Major Facilitator Superfamily, Mdr1) and ABC (ATP Binding Cassette, Cdr1/2) efflux pump proteins. Cdr1/2 proteins are natural lipid translocases and Mdr1 is H^+ antiporter, localized within the plasma membrane (PM), but these can also recognize drugs as their substrates; (iii) overexpression of ERG11, among others. The reported drug resistant mechanisms are directly or indirectly linked to alterations in lipid metabolism of *C. albcians*. Studies using genetic approaches show that any compromise in the lipid homeostasis can alter the drug resistance phenotype of *C. albcians*.[6,7] Even compositional lipid variations and distributional variations in membrane lipids can affect the drug resistance and virulence phenotypes in *C. albcians*.[6,7]

In our previous studies, using lipidomic platforms, we were able to establish the lipid composition profile of *Candida* spp., and that specific changes in lipids (along with the molecular species) can be correlated with drug susceptibilities and defects in cell wall and mitochondria.[8-12] Our group along with others has speculated that changes in plasma membrane lipid homeostasis

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can alter efflux pump activities and localization, and drug diffusion rates, thereby impacting the drug susceptibilities in *C. albicans*.[13,14] While majority of lipid analysis have been performed using total cellular lipid extracts, we wondered whether the cellular lipid changes observed are preserved in the PM. It is possible that the PM lipids might show a completely different lipid profile compared to cellular lipidomes, thereby completely changing the way in which have been explaining the drug susceptibilities of *C. albicans*.

In order to confirm the relationship between the whole cell and PM lipids, in the present study we have performed a snapshot fatty acid and sterol profile of lipids extracted from the whole cell, PM and lipid rafts (i.e. DRM or Detergent Resistant Membrane) in drug susceptible and resistant isolates of *C. albicans*. We found some interesting correlations between these lipid compositions to the observed drug resistance in *C. albicans*, which are presented in this study.

EXPERIMENTAL PROTOCOLS

Strains and culture conditions: *C. albicans* strains used in this study include: AS/AR matched pair clinical isolate Gu4/Gu5, where Cdr1 is overexpressed in Gu5; AS/AR matched pair clinical isolates G2/G5 and F2/F5, where Mdr1 is overexpressed in G5 and F5; laboratory azole adopted strains YOI-16, YOI-32, YOI-64, where Cdr1 is overexpressed in YOI-32 and YOI-64.[9,11] Cells were cultured on YPD (1% yeast extract, 2% glucose, and 2% bactopeptone) agar plates (HiMedia, India) at 30ºC. For harvesting cells for lipid extraction, ~10^6 cells/mL were inoculated into 50mL YPD broth and grown up to exponential phase (~14h). Growth of the cells was monitored by measuring the optical density (O.D) at 600 nm using a spectrophotometer plate reader (Varioskan® Flash-3001, Thermo Scientific). Cells were washed thrice with sterile water prior to further processing.

Preparation of membrane fractions: Plasma membrane fractions were prepared as described earlier.[15-17] Briefly, cells were homogenized in 50 mM Tris buffer (pH 7.5) containing 2.5 mM EDTA, 150 mM NaCl, and protease inhibitors (aprotinin, pepstatin A, leupeptin and phenylmethylsulfonyl fluoride) [TNE buffer]. Unbroken cells were removed and crude membrane (CM) was recovered by centrifugation at 1000 g for 10 min. CM was pelleted at 100000g for 1 h and resuspended in 50 mM Tris buffer (pH 7.5) containing 0.5 mM EDTA and 10% glycerol. Plasma membrane fractions could be recovered from the extracted CM by sucrose density gradient ultracentrifugation (Beckman SW28 rotor) at 100000g for 5 h, as described earlier.[15] For isolation of DRM (Detergent Resistant Membrane) fractions, resuspended CM fraction was mixed with Triton X-100 and incubated for 30 min at 4ºC. DRM fractions were recovered by resolving CM fractions on Optiprep (Sigma) density gradient ultracentrifugation (Beckman TLS55 rotor) at 259,000 × g for 2 h, as described earlier.[15] Six 1 mL fractions were collected in separate tubes. Each fraction was diluted with 4 volumes of TNE buffer and pelleted at 100000g (Beckman TLS55 rotor) for 2 h. Protein concentrations of different fractions were determined using bicinchoninic acid assay.[16] Purity of plasma membrane and DRM fractions was confirmed using Western Blot analysis as described earlier.[17] Membrane fractions were probed using anti-Pma1 antibody and detected using horseradish peroxidase (HRP) - labelled secondary antibody and HRP substrate for chemiluminescence (ECL kit; Amersham).[15,17]

Lipid extraction and analysis: Extraction of lipids from cells required breaking using glass beads (Glaperlon, 0.4–0.6 mm) in methanol and extraction into a final ratio of chloroform: methanol (2:1, v/v) using the methods described earlier.[8,18] PM and DRM fractions were directly extracted using chloroform: methanol (2:1, v/v).[18] For fatty acid and sterol analysis, extracted lipids were base hydrolyzed using methanolic KOH.[19]

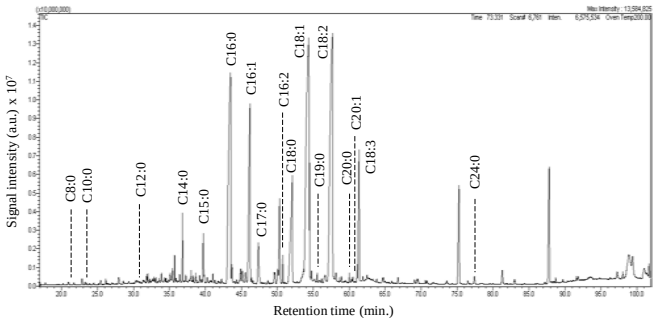

Figure 1. Representative total ion chromatogram showing fattyome of *C. albicans* obtained by GC-MS.

Prior to analysis fatty acids and sterols were derivatized using BF_3-MeOH (Sigma) and BSTFA/TMCS [N,O-Bis(trimethylsilyl) trifluoroacetamide with trimethyl-chlorosilane] (Sigma), respectively, as described earlier.[19-21] The derivatized fatty acids and sterols were then analyzed using gas chromatography-mass spectrometry (GCMS) (Shimadzu QP2010 Plus, Japan) fitted with Omegawax™ (Supelco) and Rtx™-5MS (Restek, Thermo Fisher Scientific) column respectively, using the conditions as described previously (Figure 1 and 2).[11,22,23]

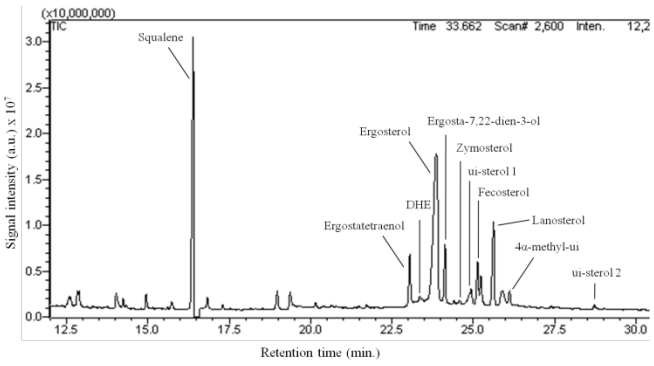

Figure 2. Representative total ion chromatogram showing sterolome of *C. albicans* obtained by GC-MS.

Peak identification was based on retention times and mass spectra comparison with external standards. FAME mix (Supelco) and purified sterols (Sigma) were used as standards.

Table 1. Fatty acid compositions from whole cell extracts of azole susceptible and resistant clinical and laboratory isolates of *C. albicans*. Values represent % of fatty acid normalized to the total fatty acid mass spectral signal. Error bars indicate ± SEM. (n = 3 for 3 independent analyses of lipid extracts from 3 independent cultures). Symbols '£' and '¥' represents that Cdr1 and Mdr1 proteins respectively, are overexpressed in these strains. '*' represents *p* value < 0.05 for comparison between each AS/AR matched pair. '†', '‡' and 'ł' represents *p* value < 0.05 for comparisons between YOI-16 versus YOI-32, YOI-16 versus YOI-64 and YOI-32 versus YOI-64, respectively.

Fatty Acid	Gu4 (AS)	Gu5 (AR)£	G2 (AS)	G5 (AR)¥	F2 (AS)	F5 (AR)¥	YOI-16 (AS)	YOI-32 (AR)£	YOI-64 (AR)£
C8:0	0.10 ± 0.04	0.04 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C10:0	0.48 ± 0.34	0.59 ± 0.43	0.01 ± 0.00	0.05 ± 0.03	2.66 ± 0.00	0.00 ± 0.00	1.01 ± 0.16	0.83 ± 0.06	1.03 ± 0.59
C12:0	0.12 ± 0.05	0.11 ± 0.06	0.00 ± 0.00	0.17 ± 0.04*	0.07 ± 0.06	0.06 ± 0.03	0.03 ± 0.00	0.02 ± 0.00 †	0.00 ± 0.00 ‡,ł
C14:0	1.21 ± 0.34	1.14 ± 0.16	0.66 ± 0.20	0.72 ± 0.05	0.66 ± 0.23	1.01 ± 0.07	2.06 ± 0.10	1.03 ± 0.16 †	1.05 ± 0.27 ‡
C15:0	1.15 ± 0.16	0.77 ± 0.17	0.37 ± 0.08	1.00 ± 0.06*	0.81 ± 0.14	0.68 ± 0.13	0.12 ± 0.04	0.12 ± 0.01	0.20 ± 0.00 ł
C16:0	16.50 ± 0.60	15.99 ± 0.70	18.75 ± 0.81	16.78 ± 0.29	13.86 ± 0.69	16.66 ± 0.39*	30.15 ± 0.21	29.97 ± 0.14	32.51 ± 1.16
C16:1	10.16 ± 0.22	8.93 ± 0.17*	7.72 ± 0.60	8.91 ± 0.20	6.15 ± 0.26	12.53 ± 0.31*	9.40 ± 0.37	9.10 ± 0.29	7.82 ± 0.85
C16:2	0.47 ± 0.11	0.53 ± 0.04	0.16 ± 0.02	0.54 ± 0.04*	0.33 ± 0.08	0.07 ± 0.00*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C17:0	1.56 ± 0.19	1.77 ± 0.01	0.66 ± 0.08	1.82 ± 0.18*	2.13 ± 0.14	0.00 ± 0.00*	0.32 ± 0.00	0.00 ± 0.00 †	0.00 ± 0.00 ‡
C18:0	7.03 ± 0.29	7.19 ± 0.52	5.06 ± 0.23	5.06 ± 0.49	6.34 ± 0.16	2.12 ± 0.25*	13.37 ± 0.12	12.17 ± 0.42	11.19 ± 0.42 ‡
C18:1	28.38 ± 0.63	32.41 ± 2.48	37.57 ± 0.28	28.56 ± 0.15*	31.64 ± 0.71	31.4 ± 0.48	28.78 ± 0.32	29.61 ± 0.26	28.61 ± 0.56
C18:2	23.51 ± 1.12	24.16 ± 0.39	24.49 ± 0.46	29.25 ± 0.5*	27.36 ± 0.24	22.18 ± 0.34*	10.93 ± 1.20	14.96 ± 0.14 †	15.29 ± 0.09 ‡
C18:3	5.65 ± 0.05	4.34 ± 0.17*	3.47 ± 0.13	5.61 ± 0.32*	5.93 ± 0.21	12.45 ± 0.28*	2.49 ± 0.11	1.97 ± 0.05 †	1.97 ± 0.01 ‡
C19:0	0.27 ± 0.03	0.22 ± 0.04	0.04 ± 0.01	0.09 ± 0.01*	0.12 ± 0.03	0.00 ± 0.00*	0.24 ± 0.00	0.00 ± 0.00 †	0.10 ± 0.00 ‡,ł
C20:0	0.14 ± 0.02	0.11 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.13 ± 0.02	0.05 ± 0.00*	0.15 ± 0.00	0.00 ± 0.00 †	0.00 ± 0.00 ‡
C20:1	0.52 ± 0.31	0.24 ± 0.15	0.23 ± 0.04	0.19 ± 0.04	0.25 ± 0.05	0.00 ± 0.00*	0.00 ± 0.00	0.00 ± 0.00 †	0.00 ± 0.00 ‡
C20:2	0.06 ± 0.04	0.13 ± 0.00	0.07 ± 0.01	0.54 ± 0.34	0.08 ± 0.00	0.13 ± 0.00	0.04 ± 0.00	0.00 ± 0.00 †	0.00 ± 0.00 ‡
C24:0	1.15 ± 0.34	0.31 ± 0.08	0.09 ± 0.01	0.10 ± 0.04	0.12 ± 0.05	0.15 ± 0.02	0.01 ± 0.00	0.04 ± 0.01 †	0.00 ± 0.00 ‡
Others	1.54 ± 0.12	0.92 ± 0.04*	0.54 ± 0.05	0.54 ± 0.09	1.10 ± 0.19	0.62 ± 0.01	0.67 ± 0.02	0.15 ± 0.06 †	0.23 ± 0.11 ‡

All data were analyzed using GCMS Solutions Software (Shimadzu), and NIST and WILEY reference mass spectral databases.

Statistical analysis: Datasets are represented as Mean ± standard error mean (SEM). *P* values < 0.05 were considered significant as determined by Student's t-test.

RESULTS

A snapshot of lipids was obtained by monitoring the fatty acid and sterol profiles using GCMS. Both cellular and membrane lipid compositions were observed.

Cellular lipid composition of drug susceptible and resistant isolates of *C. albicans*

In fatty acid analysis of cellular lipid extracts we could identify 18 or more fatty acid structures (Figure 1 and Table 1). These included fatty acyl chains ranging from 8 carbons to as long as 24 carbons, from saturated structures to structures with one or multiple double bonds. Six fatty acids were present in abundance in all tested isolates. These include C16:0 (Ranging from 13.8 – 18.7%, palmitic acid), C16:1 (Ranging from 6.1 – 12.5%, palmitoleic acid), C18:0 (Ranging from 2.1 – 7.1%, stearic acid), C18:1 (Ranging from 28.3 – 37.5%, oleic acid), C18:2 (Ranging from 22.1 – 29.2%, linoleic acid), and C18:3 (Ranging from 3.4 – 12.4%, linolenic acid). Upon comparison, we observed significant changes in the fatty acid compositions between azole susceptible and resistant strains (Table 1).

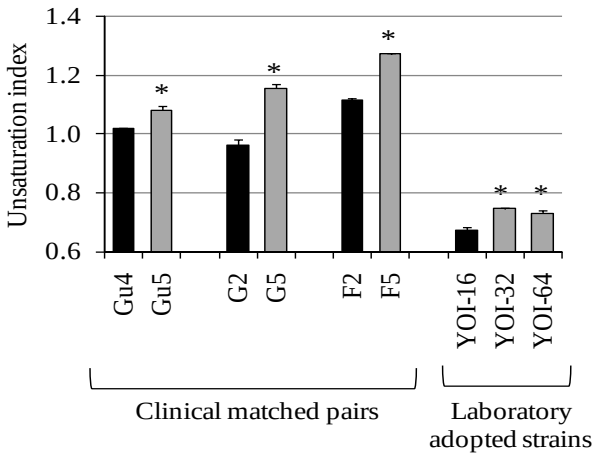

Figure 3. Unsaturation index measurements based on whole cell fattyome of various *C. albicans* isolates. Degree of unsaturation was determined by calculating the unsaturation index (UI) of the fatty acids. UI was calculated as follows: UI = [(1 x % mono-unsaturated fatty acids) + (2x % di-unsaturated fatty acids) + (3 x % tri-unsaturated fatty acids)] / 100.[10] Values are mean ± SEM of 3 independent analyses (n=3). '*' represents *p* value < 0.05 for comparison between respective AS and AR isolates.

Table 2. Sterol compositions from whole cell extracts of azole susceptible and resistant clinical and laboratory isolates of *C. albicans*. Values represent % of sterol normalized to the total sterol mass spectral signal. Error bars indicate ± SEM. (n = 3 for 3 independent analyses of lipid extracts from 3 independent cultures). Symbols '£' and '¥' represents that Cdr1 and Mdr1 proteins respectively, are overexpressed in these strains. '*' represents *p* value < 0.05 for comparison between each AS/AR matched pair. '†', '‡' and 'ǂ' represents *p* value < 0.05 for comparisons between YOI-16 versus YOI-32, YOI-16 versus YOI-64 and YOI-32 versus YOI-64, respectively. 'ui' represents unidentified sterol structure.

Sterol	Gu4 (AS)	Gu5 (AR)£	G2 (AS)	G5 (AR)¥	F2 (AS)	F5 (AR)¥	YOI-16 (AS)	YOI-32 (AR)£	YOI-64 (AR)£
Farnesol	2.62 ± 1.73	0.22 ± 0.01	4.00 ± 0.25	1.43 ± 0.61 *	1.82 ± 0.20	0.85 ± 0.47	2.67 ± 0.40	2.02 ± 1.30	1.51 ± 0.09 ‡
Squalene	0.28 ± 0.10	0.21 ± 0.04	3.92 ± 0.58	1.41 ± 0.16 *	2.34 ± 0.71	1.18 ± 0.36	1.59 ± 0.67	1.77 ± 1.33	2.43 ± 0.84
Ergostatetraenol	4.91 ± 1.42	3.02 ± 0.61	7.90 ± 0.01	5.13 ± 0.47 *	7.27 ± 0.25	4.02 ± 0.29 *	6.8 ± 0.34	7.39 ± 1.29	6.01 ± 0.50
DHE	1.99 ± 0.03	0.72 ± 0.19 *	5.41 ± 0.12	8.62 ± 1.01 *	7.00 ± 1.03	17.59 ± 1.44 *	5.82 ± 0.34	7.06 ± 0.84	7.30 ± 0.75
Ergosterol	72.52 ± 2.98	84.57 ± 2.18 *	69.64 ± 0.27	76.30 ± 0.05 *	62.03 ± 2.7	64.69 ± 0.32	71.35 ± 0.34	72.52 ± 1.91	75.29 ± 2.52
Ergosta-7,22-dien-3-ol	3.73 ± 0.15	1.55 ± 0.31 *	3.18 ± 0.41	0.92 ± 0.16 *	6.45 ± 0.32	1.46 ± 0.03 *	4.08 ± 1.47	2.89 ± 1.21	1.76 ± 0.50
Zymosterol	13.12 ± 2.44	5.48 ± 0.77 *	2.83 ± 0.25	1.65 ± 0.50	4.98 ± 0.52	1.93 ± 0.52 *	5.32 ± 0.44	2.21 ± 1.04 †	2.39 ± 1.00 ‡
ui-sterol 1	0.02 ± 0.02	2.19 ± 2.15	0.04 ± 0.00	0.31 ± 0.04 *	0.19 ± 0.01	0.53 ± 0.06 *	0.92 ± 0.19	0.99 ± 0.33	0.38 ± 0.28
Fecosterol	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lanosterol	0.11 ± 0.11	0.77 ± 0.31	0.69 ± 0.18	0.48 ± 0.21	5.33 ± 0.04	0.27 ± 0.27 *	0.53 ± 0.16	0.42 ± 0.12	0.00 ± 0.00 ‡,ǂ
4α-methyl-ui	0.69 ± 0.34	1.27 ± 0.28	2.40 ± 0.08	3.74 ± 0.20 *	2.59 ± 0.66	7.49 ± 0.06 *	0.92 ± 0.19	2.73 ± 0.86	2.93 ± 0.98
ui-sterol 2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

For example, Cdr1 overexpressed resistant strain Gu5 showed 1.14 and 1.3 fold decrease in C16:1 and C18:3 content respectively, compared to Gu4. Fatty acid changes were more prominent in Mdr1 overexpressed resistant strains. Strain G5 showed 1.3 fold decrease in C18:1, and 1.2 and 1.6 fold increase in C18:2 and C18:3 content respectively, compared to G2. Strain F5 showed 1.2 to 2.1 fold increase in C16:0, C16:1 and C18:3 contents and 1.2 to 3.0 fold decrease in C18:0 and C18:2 content, compared to F5.

Fatty acid compositional variations can provide significant insight into homeostatic changes in cell membrane fluidity.[24] Using the fatty acid compositional profiles, we determined the unsaturation index (UI) as an indicator of membrane fluidity. Upon comparison, we found that all drug resistant clinical isolates (Gu5, G5 and F5) showed a higher UI compared to drug susceptible isolates (Gu4, G2 and F2) (Figure 3).

Sterols are the key determinants of membrane fluidity. In our analysis of cellular lipid extracts, we could detect 12 different sterol pathway related structures (Table 2). These include: ergostatetraenol (Ergosta-5,7,22,24(28)-tetraen-3beta-ol), dehydroergosterol (DHE, Ergosta-5,7,9(11),22-tetraen-3beta-ol), ergosterol (ergosta-5,7,22E-trien-3beta-ol), ergosta-7,22-dien-3-ol, zymosterol (5alpha-cholesta-8,24-dien-3beta-ol), fecosterol (24-methylene-5α-cholest-8-en-3β-ol), lanosterol (lanosta-8,24-dien-3beta-ol), 4α-methyl-ui (methylated unidentified sterol), among others. Among these, ergosterol was detectable in abundance (62.0 – 84.5%) across all tested isolates. Upon comparison, we observed 1.1 – 2.9 fold increase in ergosterol, DHE and methylated sterol structures, 1.5 – 19.7 fold decrease ergostatetraenol, ergosta-7,22-dien-3-ol and methylated sterol structures, in resistant isolates, compared to susceptible isolates. Further, the ratiometric analysis showed that ergosterol/DHE ratio was higher in Gu5 compared to Gu4, and lower in G5 and F5 compared to G2 and F2. On the other hand, 4α-methyl-ui/lanosterol ratio was lower in Gu5 compared to Gu4, and higher in G5 and F5 compared to G2 and F2. Ergosterol/4α-methyl-ui ratio was invariably higher in resistant isolates.

Table 3. Fatty acid compositions from plasma membrane extracts of azole susceptible and resistant isolates of *C. albicans*. Values represent percent of fatty acid normalized to the total fatty acid mass spectral signal. Error bars indicate ± SEM. Symbols '£' represents that Cdr1 protein is overexpressed in these strains. '*' represents *p* value < 0.05 for comparison between Gu4 and Gu5 strains.

Fatty Acid	Gu4 (AS)	Gu5 (AR)
C12:0	0.00 ± 0.00	0.00 ± 0.00
C14:0	0.47 ± 0.42	0.03 ± 0.03
C15:0	0.00 ± 0.00	0.00 ± 0.00
C16:0	24.32 ± 3.26	21.35 ± 1.83
C16:1	6.98 ± 1.30	4.53 ± 0.65
C17:0	0.32 ± 0.08	0.34 ± 0.15
C18:0	4.83 ± 0.66	5.54 ± 0.56
C18:1	39.81 ± 2.88	37.74 ± 0.98
C18:2	21.72 ± 1.42	28.86 ± 0.57 *
C18:3	1.09 ± 0.16	1.56 ± 0.17
Others	0.22 ± 0.22	0.04 ± 0.02

Earlier, our group had developed fluconazole adopted strains in the laboratory, where YOI-16, YOI-32 and YOI-64, showed susceptibility, intermediate resistance and resistance to fluconazole, respectively.[11] We tested these strains for their lipid compositions as well. The major fatty acid detected include:

These include C16:0 (Ranging from 29.9 – 32.5%, palmitic acid), C16:1 (Ranging from 7.8 – 9.4%, palmitoleic acid), C18:0 (Ranging from 11.2 – 13.3%, stearic acid), C18:1 (Ranging from 28.6 – 29.6%, oleic acid), C18:2 (Ranging from 10.9 – 15.3%, linoleic acid), and C18:3 (Ranging from 1.9 – 2.5%, linolenic acid) (Table 1). Although, these fatty acid composition ranges are significantly different from those observed in clinical isolates, yet, the increasing trend of UI was observed in YOI-32 and YOI-64, compared to YOI-16 (Figure 2). Upon comparison of sterolomes, similar to clinical isolates we observed increase in ergosterol, DHE and methylated sterol structures, decrease in contents of ergosta-7,22-dien-3-ol structure, in resistant isolates, compared to susceptible isolates. Further, the ratiometric analysis showed that ergosterol/DHE ratio was lower, 4α-methyl-ui/lanosterol ratio was higher, and ergosterol/4α-methyl-ui ratio was higher in YOI-32 and YOI-64, compared to YOI-16.

Membrane lipid composition of drug susceptible and resistant isolates of *C. albicans*

In order explore the variations in the lipid compositions within the plasma membrane of azole susceptible and resistant *C. albicans* cells, we chose an isogenic match pair Gu4/Gu5.

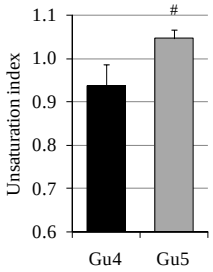

Figure 4. Unsaturation index measurements based on plasma membrane fattyome of *C. albicans* isolates. Degree of unsaturation was determined by calculating the unsaturation index (UI) of the fatty acids. UI was calculated as follows: UI = [(1 x % mono-unsaturated fatty acids) + (2x % di-unsaturated fatty acids) + (3 x % tri-unsaturated fatty acids)] / 100. Values are mean ± SEM of 2 independent analyses (n=2). '#' represents *p* value 0.08 for comparison between Gu4 and Gu5 matched pair.

Lipids were extracted from the purified plasma membrane and subjected to fattyome and sterolome analysis using methods described above. In our analysis, C16:0, C18:1 and C18:2 comprised of >85% of the total fatty acyl pool of the plasma membrane (Table 3). We observed a 25% increase in C18:2 content in Gu5, compared to Gu4 isolate (Table 5). Furthermore, we find a significant increase in the UI of plasma membrane fatty acids of the azole resistant Gu5, compared to Gu4 isolate (Figure 4). Due to low abundance of intermediate sterol species, we could only detect ergosterol in the plasma membrane fraction. The total sterol content in plasma membrane fraction of Gu4 and Gu5 isolate were found to be 25.7 and 36.7 ng/µg plasma membrane protein; here, resistant isolate Gu5 contained 30% more sterol compared to Gu4 isolate.

Membrane microdomains or lipid rafts have been implicated in azole resistance mechanisms.[25,26] We purified these lipid rafts as the DRM fractions and fattyome and sterolome analysis using methods described above. Our analysis showed that C16:0,

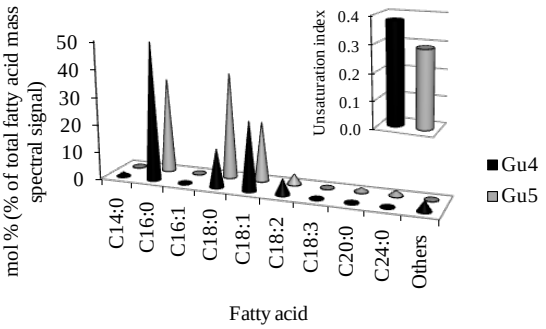

Figure 5. Fatty acid composition and unsaturation index measurements based on lipid rafts extracts of *C. albicans* isolates. Values represent percent of fatty acid normalized to the total fatty acid mass spectral signal. Degree of unsaturation (inset) was determined by calculating the unsaturation index (UI) of the fatty acids. UI was calculated as follows: UI = [(1 x % mono-unsaturated fatty acids) + (2x % di-unsaturated fatty acids) + (3 x % tri-unsaturated fatty acids)] / 100.

C18:0, C18:1 and C18:2 comprised of >93% of the total fatty acyl pool of the lipid rafts (Figure 5). While, we observed 1.2 – 1.8 fold decrease in C16:0, C18:1, C18:2 and C18:2, and 2.8 fold increase in C18:0 content in Gu5, compared to Gu4 isolate (Figure 5). Further, we could observe a significant decrease in UI of lipid raft fatty acids in Gu5, compared to the Gu4 isolate (Figure 5 inset). Unfortunately, it was tough to compare the sterol contents within the DRM fractions due to low detection. We could only see the sterol contents in the range of 13.7 – 18.2 pg/µg CM protein within different tested samples.

DISCUSSION

Membrane lipid compositions of fungi are known to respond to cellular changes like altered environment (changes in pH, temperature, growth medium), exposure to chemicals (drugs or other agents), or any other kind of stress.[27-29] Variations in lipid composition have been associated with the functional importance of several genes, impacting the overall physiology of fungi.[30] Such changes in membrane lipid compositions could either be reflected in their altered contents or molecular structure or both. In any scenario, the homeostatic environment of the cellular membranes is definitely altered. Functionally this may directly impact passive diffusion, facilitated /or active transport, or even destabilize the protein – protein interaction domains. Studies have shown in different model fungus that specific lipid structures could be associated with specific membrane functions.[31]

Towards establishing the roles of lipids in fungal lipid biology, our group, along with others has been exploring their significance in pathogenesis, virulence and drug resistance mechanisms.[14] In our earlier studies along with others on human pathogenic *Candida albicans*, we could show that: (i) deletion of genes of lipid biosynthetic pathway severely compromises the pathogenic properties;[26] (ii) perturbation of membrane lipids may compromise membrane homeostasis and fluidity;[10] (iii) alterations in membrane fluidity directly has been shown to be associated with loss of membrane protein function specifically

membrane localized efflux pumps (Cdr1, Mdr1 in plasma membrane; Cdr6 in vacuolar membrane) or proteins that maintain lipid asymmetry viz. lipid translocases (Cdr1, Cdr2, Cdr3, Rta2 in plasma membrane);[25,26,32-34] (iv) compromised lipid synthesis also compromises proper protein(s) assembly;[35] (v) many lipid changes have been associated with altered drug susceptibilities as well.[9-11]

Due to ever upcoming evidences of importance of lipids in *C. albicans*, it was imperative to develop better methods to determine the structures of these lipids at molecular level and accurately quantify amounts as well.[8-14] In this direction, from being able to analyze lipids on traditional radioactive or non-radioactive thin layer chromatography and fluorescence based methods, we have evolved to high throughput mass spectrometry based lipidomics methods.[36] Lipidomics analyses allowed us determine structures and molecular compositions of *C. albicans* lipids in detail, and correlate certain lipid compositional variations with different phenotypes including drug resistance.[9-11,23] Because majority of these studies have been performed by using total cellular lipid extracts, it is quite difficult to clearly define the specific contribution of lipids in membrane function. In studies by using cellular lipidomics of azole susceptible and resistant isolates, it was demonstrated that specific alterations in phosphoglyceride and sterol molecular species can be correlated to azole resistance.[9,11] There are a few points to consider here: (i) azole resistance in these strains is associated with overexpression efflux pumps Cdr1/Mdr1 localized within the plasma membrane;[33] (ii) Cdr1 has been shown to be associated with lipid rafts or DRMs;[14,26] (iii) Mdr1, although within the plasma membrane, is rather lipid raft or DRM independent.[14,26] With these considerations, we posed the question that whether cellular lipidomes reflect the true picture of compositional changes occurring specifically within the plasma membrane. To address this question, in present study we aimed to compare cellular and plasma membrane lipidomes using the snapshots of fatty acid and sterol structures.

Fatty acid variations can significantly impact the membrane fluidity,[37] and affect the status of azole resistance as well.[38] In our fattyome analysis of cellular lipid extracts, we could detect over 20 species of fatty acids where 16 and 18 carbon fatty acids were most abundant. We could see a high abundance of mono- and poly- unsaturated fatty acids in resistant isolates, compared to the susceptible isolates. The high levels of unsaturated fatty acids amount to high fatty acyl UI in the resistant isolates. Altered sterol content has been attributed to the development of azole resistance,[39] and to the modulation of membrane lipid homeostasis which in turn can affect the structure and function of associated proteins.[40] Our analyses show that resistant isolates showed a significant increase in the sterol content. Probably the high sterol content is an adaptive response to compensate for the increased fatty acyl mediated unsaturation. Producing more sterols would definitely be more logical in tolerating higher drug concentrations simply because azoles inhibit the sterol biosynthetic pathway. These changes were consistent in laboratory adopted strains as well.

Different azole resistant strains have shown lipid specificities. For examples, while Cdr1 is membrane raft localized, where as Mdr1 is not; both Cdr1 and Mdr1 have been attributed to azole resistance.[25,26] It is possible that lipids adapt differently to the distribution and abundance of these protein in the plasma membrane. Among the clinical isolated, we observed that Mdr1 overexpressed strains (G5 and F5) showed a much higher increase in UI, compared to the Cdr1 overexpressed strain (Gu5). On the other hand, increase in ergosterol content was much more prominent in G5 as compared to that in F5. Studies point to a complex role of sterols, rather than the emphasized structural roles. Membrane sterol composition and levels can directly impact the exocytosis and endocytosis mechanisms.[41] A balance between exocytosis and endocytosis pathways has been shown necessary to mediate azole (a sterol targeting drug) resistance in *Candida* glabrata.[42] Further, accumulation of different substituted sterol structures or ergosterol biosynthetic pathway intermediates can differentially impact these exocytosis and endocytosis mechanisms.[40] In our analysis, among the azole resistant strains, the 4α-methyl-ui/lanosterol ratio was found to be lower in Gu5 and higher in G5 and F5, compared to their susceptible counterparts. These specific imprints can be correlated to differential localization of membrane proteins such as Cdr1 or Mdr1, and therefore, to the background of specific azole resistance (i.e. ABC or MFS dependent).

Although the plasma membrane lipid functions have been evaluated by analyzing mostly the cellular lipids, it has always been a question of interest whether the cellular analysis is a true reflection of the exact scenarios present in the plasma membrane. In our analysis, we could show that the observed cellular lipid changes are consistent with plasma membrane lipid changes viz. a high UI and sterol content in the azole resistant isolate. However, upon examining the membrane raft lipid compositions, we found that rafts have a much lower UI in resistant isolate. Considering that raft is known to be formed from specific protein-protein interactions supported by specific lipid compositions, and is expected to be relatively rigid. It is highly like that the low UI of fatty acyls contributes to the required rigidity of these structures and proper localization of Cdr1 therein. This inference is also supported by the fact that alteration in raft associated lipids mislocalization of raft associated proteins. It has been argued that minor alterations in fatty acyl UI of the plasma membrane can significantly alter its thermodynamic stability.

CONCLUSION

Together, this study provides three significant inputs: (i) correlation between cellular and membrane lipidomes; (ii) correlation between fatty acid unsaturation and azole resistance; (iii) correlation between sterol composition and azole resistance. The whole range of membrane lipid changes appear to be rather more complex and intricately regulated, aspects of which are required to be explored in detail. Nonetheless, we must appreciate the extent of refined variations that have been strategically placed by *C. albicans* cells to evade azoles.

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