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Fluconazole resistant *Candida auris* clinical isolates have increased levels of cell wall chitin and increased susceptibility to a glucosamine-6-phosphate synthase inhibitor

Garima Shahi [a], Mohit Kumar [a], Andrzej S. Skwarecki [b], Matt Edmondson [c], Atanu Banerjee [a], Jane Usher [c], Neil A.R. Gow [c], Sławomir Milewski [b,*], Rajendra Prasad [a,*]

[a] Amity Institute of Integrative Science and Health and Amity Institute of Biotechnology, Amity University Gurgaon, Haryana 122413, India
[b] Department of Pharmaceutical Technology and Biochemistry, Gdansk University of Technology, 11/12 Narutowicza Street, 80-952 Gdansk, Poland
[c] Medical Research Council Centre for Medical Mycology at the University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, UK

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ABSTRACT

In 2009 *Candida auris* was first isolated as fungal pathogen of human disease from ear canal of a patient in Japan. In less than a decade, this pathogen has rapidly spread around the world and has now become a major health challenge that is of particular concern because many strains are resistant to multiple class of antifungal drugs. The lack of available antifungals and rapid increase of this fungal pathogen provides an incentive for the development of new and more potent anticandidal drugs and drug combinatorial treatments. Here we have explored the growth inhibitory activity against *C. auris* of a synthetic dipeptide glutamine analogue, L-norvalyl-N^3-(4-methoxyfumaroyl)-L-2,3- diaminopropanoic acid (Nva-FMDP), that acts as an inhibitor of glucosamine-6-phosphate (GlcN-6-P) synthase - a key enzyme in the synthesis of cell wall chitin. We observed that in contrast to FLC susceptible isolates of *C. auris*, FLC resistant isolates had elevated cell wall chitin and were susceptible to inhibition by Nva-FMDP. The growth kinetics of *C. auris* in RPMI-1640 medium revealed that the growth of FLC resistant isolates were 50–60% more inhibited by Nva-FMDP (8 μ g/ml) compared to a FLC susceptible isolate. Fluconazole resistant strains displayed increased transcription of *CHS1, CHS2* and *CHS3,* and the chitin content of the fluconazole resistant strains was reduced following the Nva-FMDP treatment. Therefore, the higher chitin content in FLC resistant *C. auris* isolates may make the strain more susceptible to inhibition of the antifungal activity of the Nva-FMDP peptide conjugate.

Introduction

C. auris is an opportunistic pathogen that colonises skin and causes systemic infections in hospital settings in patients with co-morbidities that include patients with Covid-19 (Borman et al., 2021). Notably, *C. auris* frequently display resistance to single or multiple class of commonly used antifungal drugs i.e. azoles, polyenes and echinocandins which severely limits the treatment options (Lockhart et al., 2017a). Studies of susceptibility profiles of antifungals on *C. auris* isolates revealed that up to 90% of isolates were resistant to fluconazole, 8% to amphotericin B (AMB) and 2% to echinocandins (Chowdhary et al., 2018). Increased coincident resistance to other azoles such as itraconazole, voriconazole and isavuconazole have also been reported (Sharma et al., 2015; Chowdhary et al., 2014; Magobo et al., 2020; Kumar et al., 2015). *In vivo* analyses have shown that micafungin exhibits greater potency towards invasive *C. auris* infections compared to FLC and AMB (Lepak et al., 2017). However, the extensive use of echinocandins may have contributed to the development of resistance towards this class of front line drugs (Lockhart et al., 2017b; Sharma et al., 2016; Chowdhary et al., 2017; Sharma et al., 2020).

The underlying molecular mechanisms that account for the high level of resistance to antifungals of *C. auris* are incompletely understood. However, a range of antifungal mechanisms in *C. auris* are beginning to emerge (Ademe and Girma, 2020). For example, drug efflux pumps which play crucial role in mediating drug resistance in other *Candida* species have also been shown to be upregulated in *C. auris* resistant isolates (Chaabane et al., 2019). A number of MFS and ABC-type genes have been identified in the *C. auris* genome (Chatterjee et al. 2015; Wasi

* Corresponding authors.
E-mail addresses: slawomir.milewski@pg.edu.pl (S. Milewski), rprasad@ggn.amity.edu (R. Prasad).

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et al., 2019). However, studies to date have not been able to reconcile the relatively modest levels of efflux pump protein upregulation that are observed with the high levels of antifungal resistance associated with this organism. In this paper we reveal a change in the physiology of fluconazole resistant strain of *C. auris* that provide new insights into its enhanced azole resistant phenotype. The mechanism of azole resistance in *C. auris* has been associated with point mutations in its target gene, lanosterol 14 α-demethylase and upregulation of *ERG11* and mutations in the Upc2 transcription factor (Lockhart et al., 2017a; Ben-Ami et al., 2017; Prakash et al., 2016). Common mutations in *FKS1* and *FKS2* gene associated with echinocandin resistance mechanism have also been reported in (Cortegiani et al., 2018). The increasing prevalence of *C. auris* infection and high level of resistance against major classes of antifungals has led to searches for novel compounds that have the potential to improve the existing arsenal of antifungals. In addition the use of drug combinations may provide opportunities for improved chemotherapy of *C. auris*.

N^3–(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid- (FMDP) is a potent selective inhibitor of the enzyme GlcN-6-P synthase (Milewski et al., 1988), which catalyses the first step in the cytosolic pathway of UDP-GlcNAc biosynthesis (Milewski et al., 2006) that provides the substrate for cell wall assembly. UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc) is a ubiquitous metabolite of eukaryotes and used in different ways. In fungi this a substrate is essential for chitin and mannoprotein biosynthesis which are important components of the cell wall. FMDP behaves as a glutamine analogue and blocks *N*-terminal glutamine binding domain of GlcN-6-P synthase (Andruszkiewicz et al., 1987). The limited efficacy of FMDP against intact *Candida* cells was attributed to limitations to transport it efficiently into the cell. In order to enhance the permeability of FMDP, different oligopeptide structures incorporating FMDP were explored (Andruszkiewicz et al., 1987). Among them, Nva-FMDP (L-norvalyl-N^3–(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid) showed the highest efficacy towards different fungi, including *S. cerevisiae*, *C. albicans*, *Cryptococcus neoformans* and *Aspergillus* species. Nva-FMDP was shown to be an effective inhibitor *in vitro* against many recombinant yeast cells and fluconazole resistant *C. albicans* isolates that overexpress ABC efflux pumps (Milewski et al., 2001; Wakieć et al., 2008). Nva-FMDP was also demonstrated to be an effective antifungal agent in mouse model of systemic candidiasis (Milewski et al., 1988).

Increased uptake of Nva-FMDP was mediated by the proton motive force-dependent oligopeptide permeases and stimulated by increase in membrane potential caused by an overexpression of *CDR1* and *CDR2* (Wakieć et al., 2008). Notably, the Pdr5p, which is a *S. cerevisiae* ortholog of *C. albicans* Cdr1p, has been recently shown to be a drug/proton co-transporter (Wagner et al., 2021), thus supporting the postulated mechanism. The distinctive selective potency of Nva-FMDP against FLC resistant *C. auris* strains has not been so far observed in case of other *Candida* species.

The mechanism of action of FMDP-oligopeptides includes transport of di- and tripeptide conjugates by the PTR2 and PTR22 peptide permeases. Specifically oligopeptide permease is required for tetra- penta- hexa- hepta- and octapeptide transport. Uptake of these antifungal peptides is followed by intracellular cleavage by peptidases and inactivation of GlcN-6-P synthase enzyme by released FMDP, resulting in the blockade of intracellular glucosamine supply for chitin biosynthesis. In *C. albicans* the levels of chitin and mannoprotein were reduced in Nva-FMDP treated cells and cell wall integrity was compromised as evidenced by direct imaging using scanning electron microscopy (Milewski et al., 1991).

In the present study, we examined the activity of Nva-FMDP to *C. auris* hospital isolates which displayed high level of resistance to azoles. We observed that Nva-FMDP was selectively effective against fluconazole resistant *C. auris*, but remained ineffective towards azole susceptible isolates. We therefore propose that the increased chitin levels in the cell wall increases the susceptibility of these FLC resistant isolates to the Nva-FMDP cell wall inhibitor.

Results and discussion

FLC resistant C. auris isolates are selectively susceptible to Nva-FMDP

To assess the impact of Nva-FMDP on *C. auris*, we selected five FLC resistant hospital isolates (MIC_{80} > 128μ g/ml); NCCPF_470033, NCCPF_470034, NCCPF_470035, NCCPF_470036 and NCCPF_470037 and a susceptible isolate AMR_5556. All these clinically resistant isolates belong to clade I of the *C. auris* group and were obtained from the National culture collection of Pathogenic fungi, Post-graduate Institute of Medical Education and research (PGIMER), Chandigarh, India. For comparison, we also included another FLC susceptible clade II isolate CBS10913T that was obtained from the CBS culture collection, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands. The growth inhibitory effect of Nva-FMDP on these isolates was tested by three independent methods; growth kinetic studies in liquid medium, MIC susceptibility and spot sensitivity assays as described in Materials and Methods.

MIC determination in RPMI-1640 media demonstrated that FLC resistant *C. auris* isolates were susceptible to Nva-FMDP (MIC_{80} 32μ g/ml) as compared to its susceptible strain AMR_5556 and CBS10931T that had an MIC_{80} of 128μ g/ml (Fig. 1A). Susceptibility to Nva-FMDP was further assessed by growth kinetics analysis in RPMI-1640. Doubling the MIC_{80} concentration of FLC susceptible isolates further confirmed the susceptibility of all five tested FLC resistant isolates to 8μ g/ml Nva-FMDP (Fig. 1B). Growth kinetic analysis showed up to 50–60% inhibition at 8μ g/ml Nva-FMDP. Spot analysis of tested strains also gave similar results in YPD medium (Fig. 1C). The antifungal activity of Nva-FMDP was further assessed in several other drug resistant clinical isolates of *C. auris* which were not only FLU resistant, but also resistant to amphotericin B (AMB). Nva-FMDP was again found to be effective in inhibiting growth of these multidrug resistant isolates (Supplementary Fig. S1A).

In addition to the Nva-FMDP dipeptide, the inhibitory effect of two other FMDP conjugates, Lysine-Nva-FMDP and $(Alanine)_3$-FMDP was also tested on FLC resistant *C. auris* isolates. The antifungal activity of these FMDP conjugates, Lys-Nva-FMDP and $(alanine)_3$-FMDP was tested previously against *C. albicans,* where enhanced uptake of these conjugates was correlated with their strong inhibitory effect (Wakieć et al., 2008). However, both the FMDP conjugates, Lysine-Nva-FMDP and $(Alanine)_3$-FMDP did not show any inhibitory effect against FLC resistant *C. auris* isolates compared with the CBS10931T susceptible strain (Supplementary Fig. S1B).

Having observed the selective susceptibility of FLC resistant isolates to Nva-FMDP treatment, we then wanted to see if the combination of Nva-FMDP with FLC exhibited synergistic inhibition on these azole resistant isolates. For this, we employed the chequerboard method in which inhibition in RPMI-1640 medium is expressed as the sum of fractional inhibitory concentration index (FICI) for two antifungals. Fractional inhibitory concentration (FIC) of both antifungals was calculated as the MIC of the antifungal compounds in combination divided by the MIC of antifungal alone. FICI was calculated by adding the FIC values for the two antifungals used. The starting concentration of FLC and Nva-FMDP for chequerboard assay was selected as, half of the MIC_{80} concentration of FLC (For resistant isolates = 64μ g/ml and for FLC susceptible strain = 2μ g/ml) and of Nva-FMDP (For resistant isolates = 16μ g/ml and for FLC susceptible strain = 64μ g/ml). The FICI values for CBS10931T and all the tested FLC resistant isolates were > 1 which implied indifferent interaction between Nva-FMDP and fluconazole. Therefore FLC resistant isolates are inhibited by Nva-FMDP dipeptide, however, Nva-FMDP when combined with FLC has no synergetic impact on these isolates.

A

Strains	MIC_{80} (μg/ml)	
	FLC	Nva-FMDP
CBS10931T	4	>128
AMR_5556	4	≥128
NCCPF_470033	>128	32
NCCPF_470034	>128	32
NCCPF_470035	>128	32
NCCPF_470036	>128	32
NCCPF_470037	>128	32

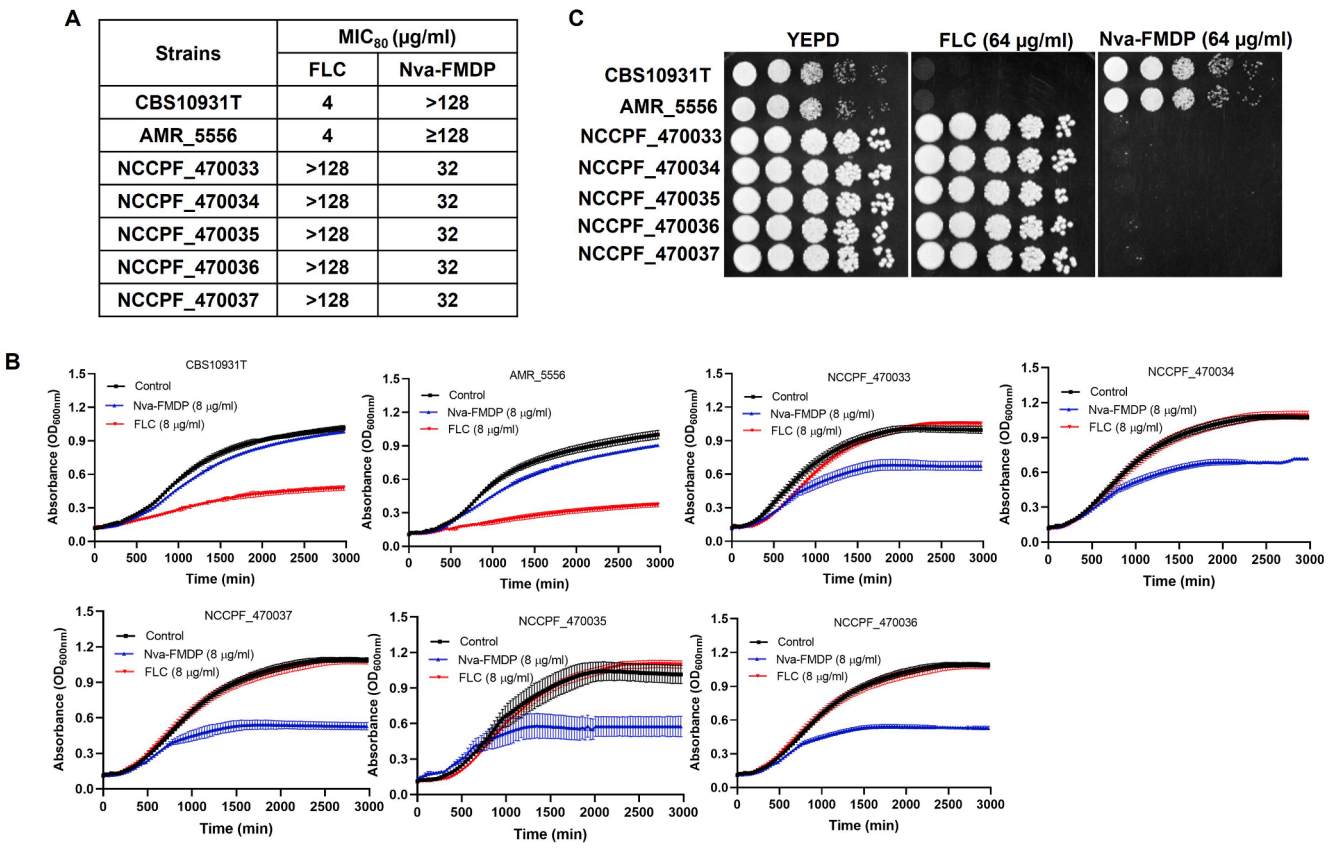

Fig. 1. FLC resistant *C. auris* isolates exhibit susceptibility to Nva-FMDP: (A) MIC_{80} values were obtained using microdilution assay in RPMI-1640 media on fluconazole and Nva-FMDP as described under "Material and Methods". (B) Growth kinetics study of each isolate was performed by using micro-cultivation method in a 96-well plate using Liquid Handling System (Tecan, Austria) in RPMI-1640 medium. (C) Spot serial dilution assay for *C. auris* isolates on FLC and Nva-FMDP at indicated concentration in YEPD agar plate. All the experiment was conducted in triplicates (n = 3), and values are expressed in mean ± standard deviation.

Supplementation of N-acetyl-D-glucosamine reversed the susceptibility of FLC resistant isolates

Nva-FMDP targets the enzymes responsible for the synthesis for UDP-GlcNAc and thereby inhibits cell growth. Therefore we then attempted to rescue the growth inhibition by supplementation of the growth media with *N*-acetyl-D-glucosamine (GlcNAc). We supplemented RPMI-1640 medium with 10 mM concentration of GlcNAc along with 8μ g/ml Nva-FMDP to the cell suspension and incubated it for 24 h at 37 °C. As shown in Fig. 2A, the growth inhibitory effect of Nva-FMDP was abolished by the addition of GlcNAc and it also supported that GlcN-6-P synthase is the only essential target for FMDP dipeptide.

qRT-PCR confirms increased basal expression of peptide transporter genes in few FLC resistant isolates

In most fungi, two types of peptide transport systems are present: (i) peptide transporters (PTR) carrying di- and tripeptides comprises of two proteins encoded by *PTR2* and *PTR22* and (ii) the oligopeptide transporters (OPT) which mediates the uptake of longer oligopeptides (Basrai et al., 1995; Dunkel et al., 2013). Since Nva-FMDP is a dipeptide and is known to be transported by peptide transporters *PTR2* and *PTR22*, we then assessed the basal expression level of these genes in FLC resistant and susceptible *C. auris* isolates. Basal expression profile of these two genes were quantified and normalised relative to the expression of *TDH1* as a housekeeping gene (Kumari et al., 2018). Basal level expression of both the peptide transporter genes, *PTR2* and *PTR22* was higher in some, but not all, of the tested FLC resistant isolates. For example, the transcript of *PTR2* in NCCPF_470034 and *PTR22* in NCCPF_470037 did not change in comparison to susceptible CBS10931T and AMR_5556 isolates (Fig. 2B). Therefore there was a partial correlation between *PTR2* and *PTR22* expression with Nva-FMDP susceptibility. Previously it was reported that an impaired transport of Nva-FMDP led to increased resistance of *C. albicans* cells to this antifungal peptide and deletion of the *PTR* genes eliminated the Nva-FMDP growth inhibitory effect on *C. albicans* cells (Hori et al., 1974; Milewski et al., 1988; Basrai et al., 1992; Schielmann et al., 2017). These results indicates that faster uptake of Nva-FMDP by FLC resistant cells through peptide transporters might explain the inhibitory effect towards these cells. The efflux of rhodamine 6G, a fluorescent substrate of Cdr1/Cdr2 showed no significant difference between untreated and Nva-FMDP treated FLC resistant *C. auris* isolates, implying no major change in the expression of the major efflux pumps encoding genes (Fig. 2C(i)). In the absence of a true homologue of *CDR2* in *C. auris* (Wasi et al., 2019), we compared the expression of *CDR1* in Nva-FMDP treated and untreated FLC resistant isolates (Fig. 2C (ii)). No significant difference in *CDR1* expression was observed between Nva-FMDP treated and untreated FLC resistant isolates. The two isolates (NCCPF_470034 and NCCPF_470035) displayed downregulation of the *CDR1* transcript upon Nva-FMDP treatment. Thus, we suggest that the potentiating effect of Nva-FMD on fluconazole resistant strains was not due to major changes in the drug efflux activity.

Calcofluor White staining reveals increased chitin content of FLC resistant isolates

It has been reported that blockage of GlcN-6-P synthase in Nva-FMDP treated *C. albicans* cells results in decreased chitin and mannoprotein biosynthesis and that this effect could be mitigated by the addition of exogenous GlcNAc (Milewski et al., 1991). We therefore stained chitin in FLC resistant and susceptible isolates using Calcofluor White (as

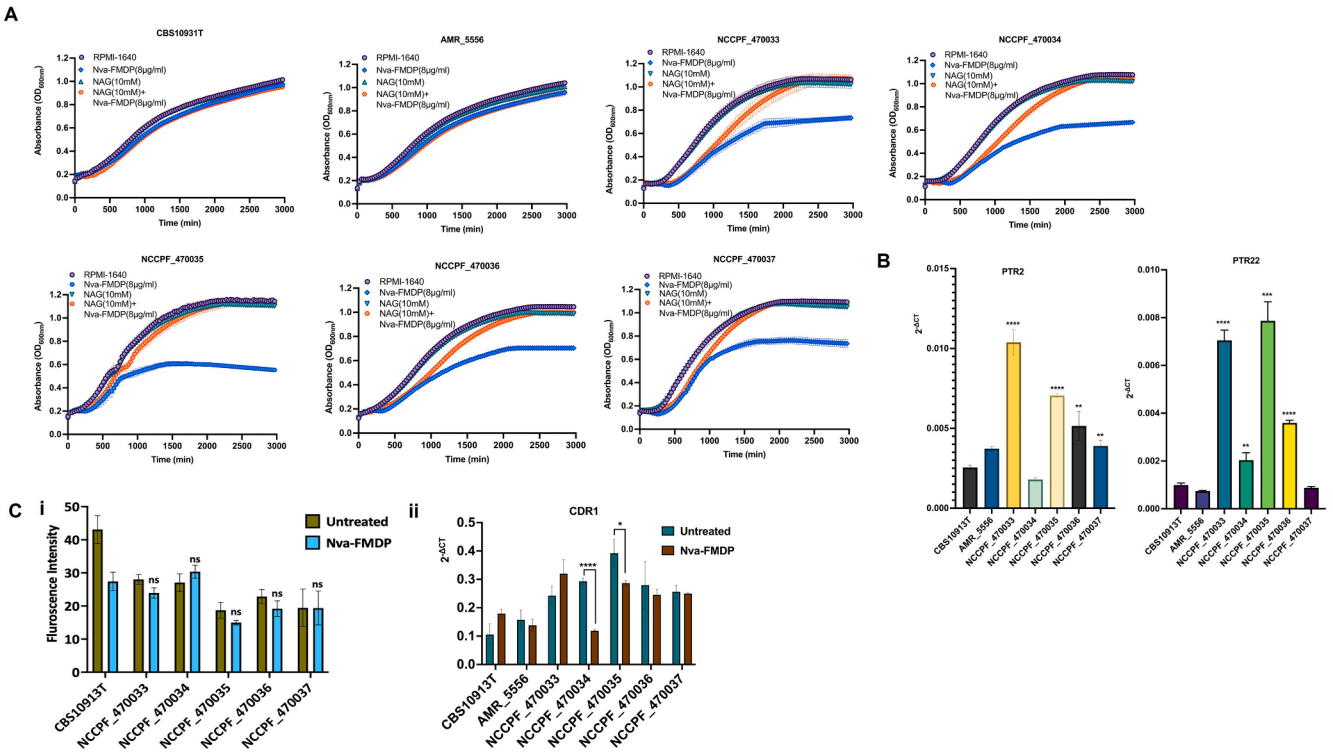

Fig. 2. (A) NAG supplementation reverses the inhibitory effect of Nva-FMDP in *C. auris* resistant isolates: Growth kinetics of all the isolates were performed in the RPMI-1640 medium supplemented with 10 mM of NAG and 8 μg/ml Nva-FMDP and both in the combination. (B) Peptide transporter gene expression in FLC resistant *C. auris* isolates: PTR2 and PTR22 expression levels were determined by qRT-PCR, and expression was estimated as $2^{-\Delta CT}$ with the TDH1 gene as a control. Data is presented as means ± standard deviation from biological duplicates with technical triplicates. (C) (i) *C. auris* FLC resistant isolates exhibit no changes in R6G efflux after Nva-FMDP treatment: R6G efflux in Nva-FMDP treated and untreated isolates were performed as described in Materials and Methods. Each bar indicates the standard deviations of mean of three sets of experiments. p-values < 0.05 were considered statistically significant and were calculated using GraphPad prism 9 and two-way ANOVA (uncorrected Fisher's LSD). (ii) No change in CDR1 expression of untreated and Nva-FMDP treated *C. auris* isolates: Expression of *CDR1* was measured by qRT-PCR as described in Material and Methods.

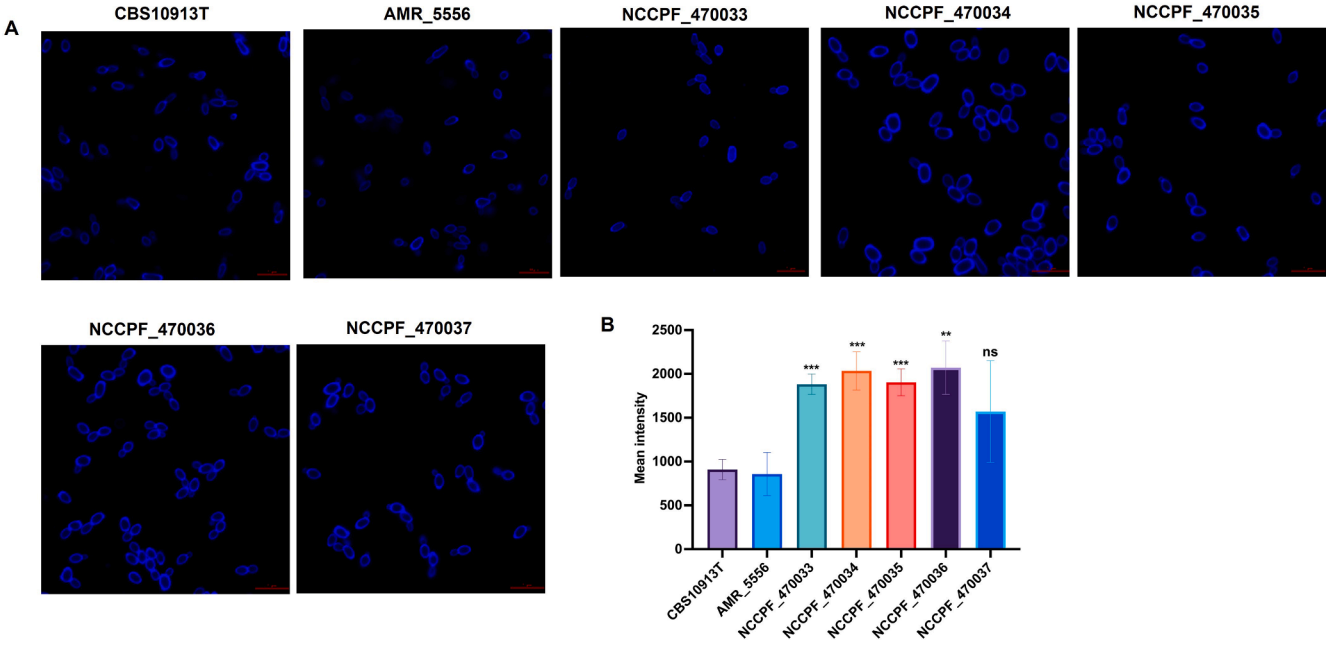

Fig. 3. *C. auris* FLC resistant isolates display increased CFW staining: Cell wall chitin staining was performed as described in the Material and Methods using 10μ g/ml Calcofluor White solution. After incubation for 10 min with final conc. of 10 μg/ml Calcofluor White solution in dark, cells were observed by confocal microscopy (Fig. 3A). Mean fluorescent intensity with respective to each strains were quantitated and graph was plotted (Fig. 3B). Increase fluorescent colour intensity depicts higher chitin content in respective isolates.

described in Material and Methods). Quantitative confocal imaging demonstrated an increased intensity of Calcofluor White staining in FLC resistant isolates as compared with both the susceptible isolates (CBS10931T and AMR_5556), suggesting an increase in cell wall chitin levels (Fig. 3A and B). The increased chitin staining in FLC resistant isolates led us to examine the basal expression levels of chitin synthase genes *CHS1, CHS2* and *CHS3* in resistant and susceptible isolates by qRT-PCR. The five tested FLC resistant isolates showed different transcript levels of these genes. Most of the FLC resistant isolates showed higher expression of chitin synthase genes compared with FLC susceptible isolates. NCCPF_470033 and NCCPF_470036 showed significantly higher expression of *CHS1,* and *CHS2* expression in all five FLC resistant isolates. *CHS3* expression was elevated in NCCPF_470033, NCCPF_470035 and NCCPF_470036 compared to CBS10931T (Fig. 4A). Expression of *CHS* genes in other susceptible AMR_5556 was also seen to be lower than FLC resistant isolates (Fig. 4A). The higher chitin content in FLC-resistant cells may therefore be due to the higher *CHS2* expression potentially indicating a major role of Chs2 in chitin biosynthesis in *C. auris*, which contracts with *C. albicans*, where *CHS3* is responsible for biosynthesis of 80% of cell wall chitin, and *CHS1* provides chitin for the primary septum (Munro, 2013). The basal expression profile of these CHS genes therefore correlated with the increased Calcofluor White staining in FLC resistant *C. auris* isolates. These intrinsic changes in cell wall chitin level between resistant and susceptible isolates may relate to the selective susceptibility profile of Nva-FMDP in FLC resistant *C. auris* isolates.

Our data reinforce and significantly extend previous studies. For example, we showed previously that the level of cell wall chitin appeared to be correlated with the extent of sterol synthesis and consequently the regulation of chitin synthesis (Chiew et al., 1982; Sekiya and Nozawa, 1983; Bossche, 1985). The isolates of *C. parapsilosis*, *C. tropicalis, C. krusei* and *C. albicans* have a higher content of chitin when treated with fluconazole that may relate to the effects of ergosterol depletion and lanosterol increase on chitin synthesis (Pfaller et al., 1990; Pfaller and Riley, 1992). Of note, the azole resistance clinical isolates used in the present study also displayed reduced susceptibility to echinocandins. Growth kinetics assays of these isolates in the presence of caspofungin (CAS) and micafungin (MFG) showed reduced inhibitory effects of these drugs on *C. auris* clinical isolates (Supplementary Fig. S2). Interestingly, as in other fungi, a recent study showed that caspofungin adapted cells exhibit enhanced chitin levels and corresponding upregulation of chitin synthase genes (Lara-Aguilar et al., 2021). Together, these studies suggest that alterations in chitin synthesis is a common response to azole treatment.

Quantitative analysis of cell wall component by HPLC show low level of chitin in Nva-FMDP treated FLC resistant isolates

Cell wall of fungi is structured as two layers in which the innermost layer is broadly conserved in structure and the outer layers can vary significantly between different species of fungi (Erwig and Gow, 2016; Lenardon et al., 2020). In *Candida* species inner layer is composed of chitin and β-glucan and the outer layer is enriched with mannoproteins - although chitin can be dispersed in the inner and outer layers under conditions of stress (Da Silva Dantas et al., 2021). Changing cell wall composition is an important mechanism facilitating survival of *Candida*

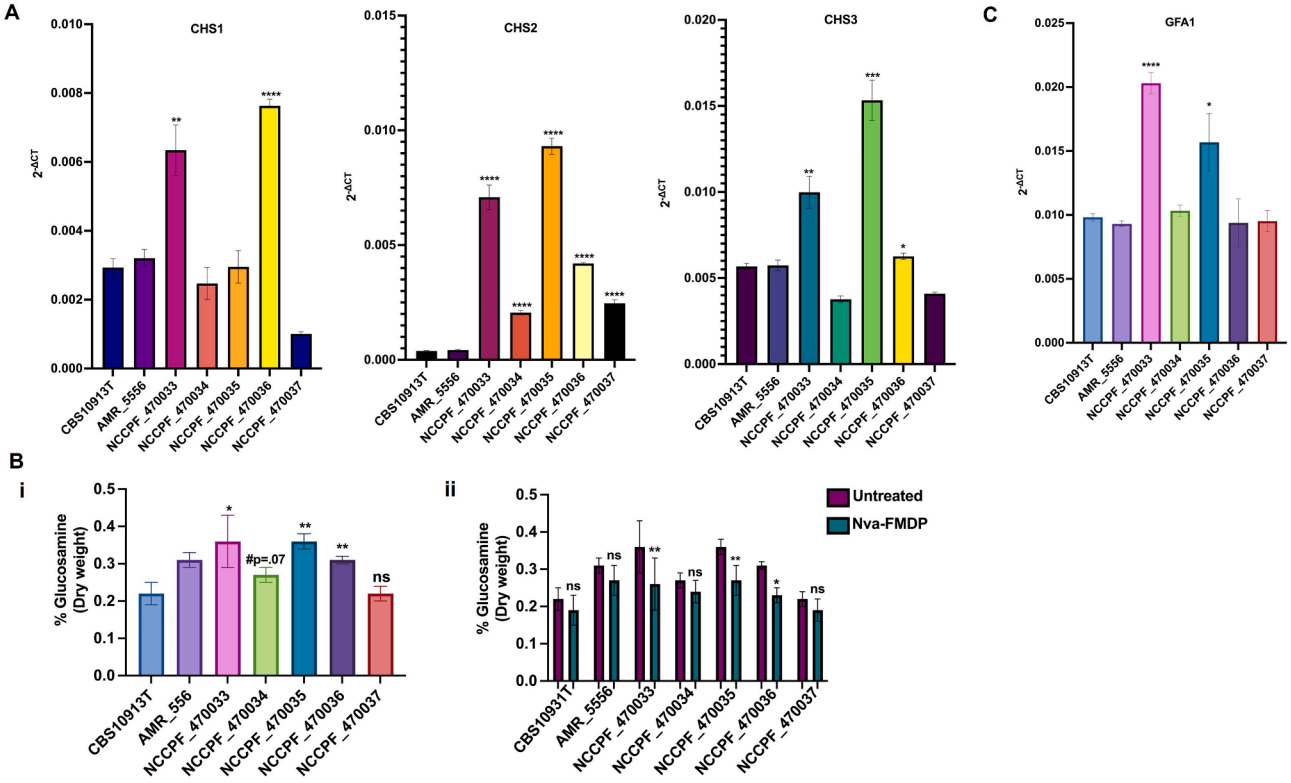

Fig. 4. (A) Basal level expression of chitin synthase genes were elevated in C. *auris* FLC resistant isolates: Basal level expression of chitin synthase genes (*CHS1*, *CHS2* and *CHS3*) was measured by qRT-PCR as described in Material and Methods. *p*-values > 0.05 were considered significant and calculated with unpaired *t*-test using GraphPad prism 9. Data represented as means ± standard deviation (n = 3) from biological duplicates and technical triplicates. (B) Glucosamine levels in *C. auris* FLC resistant isolates: Glucosamine content of cell wall was determined by HPLC as described earlier in materials and methods. Nva-FMDP treated FLC resistant isolates exhibited lower chitin level compared to untreated cells. *p*-values < 0.05 was considered to be significant and were calculated with unpaired *t*-test using GraphPad prism 9. Data represented as means ± standard deviation from three biological replicates (n = 3). (C) Gene encoding GlcN-6-P synthase enzyme display less expression in susceptible isolates: Basal level expression of *GFA1* was observed less in FLC susceptible isolates which correlates with less glucosamine content in this isolate. Significance was calculated using unpaired *t*-test and p > 0.05 was statistically considered to be significant.

spp. However, cell wall dynamics of *C. auris* has not been much explored although there have been reports of novel mannans in the outer cell wall (Bruno et al., 2020). The difference in chitin content and expression of chitin synthase genes between FLC susceptible and resistant *C. auris* isolates prompted us to analyse cell wall components quantitatively. After culturing in RPMI-1640 medium, cell walls were extracted and hydrolysed into their respective sugar components (see Material and Methods). Levels of glucosamine, glucose and mannose in the cell wall fractions were quantified using high-performance liquid chromatography (HPLC). Dry weight of recovered cell walls was quantified, and chitin, glucan and mannan contents were determined by measuring the glucosamine, glucose and mannose released (respectively) following hydrolysis of purified wall (Plaine et al., 2008). Consistent Calcofluor White staining and CHS expression, we observed significantly higher chitin content in the FLC resistant isolates compared to the FLC susceptible CBS10931T isolate. The isolate NCCPF_470037 was the exception where glucosamine content remained unchanged. In another susceptible isolate, AMR_5556 the glucosamine level was slightly higher than CBS10931T although it's level was lower when compared to FLC resistant NCCPF_470033 and NCCPF_470035 isolates (Fig. 4B-i). To further check the impact of Nva-FMDP on these isolates, we treated the cells at MIC_{50} values and observed the chitin level. We noted significantly lower chitin levels in Nva-FMDP treated FLC resistant isolates except for NCCPF_470034 where the observed low level was not statistically significant. The NCCPF_470037 isolate showed no change in chitin contents compared to the untreated controls (Fig. 4B-ii). These data imply that Nva-FMDP susceptibility of these isolates may be the result of blockage of enzyme GlcN-6-P synthase by Nva-FMDP, resulting in the attenuation of intracellular glucosamine supply for chitin synthesis. Notably, the chitin level did not change between treated and untreated cells of CBS10931T.

Lower basal expression of glutamine: fructose-6-phosphate aminotransferase (GFA1) gene in FLC susceptible isolates

N-acetyl-D-glucosamine is a component of chitin and biosynthesis of these polysaccharides depends entirely on the provision of glucosamine-6-phosphate - a product of GlcN-6-P synthase. This enzyme is encoded by *GFA1* and which is an essential gene in fungi (Whelan and Ballou, 1975). We therefore checked the basal expression of *GFA1* in FLC resistant and susceptible isolates. The mRNA level of *GFA1* in FLC susceptible isolates was considerably lower than in FLC resistant isolates (Fig. 4C). This finding was consistent with previous observations of reduced chitin and decreased basal expression of chitin synthase genes in FLC susceptible isolates. Lower expression of *GFA1* in these isolates may result in decreased GlcN-6-P synthase activity - the target of Nva-FMDP. The lack of binding of FMDP to the target enzyme may explain the absence of an inhibitory effect of Nva-FMDP in this isolate.

Glucan and mannan levels are not affected in FLC resistant C. Auris isolates

We did not measure any changes in glucan levels in FLC resistant isolates compared to FLC susceptible isolates, however, a significant increase in glucan was observed following Nva-FMDP treatment in CBS10931T cells (Fig. 5A). The glucan level increase in Nva-FMDP treated CBS10931T cells might therefore reflect the activation of compensatory mechanism responding to lower chitin levels in this isolate (Walker et al., 2008). However no significant change were recorded in Nva-FMDP treated AMR_5556 isolates in comparison to untreated. The mannan levels in FLC resistant isolates upon Nva-FMDP treatment was also unchanged, except in FLC susceptible isolate CBS10931T that showed a slight but significant decrease (Fig. 5B).

The present study demonstrates selective antifungal activity of Nva-FMDP on FLC-resistant clinical isolates of *C. auris*. Fluconazole susceptible strains, belonging to Clades I and II were not affected in their content of chitin or susceptibility towards Nva-FMDP. This difference in susceptibility, is hypothesised to be a consequence of Nva-FMDP induced inhibition of cell wall chitin biosynthesis. In this respect, it is noteworthy that *C. auris* some clinical isolates have been demonstrated to be susceptible to the chitin synthase inhibitor Nikkomycin Z (Bentz et al., 2021). These observations suggest that combinatorial treatment of *C. auris* with azoles and agents that interfere with chitin synthesis may improve clinical outcomes.

Material and Methods

Materials

The growth media RPMI-1640, YEPD (yeast extract/peptone/dextrose) and MOPS (3-(*N*-morpholino) propanesulfonic acid) were purchased from Himedia (Mumbai, India). Antifungal drug Fluconazole, *N*-acetylglucosamine (GlcNAc) and the Calcofluor White optical brightener was purchased from Sigma. Nva-FMDP, Lys-Nva-FMDP and $(Ala)_3$-FMDP were synthesised at the Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology, Poland. Primers used in the present study were obtained from IDT (Integrated DNA technologies).

Strains and culture conditions

FLC susceptible CBS10931T clade II isolate was obtained from the

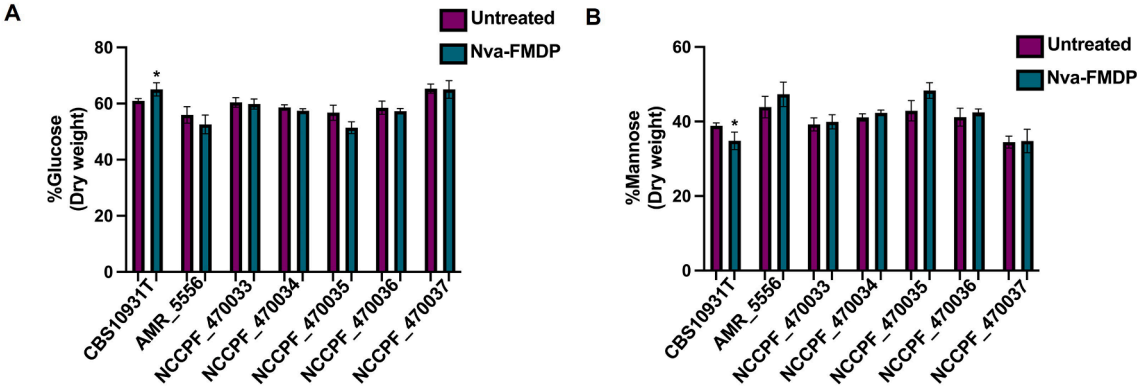

Fig. 5. (A) Nva-FMDP treated CBS10931T isolate exhibit increased glucan content: The glucan content was determined by hydrolysis and quantification of glucose as described in material and methods. After Nva-FMDP treatment glucan level was increased in FLC susceptible isolate (CBS10931T). Significance was calculated with two-way ANOVA (uncorrected Fisher's LSD). (B) Mannan levels in *C. auris* isolates upon Nva-FMDP treatment: Mannan content was determined by hydrolysis and quantification of mannose as described in Material and Methods. Statistical significance was calculated using two-way ANOVA (uncorrected Fisher's LSD).

CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands. Another susceptible isolates, AMR_5556 and clinically resistant clade I isolates NCCPF_470033, NCCPF_470034, NCCPF_470035, NCCPF_470036 and NCCPF_470037 were obtained from the National culture collection of Pathogenic fungi, Post-graduate Institute of Medical Education and research (PGIMER), Chandigarh, India. All the strains were grown and preserved in YEPD medium and freshly revived before use from −80 °C stock.

Drug susceptibility assay

Minimal inhibitory concentration (MIC): MIC values were determined by the serial two fold dilution method using 96-well microtiter plates in RPMI-1640 w/o sodium bicarbonate with L-glutamine buffered with MOPS buffer, pH adjusted to 7 as indicated in CLSI recommendations (Clinical and Laboratory Standards Institute) and described previously (Pfaller et al. 2008).

Spot assays: Spot assays were performed using 5 fold serial dilution of cell suspension in saline (0.9% NaCl) solution, and 4 µl aliquot of each dilution was spotted on YEPD plates with and without drugs as described previously (Mukhopadhyay et al., 2002).

Growth kinetic assays: Growth kinetic assays was performed using a micro-cultivation method in 96 well plate using Liquid Handling System (Tecan, Grodig, Austria) in RPMI-1640 media at 37 °C. Briefly, overnight grown cells were inoculated at a dilution of 0.1 OD_{600} in a 96-well plate with and without drug and allowed to grow at 37 °C. OD_{600} was measured every 30 min for an interval up to 48 h. Doubling times of isolates were calculated by measuring the time taken in doubling of logarithms values of the OD_{600} of the exponential phase.

Quantitative Real-Time PCR (qRT PCR)

For total RNA isolation primary culture of isolates was grown overnight from which secondary culture was inoculated in YEPD broth at 0.2 OD_{600} and grown for 4 h. The cultures were collected by centrifugation, and washed with DEPC-treated water. Total RNA was isolated following manufacturer's specifications, using a RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was carried out using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, United States) as per manufacturer's instruction. Quantitative RT PCR was performed using iTaq Universal SYBR green super mix Bio-Rad with desired gene-specific primers to estimate the expression profile. The *TDH1* gene was used for normalisation and results were compared between *Ct* values of FLC susceptible and resistant isolates. Experiments were performed in biological duplicates and technical triplicates and statistical significance was calculated using unpaired *t*-test in GraphPad Prism 9 software.

Rhodamine-6G efflux

Efflux of R6G was determined essentially using a protocol described by Kohli et al. (2002). Briefly, overnight cultures of strains were diluted to OD_{600} 0.2 in YEPD media with and without Nva-FMDP and allowed to grow for 4 h. Cells were then resuspended in phosphate-buffered saline at a cell density of 10^8 cells ml^{-1} and incubated at 37 °C for 1 h in a shaker. R6G was then added at a final concentration of 10μ M and incubated for 3 h. After incubation cells were washed and suspended in PBS containing 2% glucose and incubated for 45 min to initiate the efflux. Cells were then centrifuged and supernatant was collected and absorption was measured at 527 nm.

Calcofluor White staining to assess chitin content

Overnight cultures of strains were diluted to OD_{600} 0.2 in RPMI-1640 and grown for 4 h at 37 °C, shaking. Cells were collected by centrifugation and fixed with 3.7% formaldehyde solution to final cell density corresponding to OD_{600} 1 for 30 min. Washed the cells with phosphate buffer saline (PBS) and treated with final conc. of 10μ g/ml Calcofluor White solution for 10 min in dark. After washing with PBS cells were mounted on a glass slide and observed using a DAPI filter set on confocal microscope as described previously (Okada and Ohya, 2016).

Quantitation of cell wall components by HPLC

Cell wall of isolates were prepared from exponential cultures grown in RPMI-1640 with and without Nva-FMDP. Briefly, cell walls were extracted by disrupting cells with glass beads (Sigma, G9268) using a Fastprep cell breakage machine (Thermo Savant, Middlesex, UK). Samples were then washed five times with 1 M NaCl and cell walls were extracted using SDS-Mer-OH buffer (50 mM Tris, 2% sodium dodecyl sulphate (SDS), 0.3 M β- mercaptoethanol, 1 mM EDTA; pH 8.0) at 100 °C for 10 min. Cell wall pellets were resuspended in sterile dH2O, freeze dried, and the dry weight of recovered cell walls was measured. Chitin contents were determined by measuring the glucosamine, glucan by measuring glucose and mannan was determined by measuring mannose released by acid hydrolysis of purified cell walls as described previously (Plaine et al., 2008).

CRediT authorship contribution statement

Garima Shahi: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Mohit Kumar:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Andrzej S. Skwarecki:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Matt Edmondson:** Methodology, Investigation, Formal analysis, Data curation, Writing – review & editing. **Atanu Banerjee:** Formal analysis, Data curation, Writing – review & editing. **Jane Usher:** Methodology, Investigation, Formal analysis, Data curation. **Neil A.R. Gow:** Resources, Formal analysis, Data curation, Writing – review & editing. **Sławomir Milewski:** Conceptualization, Resources, Formal analysis, Data curation, Writing – review & editing. **Rajendra Prasad:** Conceptualization, Resources, Formal analysis, Data curation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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