Olorofim and the azoles are antagonistic in *A. fumigatus* and functional genomic screens reveal mechanisms of cross resistance.

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Abstract:

Aspergillosis, in its various manifestations, is a major cause of morbidity and mortality. Very few classes of antifungal have been approved for clinical use to treat these diseases and resistance to the first line therapeutics is increasing. A new class of antifungals, the orotomides, are currently in development with the first compound in this class olorofim in late-stage clinical trials. In this study, we characterise a network of genes that govern olorofim response in *A. fumigatus*. We reveal that the number of transcription factors that regulate olorofim susceptibility are far fewer than we have previously observed for the azoles and the change in sensitivity observed in these isolates is less extreme. Intriguingly, loss of function in two higher order transcriptional regulators, HapB a member of the heterotrimeric HapB/C/E (CBC) complex or the regulator of nitrogen metabolic genes AreA, leads to cross resistance to both the azoles and olorofim. However, a clinical azole resistant isolate with a point mutation in HapE (*hapE^{P88L}*) retains sensitivity to olorofim. Our transcriptomic analysis suggests that altered sensitivity to olorofim may emerge via modification of genes involved in the production of pyrimidine biosynthetic precursors. Finally, we also show that the action of the azoles are antagonistic to olorofim *in vitro*.

Introduction:

Invasive and chronic forms of aspergillosis affect over 3 million people resulting in excess of 300 thousand deaths per year [1]. Only three classes of antifungals are currently clinically available to treat aspergillosis, with the triazoles used as first-line therapy in most centres [2]. Resistance to the azoles is rising, which is linked to usage of triazole compounds in agri- and horticulture [3]. It is expected more resistant *A. fumigatus* will be seen due to increased azole used in farming linked to climate change [4]. In the last decade, isavuconazole is the only antifungal to have been approved by the Food and Drug Administration (FDA) for treatment of invasive aspergillosis [5], however many of the mechanisms that contribute to resistance to the other azoles also result in isavuconazole resistance. The development of novel classes of antifungals will be a key component to addressing the emerging resistance problem. Fortunately, there are a number of novel classes of antifungal currently in development for treatment of invasive aspergillosis (IA) including ibrexafungerp, which has recently (2021) gained FDA approval for treatment of vulvovaginal candidiasis and olorofim (phase 3) [6]. Olorofim (formerly known as F901318 and under development by F2G, Ltd.) is of particular interest as it is not only a member of a novel class of antifungal (the orotomides) but unlike ibrexafungerp has a novel mechanism of action that has not been exploited clinically [7]. As olorofim is orally bioavailable it presents a realistic alternative to the azoles for long-term treatment of chronic and allergic infections and especially resistant infections [8] and could potentially be used in combination therapy strategies to suppress the emergence of resistance.

Olorofim acts by inhibiting the enzyme dihydroorotate dehydrogenase (DHODH), which is a crucial enzyme within the pyrimidine biosynthesis pathway and is required for both DNA and RNA synthesis [7]. Structural and biochemical analysis of DHODH suggests olorofim competes with CoQ to bind to DHODH, preventing the oxidation of dihydroorotate to orotate.

DHODHs are grouped into 2 classes according to sequence similarity and subcellular localisation. Both mammals and most fungi have class 2 DHODH, which is bound to the inner mitochondrial membrane [9]. The human DHODH only shares 30% protein sequence identity with the *A. fumigatus* DHODH and olorofim has also been demonstrated to be >2,200-fold more potent against the *A. fumigatus* enzyme [7]. Inhibition of the pyrimidine biosynthesis pathway by olorofim prevents the germination of *A. fumigatus* conidia and causes hyphae to undergo morphological changes [10]. Prolonged exposure of germlings and vegetative hyphae to olorofim also causes extensive isotropic expansion that is then followed by cell lysis [11].

Olorofim has an effect on a wide range of fungi and has been shown to be effective against *Coccidioides immitis*, *Scedosporium* spp., *Madurella mycetomatis*, *Lomentospora prolificans* and several *Aspergillus* species [12-18]. Olorofim is also effective against triazole resistant *A. fumigatus* isolates and cryptic *Aspergillus* species [19, 20]. In several murine models of aspergillosis, olorofim treatment significantly reduced fungal burden and mortality [15]. However, olorofim has a reduced activity against *Fusarium solani* species complex and *Fusarium dimerum* and is inactive against Mucorales [21]. To date, there is no reported incidence of acquired olorofim resistance in clinical isolates of normally susceptible fungal species.

In this study, we explore potential routes to olorofim resistance for *A. fumigatus* through screening a 484-member *A. fumigatus* transcription factor null mutant library. We have uncovered a total of four transcription factors, which regulate susceptibility to olorofim. Existing published literature, and our phenotypic and transcriptomic data revealed these transcription factors regulate genes involved in upstream processes of the pyrimidine biosynthesis pathway. Notably two of these transcription factor null mutants, $\Delta hapB$ and $\Delta areA$, had elevated MICs to olorofim and are resistant to the azole class of antifungal

highlighting potential routes to cross resistance. As HapB is a member of the heterotrimeric CCAAT-binding complex (which comprises HapB/C and E) we assessed the susceptibility of a azole resistant clinical isolate with a mutation in *hapE* (*HapE^{P88L}*). Intriguingly this mutation does not change susceptibility towards olorofim. Furthermore, we demonstrate that there is an antagonism between the azoles and olorofim in three wild-type strains of *A. fumigatus* and the olorofim resistant mutants. Despite our discovery that there are mechanisms that can reduce susceptibility to both azoles and orotomides we feel these results do not preclude the development of combination therapeutics with these two agents. Our study also provides data that can be used to develop molecular diagnostic tests to identify olorofim resistant isolates in clinic.

Materials and Methods:

Fungal strains

Conidia of *Aspergillus fumigatus* MFIG001 (a derivative of CEA10) and transcription factor null mutants [22, 23] were prepared by inoculating strains in vented 25cm² tissue culture flasks with Sabouraud Dextrose agar (Oxoid, Hampshire, England) and incubating at 37°C for 48 hours. Spores were harvested in PBS + 0.01% Tween-20 by filtration through Miracloth. Spores were counted using a haemocytometer (Marienfeld Superior, Baden-Württemberg, Germany). The clinical isolate VP067-36, containing the P88L allele of HapE, was a kind gift from Willem Melchers and Paul Verweij [24]. To construct the HapE^{P88L} allele in MFIG001, the *hapE* gene was amplified from VP067-36 using primers hapE_gene_Fw and hapE_gene_Rv (Supplementary Table 1). This PCR product was transformed into *A. fumigatus* MFIG001 using CRISPR-Cas9 mediated transformation, using hapE_392_revcom as crRNA [25]. Transformants were selected using voriconazole (1 mg/L) containing YPS plates. Transformation was confirmed by PCR amplification of the complete *hapE* gene (hapE_Fw and hapE_Rv), followed by Sanger sequencing using hapE_Seq.

Olorofim MIC screening

Olorofim was a kind gift of F2G Ltd. The Minimum Inhibitory Concentration (MIC) of olorofim against *A. fumigatus* was assessed using the European Committee for Antimicrobial Susceptibility Testing (EUCAST) methodology [21, 26]. Briefly, 2x10⁴ spores/mL (in 100 µl) were added to a CytoOne® 96-well plate (StarLab, Brussels, Belgium) containing 1xRPMI-1640 medium (Sigma Aldrich, St. Louis, MO), 165 mM MOPS buffer (pH 7.0), 2% glucose, with olorofim 2-fold dilution series ranging from 0.1 µg/L to 0.25 mg/L and a drug free control (n = 4). Additionally, a serial dilution of olorofim containing 10 mM uracil and uridine was performed. 96-well plates were incubated at 37°C for 48 hours. The MIC was determined as the minimum drug concentration at which no germination was observed. Optical density was

measured at 600 nm using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT). In keeping with research laboratory based definitions, but in contract to definitions used clinically, we define *in vitro* resistance as a strain that is less susceptible to drug than the parental isolate [27].

Olorofim sensitivity screening of the A. fumigatus transcription factor null mutant library

2x10⁴ spores/mL from each of the 484 members of the transcription knockout library were added to 1x RPMI-1640 medium, 165 mM MOPS buffer (pH 7.0), 2% glucose in each well of a CytoOne® 96-well plate with 0.002 mg/L olorofim (n = 4). Plates were incubated at 37°C for 48 hours. Fitness was calculated by dividing the optical density of respective null mutants to the MFIG001 control. Relative fitness in olorofim was calculated by dividing fitness in olorofim with general growth fitness of the transcription factor null mutants using the same microculture conditions in 1x RPMI-1640 medium, 165 mM MOPS buffer (pH 7.0), 2% glucose without olorofim (n = 4). Optical density was measured at 600 nm on a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT).

RNA-extraction

1x10⁶ spores/mL of *A. fumigatus* MFIG001, $\Delta AFUB\_056620$ and $\Delta AFUB\_030440$ were inoculated into 50 mL of *Aspergillus* complete media (ACM) [28] and incubated for 18 hours at 37°C in a rotary shaker (180 rpm). Mycelia were harvested using filtration through Miracloth (Merck Millipore) and washed in 1x RPMI-1640 medium. Approximately 1g of mycelia was added to shake flasks containing 50 mL RPMI-1640 medium, 165 mM MOPS buffer (pH 7.0), 2% glucose and incubated for 1 hour at 37°C in a rotary shaker (180rpm) in the presence or absence of 0.062 mg/L olorofim (n = 3). Mycelia was filtered through Miracloth and snapfrozen using liquid nitrogen and kept at -80°C until required.

To extract RNA, 1 mL of TRIzol reagent (Sigma Aldrich) and 710-1180 µm acid washed glass beads (Sigma Aldrich) were added to frozen mycelia and placed in a TissueLyser II® (Qiagen,

Hilden, Germany) for 3 minutes at 30 Hz. The solution was centrifuged (12,000 rpm) for 1 minute at 4°C. The aqueous phase was added to 200 µL of chloroform and centrifuged (12,000 rpm) for 10 minutes at room temperature. The supernatant was added to 0.2 M sodium citrate, 0.3 M sodium chloride and 25% (v/v) isopropanol and left at room temperatures for 10 minutes. This solution was centrifuged (12,000 rpm) for 15 minutes at 4°C. The supernatant was removed; the pellet was washed in 70% (v/v) ethanol and resuspended in RNase free water (Thermo Fisher Scientific, Waltham, MA). RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI) and purified using a RNeasy Mini Kit (Qiagen). RNA quality and quantity were assessed using gel electrophoresis and using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific). All RNA extractions were carried out in triplicate.

Transcriptomic Analysis

RNA sequencing was carried out by the Genomic Technologies Core Facility (GTCF) at The University of Manchester. Sequencing libraries were prepared from mRNA using TruSeq® Stranded mRNA assay (Illumina, San Diego, CA). Samples were sequenced on a single lane on an Illumina HiSeq2500 (Illumina). Low-quality reads of resulting fastq files were removed using FastQC and trimmed using Trimmomatic (Quality >20, Sliding window average of 4 bases) [29]. Bowtie was used to align libraries to the *A. fumigatus* A1163 genome assembly GCA_000150145.1 with gene annotation from CADRE/Ensembl Fungi v24 [30]. Differential expression analysis of was performed using DESeq2 [31].

Functional category and gene ontology enrichment analysis was carried out using FungiFun2 2.2.8, converting genes to Af293 gene names to allow using the KEGG option [32]. Genes that showed over 2-fold in differential expression and Benjamin-Hochberg FDR <0.01 underwent enrichment analysis. StringsDB analysis was performed by only including genes with at least two connections.

Phenotypic analysis

For colony images, 500 spores per isolate were spotted onto solid ACM or Aspergillus Minimal Media (AMM) and left to dry. Plates were incubated at 37°C for 72 hours and imaged. Growth on solid AMM supplemented with different nitrogen sources (50 mM ammonium tartrate, 10 mM sodium nitrate, 10 mM L-glutamine, 10 mM urea or 10 mM L-proline) were assessed by spotting 500 spores from each isolate (n = 3). Plates were incubated at 37°C for 72 hours. MICs were determined using the same supplementation as the phenotypic test with a serial dilution of olorofim (ranging from 0.1 µg/L to 0.25 mg/L). 96-well plates were incubated for 48 hours at 37°C and growth was determined by microscopic evaluation.

Checkerboard assays

For assessing drug combination efficacies of itraconazole and olorofim against *A. fumigatus*, we used a checkerboard assay similar to EUCAST MIC testing described above. Twofold serial dilutions of itraconazole were prepared across the X-axis and olorofim serial dilutions across the Y-axis. The MIC was determined by microscopy by visually assessing the well containing the lowest drug concentration with non-germinated spores. The fractional inhibitory concentration index (FICI) was calculated as the MIC in combination divided by the MIC of individual drugs [33].

Disk assays

4 × 10⁴ conidia of the relevant *A. fumigatus* strain were evenly distributed on solidified 1xRPMI 1640 (Sigma), 165 mM MOPS buffer (pH 7.0), 2% glucose. One 6 mm antibiotic assay disk (Whatman) was placed on the middle of the plate or two disks at a fixed distance, and 10 µL of voriconazole (0.8 mg/mL), olorofim (0.005 mg/L) or H₂O₂ (30%) were added to each of them. The plates were incubated at 37°C for 48 hours and imaged.

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Data availability

RNA-seq data is available from ArrayExpress as experiment: E-MTAB-10590. The differential expression output from DESeq2 is included as Supplementary Data 1. (reviewer access: Reviewer_E-MTAB-10590 Password: pptwwqmj)

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Competing interests

Michael Bromley is a former employee of F2G Ltd. F2G currently funds a PhD position in the laboratory. F2G was not involved in any of the experimentation or analysis of data in this study.

Author contributions

N.v.R designed and performed the experiments, analysis, wrote and edited the manuscript. S.H. designed and performed experiments and analysis. C.V. designed and performed experiments and analysis. J.A. designed and performed experiments and analysis. M.B. provided the funding, designed the experiments, wrote and edited the manuscript

Results:

The effect of olorofim on A. fumigatus MFIG001 results in minor transcriptional changes in the pyrimidine biosynthetic pathway.

In order to standardise assays throughout, the Minimum Inhibitory Concentration (MIC) of olorofim against *Aspergillus fumigatus* MFIG001 was determined. The MIC was defined as the minimum concentration of olorofim at which no germination from *Aspergillus* spores was observed by microscopic evaluation (**Supplemental Figure 1**). Microscopic evaluation revealed the MIC of olorofim to be 0.06 mg/L for *A. fumigatus* MFIG001, consistent with previous findings of other *A. fumigatus* isolates [19]. The effect of olorofim on growth of *A. fumigatus* was further evaluated by measuring optical density of the plates used to determine the MIC (**Figure 1a**). The maximal growth observed ($OD_{600} = 0.39$) and minimal growth observed ($OD_{600} = 0.04$) was separated by a 64-fold difference in drug concentration, which is in stark contrast to the inhibitory effects of the azoles on *A. fumigatus* where the difference between maximal and minimal growth typically occurs over a drug concentration not exceeding 8-fold (**Supplemental Figure 2**). As this range is so broad, we consider it useful to measure the concentration at which growth is inhibited by 50% (herein referred to as IC₅₀ [34] to distinguish from MIC₅₀ which is an MIC determination made of populations). For MFIG001, the IC₅₀ for olorofim is 0.012 mg/L whereas for itraconazole its 0.65 mg/L [22]. As olorofim inhibits pyrimidine biosynthesis, it would be expected that the action of the drug would be fully reversed by supplementing the media with an excess of exogenous pyrimidines [7]. To confirm growth inhibition was due to directly targeting the pyrimidine biosynthesis pathway, the MIC was determined with the addition of 10 mM uridine and 10 mM uracil

(**Figure 1b**). Under these conditions there was no observed reduction in *A. fumigatus* growth, and at all olorofim concentrations the median OD₆₀₀ did not fall below control levels indicating that there are no significant off target effects of this drug.

To facilitate our understanding of how olorofim impacts the transcriptome of *A. fumigatus*, RNA-seq analysis was performed. Upon olorofim exposure (1x MIC), a modest 185 genes and 41 genes respectively were up- and downregulated (**Figure 1c**). Our expectation was that several genes in the immediate pyrimidine biosynthesis pathway would be upregulated however only the multifunctional carbamoyl-phosphate synthase/aspartate carbamoyltransferase (PyrABCN, AFUB_077330) enzyme, which is upstream of DHODH and converts carbamoyl-P to N-carbamoyl-L-aspartate, was upregulated by Log2 fold >1. Instead, genes associated with pathways that synthesise precursors of the pyrimidine biosynthetic pathway were identified including oxaloacetate metabolism and glutamate biosynthesis (**Figure 1d,e**). Genes associated with tyrosine metabolism; secondary metabolite biosynthesis, glycolysis/gluconeogenesis and valine, leucine and isoleucine degradation were enriched among downregulated genes (**Figure 1d,e**). Interestingly, several proteins involved in ergosterol biosynthesis (HmgA, Erg13a and Erg26) were differentially expressed (**Supplemental Data 1**).

Deletion of HapB, AreA, DevR and AcdX changes olorofim susceptibility.

In order to identify novel transcriptional regulators associated with differential olorofim susceptibility, the COFUN transcription factor knockout (TFKO) library was screened against olorofim at a concentration that reduces growth of the isogenic wildtype isolate (MFIG001) by c 20% (0.002 mg/L). At this concentration we were able to identify strains that have the potential to be resistant or hypersensitive (**Figure 2a**).

Three transcription factor null mutants ($\Delta areA$, $\Delta hapB$ and $\Delta devR$) showed reproducible increased relative fitness in the presence of olorofim and elevated MICs compared to MFIG001 (**Figure 2c, d and e**). Remarkably, two of these mutants ($\Delta areA$ and $\Delta hapB$) are also resistant to the azole class of antifungals. Loss of AreA, a transcription factor that has a global role in activating expression of genes involved in nitrogen acquisition and processing [35] or loss of HapB, which along with HapC and HapE, comprise the CCAAT Binding Complex (CBC) [36] resulted in a 2-fold increase in MIC to olorofim when compared to the isotype control MFIG001; IC50 values for these strains were 0.04 mg/L (4-fold increase) and 0.07 mg/L (8 fold-increase), respectively (**Figures 2c and 2e**). The $\Delta devR$ mutant showed a significant reduction in susceptibility to olorofim at concentrations ranging from 0.008 mg/L to 0.06 mg/L (MIC) and had an IC50 of 0.025 mg/L (**Figure 2d**). Although the MIC for this strain increased to >0.125 mg/L most spores did not germinate at this concentration making this phenotype reminiscent of tolerance (**Supplemental Figure 1**).

One isolate ($\Delta AFUB\_056620$, $\Delta acdX$) showed a significant increase in sensitivity to olorofim and had an MIC of 0.03 mg/L and a IC50 of 0.006 mg/L, 2-fold lower than *A. fumigatus* MFIG001 (**Figure 2b**). The *acdX* gene encodes a 612 amino acid transcription factor which, a SMART domain search showed, contains six WD40 repeat units but no other functional domains. A reciprocal BLAST of the AFUB_056620 protein sequence found a match to the *Saccharomyces cerevisiae* transcription factor Spt8. However, the proteins only share 44% identity of the entire protein sequence. In *S. cerevisiae*, Spt8 forms part of the SAGA (Spt-Ada-Gcn5-acetyltransferase) complex [37] which is known to act as a transcriptional activator under several stress conditions. While the orthologue of AcdX in other fungi generally contains six WD40 domains, in species such as *N. crassa* and *A. terreus* only five domains are present, however the significance of this is unclear. In *A. nidulans* AcdX has been described to be

functional in the SAGA complex and is involved in repressing genes in acetate metabolism and has a regulatory role in the proline metabolic pathway [38].

Transcription factor mutants with altered susceptibility to orlofin have defects in nitrogen assimilation.

Further phenotypic analysis of the null mutants with differential susceptibility to orlofin revealed that all had growth defects on *Aspergillus* Complete Medium (ACM) (**Figure 3a and b**) and *Aspergillus* Minimal Medium (AMM) (**Figure 3a and c**). The *hapB*, *devR*, *areA* and *acdX* null mutants showed a reduction of radial growth on ACM of 28%, 22%, 12% and 24% respectively when compared to the isotype control. On AMM, the *hapB* mutant showed increase radial growth (58%) however, colony growth was more diffuse than the isotype strain (**Figure 3a and c**). As orlofin inhibits DHODH, which acts within the pyrimidine biosynthetic pathway, and we observed that orlofin results in transcriptional repositioning of several pathways linked to nitrogen processing we hypothesised that these growth defects could be nitrogen source dependent. Indeed, the radial growth defect of strains were reversed by differential supplementation ($\Delta devR$ by proline; $\Delta acdX$ by glutamine or proline and $\Delta areA$ by glutamine). Furthermore, we observed an interesting correlation between radial growth on the different nitrogen containing media and orlofin sensitivity. While the radial growth of the isotype isolate differed little between nitrogen sources (**Figure 3d and Supplemental Figure 3**) the strains lacking the ability to effectively utilise nitrate as a sole nitrogen source were all less susceptible to orlofin. Taken together, this suggests that defects in nitrogen utilisation in these transcription factor null mutants could be linked to their orlofin susceptibility.

Changes in susceptibility to orlofin in $\Delta devR$ and $\Delta acdX$ mutants are caused by opposing regulation of pathways preceding pyrimidine biosynthesis

In order to characterise the basis of differential olorofim susceptibility in the $\Delta devR$ and $\Delta acdX$ mutants the transcriptomes of these two mutants were compared to the wild-type (**Supplemental Data 2, Supplemental Figure 4**). In the absence of olorofim 510 and 137 genes were respectively downregulated and upregulated in the $\Delta devR$ isolate while 212 were downregulated and 194 upregulated upon olorofim exposure. In the absence of olorofim, notable enriched functional categories included downregulation of genes involved in tyrosine metabolism and an upregulation of genes involved in the biosynthesis of branched chain amino acids and metabolism of arginine and proline, the latter of which was also seen under olorofim exposure (**Figure 4a**). A detailed pathway analysis of genes involved in the conversion of metabolites towards L-glutamate and through to orotate revealed that proline uptake and degradation were upregulated in the *devR* null mutant (**Figure 4c**). Other pathways that contribute to orotate precursors were also significantly upregulated, notably the nitrate assimilation pathway (NAP [*crnA*, *niaD*, *niiA*]), and glutamate, glutamine and carbomyl-P synthesis. Pathways that compete with orotidine biosynthesis for L-glutamate were not differentially regulated in any of the assessed mutants (**Supplemental Figure 5**). Our transcriptional data therefore suggests that nitrogen metabolism maybe altered in this strain in ways that favor the generation of precursors for orotate biosynthesis and hence could explain the reduced sensitivity of *devR* null mutant to olorofim.

The transcriptome of the olorofim hypersensitive $\Delta acdX$ mutant also revealed that proline and arginine metabolism were upregulated compared to the wild-type however genes involved in the NAP and glutamate, glutamine and carbomyl-P synthesis pathways were downregulated suggesting that *AcdX* and *DevR* have directly opposing functions on these linked pathways (**Figure 4d**) and providing further evidence to suggest that regulation of these pathways is important for olorofim sensitivity.

Our transcriptomic data led us to assess the effect of pyrimidine pathway precursors on olorofim susceptibility in the transcription factor null mutants (**Supplemental Figure 6**). Using nitrate as the most abundant, but not sole nitrogen source increases susceptibility of *A. fumigatus* MFIG001 to olorofim. Interestingly this increase in susceptibility is lost in the $\Delta devR$, perhaps as a consequence of the upregulation of the NAP in this mutant. In keeping with this, the resistance phenotype observed with the loss of AreA, a regulator that activates the NAP, is lost. The hypersensitive phenotype in the *acdX* null mutant was exaggerated when arginine and proline were used as sole nitrogen sources. These data, combined with results from our transcriptomic analysis suggest that modification of environmental nitrogen sources and or dysregulation of nitrogen metabolism is directly linked to changes in olorofim sensitivity.

A clinical isolate with an hapE allele known to contribute to azole resistance does not affect olorofim sensitivity.

HapB is a member of the heterotrimeric CBC complex along with HapE and HapC. We have previously shown that this complex negatively regulates sterol biosynthesis and loss of any member of this complex leads to azole resistance in *A. fumigatus* [22]. As the $\Delta hapB$ mutant exhibited an olorofim resistance phenotype, we assessed the olorofim susceptibility for a clinical isolate (V067-36) which has non-synonymous SNP in the *hapE* gene (resulting in a P88L substitution) and which has been demonstrated to affect azole susceptibility. The MIC of this isolate and an isogenic isolate with a wild-type *hapE* allele from the same patient were identical for olorofim. To further confirm that the *hapE*^{P88L} allele did not impact olorofim susceptibility, we reconstructed this point mutation in the MFIG001 background. While a $\Delta hapB$ mutant showed decreased susceptibility to both olorofim and itraconazole, the MFIG001 *hapE*^{P88L} strain only showed decreased susceptibility to itraconazole (**Figure 5**).

The action of the azoles and olorofim are antagonistic.

As we have identified two genes whose loss of function can result in cross resistance, we assessed if both drugs when used in combination would be synergistic against the respective mutants. We first evaluated the potential synergism of both compounds against the isotype control MFIG001. To our surprise given the distinct mechanisms of action of the orotomides and the azoles, we observed a clear antagonism between itraconazole and olorofim. The antagonism also persisted in the $\Delta hapB$, the V067-36 isolate and the *hapE*^{P88L} isolate (**Figure 5**). We evaluated our previously published RNA-seq data to assess if itraconazole increased expression of genes involved in olorofim biosynthesis however contrary to our expectations, many of the genes in the pyrimidine biosynthetic pathway were highly downregulated, even when itraconazole was used at low levels (**Supplemental Data 1**).

We assessed if the antagonism persisted in multiple genetic backgrounds and to an alternative azole (voriconazole). Consistent with our previous observations the wild-type isolate CEA10, which is the parental strain from which MFIG001 derived, the ATCC46645 isolate and an azole resistant isolate with the TR₃₄ L98H allele isolated from a patient, all showed an antagonistic relationship between olorofim and voriconazole (**Figure 6**). Interestingly our data suggests that the antagonism is predominantly unidirectional, with the azoles inhibiting the action of olorofim in both solid and liquid media for all strains.

Discussion

Olorofim is a novel antifungal, currently in phase 3 clinical trials. It has a broad spectrum of activity against most moulds and acts by inhibiting the pyrimidine biosynthetic pathway through disruption of DHODH activity [7]. Our preliminary analysis of the inhibitory effects of olorofim revealed that the MIC and the IC₅₀ were separated over a relatively large concentration range (5-fold). This contrasts with what is seen with itraconazole and other azoles where this concentration spread is typically 2-fold. The clinical implication of this finding remains unclear, however it is likely that olorofim will support clearance of an infection at doses well below the MIC. At these lower concentrations however, exposure to drug will be imparting selective pressure and has the potential to induce the production of mutagenic precursors that may drive the emergence of resistance as has been shown for several antibiotics [39]. As with other anti-infectives that act by inhibiting a single biological target there is clear potential for emergence of resistance. Understanding these mechanisms will provide a framework for development of diagnostics to detect resistance rapidly in the clinic.

Our previous survey of azole sensitivity in the *A. fumigatus* COFUN transcription factor knockout library [22] revealed 6 null mutants that had decreased sensitivity (ranging from 4 to 6-fold increase in MIC compared to the isogenic control) and 6 had increased sensitivity (4 to 8-fold decrease in MIC) to itraconazole. Here our screen revealed that only 1 mutant ($\Delta acdX$) showed increased sensitivity while 3 showed decreased sensitivity ($\Delta hapB$, $\Delta devR$, $\Delta areA$) to olorofim and the changes in sensitivity in these isolates were less extreme than seen for the azoles. It is unsurprising, given the mechanism of action of olorofim, that the transcription factors that we have identified in this screen either have well defined roles in regulating nitrogen utilisation or have been linked to this function in our study.

What is remarkable however given the distinct mechanisms of actions of the two compound classes, loss of function of either of AreA and HapB results in cross-class resistance to both

the azoles and orotomides. HapB is a member of the heterotrimeric CCAAT-binding complex (CBC) and alongside HapC and HapE regulates the expression of over a third of the genome [40] including several genes involved in ergosterol biosynthesis. The *hapB* null displayed the highest levels of resistance to olorofim and was able to germinate at 0.12 mg/L, which is 8-fold higher than the parental isolate but within the concentration range needed for clinical utility. In *A. nidulans* AreA is a positive regulator of many genes that are required for utilisation of nitrogen sources other than glutamate or ammonia [41] with loss of function resulting in an inability to utilise amongst other nitrogen sources, nitrate, nitrite, uric acid and many amino acids [42]. Reassuringly however, drug concentrations in animal models are tolerated well above the increased MIC levels of the null mutants identified in this screen. Dosing 8 mg/kg at 8 hour intervals in mice results in peak serum levels of 2.5-3 mg/L [43]. Olorofim can be tolerated at doses as high as 30 mg/kg intravenously, giving scope for higher drug levels *in vivo* if required. In cynomolgus monkeys a single oral dose of olorofim resulted in peak levels of 0.605-0.914 mg/L in serum for female and male animals, respectively [44].

Recently a clinical isolate, V067-36, has been described that harbours a mutant HapE allele (HapE^{P88L}) which directly contributes to azole resistance [24]. Even though this mutation appears to affect the way the CBC interacts with its gDNA target, independent of other co-factors [40, 45, 46] our data shows that this allele does not affect susceptibility of *A. fumigatus* to olorofim and is therefore less important at regulating a response to the orotomides. It is also noteworthy that to date, no further documented cases of azole resistance have been linked to CBC function and even though AreA is partially dispensable for virulence in *A. fumigatus* [41], no cases of clinical resistance have been linked to AreA function. Therefore, although concerning, the cross-resistance phenotypes we have observed may not have major clinical significance. If olorofim is ultimately licenced for use, monitoring for co-resistance before switching therapy would be a sensible precaution.

Our studies have shown there is a clear antagonism of the azoles and olorofim. Whilst concerning the antagonism is only evident when relatively low levels of both drugs are used. It is interesting to note that the TR₃₄ L98H isolate used in this study has reduced susceptibility to olorofim when compared to the CEA10 isolate and the antagonism drives the MIC above 0.5 mg/L. If these drugs are to be used in combination in a clinical setting, careful evaluation of respective drug levels at the site of infection to ensure sufficient concentration of drug to avoid antagonism would be sensible. The consequences of using azoles and olorofim in combination for treatment of strains harbouring the TR₃₄ L98H allele also needs further evaluation.

In summary, we have explored the mechanism behind olorofim susceptibility through a systematic analysis of the COFUN transcription factor null library. All the mutants we identified that had altered sensitivity to olorofim have associated defects in nitrogen metabolism and two of these mutants $\Delta devR$ and $\Delta acdX$ regulate genes involved in metabolic pathways immediately upstream of the pyrimidine pathway potentially leading to a differential flux of metabolites into this pathway. Although we have identified two transcription factors, the CBC and AreA, that regulate cross resistance to both the azoles and olorofim we are reassured that the only recognised incidence of clinical azole resistance linked to CBC function (HapE P88L) does not lead to olorofim resistance.

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Figure 1: The response of *A. fumigatus* to olorofim. (a) MIC of *A. fumigatus* MFIG001 to olorofim as measured by OD₆₀₀ by EUCAST methodology. (b) Addition of 10mM uracil and 10mM uridine reverses the action of olorofim to *A. fumigatus* MFIG001 (n=3). (c) Volcano plot of RNA-seq of *A. fumigatus* A1160p+ exposed to olorofim. 185 genes (blue dots) and 41 genes (red dots) were considered downregulated and upregulated, respectively (>2-fold differentially regulated, p<0.05). (d) KEGG pathways that are enriched within differentially regulated genes, blue categories are associated with downregulated genes, red with upregulated genes. (e) Interactions of proteins involved in response to olorofim as determined by StringsDB. Proteins derived from upregulated transcripts are in red, downregulated in blue.

Figure 2: Olorofim susceptibility screening of the COFUN transcription factor knockout library. (a) Relative fitness of each individual strain was assessed by normalising to fitness in non-drug condition (n=3). TF null mutants that are of particular interest are highlighted. (b-e) MIC to olorofim of the TF null mutants, (b) for $\Delta acdX$, (c) for $\Delta hapB$, (d) for $\Delta devR$, (e) for $\Delta areA$, as determined by OD₆₀₀ (n=3). Statistical difference was assessed by Two-way ANOVA with Sidaks multiple comparison test (* p<0.05, ** p<0.01, *** p<0.001, *** p<0.001).

Figure 3: Phenotypic evaluation of TF null mutants. (a) 500 spores of TF null mutants and MFIG001+ were spotted on Aspergillus Complete Medium and Aspergillus Minimal Medium and incubated for 48 hours at 37° Celsius. (b-c) Radial growth of TF null mutants and A1160p+ on ACM (b) and AMM (c), after 72 hours at 37° Celsius (n=3) (d) TF null mutants spotted on AMM supplemented with 50 mM ammonium tartrate, 10 mM sodium nitrate, 10 mM L-glutamine, 10 mM urea or 10 mM L-proline (n=3) Statistical difference was assessed by two-way ANOVA with Dunn's correction (p-values < 0.05 are shown).

Figure 4: RNA-seq analysis of $\Delta devR$ and $\Delta acdX$ exposed to olorofim. (a) KEGG pathways enriched for down- (blue) or upregulated (red) genes in RPMI-1640 or upon olorofim exposure

for $\Delta devR$ compared to *A. fumigatus* A1160p+. (b) KEGG pathways enriched for down- (blue) or upregulated (red) genes in RPMI-1640 or upon olorofim exposure for $\Delta acdX$ compared to *A. fumigatus* A1160p+. (c) Detailed analysis of genes involved in pathways upstream of and including the pyrimidine pathway. The target of olorofim, DHODH, is highlighted. Blue is more than 1-fold downregulated, yellow more than 1-fold upregulated; red is more than 5-fold upregulated. The right of each box is associated with $\Delta acdX$, left with $\Delta devR$. (d) Heatmap of genes involved in the pyrimidine pathway and component upstream of this pathway.

Figure 5: Checkerboard assays for CBC complex associated strains. Checkerboard assays were performed (n=3) by using a double dilution series of itraconazole (16-0.06 mg/L) along the X-axis and olorofim (0.5-0.003 mg/L) along the Y-axis. The $\Delta hapB$ and hapE^{P88L} isolates are in the MFIG001 background, the V067-36 isolate is a clinical isolate containing the P88L point mutation within HapE. The FICI is shown in the right top corner of each individual plot to show the antagonistic effect. [Values greater than 4 indicate antagonism.](#)

Figure 6: The antagonistic effect between voriconazole and olorofim. (a) Checkerboard assays were performed (n=2) by using a double dilution series of voriconazole (16-0.06 mg/L) along the X-axis and olorofim (0.500-0.007 mg/L) along the Y-axis. Two wild-type isolates (CEA10 and ATCC46645) were assessed and an azole resistant TR₃₄ L98H isolate. The FICI is shown in the right top corner of each individual plot to show the antagonistic effect. (b) Representative images of disk assays of the CEA10, ATCC46645 and TR₃₄ L98H isolates to voriconazole and olorofim. A clear disruption of the olorofim halo can be observed proximal to the voriconazole halo.

Supplemental Figure 1: Microscopical evaluation of *A. fumigatus* MFIG001 and transcription factor null mutants upon olorofim exposure. Representative images were taken from the MIC 96-well plates by stereomicroscope. Maximum concentration at which growth is observed is highlighted in black for each strain.

Supplemental Figure 2: Determination of IC₅₀ for itraconazole. MIC to olorofim in RPMI-1640 was determined according to EUCAST methodology for *A. fumigatus* MFIG001. OD₆₀₀ was measured after 48 hours to determine growth quantitatively.

Supplemental Figure 3: Images of nitrogen spot tests of TF null mutants. Growth of TF null mutants and wild-type was assessed on AMM supplemented with 50 mM ammonium tartrate, 10 mM sodium nitrate, 10 mM L-glutamine, 10 mM urea or 10 mM L-proline (n=3). Images were taken after 72 hours at 37 Celsius.

Supplemental Figure 4: Volcano plots of $\Delta acdX$ and $\Delta devR$ RNA-seq data. Volcano plots showing log₂(Fold-Change) compared to A1160p+ on the X-axis and $-\log_{10}$ (False Discovery Rate) from DESeq2 on the Y-axis. Genes considered differentially regulated are coloured in red, and number of genes differentially regulated are noted in each respective corner of volcano plots.

Supplemental Figure 5: Pathways utilising L-glutamate. Additional pathways utilising L-glutamate. Genes involved in these reactions are shown, none of these were differentially regulated in $\Delta acdX$ or $\Delta devR$.

Supplemental Figure 6: Olorofim MICs in supplemental RPMI-1640. MICs according to EUCAST methodology in RPMI-1640 supplemented with 20 mM arginine, 10 mM nitrate, 20 mM proline or 50 mM glutamine. Addition of nitrate changed Olorofim susceptibility by 2-fold for all strains except $\Delta devR$.

Supplemental Table 1: Oligos and crRNA used in this study.

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(a)

(b)

CEA10

ATCC46645

TR₃₄ L98H

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Oligo name	Oligo sequence (5 to 3')
hapE_gene_Fw	AGCTCTATCCAGCTGAAGTG
hapE_gene_Rv	AGATTTCTCTCGCTGACTGA
hapE_Fw	TCAAGGGCTACAAGGCACAG
hapE_Rv	AGATATCGCAGCCTTTCGCA
hapE_Seq	CCGCGACATACTAACGACCT
hapE_392_revcom	ATAGGTCGTTAGTATGTCGC

655