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Short Communication

Impairing fluoride export of *Aspergillus fumigatus* mitigates its voriconazole resistance

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ABSTRACT

Fungi have evolved specific export activities to balance intracellular levels of the toxic ion fluoride, while the first-line antimycotic voriconazole contains fluorine. This study aimed to explore whether impaired fluoride export might result in altered susceptibilities of the human pathogenic mould *Aspergillus fumigatus* towards this antifungal compound. Functional characterization of the putative fluoride exporter in *A. fumigatus* was performed in the context of azole resistance by generating deletion strains that were assessed for their resistance against fluoride and voriconazole. The FexA fluoride exporter of *A. fumigatus* appears to be expressed constitutively, and targeting its encoding gene results in significantly increased sensitivity towards this halide. Impaired fluoride export correlates with increased susceptibility of an azole-resistant *fexA*Δ strain. These results demonstrate that the *fexA*-encoded gene product is the major fluoride export activity of *A. fumigatus*, and that voriconazole serves as a source of fluoride. However, these data do not support the application of voriconazole based on fluoride toxicity.

1. Introduction

Infections by human pathogenic fungi pose a permanent threat, not only in endemic areas but also in distinct clinical settings [1]. Given the eukaryotic nature of the infectious agent, validated targets for antifungal therapy are relatively rare, and established compounds to treat fungal infections are limited, may have severe side effects, or might become ineffective due to emerging resistance [2–4]. The pharmaceutical pipeline of antifungal substances is sparsely filled, and considering timelines of target and drug evaluation, there is an urgent need for the identification of novel strategies in antifungal therapy [5]. Repurposing existing substances can be a feasible approach, based on the exploitation of pre-established substances that might exert a previously unexploited activity in a novel context or indication, shortening the drug development process significantly [6]. In addition, using existing compounds in different contexts or combining them with unrelated modes of action might open further avenues for the development of antifungal substances.

Fungi have evolved mechanisms to withstand the toxicity of environmental compounds and ions by means of detoxification, reduced uptake or increased export. At high concentrations, the ubiquitous and abundant halide ion fluoride interferes with the basic physiology of living organisms, and therefore intracellular levels of this anion need to be carefully adjusted and maintained [7–9]. As an antimicrobial agent, fluoride has been characterized to inhibit growth of several micro-organisms, including fungi. In many eukaryotes, but not in mammals, resistance towards fluoride relies on its export by conserved efflux pumps that constitute the FEX (fluoride exporter) family [10]. Functional characterization of the *FEX*-encoded proteins has been undertaken in the fungal model organisms *Saccharomyces cerevisiae* and *Neurospora crassa*, and in the human commensal and opportunistic pathogen *Candida albicans*, demonstrating a significant increase in fluoride sensitivity of the corresponding deletion strains [7,10]. Furthermore, a positive effect of fluoride on antifungal compounds has been described based on synergies that interfere with the integrity of the cell membrane [11]. Given that the first-line azole to treat aspergillosis voriconazole (VFend) contains fluorine which serves as a source of fluoride [12], this study investigated whether impaired fluoride export might result in altered susceptibilities of the human pathogenic mould *Aspergillus fumigatus* towards this antifungal substance.

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2. Materials and methods

2.1. Strains and culture conditions

Fungal strains were cultured at 37°C in/on supplemented nitrate-based minimal medium. Selection for the presence of the chlorimuron-ethyl resistance marker was performed by adding 50 μg/mL of this compound. The absence of any influence of the resistance marker on the response of *A. fumigatus* to fluoride was tested and confirmed with independent isolates carrying the resistance marker integrated ectopically (data not shown).

2.2. Generation of recombinant fungal strains

Recombinant plasmids harbouring replacement cassettes for gene targeting were cloned following standard protocols. A detailed description of plasmids and strains generated in the course of this study is presented as online supplementary material, as are protocols for nucleic acid manipulation.

2.3. Expression analyses

Protocols including primer sequences for transcript quantification by quantitative real-time polymerase chain reaction are described in the online supplementary material, accompanied by specifics of protein expression analysis by Western blot hybridization.

2.4. Determination of minimum inhibitory concentrations

For quantifications in liquid or on solid culture media, serial dilutions of the respective substance were prepared in 96-well plates in volumes of 200 μL each and inoculated with 1000 conidia of the relevant *A. fumigatus* strains. After incubation at 37°C for 24 h and 48 h, wells were inspected and scored for germination and fungal growth. Minimum inhibitory concentrations (MICs) of antimycotic substances on solid media were quantified using E-test strips (bioMérieux, Marcy l'Etolie, France) placed on agar plates inoculated with 10^6 freshly harvested spores of the strains in question. MIC values for voriconazole in liquid culture were quantified in Micronaut antimicrobial susceptibility testing microplates (Merlin GmbH, Forchtenberg, Germany) in accordance with the EUCAST protocol.

3. Results

Genome sequence analysis was used to search for the closest orthologue of fungal FEX proteins in *A. fumigatus*, and the annotated gene locus Afu2g16210 of 1.3 kb, accordingly named '*fexA*', was identified. The deduced protein sequence of 436 amino acids with a calculated mass of 46.9 kDa shows significant similarity to fungal FEX proteins, such as from *S. cerevisiae* (YOR390W: 26.7% identity and 41.4% similarity; YPL279C: 26.7% identity and 41.2% similarity), *N. crassa* (NCU06262: 48.7% identity, 59.1% similarity) or *C. albicans* (CaO19.7095: 35.9% identity, 48.5% similarity). The presumed FexA protein contains domains characteristic for eukaryotic fluoride exporters, such as a tandem arrangement of Fluc/FEX domains – formerly coined 'CRCB domain' (Pfam family PF02537) – of 150 and 123 residues length, respectively, and each of these comprising four transmembrane segments (Fig. 1A). Expression of the *fexA* gene was investigated by monitoring transcript steady-state levels after a shift to intermediate and high concentrations of fluoride, which revealed constitutive transcription of the encoding locus (Fig. 1B and C). To check for any post-transcriptional means of regulation, expression levels of an *fexA* allele in which the gene product is tagged by the HA epitope were determined (Fig. 1D). While the membrane protein fraction isolated from the untagged wild-type progenitor strain or its *fexA*Δ derivative did not yield any distinct signal in Western blot analyses, two cross-reacting proteins could be detected for the HA_3::FexA strain with an apparent molecular mass of approximately 50 kDa and 60 kDa. While the former is likely to represent the HA-tagged *fexA* gene product, the nature of the latter is elusive and might relate to post-translational modification. Correlating with equimolar treatment with sodium chloride as control, no fluoride-specific regulation of FexA expression on the protein level was identified (Fig. 1D). However, decreased levels of the 50-kDa protein became evident after prolonged incubation, independent from the absence or presence of sodium chloride or fluoride, while the detected protein of higher mass was constitutively expressed, albeit at slightly lower levels in comparison with earlier samples.

In contrast to the bakers' yeast, only one FEX domain-encoding gene appears to present in *A. fumigatus*, making its product a valid target for further investigations. In order to reveal its cellular function, the *fexA* gene was deleted in an non-homologous-end-joining-deficient derivative of the clinical isolate ATCC 46645, serving as a wild-type reference strain, by gene replacement with a selection marker conferring resistance to the sulfonylurea chlorimuron-ethyl [13], accompanied by generation of a reconstituted *fexA*$^+$ isolate for comparison (Fig. 2A). Any influence of the resistance gene on the response of *A. fumigatus* to fluoride was tested and excluded with independent isolates carrying the marker integrated ectopically (not shown). Phenotyping this set of strains revealed no obvious deficiencies in growth or sporulation on standard media, with the exception of significantly increased sensitivity towards fluoride ions (Fig. 2B). Quantification of fluoride MICs revealed an approximately 100-fold reduction in F^- resistance on solid (MIC of 1 vs 100 mM) and 80-fold reduction in liquid (0.4 vs 32.5 mM) growth medium for the *fexA*Δ deletion strain AfS197, which was completely reversed to wild-type levels in the reconstituted strain AfS198. Sensitivity towards fluoride is pH dependent due to the formation of hydrofluoric acid which has considerably higher membrane permeability [10,14]. Accordingly, MIC values were significantly lower at acidic pH for both the wild-type and the *fexA*Δ strain, with a more pronounced effect for the latter as indicated by an increase of the MIC ratio wild-type vs *fexA*Δ (Fig. 2C). Given the prominent phenotype of the deletion strain, this identified the major fluoride exporting activity expressed by this human pathogenic mould. To test whether the significant sensitivity of an *fexA*-deleted strain towards fluoride might be translated to increased susceptibility for the fluorine-containing antimycotic voriconazole, the MIC values for this azole in the deletant were determined in comparison with its wild-type progenitor. In the absence of additional fluoride, there was no significant difference between the *fexA*Δ mutant and the wild-type strain as monitored by E-tests. However, the addition of 400 μM NaF resulted in increased sensitivity of the mutant strain towards voriconazole (Fig. 2D). This effect was further elaborated by determining the fractional inhibitory concentration index (FICI) [15] for simultaneous treatment of the *fexA*Δ mutant with increasing levels of fluoride and voriconazole in a 9×7 chequerboard design and visual inspection after 48 h. When scoring hyphal growth accompanied by sporulation on culture medium containing F^- concentrations in the range of 0.03125 to 2 mM and voriconazole between 0.0156 and 4 mM as established by serial two-fold dilutions, halved MIC values became evident for each agent to result in a calculated FICI of 1, which indicates no significant interaction for the antifungal activities exerted by fluoride and voriconazole.

Resistance towards azole antimycotics such as voriconazole is based on various molecular mechanisms [16]. The most prominent mechanism is mediated by overexpression of the target enzyme, which is predominantly achieved by mutations in the *cyp51A*

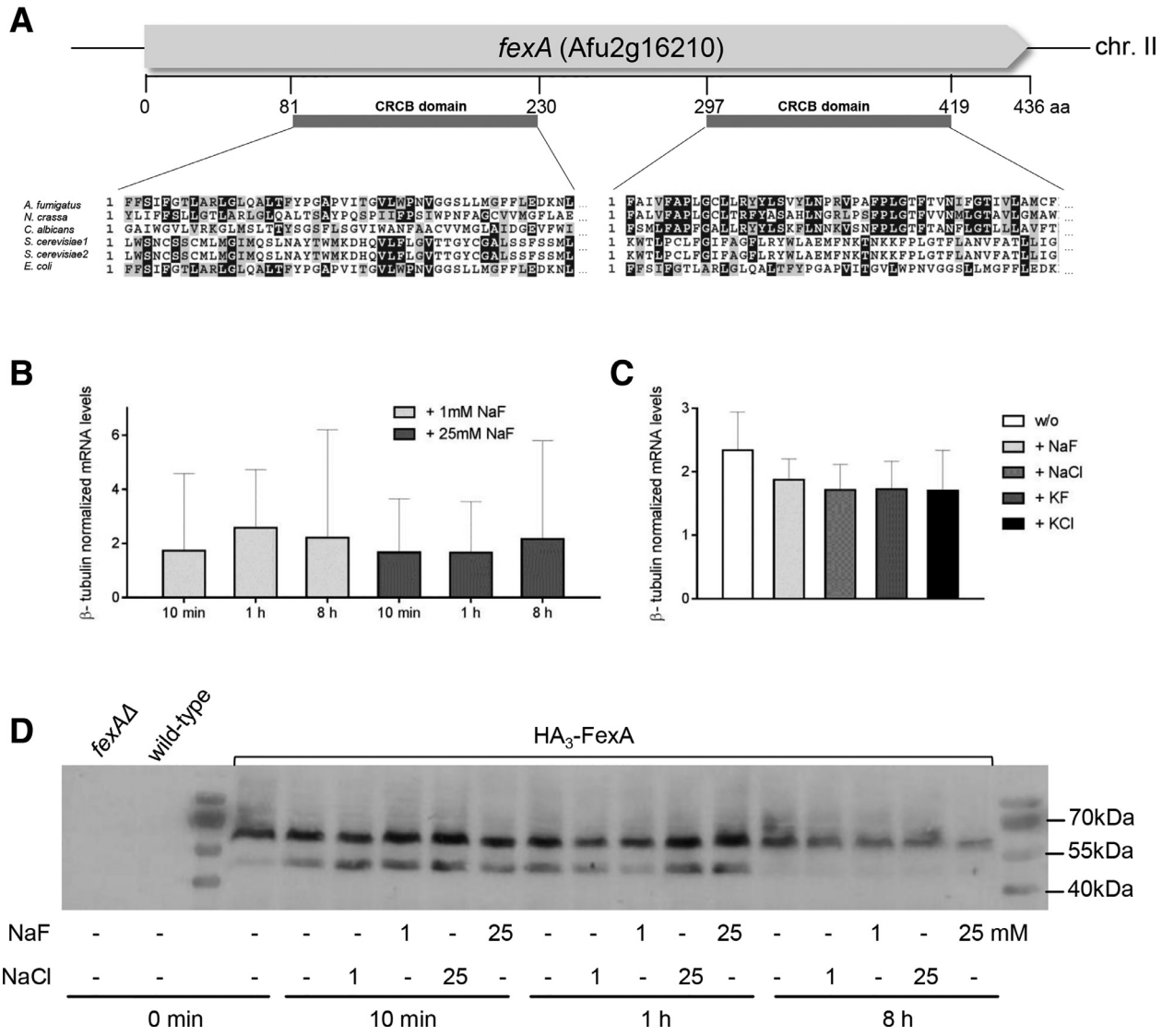
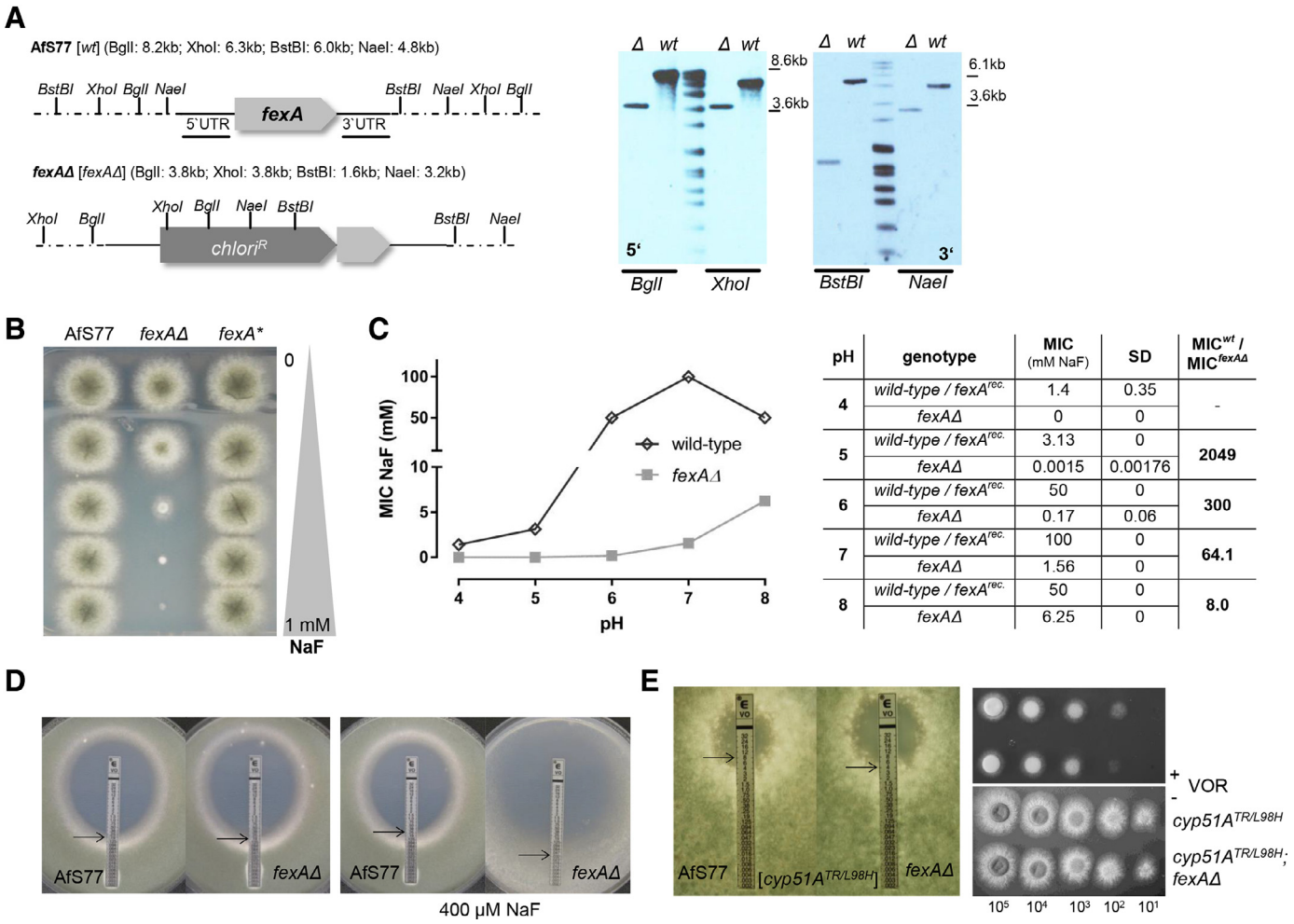


Fig. 1. *Aspergillus fumigatus* expresses one single Fex orthologue, FexA. (A) The *A. fumigatus* fluoride exporter FexA contains two CRCB domains that are characteristic for FEX fluoride exporter proteins, as revealed by protein sequence alignment with the corresponding gene products of *Saccharomyces cerevisiae* (YOR390W and YPL279C), *Neurospora crassa* (NCU06262), *Candida albicans* (CaO19.7095) and *Escherichia coli* (CrcB). (B) Transcript levels determined after a shift of wild-type strain AfS77 to fresh culture medium containing sodium fluoride at intermediate (1 mM) and high (25 mM) concentrations and incubated for different time periods do not reveal regulation of *fexA* transcription. (C) Fluoride-independent transcription of *fexA* is supported by quantification of transcript levels from cultures in medium supplemented with various salts at 1 mM concentration. (D) Expression analysis of FexA shows no fluoride-specific regulation of FexA protein levels after a shift to intermediate (1 mM) and high (25 mM) concentrations of fluoride, as evidenced by Western blots derived from the fungal cell wall and membrane protein fraction using a functional *fexA* allele to tag the gene product with the HA epitope and a corresponding anti-HA antibody. Samples were loaded according to equal protein content due to the lack of a validated, constitutively expressed control protein.

gene in *A. fumigatus*. Several mutations in its promoter region and coding sequence have been identified in this respect, with the promoter tandem repeat duplication TR34 combined with the single nucleotide substitution that results in a leucine-to-histidine exchange at position 98 (L98H) representing the most prominent pan-azole resistance mechanism [17]. When comparing a voriconazole-resistant *A. fumigatus* strain carrying the TR34/L98H allele of the *cyp51A* gene [18,19] with its *fexA*-deleted counterpart, a decrease in the voriconazole MIC became evident in E-tests (Fig. 2E), which could be supported by growth monitored for serial 10-fold dilutions of conidial suspensions as well as by susceptibility tests using EUCAST-compliant Micronaut microtitration plates at conidial densities of $2.5 \cdot 10^6$/mL: the MIC of voriconazole determined for the *cyp51A*$^{TR34/L98H}$ strain was 2 mg/L, and this reduced to 1 mg/L for the *cyp51A*$^{TR34/L98H}$*; fexA*Δ strain. Interestingly, this effect became diminished using less dense inocula in the range of 10^5/mL. Taken together, these data indicate that the fluorine-containing molecule voriconazole exerts a growth inhibitory effect in the absence of proper fluoride export, which becomes apparent when its actual mode of action (i.e. interference with ergosterol biosynthesis) is not functional.

4. Discussion

This paper presents functional characterization of a fluoride exporter from a human pathogenic mould in the context of antifungal therapy. In *A. fumigatus*, the *fexA*-encoded efflux pump appears to be the major fluoride-transporting activity, although the possibility of additional, less effective transport mechanisms cannot be excluded. Pathogenesis of aspergillosis strictly depends on growth of the opportunistic pathogen, which needs to acquire nutrients, and macro- and micro-elements at the site of infection and during



pH	genotype	MIC (mM NaF)	SD	MIC^wt / MIC^fexAΔ
4	*wild-type / fexA*$^{rec.}$	1.4	0.35	-
	fexAΔ	0	0	
5	*wild-type / fexA*$^{rec.}$	3.13	0	**2049**
	fexAΔ	0.0015	0.00176	
6	*wild-type / fexA*$^{rec.}$	50	0	**300**
	fexAΔ	0.17	0.06	
7	*wild-type / fexA*$^{rec.}$	100	0	**64.1**
	fexAΔ	1.56	0	
8	*wild-type / fexA*$^{rec.}$	50	0	**8.0**
	fexAΔ	6.25	0	

Fig. 2. Absence of *fexA* in *Aspergillus fumigatus* results in significant fluoride sensitivity that mitigates voriconazole resistance. (A) Schematic outline of the genomic situation for the *fexA*Δ and wild-type strain AfS198 and AfS77, respectively, accompanied by Southern hybridization analyses demonstrating successful deletion of *fexA*. (B) Growth analysis of *fexA*Δ on a sodium fluoride gradient reveals its increased sensitivity towards fluoride. (C) Fluoride sensitivity of *fexA*Δ, *fexA*$^{+}$ and wild-type strains increases at low pH (left panel), and this effect is significantly more pronounced for the *fexA*Δ deletant to result in an increase in the minimum inhibitory concentration (MIC) ratio monitored for the wild-type vs the mutant as presented in the table on the right. (D) The inhibitory effect of voriconazole on the *fexA*Δ strain increases in the presence of 400 µM fluoride when assessed by E-test (left), while the MIC of voriconazole in a resistant isolate deleted for *fexA* [*fexA*Δ*; cyp51A*$^{TR/L98H}$] decreased from 8 to 6.25 mg/L compared with its [*fexA; cyp51A*$^{TR/L98H}$] counterpart in this assay (E). This alleviation of voriconazole resistance is further evidenced in spot growth tests of serially diluted conidial suspensions in the presence of 1.5 mg/L voriconazole (VOR).

dissemination. In this respect, *A. fumigatus* as a prime saprobe has proven to be versatile by mobilizing the growth substrate in a highly efficient manner by extracellular hydrolytic activities and transporters for uptake [20]. This dietary flexibility counteracts the host's nutritional immunity to some degree and contributes to virulence. In line with this, *A. fumigatus* needs to secure efficient efflux of toxic compounds and ions, including fluoride. The fact that fluoride export appears to be mediated predominantly in *A. fumigatus* by a single FEX protein, which is in contrast to the highly redundant arsenal of nutrient uptake activities, underscores this cellular activity as a potential target for antifungal therapy. This is even more evident given the absence of orthologous exporters in mammals [10], while the majority of *Aspergillus* spp., with the non-fumigatus pathogen *A. terreus* as a rare exception, appear to encode an orthologue as deduced from BLAST analyses (authors' unpublished results). However, the notion that fluoride has a fungistatic effect on *A. fumigatus* conidia, as growth was resumed after prolonged cultivation in the presence of fluoride at multi-fold MIC (authors' unpublished observation), limits its therapeutic potential. Considering that numerous therapeutic substances contain fluorine, the aspect of drug repositioning might become of interest in the context of impaired fluoride export. This study has taken the first step in that direction by testing voriconazole, a first-line antimycotic containing three fluorine atoms. In line with the divergent concentration ranges, no significant effect on voriconazole sensitivity of an *fexA*Δ deletant strain became evident. Introducing a widespread resistance allele of the target-encoding *cyp51A* gene revealed an increase in sensitivity, demonstrating that the catabolized azole could serve as a source of fluoride. This is in line with clinical observations of fluorosis under long-term voriconazole treatment [12]. However, due to the different concentration ranges of fluoride and voriconazole that would interfere with fungal growth, the study data do not support antimycotic application of voriconazole based on fluoride toxicity given the mild effect detected upon limiting efflux of this halide. For the mucorales, another relevant group of human pathogenic fungi, alignment of the FexA sequence with the annotated genomes of *Mucor* spp., *Rhizopus* spp. and *Lichtheimia* spp. revealed the presence of deduced orthologues in several species (data not shown). Given that these moulds are considered to be voriconazole resistant, interfering with fluoride export might be an approach to extend the action spectrum of this fluorine-containing compound. Combining

impaired fluoride export with fluorine-containing drugs has been suggested previously for antimicrobial therapy [10], but given the rather high MIC of fluoride for fungi, it seems unlikely that this will provide a solution to bolstering the antifungal pipeline.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.02.003.

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