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Intermicrobial interaction: *Aspergillus fumigatus* siderophores protect against competition by *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa and *Aspergillus fumigatus* are pathogens frequently co-inhabiting immunocompromised patient airways, particularly in people with cystic fibrosis. Both microbes depend on the availability of iron, and compete for iron in their microenvironment. We showed previously that the *P*. *aeruginosa* siderophore pyoverdine is the main instrument in battling *A*. *fumigatus* biofilms, by iron chelation and denial of iron to the fungus. Here we show that *A*. *fumigatus* siderophores defend against anti-fungal *P*. *aeruginosa* effects. *P*. *aeruginosa* supernatants produced in the presence of wildtype *A*. *fumigatus* planktonic supernatants (Afsup) showed less activity against *A*. *fumigatus* biofilms than *P*. *aeruginosa* supernatants without Afsup, despite higher production of pyoverdine by *P*. *aeruginosa*. Supernatants of *A*. *fumigatus* cultures lacking the *sidA* gene (AfΔ*sidA*), unable to produce hydroxamate siderophores, were less capable of protecting *A*. *fumigatus* biofilms from *P*. *aeruginosa* supernatants and pyoverdine. AfΔ*sidA* biofilm was more sensitive towards inhibitory effects of pyoverdine, the iron chelator deferiprone (DFP), or amphothericin B than wildtype *A*. *fumigatus* biofilm. Supplementation of *sidA*-deficient *A*. *fumigatus* biofilm with *A*. *fumigatus* siderophores restored resistance to pyoverdine. The A. fumigatus siderophore production inhibitor celastrol sensitized wildtype *A*. *fumigatus* biofilms towards the anti-fungal activity of DFP. In conclusion, *A*. *fumigatus* hydroxamate siderophores play a pivotal role in *A*. *fumigatus* competition for iron against *P*. *aeruginosa*.

Introduction

Ecosystems of pathogens have been described with regard to a multitude of diseases [1–3]. The bacterium *Pseudomonas aeruginosa* and the fungus *Aspergillus fumigatus* form such an ecosystem, e.g. when chronically colonizing the lungs of cystic fibrosis (CF) individuals [4–7]. Both pathogens have been associated with deterioration of lung function [4–17], and their combined presence in airways of CF patients seems to aggravate disease progression [18,19]. *P*.

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Abbreviations: Af, *Aspergillus fumigatus*; Pa, *P. Aeruginosa*; PS, planktonic *P. aeruginosa* culture filtrate; PYOV, pyoverdine; BCAM, metabolic assay of *A. fumigatus* biofilm formation on agar; DFP, deferiprone; AmB, amphotericin B; FC, ferricrocin; HFC, hydroxy-ferricrocin; FsC, fusarinin C; TAFC, triacetylfusarinine C; DF-TAFC, desferri-triacetylfusarinine C; CAS, chrome azurol S.

aeruginosa and *A. fumigatus* also are prominent opportunistic pathogens in immune-compromised patients, particularly in those with neutropenia [20,21].

Previous studies have focused on *A. fumigatus* inhibition caused by *P. aeruginosa* products such as pyocyanin (5-N-methyl-1-hydroxyphenazine) [22–25], 1-hydroxyphenazine [22,24,25], phenazine-1-carboxamide and phenazine-1-carboxylic acid [25]. We recently reported that the *P. aeruginosa* product pyoverdine is the major mediator of *P. aeruginosa* inhibitory function towards *A. fumigatus* biofilms [26]. Pyoverdine, the major siderophore of *P. aeruginosa* [27,28], strongly binds to iron, which is an essential co-factor for both *P. aeruginosa* and *A. fumigatus* [29–31]. Pyoverdine-bound iron is no longer available for *A. fumigatus*, starving *A. fumigatus* of iron, and resulting in fungistasis [26]. The question arose whether *A. fumigatus* could counteract *P. aeruginosa* inhibition. Here we provide evidence that *A. fumigatus* hydroxamate siderophores in times of iron shortage, created by a competing microbe, ensure availability of the essential co-factor iron exclusively to the fungus. Concomitantly, interference with *A. fumigatus* siderophore production renders the fungus more sensitive to anti-fungal effects of iron chelators, and possibly more sensitive even to effects of anti-fungal drugs not involved in iron chelation, like amphotericin B.

Materials and methods

Materials

Pyoverdine (PYOV), 3-hydroxy-1,2-dimethyl-4(1H)pyridine (deferiprone, DFP), celastrol, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT), and menadione were purchased from Sigma-Aldrich (St. Louis, MO). Amphotericin B (AmB) was derived from X-Gen Pharmaceuticals Inc. (Horseheads, NY). Chrome Azurol S (CAS) was purchased from MP Biomedicals (Solon, OH). Ferri- and desferri-triacetylfusarinine C (TAFC, DF-TAFC) were purified as described previously [32].

Isolates

All isolates used in this study are summarized in Table 1.

Table 1. Isolates used in this study.

Organism	Isolate	Description	ATCC	Reference
A. fumigatus	10AF	Virulent patient isolate	90240	[33,34]
A. fumigatus	AF13073	Parental strain for AfΔ*sidA*	13073	
A. fumigatus	AfΔ*sidA*	L-ornithine-N^5-mono-oxygenase deficient *A. fumigatus* mutant strain		[35]
A. fumigatus	AF46645	Parental strain for AfΔ*sidC* and AfΔ*sidF*	46645	
A. fumigatus	AfΔ*sidC*	Deficient for the hydroxamate siderophores ferricrocin (FC) and hydroxy-FC (HFC)		[36]
A. fumigatus	AfΔ*sidF*	Deficient for the hydroxamate siderophores fusarinine C (FsC) and triacetylfusarinine C (TAFC).		[36]
A. fumigatus	AfS77	Derivate of ATCC 46645		[37]
P. aeruginosa	PA14	Parental strain for *pvdD-* and *pvdD-pchE-*		[38]
P. aeruginosa	*pvdD-*	Pyoverdine deficient mutant		[39]
P. aeruginosa	*pvdD-pchE-*	Pyoverdine/pyochelin deficient mutant		[26]

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The work flow for the following procedures is summarized in S1 Fig.*A. fumigatus* supernatant production

A. fumigatus conidia were inoculated into RPMI 1640 medium (RPMI, Lonza, Walkersville, MD) at 2.5×10^4 conidia/ml. *A. fumigatus* suspensions were incubated at 37˚C for 48h

(S1 Fig). *A. fumigatus* supernatants (Afsup) were filtered (0.22 μm) for sterility after the growth period.

Pseudomonas supernatant production and pyoverdine measurement

PA14 supernatants were prepared as detailed previously [40]. Briefly, *P. aeruginosa* [5 x 10^7 cells/ml] was inoculated into RPMI 1640 medium, or mixtures of RPMI and Afsup, and incubated at 37˚C for 24h. Bacterial growth was measured at 600 nm with a spectrophotometer (Genesys 20, Thermo Fisher Scientific Inc., Waltham, MA). Bacterial cultures were centrifuged at 200 x *g* for 30 min at room temperature, and filtered (0.22 μm). Pyoverdine production in the supernatant was measured as described previously [41] at 405 nm. Pyoverdine measurements were normalized to bacterial growth using the formula: Relative PYOV expression = OD405 / OD600. At the concentrations used in this study, pyoverdine, a colored substance, did not interfere with the colorimetric XTT assay used for determination of fungal metabolism. PYOV concentrations in undiluted *P. aeruginosa* supernatants are about 30 μM. Pyoverdine concentrations in sputum have been shown to be between 0.3 and 51 μM [42].

Assay for the measurement of metabolism of *A. fumigatus* forming (BCAM assay, Bioassay-Conidia-Agar-Metabolic) or preformed (BHAM assay, Bioassay-Hyphae-Agar-Metabolic) biofilms

BCAM and BHAM assays were performed as described previously [26]. In these assays, *A. fumigatus* grows out into biofilms covering the agar surface. Briefly, RPMI agar containing 2.5x10^4 to 10^5 *A. fumigatus* conidia/ml agar (as specified for different experiments in the Results section) was distributed into sterile flat-bottom 96 well cell culture plates (COSTAR, Corning, NY) at 100 μl/well. Upon agar solidification, wells were either incubated at 37˚C for 24 hours before loading (= BHAM assays), or immediately loaded with 100 μl of test substances (= BCAM assays). Control wells on each test plate contained 100 μl of RPMI 1640 medium, allowing the conversion of test results to % of the RPMI control (= 100%). Loaded plates were incubated at 37˚C for 24 hours. Fungal metabolism was determined by XTT metabolic assay at 490 nm [40,43]. Menadione (vitamin K3) was used as an ingredient in the XTT metabolic assay, boosting the reduction of tetrazolium salts to formazans. XTT assays were evaluated using a plate reader (Opsys MR, DYNEX Technologies, Chantilly, VA). Although XTT is a measure of metabolic activity of cells, previous studies of *A. fumigatus* have indicated that XTT results are linear with mass, and equated XTT result with dry weight [44–46].

Aspergillus growth assays

AfΔ*sidA* (10^4conidia) was point-inoculated on 2 ml solid minimal medium [47] in the presence of 50–600 μl PA14 wildtype or PA14 PaΔ*pvdD* bacterial supernatant with or without supplementation of $FeSO_4$ [1 μM]. Radial fungal growth was scored after incubation of the plates for 48 hours at 37˚C.

Chrome azurol S (CAS) assay

For measurement of siderophore production 10x CAS assay reagent was prepared as described previously [48]. One part 10x CAS reagent was combined with 9 parts Afsups in RPMI, and incubated at 37˚C for 24 hours. Mixtures were measured using the plate reader, and compared to RPMI not containing CAS reagent or RPMI/1x CAS reagent as reference points.

Statistical analysis

Results were analyzed using Student's *t* test, if two groups were compared, and by 1-way ANOVA, combined with a Tukey's post-test for multiple comparisons. Data reported as percentages of the control value were compared after arcsin transformation of the proportions [26]. All data in this study are expressed as a mean ± SD.The number of replicates in each assay is four or higher. Assays were repeated at least twice, and a representative experiment is shown. Supporting information on data sets used in this study is provided in S1 Table.

Results

A. fumigatus supernatants induce pyoverdine production by *P. aeruginosa*

Fungal supernatants (Afsup), produced by planktonic growth of *A. fumigatus* strain 10AF in RPMI (experimental setup described in S1 Fig), induced pyoverdine production by *P. aeruginosa* in a dose-dependent manner, with 10% Afsup still significantly inducing pyoverdine production. As pyoverdine is induced in response to iron shortage, increased pyoverdine production here suggests sequestration of iron from the growth medium by Afsup (Fig 1A).

Concentrations of Afsup higher than 10% interfered with bacterial growth in a concentration-dependent manner (Fig 1B). As iron is a major co-factor for microbial growth, the reason for inhibitory effects of Afsup on *P. aeruginosa* growth might be a reaction to iron denial. Although gliotoxin has been suggested as an anti-microbial factor [49], in our hands supernatants produced by an *A. fumigatus* mutant unable to produce gliotoxin [50] affected *P. aeruginosa* growth to a similar degree as supernatants produced by its parent (S2 Fig). Distilled water (25%), instead of Afsup (25%) during *P. aeruginosa* supernatant preparation did not result in interference with *P. aeruginosa* effects on *A. fumigatus* biofilm metabolism, indicating that *P. aeruginosa* supernatant dilution by Afsups was not the reason for the protective effects of Afsups (S3 Fig).

In order to verify that Afsup indeed induced production of pyoverdine, we used a PA14 mutant not able to produce pyoverdine (PaΔ*pvdD*) [39]. With or without the presence of Afsup, PaΔ*pvdD* supernatant did not absorb at 405 nm, confirming that Afsup did not induce production of an unknown *P. aeruginosa* product detectable at 405 nm (S4A Fig).

Afsup protects *A. fumigatus* forming biofilm from *P. aeruginosa* anti-fungal activity and pure pyoverdine

Pyoverdine has detrimental effects on Af biofilm metabolism [26]. Surprisingly, although containing high concentrations of pyoverdine (Fig 1A), *P. aeruginosa* supernatants produced in the presence of Afsup were less inhibitory for forming (Fig 1C) or preformed (Fig 1D) *A. fumigatus* biofilms than *P. aeruginosa* supernatants produced without Afsups,whereas Afsups up to 50% did not affect *A. fumigatus* biofilms when administered alone. Similarly, when *P. aeruginosa* supernatants and Afsups were prepared separately, their combination was less inhibitory to *A. fumigatus* biofilms than *P. aeruginosa* supernatant alone (Fig 1E).

Presumably owing to their lack of pyoverdine production, supernatants of PaΔ*pvdD* were less inhibitory to 10AF biofilms than PA14 supernatants. The presence of Afsup further decreased the inhibitory activity of PaΔ*pvdD* supernatants to RPMI control levels (S4B Fig). Protective Afsup effects were also observed when Afsups were combined with pure pyoverdine (Fig 1F).

Taken together, these data indicate that despite its ability to induce pyoverdine production by *P. aeruginosa*, Afsup protects *A. fumigatus* biofilms.

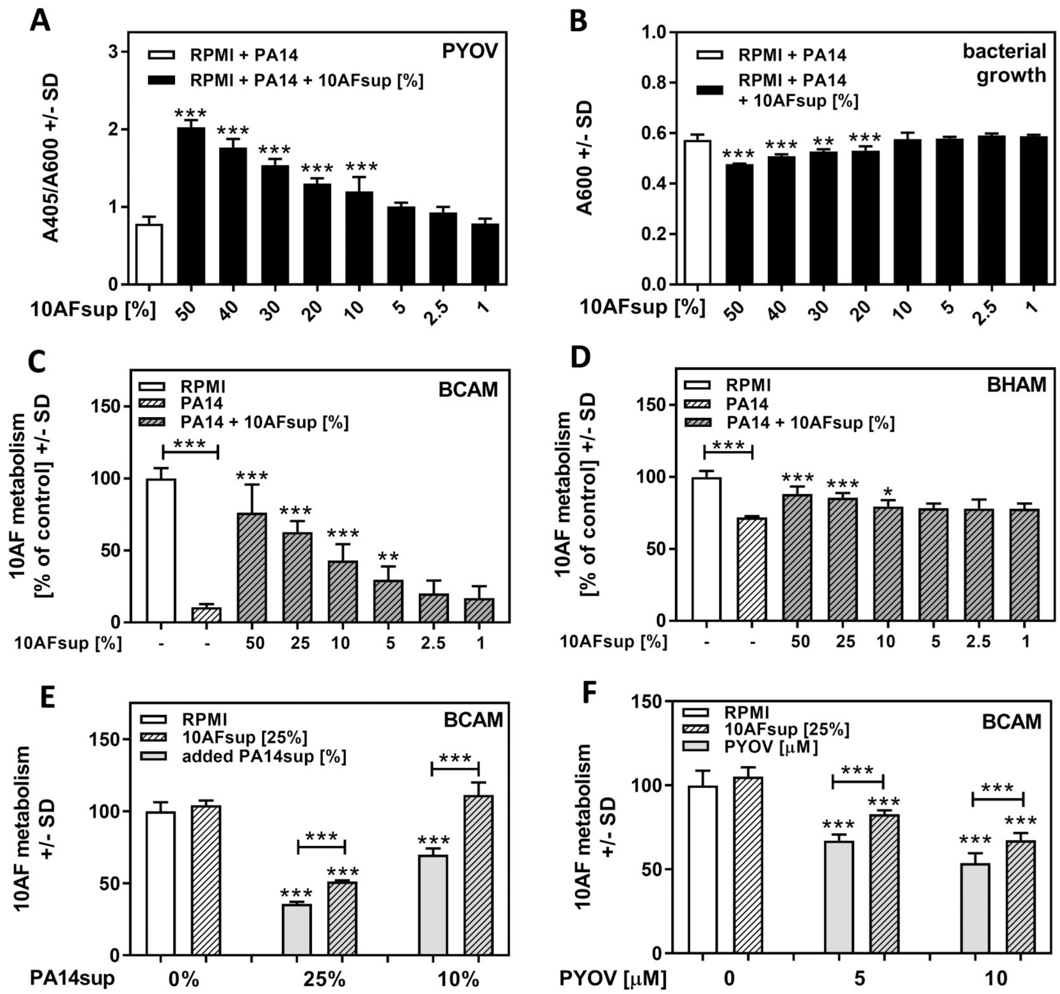

Fig 1. *A. fumigatus* supernatant effects on *P. aeruginosa* growth and pyoverdine production. Planktonic 10AF supernatant (10AFsup) was diluted in RPMI from 50% to 0% 10AFsup, and incubated with *P. aeruginosa* cells ($5x10^7$/ml) at 37˚C for 24h. Relative pyoverdine (PYOV) concentrations (A) were calculated using the quotient A405 (PYOV)/A600 (bacterial growth: B). Supernatants shown in A and B, as well as PA14 supernatant not containing 10Afsup, were compared with respect to their activities on 10AF forming biofilm (C: BCAM) or preformed biofilm (D: BHAM) metabolism. E: 10AF forming biofilm was incubated with 10 or 25% PA14 wildtype supernatant with or without the addition of 25% 10AFsup for 24 hours. Effects on 10AF forming biofilm metabolism were evaluated by XTT assay. F: 10AF forming biofilm was incubated with 5 or 10 μM pyoverdine (PYOV) with or without the addition of 25% 10AFsup for 24 hours. Effects on 10AF forming biofilm metabolism were evaluated by XTT assay. Statistics for A and B: 1way ANOVA, RPMI (white bar) vs. all groups containing Afsup (black bars). Statistics for C and D: 1way ANOVA, PA14 supernatant (white striped bar) vs. PA14 supernatant containing 10AFsup (grey striped bars). Other comparisons by t-Test as indicated by the ends of the brackets. Statistics for E and F: t-Test, RPMI (white bar) vs. all other bars. Other comparisons as indicated by the ends of the brackets. One, two or three asterisks = $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

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Stability of protective Af supernatant effects

In order to determine the reason for protection of A. fumigatus biofilms by Afsup, we first tested stability of 10AFsup to heat, and long-term storage. 10AFsup was heated to 56˚C or 90˚C for 30 minutes, or subjected to three freeze-thaw cycles. Treated or untreated 10AFsups were diluted to 25%, combined with *P. aeruginosa* supernatants, and tested for effects on *A. fumigatus* biofilm metabolism. Our results show that heat does not destroy the protective compound in Afsup (Fig 2A). Repeated freeze-thaw cycles diminished, but did not abolish protection (Fig 2A). We also kept 10AFsup at 4˚C for 12 months, and measured protection from

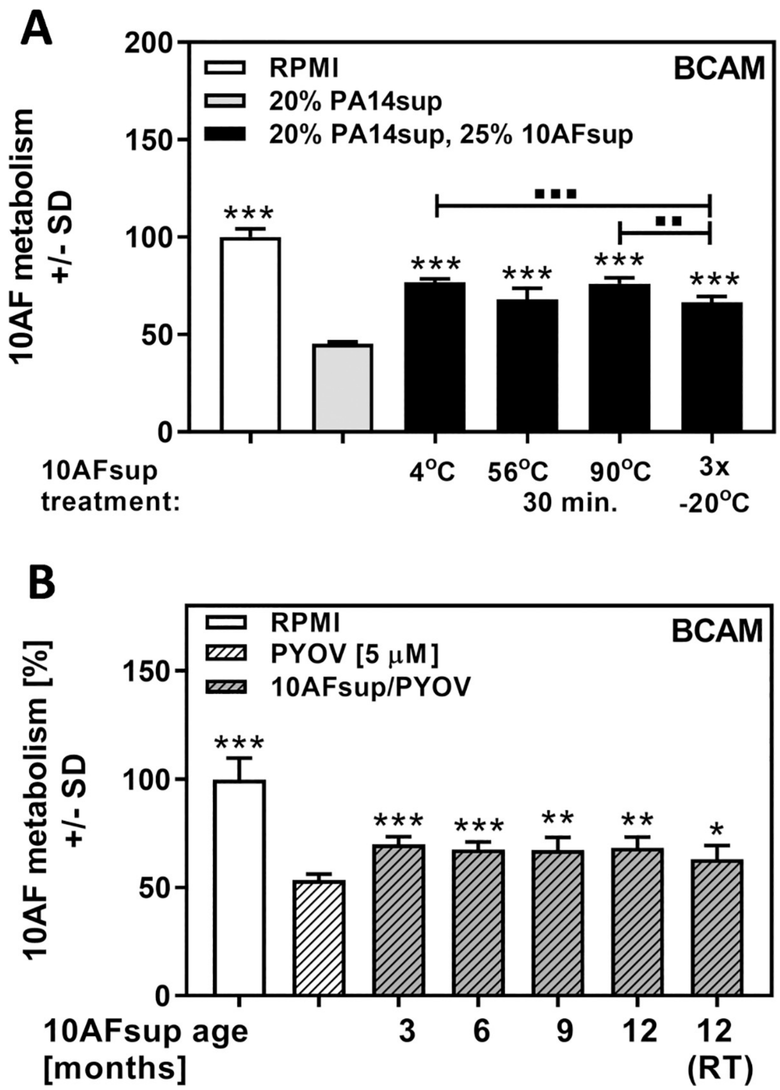

Fig 2. Stability of Afsup. A: Mixtures (25%) of freshly prepared 10AFsup in RPMI were kept at 4˚C, or heated to 56˚C or 90˚C for 30 minutes, or subjected to 3 freeze-thaw cycles. Treated 10AFsups (25% in RPMI), were combined with 20% *P. aeruginosa* supernatants, and tested for effects on *A. fumigatus* forming biofilm metabolism. B: 10AFsup was stored at 4˚C for up to 12 months, and tested for protective activity against 5 μM pyoverdine (PYOV) every 3 months. A portion of the 10AFsup was stored at room temperature (RT), and tested after 12 months of storage. Protective activity was tested using a BCAM assay. Statistics for A: t-Test, PA14 supernatant (grey bar) vs. all other bars. Other comparisons as indicated by the ends of the brackets. * indicate significant increases, ■ indicate significant decreases. Statistics for B: t-Test, pyoverdine (white striped bar) vs. all other bars. One, two or three asterisks or squares = $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

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pyoverdine every 3 months. The protective potential of Afsup was almost constant over the 12 months period (Fig 2B). When 10AFsup was kept at room temperature for 12 months, protection was marginally lower, but still significant (Fig 2B). Taken together, our data suggest that the protective compound in 10AFsup is stable.

A. fumigatus siderophores protect *A. fumigatus* biofilm from *P. aeruginosa* anti-fungal activity

Knowing that iron is a crucial factor for *A. fumigatus* biofilm, that pyoverdine inhibitory activity is owing to withholding iron from the fungus, and that the protective compound in Afsup

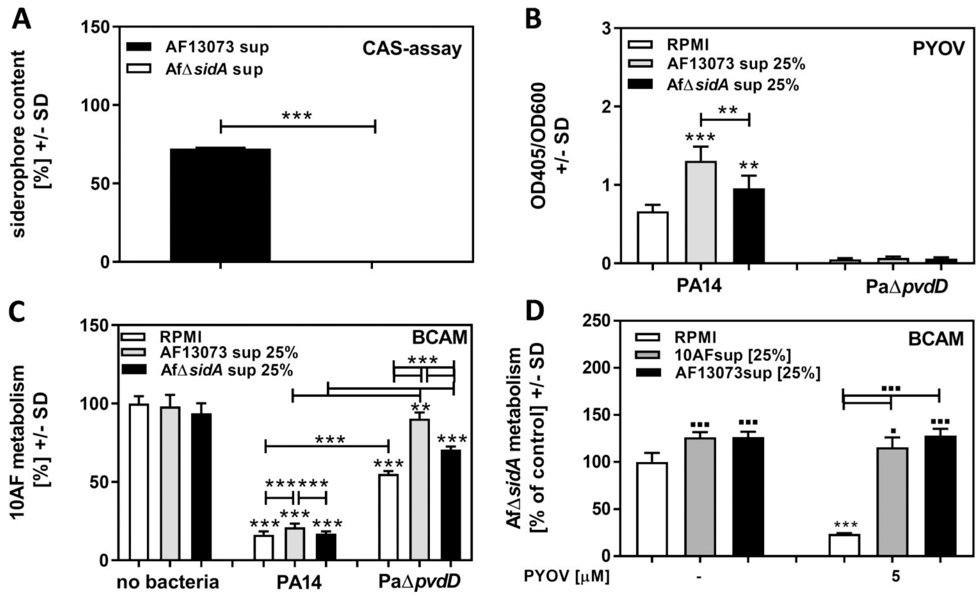

Fig 3. *A. fumigatus* siderophores protect *A. fumigatus* biofilm from *P. aeruginosa* anti-fungal activity. A: Planktonic supernatants produced by an *A. fumigatus* mutant lacking hydroxamate siderophore production (AfΔ*sidA*) or its parental strain (AF13073) were subjected to siderophore production measurement by CAS assay. B: RPMI, or 25% AfΔ*sidA* or AF13073 supernatant in RPMI, were inoculated with PA14 wildtype or the PA14 mutant PaΔ*pvdD* [$5x10^7$ cells/ml], and incubated at 37˚C for 24h. Pyoverdine production was measured. C: Supernatants obtained in B (middle and right sets of 3 bars), as well as 25% AfΔ*sidA* or AF13073 supernatants in RPMI (left 3 bars) were tested for activity against *A. fumigatus* biofilm formation. D: AfΔ*sidA* forming biofilm was incubated with 5 μM pyoverdine (PYOV) with or without the addition of 25% 10AFsup or AF13073sup for 24 hours. Effects on forming biofilm metabolism were evaluated by XTT assay. Statistics: t-Test. Comparisons without brackets: B: RPMI vs. *A. fumigatus* supernatants for each bacterial strain. C: RPMI vs. all other bars. D: RPMI (leftmost white bar) vs. all other bars. Other comparisons as indicated by the ends of the brackets. * indicate significant decreases, ■ indicate significant increases. One, two, or three asterisks or squares = $p \leq 0.05$, or $p \leq 0.01$, or $p \leq 0.001$, respectively.

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is stable (Fig 2), we investigated the hypothesis that the protective compound might be an *A. fumigatus* siderophore. We produced supernatant of an *A. fumigatus* mutant lacking *sidA*, a gene crucial for the production of all four hydroxamate siderophores (AfΔ*sidA*sup), and compared to wildtype Afsup, produced by the AfΔ*sidA* parent AF13073 (AF13073sup). A CAS assay confirmed the lack of siderophores in AfΔ*sidA*sup (Fig 3A). Dilutions (25%) of AF13073sup and AfΔ*sidA*sup were incubated with PA14 or PaΔ*pvdD*. AF13073sup stimulated pyoverdine production by PA14 significantly more than AfΔ*sidA* sup (Fig 3B). AfΔ*sidA*sup also showed less protection for *A. fumigatus* biofilm against *P. aeruginosa* anti-fungal activity than AF13073sup (Fig 3C). When siderophore-deficient fungus was treated with pyoverdine, significant damage was induced (Fig 3D), whereas Afsup derived from either 10AF or AF13073 wildtype strains protected AfΔ*sidA* from pyoverdine-induced damage (Fig 3D). It has to be noted that neither the absence of pyoverdine nor the presence of Afsup from siderophore-deficient fungus prevented *P. aeruginosa* anti-fungal activity completely.

In comparison to wildtype *A. fumigatus*, AfΔ*sidA* has a growth disadvantage due to missing Fe^{3+} uptake, which requires siderophores. 10AF or AF13073 wildtype supernatants, containing siderophores and iron, partially compensated AfΔ*sidA* disadvantages, as indicated by higher XTT values for AfΔ*sidA* in the presence of Afsups (Fig 3D). In conclusion, *A. fumigatus* siderophores are able to protect *A. fumigatus* biofilms against *P. aeruginosa* anti-fungal activity. Fig 3C also shows that AfΔ*sidA* sup was able to provide protection for *A. fumigatus* biofilm from PaΔ*pvdD* supernatant, whereas there was no protection against PA14 wildtype sup by either

Afsup. This finding indicates that *A. fumigatus* hydroxamate siderophores are crucial for protection from detrimental pyoverdine effects, but that Afsup seems to contain other compounds which are able to protect *A. fumigatus* biofilm when the Pasup challenge lacks the powerful inhibitor pyoverdine.

AfΔ*sidA* is more sensitive to *P. aeruginosa* anti-fungal activity and pyoverdine than its wildtype

AfΔ*sidA*-derived supernatants were significantly less protective against pyoverdine than wildtype supernatants (Fig 4A). AfΔ*sidA* is lacking the intracellular hydroxamate siderophores ferricrocin (FC) and hydroxy-ferricrocin (HFC), as well as the extracellular hydroxamate siderophores fusarinin C (FsC) and triacetylfusarinine C (TAFC). Using *A. fumigatus* mutants with specific mutations in intracellular (AfΔ*sidC*), or extracellular hydroxamate siderophores (AfΔ*sidF*), we found that a lack of extracellular siderophores significantly interfered with protection from pyoverdine by *A. fumigatus* supernatants (Fig 4A). Protective effects of AfΔ*sidF* sup were significantly higher than protective effects of AfΔ*sidA* sup (Fig 4A), indicating that there might be some contribution to protection by other molecules missing in AfΔ*sidA* sup. Fig 4A also shows that supernatants, derived from three different *A. fumigatus* wildtypes (AF13073, AF46645, AfS77) protected forming biofilm of a fourth *A. fumigatus* wildtype (10AF), indicating that protection is not strain specific.

PA14 supernatants, prepared in RPMI, as well as pure pyoverdine, were significantly more inhibitory during the formation of *A. fumigatus* biofilms derived from AfΔ*sid*A conidia than they were for biofilms derived from AF13073 conidia (Fig 4B). *A. fumigatus* mutants lacking either intracellular (AfΔ*sidC*), or extracellular hydroxamate siderophores (AfΔ*sidF*) showed increased sensitivity to PA14 supernatants or pure pyoverdine, compared to their wildtype AF46645 (Fig 4B). The loss of extracellular hydroxamate siderophores was more important for sensitivity than the loss of intracellular hydroxamate siderophores (Fig 4B). Using pure TAFC, or desferri-TAFC (DF-TAFC) we found complete protection from pyoverdine anti-fungal activity (Fig 4C), confirming the importance for *A. fumigatus* siderophores for protection from *P. aeruginosa* anti-fungal activity.

As observed in Fig 2, the protective compound in Afsup was stable to prolonged heat treatment (90˚C, 30 min.). After being subject to the same treatment pure TAFC and DF-TAFC still significantly protected from pyoverdine toxicity (S5 Fig), further supporting the assumption that *A. fumigatus* siderophores are the protective compound in Afsup. It was also noted that pyoverdine was heat stable (S5 Fig).

The absence of A. fumigatus hydroxamate siderophores might have therapeutic relevance

Compared to wildtype *A. fumigatus* (AF13073), AfΔ*sid*A growth on plate was more affected with the highest concentration (600 μl) of PA14 supernatant blocking growth (Fig 5A). Likewise, the IC50 of pyoverdine for AF13073-derived forming biofilm was about 4 times higher than the IC50 for AfΔ*sid*A-derived forming biofilm (Fig 5B). Recently, the iron chelator deferiprone (DFP), which similar to pyoverdine, exerts anti-fungal activity by denying iron from *A. fumigatus* biofilms, has been proposed to be useful in anti-fungal therapy [51,52]. We tested effects of DFP on AfΔ*sidA*, or its wildtype, and found significantly higher sensitivity of *A. fumigatus* biofilms to DFP when siderophore production was missing (Fig 5C). Genetic inhibition of siderophore production also increased anti-fungal effects of amphotericin B (AmB), an anti-fungal agent used against serious fungal infections, not only by *Aspergillus*, but also by other fungi [53], on *A. fumigatus* forming biofilm metabolism (Fig 5D).

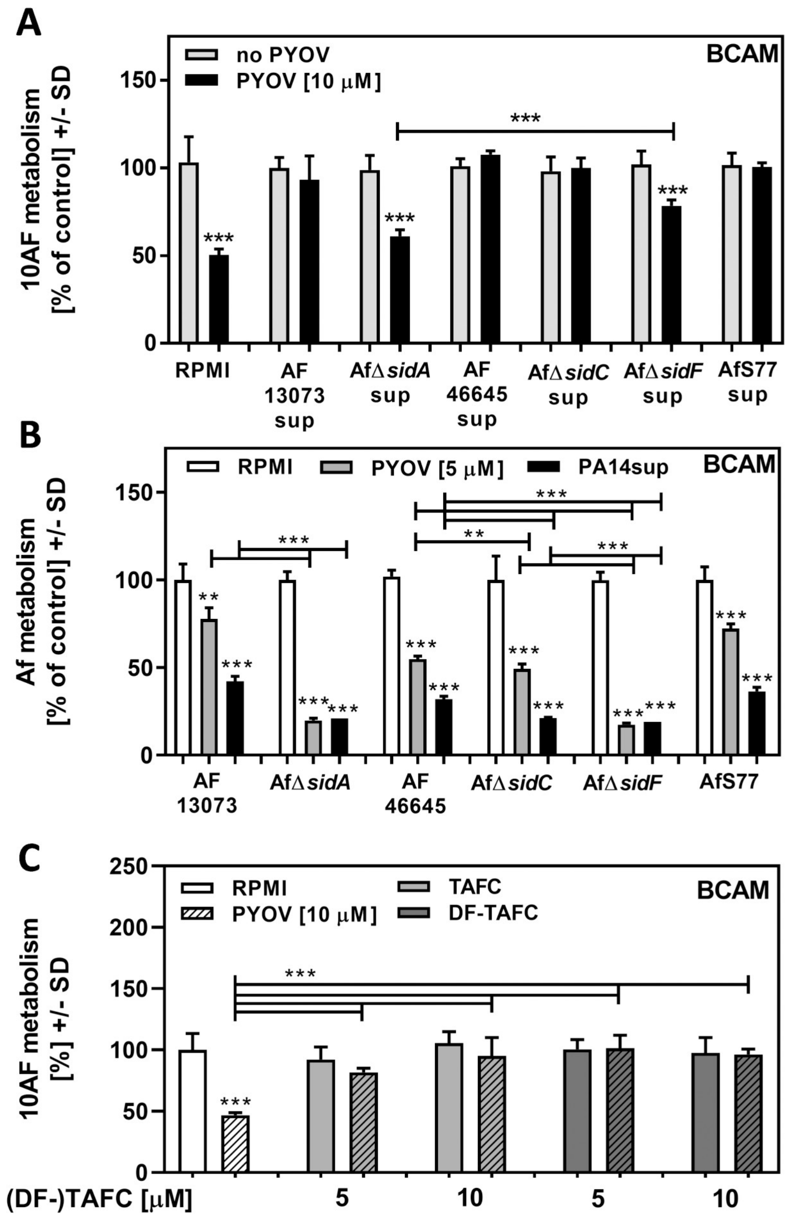

Fig 4. AfΔ*sidA* is more sensitive towards PA14 or pure pyoverdine than its wildtype. A: Mixtures (25%) of freshly prepared AF13073, AfΔ*sidA*, AF46645, AfΔ*sidC*, AfΔ*sidF*, or AfS77 supernatants were combined with pyoverdine [10 μM], and tested for effects on 10AF forming biofilm metabolism. Fungal metabolism was measured by XTT assay. Measurements for controls (no pyoverdine) in each group were regarded as 100%. Statistics: t-Test, for each group: no pyoverdine (grey bar) vs. pyoverdine (black bar). Other comparison as indicated by the ends of the bracket. B: AF13073, AfΔ*sidA*, AF46645, AfΔ*sidC*, AfΔ*sidF* or AfS77 BCAM assays were incubated with either RPMI, PA14 supernatant, or 5 μM pyoverdine. Fungal metabolism was measured by XTT assay. For each fungus RPMI control measurements were regarded as 100%. Statistics: t-Test, comparison: RPMI (white bars) vs. PA14 supernatant (grey bars), or pyoverdine (black bars) for each fungus. Other comparisons as indicated by the ends of the brackets. C: A 10AF BCAM assay was incubated with either RPMI, pyoverdine [10 μM], TAFC [5 or 10 μM], DF-TAFC [5 or 10 μM], or combinations of pyoverdine and TAFC or DF-TAFC. Fungal metabolism was measured by XTT assay. RPMI control measurements were regarded as 100%. Statistics: t-Test, comparison: RPMI (white bar) vs. all other bars. Other comparisons as indicated by the ends of the brackets. One, two or three asterisks = $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

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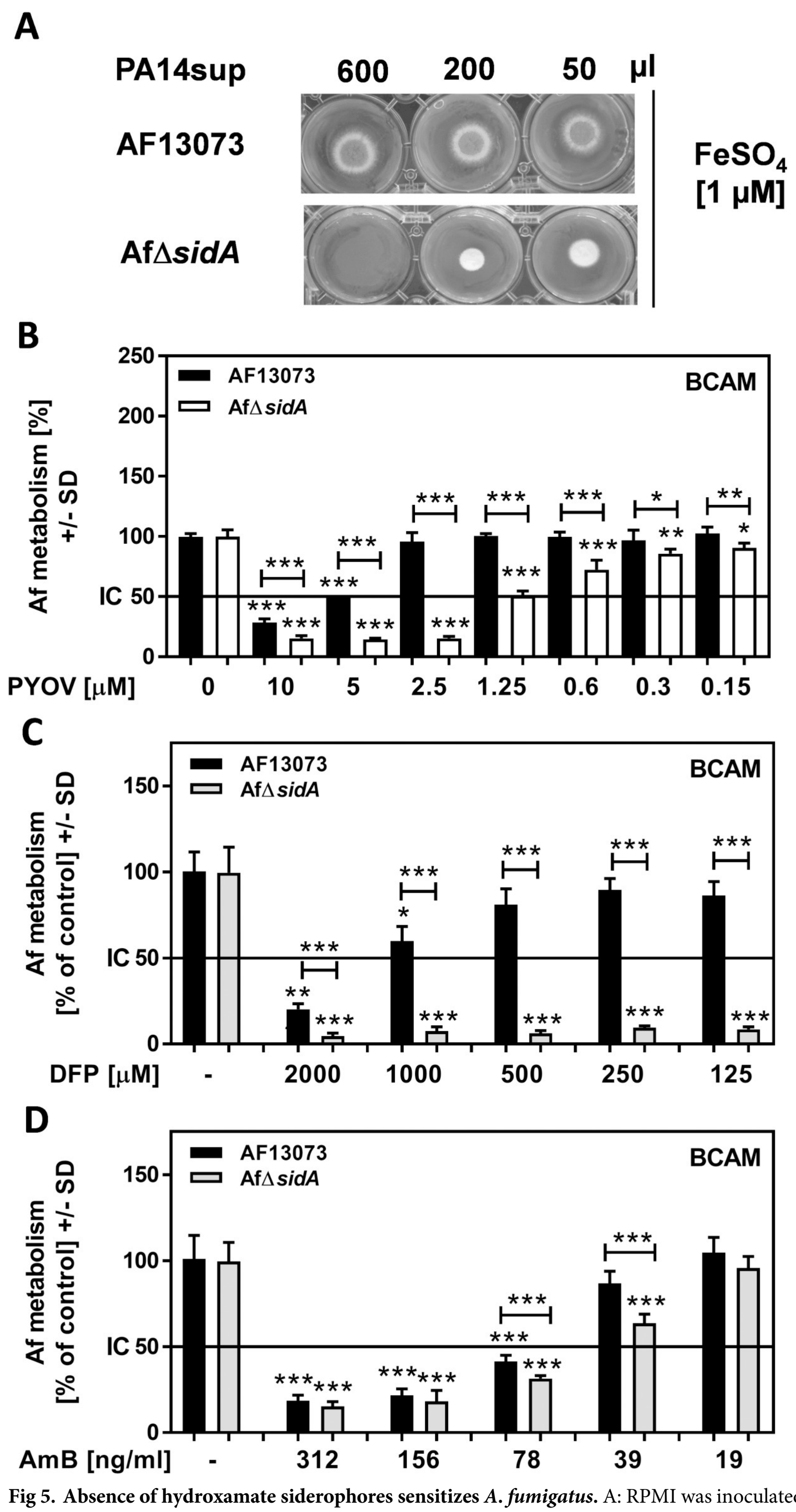

Fig 5. Absence of hydroxamate siderophores sensitizes *A. fumigatus*. A: RPMI was inoculated with PA14 [5x10^7 cells/ml], incubated for 24 hours, and the culture supernatant was sterile filtered. Growth of point inoculated AF13073 or AfΔ*sidA* (10^{4}conidia) on 3 ml solid minimal medium in the presence of 1 μM $FeSO_4$ plus 50–600 μl of the sterile filtered supernatants was compared after incubation for 48 h at 37˚C. B: AfΔ*sidA* (white bars) or AF13073 (black bars) BCAM assays were incubated with either RPMI or different concentrations of pyoverdine. Fungal metabolism was measured by XTT assay. Statistics: t-Test. For each fungus RPMI controls were regarded as 100%. RPMI controls for each fungus vs. all pyoverdine concentration. Other comparisons as indicated by the ends of the brackets. C: Wildtype

(AF13073) or AfΔ*sidA* forming biofilms were incubated with DFP [0.125–2 mM] at 37˚C for 24 hour. Fungal metabolism was measured by XTT assay. Statistics: t-Test, RPMI vs. all other bars of the same group. Other comparisons as indicated by the ends of the brackets. D: Wildtype (AF13073) or AfΔsidA forming biofilms were incubated with AmB [19–312 ng/ml] at 37˚C for 24 hour. Fungal metabolism was measured by XTT assay. Statistics: t-Test, RPMI vs. all other bars of the same group. Other comparisons as indicated by the ends of the brackets. One, two or three asterisks = $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

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As a pharmacological complementation of our data obtained using AfΔ*sidA*, we investigated effects of the SidA-biosynthesis inhibitor celastrol [54]. Celastrol showed anti-fungal activity when used alone at concentrations above 5 μM (Fig 6). When combined with DFP, celastrol significant enhanced anti-fungal effects by DFP (Fig 6).

Discussion

Fungal and bacterial biofilms e.g. frequently found co-inhabiting lungs of persons suffering from cystic fibrosis, represent a potentially severe pathogenicity factor. The present study mainly focuses on events during formation of *A*. *fumigatus* biofilm. In previous studies [40] and in studies by many others, it has been shown that biofilm formation by *A*. *fumigatus* is substantial within the first 16 hours of incubation. We have also performed many of the studies described in the present communication against fully formed *A*. *fumigatus* biofilms that develop over the subsequent 24 hours of incubation, and found the same phenomena, although to a lesser degree than in the earlier phase of *A*. *fumigatus* biofilm formation, as illustrated in Fig 1C vs. 1D. This may suggest that iron is more important for the initial development of *A*. *fumigatus* biofilms.

The human body contains free iron levels of 10^{-24} M [55]. Free iron levels are decreased during infections due to increased levels of ferritin and the release of lactoferrin from neutrophils [56]. In the lungs of cystic fibrosis patients, *P*. *aeruginosa* and *A*. *fumigatus*, which both are crucially dependent on the availability of free iron for metabolism and growth, aggravate disease pathology [4–7]. Under low iron conditions, these organisms are forced to compete for resources in the same environment [29,30].

As summarized in Fig 7, for *P*. *aeruginosa* as well as *A*. *fumigatus* a lack of iron is the signal to increase production of siderophores [27,28]. Siderophores specifically chelate ferric iron with a high affinity [57]. Siderophores are of different types, based on the way the iron is

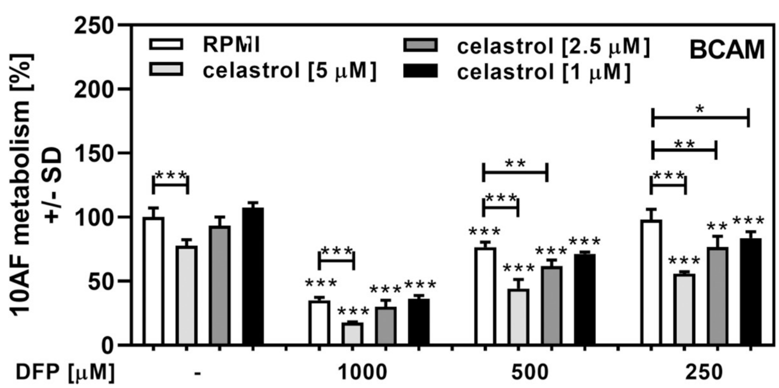

Fig 6. Celastrol sensitizes *A*. *fumigatus* for anti-fungal activity of DFP. Forming wildtype *A*. *fumigatus* biofilm (10AF) was incubated with 1, 2.5, or 5 μM celastrol, 0.25–1 mM of DFP, or combinations of these two substances at 37˚C for 24 hour. Fungal metabolism was measured by XTT assay. Statistics: t-Test. Bars without DFP (leftmost group) vs. all other bars with the same celastrol concentration. Other comparisons as indicated by the ends of the brackets. One, two or three asterisks = $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

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complexed: phenolate-, catecholate-, hydroxamate-, carboxylate-, or mixed type of siderophores have been described [58].

The *P. aeruginosa* siderophore pyoverdine is a composite (mixed) siderophore comprising a peptide chain and a chromophore [59]. Pyoverdines bind iron with very high affinity, are able to acquire iron from transferrin, and their production is absolutely needed in mouse pulmonary infections [60–62]. We have described pyoverdine to be the *Pseudomonas*-derived key inhibitor of *A. fumigatus* in their intermicrobial competition via iron sequestration under low iron conditions [26]. We note that the loss of pyoverdine did not prevent *P. aeruginosa* antifungal activity completely, however. Pyochelin, the second siderophore of *P. aeruginosa*, is produced by all *P. aeruginosa* isolates, but its affinity for iron is much lower compared to pyoverdine [63,64]. Pyoverdine does not act as a xenosiderophore for *A. fumigatus* [26], thus withholding iron from the fungus, and inducing anti-fungal effects [26]. *P. aeruginosa* does not seem to be able to use *A. fumigatus* siderophores either. Our results show that Afsups provoke increased pyoverdine production by *P. aeruginosa*, indicating that there is a paucity of iron in the medium. If *P. aeruginosa* could use iron bound to *A. fumigatus* siderophores, there would be an abundancy of iron available to the bacterium, and hence no increase in pyoverdine production. We here for the first time provide evidence that *A. fumigatus* is able to use iron bound to its hydroxamate siderophores as the main defense against *P. aeruginosa* competition for iron. These findings are summarized in Fig 7. Our results using *A. fumigatus* mutants defective in hydroxamate siderophore production also indicate that additional defense mechanisms might be in place, since supernatants derived from these mutants still partially protected from *P. aeruginosa* toxicity. Other microorganisms have developed defense mechanisms against *P. aeruginosa* not based on protective siderophore production. *Candida albicans* appears to defend itself against *P. aeruginosa* in part by down-regulating *P. aeruginosa* siderophore production [65].

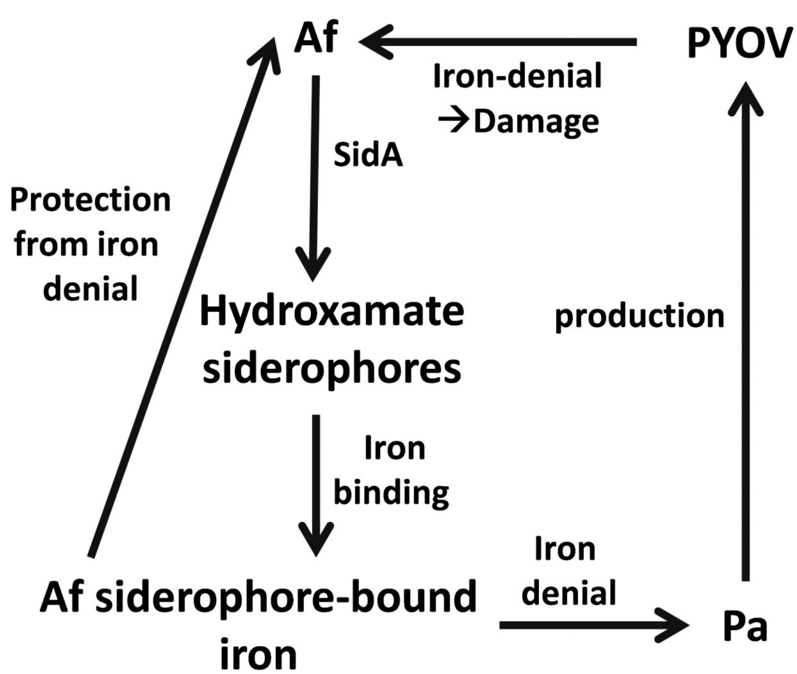

Fig 7. Summary. In need for iron *P. aeruginosa* (Pa) produces its siderophore pyoverdine (PYOV). PYOV-chelated iron is not available to *A. fumigatus* (Af), resulting in iron deficiency and damage to the fungus. Anti-fungal activity in part is counter-balanced by SidA-dependent *A. fumigatus* hydroxamate siderophores, providing iron to the fungus, further denying iron from *P. aeruginosa*.

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Anti-bacterial *A. fumigatus* supernatant effects as a reason for protective effects against *P. aeruginosa* anti-fungal activity are highly unlikely. In the presence of Afsup, *P. aeruginosa* is able to even produce more pyoverdine, which requires functional bacterial metabolism. Also, Afsup protects from *P. aeruginosa* supernatants produced without Afsup being present, and Afsup, as well as pure *A. fumigatus* siderophores, protect from pure pyoverdine. Additionally, Afsups derived from a giotoxin mutant affected bacterial growth To the same degree as wild-type Afsups. The most plausible explanation for anti-bacterial effects of Afsups is depletion of essential factors in the medium, especially that of iron.

To overcome iron starvation, *A. fumigatus* produces its own siderophores [35]. *A. fumigatus* is able to produce four hydroxamate-containing siderophores: ferricrocin (FC) as well as hydroxyferricrocin (HFC) for intracellular iron trafficking, and fusarinine C (FsC) as well as its derivative triacetylfusarinine C (TAFC) for extracellular iron scavenging [36,66,67]. The first step in the biosynthesis of all four hydroxamate-containing siderophores is catalyzed by the enzyme L-ornithine N5-monooxygenase, termed SidA [30,67]. SidA catalyzes oxygen and NADPH-dependent hydroxylation of L-ornithine to N5-L-hydroxyornithine, a crucial step for the biosynthesis of hydroxamate-containing siderophores [35]. In a similar fashion to *P. aeruginosa* siderophores, *A. fumigatus* siderophores are essential for pathogenesis, as the AfΔ*sidA* strain is unable to establish invasive aspergillosis in a mouse model [35,56]. We show that in contrast to wildtype *A. fumigatus* supernatants, supernatants derived from AfΔ*sidA* were unable to protect *A. fumigatus* biofilms from detrimental effects of *P. aeruginosa* supernatants, or pyoverdine. This finding indicates the relevance of *A. fumigatus* siderophores for protection of *A. fumigatus* from *P. aeruginosa*-induced iron denial, and was supported by our finding that *A. fumigatus* siderophores (FC as well as TAFC) each could partially protect *A. fumigatus* from detrimental *P. aeruginosa* effects (Fig 4A and 4B). Pure preparations of the sideropore TAFC protected *A. fumigatus* from pyoverdine, even after heat treatment (Fig 4C, and S5 Fig). Protection by TAFC and its desferri form DF-TAFC was about equal, indicating that TAFC very efficiently binds free iron in medium, before pyoverdine can do the same. TAFC-bound iron does not seem to be transferable to pyoverdine, and exclusively is available to *A. fumigatus*.

A. fumigatus lacking hydroxamate siderophores, especially of the extracellular type, was more susceptible to pyoverdine (Fig 4B). The most pronounced detrimental effects of pyoverdine were observed when all four siderophores were missing.

A lack of siderophores, especially owing to a loss in SidA, renders the fungus more sensitive to iron denial by either pyoverdine (Fig 5B), or the clinically used iron chelator deferiprone (DFP, Fig 5B). Siderophore deficiency even sensitized the fungus to effects of amphotericin B (AmB, Fig 5C). While sensitization to DFP might be expected knowing that iron chelation by pyoverdine powerfully inhibits the fungus, sensitization to AmB is more surprising. It might be that a struggle for iron takes away energy from the fungus, and dampens intrinsic defense mechanisms, or that the membrane action of AmB [53] may adversely affect iron flux in the fungus.

As a pharmacological analog to *sidA* knockout we used celastrol treatment [54]. Celastrol, a pentacyclic triterpenoid that belongs to the family of quinone methides, exerts potent anti-cancer and anti-metastatic [68,69], anti-inflammatory [70,71], and antioxidant [72] activities. Recently celastrol was identified as a noncompetitive inhibitor of SidA production [54]. Inhibition of SidA production by celastrol is detrimental to *A. fumigatus* growth [54]. We observed inhibitory effects of celastrol on *A. fumigatus* metabolism as well (Fig 6). Since celastrol has numerous effects [68–71] it can't be excluded that effects on *A. fumigatus* are not solely owed to inhibition of siderophores production.

SidA-deficiency or addition of celastrol to *A. fumigatus* wildtype cultures resulted not only in reduced fungal growth [54], and reduced *A. fumigatus* biofilm metabolism (Fig 6), but also in increased sensitivity towards the iron chelator DFP. DFP is clinically used to treat iron overload, as in thalassemia major [73], but also interferes with iron needs of bacteria [74], and *A. fumigatus* biofilms [51]. Given that celastrol does not have unwanted effects on the host it might be quite useful in supporting anti-fungal therapy.

Previous studies have focused on *P. aeruginosa* products and their inhibition of *A. fumigatus*, at high *P. aeruginosa* product concentrations. Such studies have not considered the possible response of *A. fumigatus* at the onset of *P. aeruginosa* competition. We here show that *A. fumigatus* uses its siderophores to counter-balance iron denial by *P. aeruginosa*. *In vivo*, the winning microbe in this competition might be the one which unleashes its products first, and in the greatest quantity. *A. fumigatus* siderophores seem to also strengthen the fungus against certain types of therapy. Therefore, interference with siderophore production might boost existing therapy against *A. fumigatus*.

Supporting information

S1 Fig. Overview of the experimental setup. Af: *Aspergillus fumigatus*; Afsup: planktonic *A. fumigatus* supernatant, Pa: *Pseudomonas*; Pasup: planktonic *P. aeruginosa* supernatant, PYOV: pyoverdine;
(TIF)

S2 Fig. Gliotoxin content in Afsup is not likely to affect *P. aeruginosa*. *P. aeruginosa* cells (5 x 10^7 /ml) were incubated with planktonic supernatants (25%) derived from AF5322 wildtype, AFgliΔP (gliotoxin mutant), or AFgliPR (reversion of the gliotoxin mutant) at 37˚C for 24h. Bacterial growth (A600: A), and pyoverdine (PYOV; A405) were measured, and relative pyoverdine concentration (B) was calculated using the quotient A405/A600. Statistics by t-Test: PA14 supernatant, not containing Afsup (white bar) vs. PA14 supernatants containing Afsup. Two or three asterisks = $p \leq 0.01$ or $p \leq 0.001$, respectively.
(TIF)

S3 Fig. Reduction of nutrients affects bacterial growth, but does not result in protection of *A. fumigatus* from *P. aeruginosa* toxicity. *P. aeruginosa* cells (5 x 10^7 /ml) were incubated in RPMI 1640 medium containing 25% 10AFsup, or 25% sterile water, at 37˚C for 24h. A: Bacterial growth (A600) was measured. Supernatants derived from A were tested for toxicity against *A. fumigatus* biofilm formation (XTT assay: B). Statistics by t-Test: A: PA14 supernatant prepared without Afsup or water addition (white bar) vs all other bars. B: RPMI (while bar) vs. all other bars. Other comparisons as indicated by the ends of the brackets. Two or three asterisks = $p \leq 0.01$ or $p \leq 0.001$, respectively.
(TIF)

S4 Fig. Afsup induces pyoverdine and protects from *P. aeruginosa* anti-fungal activity. A: RPMI was inoculated with PA14 wildtype or the PA14 mutant PaΔ*pvdD* (5x10^7 cells/ml), with (black bars) or without (white bars) the presence of 25% 10AFsup, and incubated at 37˚C for 24h. Pyoverdine production was measured. B: Samples produced in A were used in a BCAM assay, and compared to metabolism of 10AF forming biofilm in the presence of RPMI or 25% 10AFsup, incubated without bacteria. Statistics: t-Test, as indicated by the ends of the brackets. Two or three asterisks = $p \leq 0.01$ or $p \leq 0.001$, respectively.
(TIF)

S5 Fig. TAFC and DF-TAFC are stable to prolonged heat treatment. A 10AF BCAM assay was incubated with RPMI, TAFC [10 μM], DF-TAFC [10 μM], either fresh or heat treated (90˚C for 30 min), and combined with pyoverdine (not heated) [PYOV, 10 μM]. Fungal

metabolism was measured by XTT assay. Control (RPMI incubation without heat treatment) was regarded as 100%. Statistics: t-Test, comparison: PYOV without heat treatment vs. all other PYOV-containing bars. Other comparisons as indicated by the ends of the brackets. One, two or three asterisks = $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively. Comparison of heat treatment of PYOV to unheated PYOV is also shown.
(TIF)

S1 Table. Data sets used in this study.
(PDF)

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References

1. Lerner A, Arleevskaya M, Schmiedl A, Matthias T. Microbes and viruses are bugging the gut in celiac disease. Are they friends or foes? Front Microbiol. 2017; 8: 1392. eCollection 2017. Review. https://doi.org/10.3389/fmicb.2017.01392 PMID: 28824555
2. de Souza HSP, Fiocchi C, Iliopoulos D. The IBD interactome: an integrated view of aetiology, pathogenesis and therapy. Nat Rev Gastroenterol Hepatol. 2017; 14: 739–749. https://doi.org/10.1038/nrgastro.2017.110 PMID: 28831186
3. Wang J, Li F, Tian Z. Role of microbiota on lung homeostasis and diseases. Sci China Life Sci. 2017; 60: 1407–1415. Review. https://doi.org/10.1007/s11427-017-9151-1 PMID: 29019144
4. Williams HD, Davies JC. Basic science for the chest physician: *Pseudomonas aeruginosa* and the cystic fibrosis airway. Thorax. 2012; 67: 465–467. https://doi.org/10.1136/thoraxjnl-2011-201498 PMID: 22382597
5. Smyth AR, Hurley MN. Targeting the *Pseudomonas aeruginosa* biofilm to combat infections in patients with cystic fibrosis. Drugs Fut. 2010; 35: 1007–1014.

6. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N, et al. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat Rev Microbiol. 2012; 10: 841–851. Review. https://doi.org/10.1038/nrmicro2907 PMID: 23147702
7. Sabino R, Ferreira JA, Moss RB, Valente J, Veríssimo C, Carolino E, et al. Molecular epidemiology of *Aspergillus* collected from cystic fibrosis patients. J Cyst Fibros. 2015; 14: 474–481. https://doi.org/10.1016/j.jcf.2014.10.005 PMID: 25459562
8. Fillaux J, Brémont F, Murris M, Cassaing S, Rittié JL, Tétu L, et al. Assessment of *Aspergillus* sensitization or persistent carriage as a factor in lung function impairment in cystic fibrosis patients. Scand J Infect Dis. 2012; 44: 842–847. https://doi.org/10.3109/00365548.2012.695454 PMID: 22831545
9. Speirs JJ, van der Ent CK, Beekman JM. Effects of *Aspergillus fumigatus* colonization on lung function in cystic fibrosis. Curr Opin Pulm Med. 2012; 18: 632–638. https://doi.org/10.1097/MCP.0b013e328358d50b PMID: 22965276
10. Ramsey KA, Ranganathan S, Park J, Skoric B, Adams AM, Simpson SJ, et al. Early respiratory infection is associated with reduced spirometry in children with cystic fibrosis. Am J Respir Crit Care Med. 2014; 190: 1111–1116. https://doi.org/10.1164/rccm.201407-1277OC PMID: 25321321
11. de Boer K, Vandemheen KL, Tullis E, Doucette S, Fergusson D, Freitag A, et al. Exacerbation frequency and clinical outcomes in adult patients with cystic fibrosis. Thorax. 2011; 66: 680–685. https://doi.org/10.1136/thx.2011.161117 PMID: 21680566
12. Nicolai T, Arleth S, Spaeth A, Bertele-Harms RM, Harms HK. Correlation of IgE antibody titer to *Af* with decreased lung function in cystic fibrosis. Pediatr Pulmonol. 1990; 8: 12–15. PMID: 2405341
13. Forsyth KD, Hohmann AW, Martin AJ, Bradley J. IgG antibodies to *Aspergillus fumigatus* in cystic fibrosis: a laboratory correlate of disease activity. Arch Dis Child. 1988; 63: 953–957. PMID: 3046514
14. Schønheyder H, Jensen T, Høiby N, Andersen P, Koch C. Frequency of *Aspergillus fumigatus* isolates and antibodies to *Aspergillus* antigens in cystic fibrosis. Acta Pathol Microbiol Immunol Scand B. 1985; 93: 105–112. PMID: 3893030
15. Coughlan CA, Chotirmall SH, Renwick J, Hassan T, Low TB, Bergsson G, et al. The effect of *Aspergillus fumigatus* infection on vitamin D receptor expression in cystic fibrosis. Am J Respir Crit Care Med. 2012; 186: 999–1007. https://doi.org/10.1164/rccm.201203-0478OC PMID: 22904183
16. Mirković B, Lavelle GM, Azim AA, Helma K, Gargoum FS, Molloy K, et al. The basophil surface marker CD203c identifies *Aspergillus* species sensitization in patients with cystic fibrosis. J Allergy Clin Immunol. 2016; 137: 436–443. https://doi.org/10.1016/j.jaci.2015.07.045 PMID: 26388311
17. Baxter CG, Moore CB, Jones AM, Webb AK, Denning DW. IgE-mediated immune responses and airway detection of *Aspergillus* and *Candida* in adult cystic fibrosis. Chest. 2013; 143: 1351–1357. https://doi.org/10.1378/chest.12-1363 PMID: 23139075
18. Shoseyov D, Brownlee KG, Conway SP, Kerem E. *Aspergillus* bronchitis in cystic fibrosis. Chest. 2006; 130: 222–226. https://doi.org/10.1378/chest.130.1.222 PMID: 16840406
19. Amin R, Dupuis A, Aaron SD, Ratjen F. The effect of chronic infection with *Aspergillus fumigatus* on lung function and hospitalization in patients with cystic fibrosis. Chest. 2010; 137: 171–176. https://doi.org/10.1378/chest.09-1103 PMID: 19567494
20. de Bentzmann S, Plésiat P. The P*seudomonas aeruginosa* opportunistic pathogen and human infections. Environ Microbiol. 2011; 13: 1655–1665. https://doi.org/10.1111/j.1462-2920.2011.02469.x PMID: 21450006
21. Walsh TJ, Stevens DA. Aspergillosis. Chapter 347, Cecil Textbook of Medicine, 24th ed. Goldman L, Schafer A, eds.; Elsevier; 2011.
22. Mangan A. Interactions between some aural *Aspergillus* species and bacteria. J Gen Microbiol. 1969; 58: 261–266. https://doi.org/10.1099/00221287-58-2-261 PMID: 4982824
23. Blyth W, Forey A. The influence of respiratory bacteria and their biochemical fractions on *Aspergillus fumigatus*. Sabouraudia. 1971; 9: 273–282. PMID: 5002653
24. Kerr JR, Taylor GW, Rutman A, Høiby N, Cole PJ, Wilson R. *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. J Clin Pathol. 1999; 52: 385–387. PMID: 10560362
25. Briard B, Bomme P, Lechner BE, Mislin GL, Lair V, Prévost MC, et al. *Pseudomonas aeruginosa* manipulates redox and iron homeostasis of its microbiota partner *Aspergillus fumigatus* via phenazines. Sci Rep. 2015; 5: 8220. https://doi.org/10.1038/srep08220 PMID: 25665925
26. Sass G, Nazik H, Penner J, Shah H, Ansari SR, Clemons KV, et al. Studies of *Pseudomonas aeruginosa* mutants indicate pyoverdine as the central factor in inhibition of *Aspergillus fumigatus* biofilm. J Bacteriol. 2017. pii: JB.00345-17. https://doi.org/10.1128/JB.00345-17
27. Cornelis P, Dingemans J. *Pseudomonas aeruginosa* adapts its iron uptake strategies in function of the type of infections. Front Cell Infect Microbiol. 2013; 3: 75. Review. https://doi.org/10.3389/fcimb.2013.00075 PMID: 24294593

28. Schalk IJ, Guillon L. Pyoverdine biosynthesis and secretion in *Pseudomonas aeruginosa*: implications for metal homeostasis. Environ Microbiol. 2013; 15: 1661–73. Review. https://doi.org/10.1111/1462-2920.12013 PMID: 23126435
29. Minandri F, Imperi F, Frangipani E, Bonchi C, Visaggio D, Facchini M, et al. Role of iron uptake systems in *Pseudomonas aeruginosa* virulence and airway infection. Infect Immun. 2016; 84: 2324–2335. https://doi.org/10.1128/IAI.00098-16 PMID: 27271740
30. Haas H. Iron—a key nexus in the virulence of *Aspergillus fumigatus*. Front Microbiol. 2012; 3: 28. eCollection 2012. https://doi.org/10.3389/fmicb.2012.00028 PMID: 22347220
31. Matthaiou EI, Sass G, Stevens DA, Hsu JL. Iron: an essential nutrient for *Aspergillus fumigatus* and a fulcrum for pathogenesis. Current Opinion Infect Dis. 2018; 31: 506–511.
32. Oberegger H, Schoeser M, Zadra I, Abt B, Haas H. SREA is involved in regulation of siderophore biosynthesis, utilization and uptake in *Aspergillus nidulans*. Mol Microbiol. 2001; 41: 1077–89. PMID: 11555288
33. Denning DW, Clemons KV, Hanson LH, Stevens DA. Restriction endonuclease analysis of total cellular DNA of *Aspergillus fumigatus* isolates of geographically and epidemiologically diverse origin. J Infect Dis. 1990; 162: 1151–1158. PMID: 1977804
34. Denning DW, Stevens DA. Efficacy of cilofungin alone and in combination with amphotericin B in a murine model of disseminated aspergillosis. Antimicrob Agents Chemother. 1991; 35: 1329–1333. PMID: 1929289
35. Schrettl M, Bignell E, Kragl C, Joechl C, Rogers T, Arst HN Jr, et al. Siderophore biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus* virulence. J Exp Med. 2004; 200: 1213–1219. https://doi.org/10.1084/jem.20041242 PMID: 15504822
36. Schrettl M, Bignell E, Kragl C, Sabiha Y, Loss O, Eisendle M, et al. Distinct roles for intra- and extracellular siderophores during *Aspergillus fumigatus* infection. PLoS Pathog. 2007; 3:e128.
37. Hartmann T, Dümig M, Jaber BM, Szewczyk E, Olbermann P, Morschhäuser J, et al. Validation of a self-excising marker in the human pathogen *Aspergillus fumigatus* by employing the beta-rec/six site-specific recombination system. Appl Environ Microbiol. 2010; 76: 6313–6317. https://doi.org/10.1128/AEM.00882-10 PMID: 20656854
38. Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM. Common virulence factors for bacterial pathogenicity in plants and animals. Science 1995; 268: 1899–1902. PMID: 7604262
39. Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, et al. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc Natl Acad Sci U S A. 2006; 103: 2833–2838. https://doi.org/10.1073/pnas.0511100103 PMID: 16477005
40. Ferreira JA, Penner JC, Moss RB, Haagensen JA, Clemons KV, Spormann AM, et al. Inhibition of *Aspergillus fumigatus* and its biofilm by *Pseudomonas aeruginosa* is dependent on the source, phenotype and growth conditions of the bacterium. PLoS One. 2015; 10: e0134692. https://doi.org/10.1371/journal.pone.0134692 PMID: 26252384
41. Wilderman PJ, Vasil AI, Johnson Z, Wilson MJ, Cunliffe HE, Lamont IL, et al. Characterization of an endoprotease (PrpL) encoded by a PvdS-regulated gene in Pseudomonas aeruginosa. Infect Immun. 2001; 69: 5385–5394. https://doi.org/10.1128/IAI.69.9.5385-5394.2001 PMID: 11500408
42. Martin LW, Reid DW, Sharples KJ, Lamont IL. P*seudomonas* siderophores in the sputum of patients with cystic fibrosis. Biometals. 2011; 24: 1059–67. https://doi.org/10.1007/s10534-011-9464-z PMID: 21643731
43. Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, et al. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res. 1988; 48: 4827–4833. PMID: 3409223
44. Meletiadis J, Mouton JW, Meis JFGM, Bouman BA, Donnelly JP, Verweij PE, et al. Colorimetric assay for antifungal susceptibility testing of Aspergillus species. J Clin Microbiol. 2001; 39: 3402–3408. https://doi.org/10.1128/JCM.39.9.3402-3408.2001 PMID: 11526191
45. Moss BJ, Kim Y, Nandakumar MP, Marten MR. Quantifying metabolic activity of filamentous fungi using a colorimetric XTT assay. Biotechnol Prog. 2008; 24: 780–783. https://doi.org/10.1021/bp070334t PMID: 18386938
46. Pierce CG, Uppuluri P, Tristan AR, Wormley FL, Mowat E, Ramage G, et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nature Protocols. 2008; 3: 1494–1500. https://doi.org/10.1038/nport.2008.141 PMID: 18772877
47. Pontecorvo G, Roper JA, Hemmons LM, Macdonald KD, Bufton AWJ. The genetics of *Aspergillus nidulans*. Adv Genet. 1953; 5: 141–238. PMID: 13040135

48. Andrews MY, Santelli CM, Duckworth OW. Layer plate CAS assay for the quantitation of siderophore production and determination of exudation patterns for fungi. J Microbiol Methods. 2016; 121: 41–43. https://doi.org/10.1016/j.mimet.2015.12.012 PMID: 26712125

49. Reece E, Doyle S, Greally P, Renwick J, McClean S. *Aspergillus fumigatus* inhibits *Pseudomonas aeruginosa* in co-culture: Implications of a mutually antagonistic relationship on virulence and inflammation in the CF airway. Front Microbiol. 2018; 9: 1205. eCollection 2018. https://doi.org/10.3389/fmicb.2018.01205 PMID: 29922270

50. Sugui JA, Pardo J, Chang YC, Zarember KA, Nardone G, Galvez EM, Müllbacher A, Gallin JI, Simon MM, Kwon-Chung KJ. Gliotoxin is a virulence factor of *Aspergillus fumigatus*: gliP deletion attenuates virulence in mice immunosuppressed with hydrocortisone. Eukaryot Cell. 2007; 6: 1562–1569. https://doi.org/10.1128/EC.00141-07 PMID: 17601876

51. Nazik H, Penner JC, Ferreira JA, Haagensen JA, Cohen K, Spormann AM, et al. Effects of iron chelators on the formation and development of *Aspergillus fumigatus* biofilm. Antimicrob Agents Chemother. 2015; 59: 6514–6520. https://doi.org/10.1128/AAC.01684-15 PMID: 26239975

52. Hsu JL, Clemons KV, Manouvakhova O, Inayathulla M, Tu AB, Sobel RA, et al. Microhemorrhage-associated tissue iron enhances the risk for *Aspergillus fumigatus* invasion in murine airway transplantation. Sci Transl Med. 2018; 10(429). pii: eaag2616. https://doi.org/10.1126/scitranslmed.aag2616

53. Stevens DA. Systemic antifungal agents. Cecil Textbook of Medicine, 25th ed. Chapter 331; Goldman L Schafer A, eds; Elsevier; 2015.

54. Martín Del Campo JS, Vogelaar N, Tolani K, Kizjakina K, Harich K, Sobrado P. Inhibition of the flavin-dependent monooxygenase siderophore A (SidA) blocks siderophore biosynthesis and *Aspergillus fumigatus* growth. ACS Chem Biol. 2016; 11: 3035–3042. https://doi.org/10.1021/acschembio.6b00666 PMID: 27588426

55. Potrykus J, Ballou ER, Childers DS, Brown AJP. Conflicting interests in the pathogen−host tug of war: Fungal micronutrient scavenging versus mammalian nutritional immunity. PLoS Pathog. 2014; 10: e1003910. https://doi.org/10.1371/journal.ppat.1003910 PMID: 24626223

56. Hissen AHT, Chow JMT, Pinto LJ, Moore MM. Survival of *Aspergillus fumigatus* in serum involves removal of iron from transferrin: the role of siderophores. Infect Immun. 2004; 72: 1402−1408. https://doi.org/10.1128/IAI.72.3.1402-1408.2004 PMID: 14977945

57. Braun V, Killmann H. Bacterial solutions to the iron-supply problem. Trends Biochem Sci. 1999; 24: 104–109.doi:10.1016/S0968-0004(99) 01359–6 PMID: 10203757

58. Khan A, Singh P, Srivastava A. Synthesis, nature and utility of universal iron chelator—Siderophore: A review. Microbiol Res. 2017. pii: S0944-5013(17)30673-0. Review.

59. Visca P, Imperi F, Lamont IL. Pyoverdine siderophores: from biogenesis to biosignificance. Trends Microbiol. 2007; 15: 22–30. Review. https://doi.org/10.1016/j.tim.2006.11.004 PMID: 17118662

60. Albrecht-Gary AM, Blanc S, Rochel N, Ocacktan AZ, Abdallah MA. Bacterial iron transport: coordination properties of pyoverdin PaA, a peptidic siderophore of *Pseudomonas aeruginosa*. Inorg Chem. 1994; 33: 6391–6402. https://doi.org/10.1021/ic00104a059

61. Meyer JM. Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* species. Arch Microbiol. 2000; 174: 135–142. https://doi.org/10.1007/s0020300001 PMID: 11041343

62. Imperi F, Massai F, Facchini M, Frangipani E, Visaggio D, Leoni L, et al. Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity. Proc Natl Acad Sci USA. 2013; 110: 7458–7463. https://doi.org/10.1073/pnas.1222706110 PMID: 23569238

63. Ankenbauer RG, Toyokuni T, Staley A, Rinehart KL Jr, Cox CD. Synthesis and biological activity of pyochelin, a siderophore of *Pseudomonas aeruginosa*. J Bacteriol. 1988; 170: 5344–5351. PMID: 3141386

64. Brandel J, Humbert N, Elhabiri M, Schalk IJ, Mislin GL, Albrecht-Gary AM. Pyochelin, a siderophore of *Pseudomonas aeruginosa*: physicochemical characterization of the iron(III), copper(II) and zinc(II) complexes. Dalton Trans. 2012; 41: 2820–2834. https://doi.org/10.1039/c1dt11804h PMID: 22261733

65. Lopez-Medina E, Fan D, Coughlin LA, Ho EX, Lamont IL, Reimmann C, et al. Candida albicans inhibits *Pseudomonas aeruginosa* virulence through suppression of pyochelin and pyoverdine biosynthesis. PLoS Pathog. 2015; 11: e1005129. eCollection 2015 Aug. https://doi.org/10.1371/journal.ppat.1005129 PMID: 26313907

66. Haas H. Fungal siderophore metabolism with a focus on *Aspergillus fumigatus*. Nat Prod Rep. 2014; 31: 1266–1276. https://doi.org/10.1039/c4np00071d PMID: 25140791

67. Blatzer M, Schrettl M, Sarg B, Lindner HH, Pfaller K, Haas H. SidL, an *Aspergillus fumigatus* transacetylase involved in biosynthesis of the siderophores ferricrocin and hydroxyferricrocin. Appl Environ Microbiol. 2011; 77: 4959−4966. https://doi.org/10.1128/AEM.00182-11 PMID: 21622789

68. Tiedemann RE, Schmidt J, Keats JJ, Shi C-X, Zhu YX, Palmer SE, et al. Identification of a potent natural triterpenoid inhibitor of proteosome chymotrypsin-like activity and NF- B with antimyeloma activity in vitro and in vivo. Blood. 2008; 113: 4027–4037. https://doi.org/10.1182/blood-2008-09-179796 PMID: 19096011
69. Zhu H, Liu XW, Cai TY, Cao J, Tu CX, Lu W, et al. Celastrol acts as a potent antimetastatic agent targeting beta1 integrin and inhibiting cell-extracellular matrix adhesion, in part via the p38 mitogen-activated protein kinase pathway. J Pharmacol Exp Ther. 2010; 334: 489–499. https://doi.org/10.1124/jpet.110.165654 PMID: 20472666
70. Kim DH, Shin EK, Kim YH, Lee BW, Jun JG, Park JH, et al. Suppression of inflammatory responses by celastrol, a quinone methide triterpenoid isolated from *Celastrus regelii*. European Journal of Clinical Investigation. 2010; 39: 819–827.
71. Venkatesha SH, Yu H, Rajaiah R, Tong L, Moudgil KD. *Celastrus*-derived celastrol suppresses autoimmune arthritis by modulating antigen-induced cellular and humoral effector responses. Journal of Biological Chemistry. 2011; 286: 15138–15146. https://doi.org/10.1074/jbc.M111.226365 PMID: 21402700
72. Allison AC, Cacabelos R, Lombardi VRM, Alvarez XA, Vigo C. Celastrol, a potent antioxidant and anti-inflammatory drug, as a possible treatment for Alzheimer's disease. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2001; 25: 1341–1357. PMID: 11513350
73. Savulescu J. Thalassaemia major: The murky story of deferiprone. BMJ. 2004; 328: 358–359. https://doi.org/10.1136/bmj.328.7436.358 PMID: 14962851
74. Paradkar PN, De Domenico I, Durchfort N, Zohn I, Kaplan J, Ward DM. Iron depletion limits intracellular bacterial growth in macrophages. Blood. 2008; 112: 866–874. https://doi.org/10.1182/blood-2007-12-126854 PMID: 18369153