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PEARLS

A call to arms: Mustering secondary metabolites for success and survival of an opportunistic pathogen

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Introduction

Aspergillus fumigatus is a ubiquitous saprophytic mold able to grow on a diversity of material ranging from decayed organic matter in the environment to space station cupolas [1]. Yet this fungus is equally adept as a serious opportunistic pathogen, causing pulmonary aspergillosis and the more deadly invasive aspergillosis (IA). There are an estimated 3,000,000 cases of pulmonary aspergillosis annually and more than 200,000 cases of IA each year reaching a mortality rate of up to 90% in the most susceptible populations [2]. Difficulties in treating IA include delayed detection and increasing resistance to antifungal treatment. Like many opportunistic fungi, there is no one gene that makes *A. fumigatus* such a threatening pathogen. One unique feature of this pathogen is its arsenal of small molecules that impact disease development. Secondary metabolites are characterized as bioactive molecules of low molecular weight that are not required for growth of the organism but instead aid survival in harsh environments, resisting desiccation and UV stress and improving competition with other microbes. For *A. fumigatus*, these benefits extend to aiding growth not only in the environment but in the human body as well. Some secondary metabolites combat the host immune system by affecting immune cell function or by shielding the fungus against host attack, whereas others allow the fungus to acquire essential, scarce cofactors. The following synopsis of secondary metabolites produced by the opportunistic human pathogen *A. fumigatus* highlights how microbial metabolites, although undoubtedly evolved as environmental protectants, can impact infectious disease development (Fig 1). Although we delineate the roles of each metabolite by category for ease of discussion (e.g., "on the offensive," "scavenging the battlefield," "arms race"), the reader should note that each metabolite may have several biological roles for the fungus, in part illustrated in Fig 1.

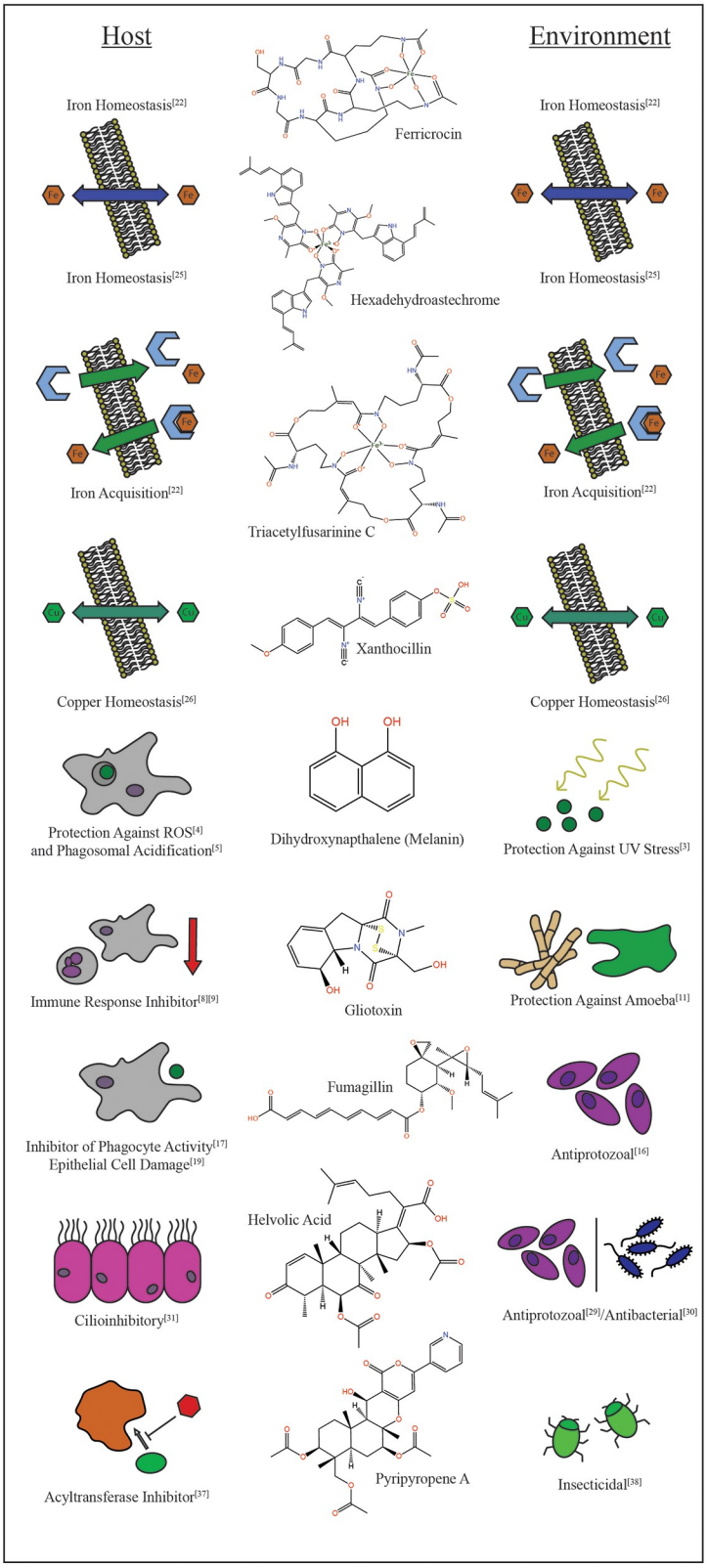
On the offensive: How *A. fumigatus* combats the immune system

Once inside the host, *A. fumigatus* must survive interactions with components of the immune system by avoiding, suppressing, or weakening the immune response. The following secondary metabolites have been shown to impact disease or interactions with the immune system through such mechanisms.

Dihydroxynapthalene melanin

Dihydroxynapthalene (DHN) melanin is a polymer consisting of 1,8-dihydroxynapthalene, found on the conidial surface. As an environmental benefit, DHN melanin helps to prevent

Roles of *Aspergillus fumigatus* Secondary Metabolites



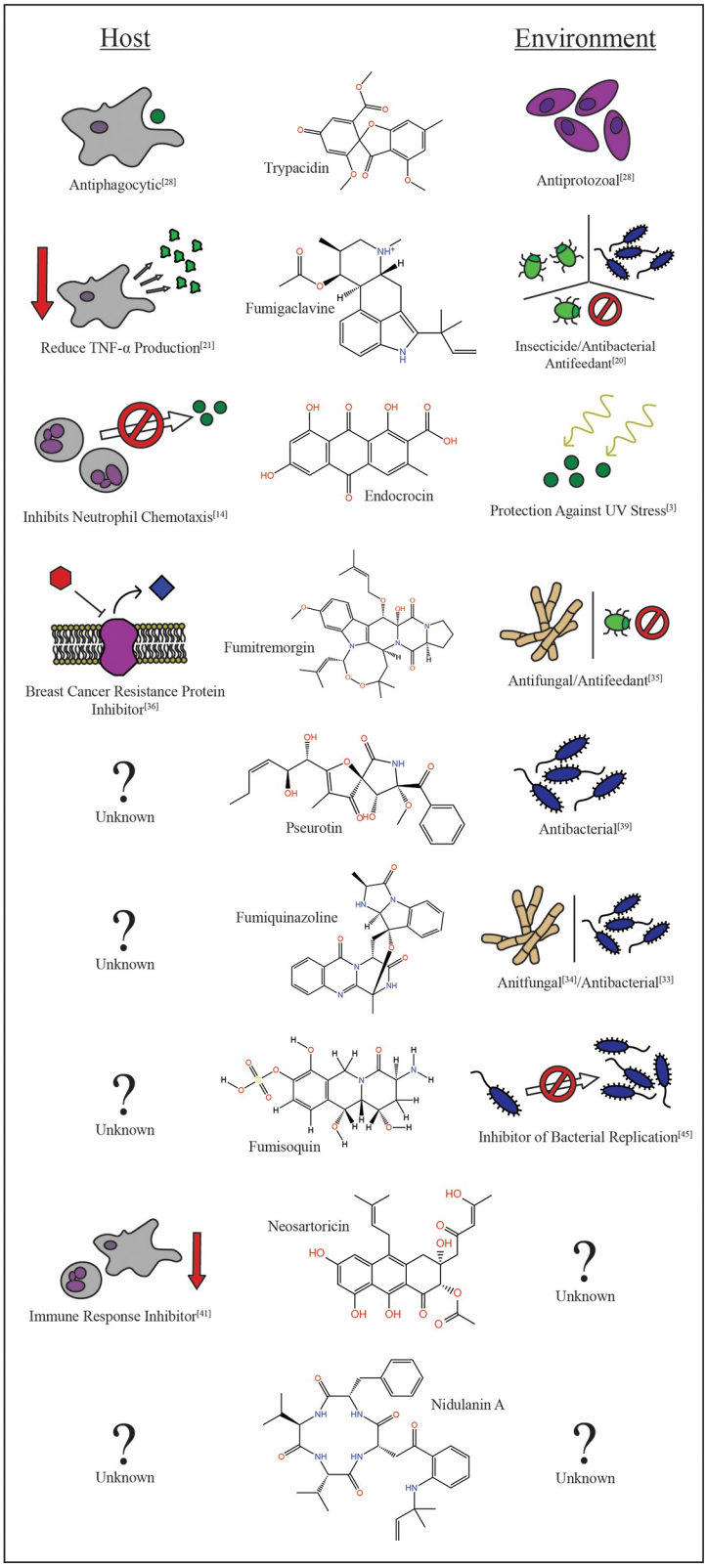


Fig 1. Roles of *Aspergillus fumigatus* secondary metabolites. A list of the secondary metabolites produced by *A. fumigatus*, flanked by their proposed roles in the environment (right) and the host (left). Metabolites with a "?" indicate that the compound has not been examined in a niche. Bracketed numerals (e.g., [22]) indicate the reference associated with the role of the metabolite. Nidulanin A is a proposed metabolite produced by *A. fumigatus*, whereas all other metabolites are characterized end-product metabolites from a biosynthetic gene cluster. ROS, reactive oxygen species; TNF-α, tumor necrosis factor alpha.

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desiccation of spores and confers resistance to UV radiation [3]. In the host, DHN melanin protects the conidia by scavenging reactive oxygen species [4], reducing phagosomal acidification in alveolar macrophages [5], and inhibiting apoptosis in epithelial cells [6]. When the polyketide synthase gene (*pksP*/*alb1*) responsible for the initial step of melanin production is deleted, there is a loss of spore pigment, a defect in virulence in intravenously injected immunocompetent murine models, and rapid killing spores in macrophage models [4]. Recently, DHN melanin has been described as a pathogen-associated molecular pattern, in that a C-type lectin receptor expressed in myeloid cells and CD31+ endothelial cells in humans recognizes DHN melanin and has been shown to have a protective role against disseminated infection in immunocompetent mice and recipients of stem cell transplants [7].

Gliotoxin

Gliotoxin is an epidithiodioxopiperazine that has been extensively studied in the context of infection. Gliotoxin inhibits activity of proteins that contain susceptible free thiols such as the host NADPH oxidase, a protein complex necessary for the generation of antimicrobial reactive oxygen species [8]. Gliotoxin has also been shown to inhibit nuclear factor-kappa B (NF-κB)-mediated transcription of cytokine genes and decrease cytotoxic activities of T lymphocytes [9]. *A. fumigatus* is resistant to its own toxin through a protective enzyme encoded in the gliotoxin cluster [10]. More recently, this metabolite has been shown to suppress the macrophage immune response by preventing integrin activation, interfering with actin dynamics, and impairing phagocytosis through affecting phosphoinositide metabolism [11]. When the gliotoxin nonribosomal peptide synthetase gene, *gliP*, is deleted, there is an attenuation of virulence in non-neutropenic murine models of IA but not in neutropenic murine models [12].

Endocrocin

Endocrocin is a polyketide that is localized to the conidia during growth [13]. Using an in vivo zebrafish assay, endocrocin was found to directly affect immune cells by inhibiting neutrophil chemotaxis [14]. When the polyketide synthase gene *encA* is deleted, there is an attenuation of virulence using the *Drosophila melanogaster* IA model [15]. Endocrocin belongs to a common class of anthraquinones and is closely related to emodin, a precursor in the trypacidin pathway that has been associated with mediating neutrophil apoptosis [15]. Although an exact role for endocrocin has not been established in nature, several related metabolites provide UV protection to fungi, similar to the role of DHN melanin [3].

Fumagillin

Fumagillin is a monoterpenoid, amoebicidal toxin with valuable pharmaceutical potential due to its inhibitory activity against methionine aminopeptidase-2, making it useful for the treatment of microsporidiosis [16]. The toxin has been found to suppress the immune response of *Galleria mellonella* by inhibiting the activity of phagocytes [17] and reduces the ability of the insect immune cells to kill opsonized *Candida albicans* cells and phagocytose *A. fumigatus* conidia [17]. In addition, fumagillin also reduces the ability of hemocytes to take up oxygen and inhibits the translocation of p47 protein [17], an essential component of the NADPH

oxidase complex. Fumagillin administered to insect larvae increases the susceptibility of the larvae to *A. fumigatus* [18]. Recently, virulence assays with an *A. fumigatus* fumagillin deletion mutant strongly support a role for this toxin in epithelial cell damage during IA [19].

Fumigaclavines

Fumigaclavines are ergot alkaloids, a class of compounds known to act as feeding deterrents and exhibit insecticidal and bactericidal activities [20]. Using the *G. mellonella* insect model for IA, it was found that a strain of *A. fumigatus* deficient in all ergot alkaloid production, Δ*dmaW*, resulted in a significantly reduced virulence. Strains that were still able to produce ergot alkaloids, but not fumigaclavine C, were significantly less virulent than wild type but still more virulent than the strain in which there was no production of ergot alkaloids, suggesting a role of the end product fumigaclavine C in virulence [20]. Fumigaclavine C has also been shown to inhibit the production of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα), suggesting a mechanism of action for the molecule [21].

Scavenging the battlefield: How *A. fumigatus* acquires essential micronutrients

Secondary metabolites regulate key aspects of micronutrient homeostasis and allow *A. fumigatus* to continue normal cellular function by meeting the needs for the trace elements such as copper and iron. Both are toxic in high doses but are necessary for essential cellular processes such as respiration and branched-chain amino acid biosynthesis. The ability to acquire these micronutrients is directly related to the ability of *A. fumigatus* to cause disease.

Siderophores

Siderophores produced by *A. fumigatus* are characterized by their hydrodroxamate moieties and function in high-affinity iron uptake and storage mechanisms. Extracellular siderophores fusarinine C and triacetlyfusarinine C are secreted into the environment, where they bind Fe^{3+} and transport it back into the cell. Intracellular siderophores ferricrocin and hydroxyferricrocin are responsible for iron storage and homeostasis. When the enzyme responsible for the first step in siderophore biosynthesis *sidA* is deleted, both extracellular and intracellular siderophore production is abolished. The *sidA* deletion grows poorly under iron-limiting conditions [22] and displays increased sensitivity to hydrogen peroxide. In addition, this mutant was found to be highly attenuated in virulence using a neutropenic murine model [23], suggesting that proper iron acquisition is essential for disease progression in the host.

Hexadehydroastechrome

Hexadehydroastechrome (HAS) is a tryptophan-derived secondary metabolite that binds to iron. Overexpressing the transcription factor present within the HAS biosynthetic gene cluster results in an increase in both siderophore and HAS production in addition to increased virulence in a neutropenic murine model [24]. HAS regulates fungal iron homeostasis circuitry, aligning iron acquisition and consumption pathways with secondary metabolite expression [25], including the newly discovered xanthocillin gene cluster [26].

Xanthocillins

Xanthocillins are tyrosine-derived metabolites that contain a characteristic isocyanide functional group and have been recently shown to be produced by the *xan* cluster in *A. fumigatus*. Overexpression of the transcription factor present within the cluster results in an increased

production of isocyanides and a defect in copper-dependent pigmentation indicating a possible link of this cluster to copper homeostasis [26]. The isocyanides produced by *A. fumigatus* may represent a unique mechanism, on top of the canonical copper regulatory system [27], to maintaining copper homeostasis for this pathogen.

Arms race: How *A. fumigatus* uses secondary metabolites to compete in the environment and host

Several secondary metabolites have no known effect or have not been tested for effects on virulence or interactions with the immune system but have only been shown to provide an advantage to *A. fumigatus* when competing with other microbes in the environment.

Trypacidin

Trypacidin is an anthraquinone that has been found to have antiprotozoal, cytotoxic, and antiphagocytic properties. The compound displays activity against *Toxoplasma gondii* and *Trypanosoma cruzi* in vitro that causes toxoplasmosis and Chagas disease, respectively. Deleting the polyketide synthase essential for trypacidin production eliminates production of the metabolite and coincides with an increase in phagocytosis when challenged with *Dictyostelium discoideum* and macrophages, indicating that trypacidin acts as an antiphagocytic metabolite [28]. The trypacidin pathway shows redundant synthesis to the endocrocin pathway, where both contribute to final endocrocin synthesis in some strains of *A. fumigatus* [15].

Helvolic acid

Helvolic acid is a fusidane antibiotic that exhibits in vitro antiprotozoal activity against the trypanosome *Trypanosoma brucei brucei* GUTat3.1, the causative agent of African sleeping sickness [29], and helvolic acid derivatives exhibit antibacterial activity against *Streptococcus agalactiae* and *Staphylococcus aureus* [30]. In addition, helvolic acid also affects mammalian cell lines, decreasing the beat frequency of ciliated respiratory epithelium, a process important in preventing colonization by *A. fumigatus* [31].

Fumiquinazolines

Fumiquinazolines are tryptophan-derived peptidyl alkaloids that have a broad range of activity and accumulate in *A. fumigatus* conidia [32]. Fumiquinazoline F isolated from cultures of *Penicillium coryphilum* exhibited activity against *S. aureus* and *Micrococcus luteus* [33]. Fumiquinazolines also exhibit antifungal activity with fumiquinazoline H and I isolated from *Acremonium sp*. showing weak antifungal activity against *C. albicans* [34].

Fumitremorgins

Fumitremorgins belong to the diketopiperazine alkaloids class of compounds and contain a unique, 8-membered endoperoxide ring. Fumitremorgin B has been found to have in vitro antifungal activity against a variety of phytopathogenic of fungi [35]. In addition, fumitremorgin B was found to be lethal to brine shrimp and displayed antifeedant activity towards armyworm larvae [35]. Fumitremorgins have also been shown to affect mammalian cells. Fumitremorgin C displays inhibitory activity towards the breast cancer resistance protein, an ATP-binding cassette transporter that is implicated in cellular resistance to anticancer drugs [36].

Pyripyropene A

Pyripyropene A was discovered during an investigation into inhibitors of acyl-coenzyme A (CoA):cholesterol acyltransferase, a mechanism by which to treat hypercholesterolemia and atherosclerosis [37]. Pyripyropenes were further shown to exhibit in vivo aphicidal activity against the green peach aphid (*Myzus persicae*) during a screen of compounds that act as insecticides [38]. How these activities may relate to aspergillosis has not been assessed.

Pseurotin

Pseurotin has been shown to be have several antimicrobial and cytotoxic properties. It has been demonstrated to have antibacterial properties when screened against both gram-positive and gram-negative organisms [39]. This metabolite is encoded by an intertwined biosynthetic gene cluster with fumagillin [40] but, unlike fumagillin, was not implicated in epithelial tissue damage [19].

Neosartoricin

Neosartoricin is a prenylated anthracenone and was discovered following activation of the gene cluster from *A. fumigatus* and *Neosartorya fischeri* [41]. The compound was found to have T-cell antiproliferative activity suggesting that the compound functions as an immunosuppressive [41]. Like several metabolites synthesized by *A. fumigatus*, the biosynthetic gene cluster is conserved in several pathogenic fungi [42].

Fumisoquin

Fumisoquin is an isoquinolone alkaloid with biosynthetic machinery that bears a striking similarity to plant berberine bridge enzyme and tetrahydrocannabinol biosynthesis [43]. Deletion of the fumisoquin transcription factor did not impact virulence in a murine infection model [44]. A related isoquinalone metabolite produced by *Aspergillus flavus* stimulates *Aspergillus* species spore germination while inhibiting bacterial growth [45], possibly hinting at a function for fumisoquin.

Nidulanin A

Nidulanin A is a tetracyclopeptide/isoprene isolated from *Aspergillus nidulans* [46]. The nidulanin A gene cluster is conserved in all *Aspergillus* spp., including *A. fumigatus*, although it has not been detected in this fungus [42]. At present, nidulanin A has yet to be tested for any antimicrobial or virulence-related properties.

Prospective

A. fumigatus produces a wide variety of small molecules, many of which are demonstrated to impact virulence, others of which have not been investigated, and likely still some of which have yet to be discovered. These molecules are the weapons that *A. fumigatus* uses to do battle with the immune system, facilitate the acquisition of essential micronutrients in their environment, and compete with other microbes. It is important to note, however, that *A. fumigatus* isn't alone in producing secondary metabolites that affect virulence. Many of these secondary metabolites are conserved in other pathogenic fungi [38]. Studying secondary metabolites produced by *A. fumigatus* will provide insight into understanding not only the chemical arsenal of *A. fumigatus* but the chemical arsenal of other pathogenic fungi as well.

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