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Review Article

Review Article

The anti-*Aspergillus* drug pipeline: Is the glass half full or empty?

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Abstract

Aspergillosis has emerged as important human mycoses, in view of the ever expanding population at risk. The emergence of resistance to the most commonly used drugs for aspergillosis, the azoles, the mediocre activity, and frequent toxicity of the current antifungal armamentarium, support the need for development of novel antifungals for treatment of this disease. In this minireview, we describe recent efforts by small drug companies and University research labs to develop novel therapies for invasive aspergillus infections. We specifically discuss four small-molecule antifungals (T-2307, E1210/APX001, ASP2397, and F901318) with novel modes-of-action, which are currently entering phase I clinical trials. In addition, we provide a nonexhaustive discussion of some interesting, yet early developments in the quest for improved therapeutic strategies such as (i) novel formulations of amphotericin B including AMB nanoparticle suspensions and AMB-arabinogalactan or AMB-PEG conjugates that show low toxicity and high efficacy in preclinical animal models, (ii) repurposed drugs that synergize with existing antifungals (clozafimine, trichostatin A, MGCD290, geldanamycin, tacrolimus, cyclosporin), (iii) natural products (psoriasin, humidimycin), and (iv) immunotherapy using adoptive transfer of activated immune cells with antifungal activity. We argue that despite the plethora of candidates, the extremely low success rates of drug development leading to clinically useful drugs reinforces the need for continued clinical reliance on mainstream antifungals and their improved derivatives.

Key words: Antifungal drug discovery, natural products, immunotherapy.

Introduction

Aspergillus fumigatus is the most common mould pathogen of humans causing severe and frequently fatal infection in patients with immunosuppression or underlying structural lung disease. An estimated 200,000 cases occur each year worldwide.[1] Left untreated, mortality rates from invasive pulmonary aspergillosis (IPA) exceed 90% and even following aggressive antifungal treatment, fatality rates of 50%

are common in heavily immunosuppressed hosts such as those with leukemia and transplant recipients. This high rate of mortality following *A. fumigatus* infection is a result of the suboptimal diagnostic tools available, leading to late diagnosis and the relative ineffectiveness of existing antifungal drugs against established Aspergillus infections.

Three classes of antifungal drugs are mainly used for the treatment of aspergillosis- triazoles that inhibit ergosterol biosynthesis (itraconazole, voriconazole, posaconazole, isavuconazole), the polyene amphotericin B-deoxycholate (and its lipid formulations) that binds fungal membrane ergosterol leading to cell lysis, and the echinocandins (caspofungin, micafungin, anidulafungin) that inhibit fungal glucan cell-wall biosynthesis. The most commonly used drugs in the treatment of aspergillosis, the azoles have significant drug-drug interactions, acute (e.g., hepatic and central nervous system toxicity) and chronic (e.g., neuropathy, alopecia) side effects. Chronic voriconazole use in particular has been associated with bone fluorosis as well as with photoaging and skin cancers. Voriconazole treatment necessitates costly and technically complex therapeutic drug monitoring due to its nonlinear and saturable pharmacokinetics.[2] Importantly, the broad spectrum of resistance reported from many countries around the globe threatens to devitalize in the long term the impact of these agents.[3] The other two classes of anti-*Aspergillus* drugs, the echinocandins and Amphotericin B formulations, are restricted to intravenous use only. In addition, echinocandins have a narrow spectrum of activity, while amphotericin B formulations have frequent treatment-limiting side effects, especially nephrotoxicity.[4] Combination antifungal therapy in clinical practice is limited to amphotericin B and flucytosine in the treatment of cryptococcal meningitis or voriconazole and anidulafungin for invasive aspergillosis. Although there is a pressing need to develop new drugs that inhibit novel fungal-specific targets, no new classes of antifungals have been commercialized since the launch of the echinocandins in 2001. Most of the large pharmaceutical companies have downsized or even halted their antifungal discovery efforts. Development of treatments for much more frequent and chronic diseases (e.g., diabetes mellitus) is far more lucrative. Discovering new antifungals when screening established compound libraries or natural products has been proven difficult for a variety of reasons, chiefly because fungi are eukaryotes and far more closely related to humans as compared to bacteria or viruses[3]. In the 1990s, following the introduction of new tools in genomics and molecular biology, the large pharmaceutical companies did perform expensive large-scale antifungal drug screening projects.[3,4] However, these efforts led to the rediscovery of known compounds (and in particular azoles and glucan-synthase inhibitors, known as "the low-hanging" fruit) or "hits" with unacceptable toxicity in mammals.[5,6] The playing field has been largely left to the efforts of small biotech startups and individual research labs, which lack the tremendous resources needed to push a drug through human trials and the extensive regulatory oversight associated with phase I–III drug development. As this review will illustrate, the scientific literature may generate the false impression of a veritable bounty of promising new antifungals in the pipeline.[7] However, almost all have at best been tested in mouse models of infection. As has been clearly shown in the field of cancer therapy, success in treating mice rarely corresponds to success in curing humans, and less than 10% of drug candidates successfully navigate the "valley of death" and gain FDA approval after vigorous testing in phases I–III of clinical trials.[8]

In this review, in addition to describing the most promising recently described antifungals with novel modes of action and activity against *Aspergillus* species, we will briefly address developments of new formulations of existing antifungals, the repurposing of old drugs that show antifungal activity, combination therapy and immunotherapy (Table 1). We will not discuss recently released improved azole and candin drugs, as these have been reviewed elsewhere.[5]

Selected new agents with potential in *Aspergillus* treatment

Although many novel compounds with anti-*Aspergillus* activity have been recently described,[9–12] we will focus on four (T-2307, E1210/APX001, ASP2397, and F901318) compounds that have reached early stages of human clinical testing (Table 1).

T-2307, developed by the Toyama Chemical Company (Tokyo, Japan), is an arylamidine compound similar to the antimicrobial drug pentamidine. It selectively targets fungal mitochondria, leading to loss of membrane potential.[13] In *Candida*, T-2307 is selectively and actively transported into the cell by a high-affinity spermine and spermidine carrier regulated by Agp2 leading to its accumulation at high concentrations added.[14,15] T-2307 exhibits potent in vitro (MIC ranges of 0.001–1 μg/ml) and in vivo activity in a murine model of systemic infection by *Candida albicans* (100% two-week survival, 0.02 mg/kg), *Cryptococcus neoformans* (>70% two-week survival, 0.3 mg/kg), and *A. fumigatus* (>80% two-week survival, 1 mg/kg), by subcutaneous injection.[16] T-2307 underwent successful phase I safety trials in healthy volunteers to assess safety, tolerability, and pharmacokinetics in 2015 (https://clinicaltrials.gov/ct2/show/NCT02289599).

Table 1. Summary of antifungal compounds described in this review.

Antifungal:	Mode of Action	In vitro MIC *A. fumigatus*	In vivo murine IA infection: active dose	Human trials Phase I	Refs.
Small molecules: T-2307	Mitochondrial function	0.01–1 μg/ml	1 mg/kg subcutaneous	Yes, 2015	13,14,16
E1210/APX001	GPI-anchor inhibitor	0.03-0.13 μg/ml	25 mg/kg oral	Yes, ongoing	17–22
ASP2397	Unknown	0.06-0.5 μg/ml	4 mg/kg	Yes, ongoing	23,24
F90138	DHODH inhibitor, pyrimidine biosynthesis	< 0.06 μg/ml	10 mg/kg oral	Yes, ongoing	25
Amphotericin B (AMB) formulations and conjugates:	Disruption of fungal membrane	0.125–0.75 μg/ml	10 mg/kg	No	30
AMB-nanoparticles		0.25–1 μg/ml	5 mg/kg	No	29
AMB-arabinogalactan AMB-PEG		1–8 μg/ml	7 mg/kg	No	28
Repurposed drugs					
Clofazimine	Antimycobacterial DNA binding	Only effective in combination with caspofungin or posaconazole	Not determined	No	32
Trichostatin A	HDAC inhibitor	4 μg/ml, synergy with caspofungin	Not determined	No	34
MGCD290	HDAC inhibitor	8->32 μg/ml, synergy with azoles	Not determined	No	35
Geldanamycin	Hsp90 inhibitor	4 μg/ml (MEC), synergy with caspofungin	Not determined	No	36
Tacrolimus (FK506)	Calcineurin inhibitor	0.01–0.6 μg/ml (MEC), synergy with caspofungin	Not determined	No	37,38
Cyclosporin	Calcineurin inhibitor	0.5–1 μg/ml (MEC)	Not determined	No	37
Natural Products					
Psoriasin	Zinc chelation	1 μM	5 mg/kg	No	39
Humidimycin	HOG pathway inhibitor	Only active in combination with caspofungin	Not determined	No	41

The fungal cell wall is an excellent fungal-specific drug target. Surprisingly, however, among currently available drugs, only the echinocandins directly target the cell wall by inhibiting glucan synthase activity. **E1210/APX001** is a novel wall-active antifungal compound discovered by the Eisai Company (Tokyo, Japan), which is being further developed by Amplyx Pharmaceuticals Inc. (San Diego, CA). E1210/APX001 inhibits an early step in the glycosylphosphatidylinositol (GPI)-dependent anchoring of mannosylated cell wall proteins attached to the polysaccharide wall component. Lacking these proteins, the cell wall weakens, resulting in the fungistatic arrest of growth. The target of E1210/APX001is Gwp1p, the fourth enzyme in the GPI-anchoring pathway, responsible for inositol acylation.[17] Mammals also contain the Gwp1 homolog PigW; however, it is only 28% identical to the fungal gene and is not inhibited by E1210.[17] *In vitro*, E1210/APX001 was highly effective (MIC90 ranges of 0.05–0.2 μg/ml) against most fungi, including yeasts (*Candida* species except *Candida krusei*) and moulds (*Aspergillus* species, *Fusarium* spp., black moulds), as well as strains resistant to azoles and polyenes. E1210/APX001 was moderately effective against Mucorales species (MIC90 ranges of 1–8 μg/ml).[18–21] *In vivo*, orally administered E1210/APX001 was effective (>80% two-week survival, 2.5–25 mg/kg/day) in the treatment of murine models of disseminated candidiasis (*C. albicans*), pulmonary aspergillosis (*A. fumigatus* or *Aspergillus flavus*), and disseminated fusariosis (*Fusarium solani*). E1210/APX001 was nontoxic at 100 mg/kg and was generally well tolerated at doses of up to 300 mg/kg in rats.[22] Following successful pharmacodynamic and metabolic studies of E1210/APX001 in

rats, dogs, and monkeys, E1210/APX001 has now entered phase I clinical trials.

ASP2397 is a novel antifungal isolated by Astellas Pharma (Tokyo, Japan) from the fungus *Acremonium* and now under clinical development by Vical Inc. (San Diego, CA). Similar in structure to other characterized fungal siderophores, ASP2397 is transported into the *A fumigatus* cell by the Sit1 siderophore transporter. Inactivation of Sit1 results in resistance toward ASP2397[23]. The target of this compound has not been identified to date (personal communication Andrew Hopkins, Vical Inc.). In vitro, ASP2397 was active (MIC < 2 μg/ml) against most aspergilli (except *Aspergillus niger)*, *C. neoformans* and *Candida krusei*, but only weakly active (MIC > 16 μg/ml) against other *Candida* spp. and the *Mucorales*.[23,24] *In vitro*, ASP2397 halted hyphal elongation of germinated *A. fumigatus* conidia as soon as treatment was initiated (1–2 h post-germination) and exhibited faster fungicidal activity (1–2 h post-treatment) against azole-resistant *A. fumigatus* compared to the existing licensed drugs-voriconazole, posaconazole and AMB. *In vivo*, ASP2397 at 4 mg/kg showed enhanced efficacy (100% survival) in a delayed (24 h post-infection) murine model of invasive aspergillosis compared to posaconazole at 10 mg/kg (40% survival).[23] ASP2397 is now entering phase I clinical trials (personal communication Andrew Hopkins, Vical Inc.)

F901318 was developed by F2G Ltd (Manchester, UK) (http://www.f2g.com/) from a hit found in a compound library screen. It inhibits the enzyme dihydroorotate (*DHODH/URA1*) catalyzing the fourth enzymatic step of pyrimidine biosynthesis.[25] Interestingly, although this pathway is conserved in man, human *DHODH* is only ~20% identical to its fungal homolog and is inhibited 2000-fold less effectively by F901318. In vitro, F901318 is highly active against azole-resistant mould pathogens (MIC *A. fumigatus* <0.06 μg/ml, *Scedosporium* spp. <0.06 μg/mL, *Fusarium* spp. 0.25–1 μg/ml, dimorphic fungi- 0.06–0.125 μg/ml) but is inactive against the *Mucorales* and *Candida* spp. *In vivo*, F901318 was effective (100% two-week survival, 10 mg/kg oral administration) in the treatment of invasive aspergillosis in a neutropenic murine model of infection.[26] In phase I human safety trials, F901318 administered IV at up to 4 mg/kg as a single dose was well tolerated with no adverse events noted and high levels in the blood. Dose-escalation studies in human volunteers are ongoing.

In summary, three of the four antifungal candidates described have a novel mode of action. Interestingly, none inhibits a fungal-specific target (T-2307- mitochondria, E1210-Gwp1/GPI-anchoring, F90138- DHODH/pyrimidine biosynthesis), yet apparently there are sufficient differences between the human and fungal targets to allow specificity. Two of the candidates, ASP2397 and F90138, have a narrow spectrum of activity that may limit their use and applicability. In addition, in view of the uncertainty of etiology of presumed fungal infection, studying ASP 2397 and F90138 in humans would require an innovative study design with the use of another antifungal until the specific diagnosis of aspergillosis is made.

Novel AMB formulations

AMB-deoxycholate, now entering its seventh decade of use, is a potent fungicidal drug with a broad spectrum of activity. However, the drug has frequent and significant side effects such is acute infusion reactions and nephrotoxicity. This has prompted the development of less toxic but expensive lipid formulations (ABLC, liposomal AMB), now in widespread use.[27] Novel formulations now under development include AMB nanoparticle suspensions and AMB-arabinogalactan or AMB-PEG conjugates with low toxicity and high efficacy in vivo (Table 1).[27–30] However, none of these formulations has entered human clinical trials. http://www.ncbi.nlm.nih.gov/pubmed/?term=amphotericin+umbrella)

Repurposing drugs as anti-*Aspergillus* agents

Considerable efforts have been made to screen and identify available drugs that synergize with the existing antifungals (Table 1). Some experts have effectively argued that off patent use of known drugs might be more effective for antifungal drug discovery compared to traditional approaches of structural biology and genome mining.[31] For example, Robbins et al.[32] screened a compound library in combination with sublethal concentrations of known antifungals in diverse fungal species. This resulted in the identification of the antimycobacterial drug clofazimine, a compound with unreported antifungal activity that synergized with caspofungin and posaconazole against *C. albicans* and *A. fumigatus*.

Targeting the fungal histone deacetylase (HDAC)-Heat shock protein 90 (Hsp90)-calcineurin axis with existing drugs is another promising antifungal strategy.[33] HDACs deacetylate and activate Hsp90. Hsp90 activates its target protein calcineurin phosphatase, which plays a key role in stress responses and cell wall repair mechanisms, which are activated upon antifungal exposure.[33] Therefore, inhibition of this pathway will enhance the activity of different classes of antifungal drugs such as the cell wall acting-echinocandins and the ergosterol biosynthesis inhibitors azoles. Inhibitors of HDAC (trichostatin A, MGCD290), Hsp90 (geldanamycin) and calcineurin (tacrolimus, cyclosporin) initially developed as anticancer and

immunosuppression/organ transplant drugs in humans have been tested for antifungal activity, either alone or in combination with existing antifungals.[33] Trichostatin A has potent *in vitro* activity against *A. fumigatus* and other moulds and enhances the efficacy of caspofungin.[34] MGCD290 alone is inactive against filamentous fungi, but it exhibits synergy *in vitro* in combination with azoles, including against azole-resistant fungal isolates and genera.[35] Geldanamycin has modest in vitro activity against moulds, but its combination with caspofungin in *A. fumigatus* results in fungicidal activity, including in azole-resistant strains.[36] Tacrolimus (FK506) or cyclosporin in combination with the antifungals caspofungin or itraconazole showed in vitro synergy against *A. fumigatus*.[37] Tacrolimus synergizes with posaconazole in the treatment of murine cutaneous mucormycoses.[38]

In summary, although the Hsp90-calcineurin axis inhibitors have shown promise *in vitro*, they have not been adequately tested (alone or in combination with existing antifungals) in mammalian models of systemic infection, nor have they been developed in humans for this use. Significant limitations to their utility as antifungals are their potent pharmacological effects in humans and the high degree of conservation of the Hsp90-calcineurin pathway proteins between humans and fungi.

Natural products as anti-*Aspergillus* agents

Natural products are an unparalleled source of bioactive molecules including antifungals. Indeed, two existing antifungal drug classes, the polyenes and echinocandins, were discovered by screening of natural products. Surprisingly, however, most drug companies have halted their natural product screens due to repeated rediscovery of existing leads. For example, in a high throughput "fitness-test" screen of over 1,800 biological extracts performed by Merck, less than 15% of the ~60 antifungal molecules identified were novel, and none of these have reached clinical trials due to low specificity, toxicity, or chemical challenges in synthesizing improved derivatives.[6] Research laboratories, on the other hand, have continued to identify new antifungal compounds from natural products (Table 1). The observation that psoriatic skin lesions are rarely infected by fungi led to the isolation of the skin-associated antifungal psoriasin.[39] It is a small (~10 kDa) fungicidal protein that works by chelating free intracellular zinc leading to fungal apoptosis. Interestingly, psoriasin significantly reduced mortality in a murine model of pulmonary aspergillosis.

The finding that the lungs of patients with cystic fibrosis are often colonized by a mixed biofilm of *Pseudomonas aeruginosa* bacteria and *A. fumigatus*, and that mice infected with both organisms have a higher rate of survival compared to those infected with the fungus alone, led to the hypothesis that these bacteria secrete antifungal compounds. Analysis of *P. aeruginosa* culture filtrates showed that they inhibit *A. fumigatus* biofilms by inducing apoptosis. The apoptotic effect involves mitochondrial membrane damage associated with metacaspase activation.[40] The identity of the bacterial antifungal compound remains unknown.

A screen of 20,000 diverse biological extracts for compounds that potentiate the activity of sublethal concentrations of caspofungin identified the bacterial ring peptide humidimycin.[41] It is inactive on its own, but in combination with caspofungin it increases the antifungal activity of this drug ~10-fold. Humidimycin apparently works by inhibiting the fungal high osmolarity glycerol (HOG) response pathway, thereby reducing the protective stress response induced by caspofungin, rendering it more lethal.[41] Because humidimycin is nontoxic to human cells in culture, and targets a pathway (HOG) not found in mammals, it is a promising lead for further development.

Antifungal immunotherapy

While immunotherapy is a relatively commonplace modality for treating bacterial and viral infections, this field is still largely at the research stage in regards to fungal infections. The complexity of the immune system provides several avenues for therapeutic intervention [reviewed in [42] and [43]]. These include the (i) adoptive transfer of activated immune cells with antifungal activity, (ii) the administration of cytokines that activate the immune system, and in particular GM-CSF (leucomax/sargramostim), G-CSF (filgrastim), IFN-γ, IL-12, or IL-17 in combination with antifungal therapy. However, while demonstrating promising results in small pilot studies in humans, this approach is still not used in standard therapy (iii) the use of fungal-specific monoclonal antibodies including Mab JF5 against *A. fumigatus*, Efungumab/Mycograb for *C. albicans*, or wide-spectrum anti-idiotype KT antibodies and anti-laminarin/β-glucan specific antibodies. Despite sustained and significant efforts, this approach has not generated an acceptable therapy (iv) the development of vaccines. Due to the breadth of this topic, we will highlight a few recent publications describing immune cell therapy that show clinical promise. Several new studies have demonstrated the protective utility of granulocyte transfusions to prevent fungal infections into stem cell transplant patients provided they are administered rapidly and with a sufficient dose of cells.[44,45] A pioneering clinical study using adoptive transfer of Aspergillus-stimulated T cells by Perrucio et al.[46] showed significant efficacy in treating stem cell transplant patients with invasive aspergillosis (9 of 10 treated patients cleared the

infection vs. 6 of 13 patients succumbing in the control cohort). Recently, Kumaresan et al.[47] generated T cytotoxic cells expressing Dectin-1 chimeric antigen receptors (CAR) that recognize surface fungal glucans. *In vitro* these D-CAR+T cells specifically damaged fungal hyphae and germinating spores. *In vivo*, infusion of D-CAR+T cells reduced both fungal burden and mortality in two disparate (pulmonary, cutaneous) immunosupressed murine models of aspergillosis.

Perspectives

While at a first glance, it appears that the antifungal drug discovery pipeline is alive and well, it is important to note that only four of the lead novel compounds described in this review (see Table 1) are in early clinical trials. To succeed, they will have to stand up to the congested field of current drugs that have been developed and finetuned over decades of use. New drugs will need to have a wide therapeutic index (= low toxicity), while having a broad spectrum and an IV to oral transition. Whether these compounds will have a "niche" and commercial viability remains to be seen. For example, if the competitive advantage of a new compound is to target specific rare resistant fungi (e.g., azole-resistant moulds), the market would be small, and profitability of the manufacturer would be limited. Perhaps a new economic model that disassociates sales volume with price is needed. On the other hand, if the new compounds are to be overused without thoughtful integration into the existing antifungal armamentarium, resistance will threaten their shelf life. Furthermore, future research for development of "theragnostics" is needed, in order to address simultaneously the problems of improving drug delivery at the site of infection and early site-specific diagnosis of infection.[48]

Promoting responsible use of existing antifungals through stewardship and education until the pipeline can be refilled is currently the most reasonable approach for the short-term future. The time is ripe for clinical mycologists, basic scientists, industry, policy makers, health economists, and funding agencies to come together in an effort to ensure rational drug development for the management of aspergillosis.

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