Analysis of Current Antifungal Agents and Their Targets within the *Pneumocystis carinii* Genome

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Abstract

*Pneumocystis* pneumonia (PCP) remains a leading opportunistic infection in patients with weakened immune system. The fungus causing the infection belongs to the genus, *Pneumocystis*, and its members are found in a large variety of mammals. Adaptation to the lung environment of a host with an intact immune system has been a key to its successful survival. Unfortunately, the metabolic strategies used by these fungi to grow and survive in this context are largely unknown. There were considerable impediments to standard approaches for investigation of this unique pathogen, the most problematic being the lack of a long term *in vitro* culture system. The absence of an *ex vivo* cultivation method remains today, and many fundamental scientific questions about the basic biology, metabolism, and life cycle of *Pneumocystis* remain unanswered. Recent progress in sequencing of the *Pneumocystis carinii* genome, a species infecting rats, permitted a more informative search for genes and biological pathways within this pathogen that are known to be targets for existing antifungal agents. In this work, we review the classes of antifungal drugs with respect to their potential applicability to the treatment of PCP. Classes covered in the review are the azoles, polyenes, allylamines, and echinocandins. Factors limiting the use of standard antifungal treatments and the currently available alternatives (trimethoprim-sulfamethoxazole, atovaquone, and pentamidine) are discussed. A summary of genomic sequences within *Pneumocystis carinii* associated with the corresponding targeted biological pathways is provided. All sequences are available via *Pneumocystis* Genome Project at http://pgp.cchmc.org/.

Keywords

Antifungal agents; Antifungal drug resistance; Antifungal drug targets; *Pneumocystis* biological pathways; *Pneumocystis* genome project; *Pneumocystis* pneumonia

Introduction

*Pneumocystis jirovecii* is a fungus that causes *Pneumocystis* pneumonia (PCP) in humans, which remains a leading opportunistic infection associated with AIDS patients, even in the era of Highly Active Anti-Retroviral Therapy (HAART)(1). Moreover, PCP increasingly targets new groups of patients with underlying chronic diseases states, such as Chronic Obstructive Pulmonary Disorder (COPD)(2); patients receiving anti-TNF therapy (3); and

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other immunosuppressive agents (4). However, despite concerted efforts towards identification of new chemotherapeutic agents, trimethoprim-sulfamethoxazole (TMP-SMX) remains the standard prophylactic and therapeutic modality in use today (5), with clindamycin-primaquine, atovaquone and pentamidine being standard secondary PCP treatments. With such a limited repertoire of therapeutic options, there is a great potential for developing resistance to these compounds by the pathogen (6). In this regard, mutations identified in the *P. jirovecii* dihydropteroate synthase and cytochrome bc1 genes, the targets of SMX and atovaquone, have already been associated with resistance in other infections (7–10).

Prior to the AIDS epidemic, PCP was an infrequent infection and attracted little attention from the scientific or clinical communities, subsequently there was a poor understanding of its basic biology, pathogenic mechanisms, and few treatment options. There were considerable impediments to standard experimental approaches for investigation of this unique pathogen, the most problematic being the lack of a long term culture system. This problem remains today, and many fundamental scientific questions about the basic biology, metabolism, and life cycle of *Pneumocystis* remain unanswered.

At present, *Pneumocystis* species have been found in a large variety of mammalian species, each of which has its own species of the fungus, i.e. species of *Pneumocystis* do not proliferate when transferred from the host in which they are found to a different host. When a host immune system is compromised, oftentimes the fungi grow to fill the alveolar lumens and effectively block oxygenation, leading to host death.

Recent progress in sequencing of the *Pneumocystis carinii* genome (11–14), a species infecting rats, allows a more informative and comprehensive search for genes and biological pathways within this pathogen that are known to be targets for existing antifungal agents. In this work, we review classes of antifungal drugs with respect to their potential applicability to the treatment of the PCP infection.

**Classes of antifungals and *Pneumocystis***

Such features as host specificity, lack of growth in culture, and extreme antigenic variation suggest that *Pneumocystis* species are dependent on their mammalian hosts, not highly pathogenic at least for individuals with intact immune systems, and have co-evolved with their hosts, perhaps in a commensal-like relationship (15). Studies in animals and humans have shown that *Pneumocystis* are sometimes present in the lungs of non-immunocompromised hosts, and it is currently thought that the non-compromised host is the natural environment of *Pneumocystis* spp. (15). Adaptation to the lung environment of a host with an intact immune system has been a key to its successful survival. Unfortunately, the metabolic strategies used by these fungi to grow and survive in this context are largely unknown.

In light of the above, it is important to investigate whether alternative strategies for PCP treatment can be found using known antifungal agents and their modes of action. Using BLAST (16), we searched for homology of *Pneumocystis* genomic sequences to the known targets of common fungicides. These putative orthologs were then mapped to respective biological pathways as defined in the KEGG Pathways database (17). Other members of these targeted pathways, found using sequence homology in the *Pneumocystis* genome, were also mapped and summarized in Table 1.
Azoles

Azoles are a class of fungicides that can be subdivided into imidazoles (clotrimazole, econazole, ketoconazole, and others), triazoles (fluconazole, itraconazole, voriconazole, and others), and thiazoles (abafungin). They are used for treatment or prevention of aspergillosis, cryptococcosis, histoplasmosis, candidiasis, and other fungal infections. Azoles target the cytochrome P450 enzyme, 14α-lanosterol demethylase (also known as Erg11 or CYP51). Specifically, they inhibit the transformation of lanosterol to 4,4′-dimethyl-cholesta-8,14,24-trienol, the first step of conversion to ergosterol or cholesterol (Figure 1, dark gray). Inhibition of ergosterol production impedes fungal cell growth and division.

Previous studies indicated that Pneumocystis does not synthesize ergosterol, a normally observed sterol in the vast majority of fungi. Instead, it utilizes cholesterol, a mammalian-associated sterol (18, 19). It has been hypothesized that some sterols are scavenged from the mammalian host by the fungus and then further modified by its own enzymes. In support of this hypothesis is the fact that Pneumocystis spp. are not susceptible to azole-based fungicides. However, there is a solid body of evidence suggesting that metabolic sterols are essential for Pneumocystis viability (18), and a significant portion of the sterol biosynthetic pathway is active during growth within the mammalian lung (13). Such evidence for an active sterol biosynthetic pathway includes: lovastatin-sensitive 3-hydroxy-3-methylgluatryl-coA reductase activity in P. carinii cytoplasmic preparations (20); incorporation of radiolabeled mevalonic acid and squalene into P. carinii-specific sterols (21, 22); and complementation of function within Saccharomyces cerevisiae mutants with P. carinii Erg11 (23) and more recently with the Erg7 gene, the deletion of which is lethal in yeast (24).

Therefore, sterol biosynthesis pathway represents a potential target for new anti-Pneumocystis therapeutics, especially given a number of Pneumocystis genes from this pathway that had been sequenced (Figure 1, Pneumocystis Genome Project). Of note, there have already been identified several inhibitors to specific enzymes in sterol biosynthesis that reduced the viability of treated Pneumocystis populations and correlated with presence of the expressed genes (25). Furthermore, resistance of Pneumocystis infection to standardazole-based drugs used clinically was recently hypothesized to be due to the amino acid sequence of P. carinii Erg11. It was shown that the sequence of the Pc 14α-lanosterol demethylase contains amino acid composition at substrate recognition sites (SRS) similar toazole-resistant Candida strains. Once SRS-1 was reversed to amino acids corresponding to an azole-sensitive strain, PcErg11 increased the susceptibility to azole-based drugs of the ScErg11 deletion S. cerevisiae strain (23).

Polyene antifungals

Polyene fungicides are based on a macrocyclic molecule with multiple conjugated double bonds on one side, and polyhydroxylated chain on the other side (Figure 2). The resulting chemical structure exerts amphipathic properties. Polyenes bind to hydrophobic sterols in the lipid environment and, at the same time, change cell membrane towards a more crystalline state forming pores. This distorts the homeostasis of the cell, when monovalent ions and small organic molecules begin leaking outside through the membrane, thus leading to cell death.

While the primary target of polyene antifungals is ergosterol, at therapeutic doses they may also bind to mammalian cholesterol causing considerable cytotoxicity and severe side effects in humans, such as nephrotoxicity. Binding affinity to cholesterol increases with the shortening of polyene’s hydrophobic part of a macrocycle (26).
Polyene antifungals have been used in the treatment of systemic fungal infections (such as candidiasis or aspergillosis) for over half a century. Those most commonly used are amphotericin B (27), natamycin (28), nystatin (29) (Figure 2). Because *Pneumocystis* does not appear to produce biochemically detectable amounts of ergosterol, the use of this class of fungicides offers little if any potential for the treatment of PCP.

### Allylamines

Allylamine-based fungicides include terbinafine (30), naftifine (31), butenafine (32), and others. They target squalene 2,3-epoxidase, an enzyme upstream of sterol biosynthesis pathway (Figure 3, dark gray). Towards elucidation of the mode of action, a recent *in silico* study suggests that terbinafine occupies part of the squalene epoxidase binding pocket and induces conformational change of the active site, thus inhibiting the binding of the natural substrate (33).

The binding of allylamines to squalene epoxidase shows different kinetics depending on the fungi probed, being non-competitive with respect to the substrate squalene in the pathogenic yeasts *Candida albicans* and *Candida parapsilosis*, but competitive in case of the rat liver epoxidase (34). This differential sensitivity to allylamines between pathogens and hosts might be exploited to develop a drug administration course with better efficacy. However, a recent *in vitro* study showed that IC\textsubscript{50} levels of terbinafine against the rat *P. carinii* were not achievable in serum to be practical for PCP treatment (35). In contrast, earlier studies showed modest reductions in *P. carinii* burdens in rats (36, 37), suggesting this compound may be used in other settings, such as in two-compound therapies.

### Echinocandins

Echinocandins (anidulafungin, caspofungin, and micafungin) are currently used for the treatment and prophylaxis of aspergillosis (38) and candidiasis (39). One more compound from this class of fungicides, aminocandin, was found to be active against *Candida* and *Aspergillus* spp. (40) and is in clinical trials (41). Their structure is a cyclic polypeptide with an additional extended side chain moiety (Figure 4). Echinocandins inhibit 1,3-β-glucan synthase (FKS1) that produces (1,3)-β-D-glucan, an important component of the fungal cell wall.

These fungicides demonstrate significant advantages over other antifungal agents. Since they target an enzyme from a pathway distinct from other antifungal therapies, there is no potential risk for cross-resistance developed after treatment by other fungicides. Mammals do not synthesize glucan and thus few toxic side effects are observed with even high doses of these compounds. Long term use over a large set of patients displayed excellent safety and tolerability. Moreover, since echinocandins have low metabolic rate by the cytochrome P450s, they can be safely co-administered with other drugs (42). However, there are some emerging trends that are of concern with the use of this class of antifungals. Mutations in the “hot spot” regions of FKS1 targeted by the echinocandins, unfortunately, can confer resistance to all clinically available echinocandins (43).

An ortholog to fungal 1,3-β-glucan synthase (GSC1) was identified in the *Pneumocystis* genome and subsequently sequenced. Furthermore, PcGSC1 mRNA was shown to have minimal expression in trophic forms, but to be highly expressed in cystic forms of the organism (44). Consistently, treatment of rodent models of PCP by echinocandins showed depletion of cysts, with the trophic forms of the pathogen remained largely unaffected (45). These findings underscore the distinct life cycle of *Pneumocystis* which alternates between an asexual cycle resulting in trophic forms that do not contain glucan and the cycle leading to formation of 8-spored asci, which contains abundant glucan. *In vitro* assays on...
Pneumocystis cultures, in suspension and biofilms, showed different susceptibility to the three compounds, with anidulafungin being the most active against Pneumocystis in suspension culture and against biofilm formation, which was then lost when treating mature, formed biofilms (46). Observed variation of activity against biofilms and suspension cultures is in line with other results observed for different fungi treated by echinocandins (40, 47).

Alternatives

Trimethoprim-sulfamethoxazole is a two component antibacterial agent that targets folic acid biosynthesis (Figure 5). Folate (vitamin B<sub>9</sub>) is essential for many functions in the organism, including DNA synthesis, repair, and modification. Sulfamethoxazole inhibits dihydropteroate synthase (DHPS, Figure 5, dark gray, EC 2.5.1.15) that produces dihydropteroate, an important intermediate in the folate biosynthesis. Dihydropteroate synthase is not expressed in humans, what makes it a convenient target for sulfonamide antibiotics. In Pneumocystis, it is found as part of the folic acid synthesis protein fol1 (FOL1), which also comprises of two other domains: dihydroneopterin aldolase (DHNA, Figure 5, light gray, EC 4.1.2.25) and 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine pyrophosphokinase (PPPK, Figure 5, light gray, EC 2.7.6.3) (48).

Trimethoprim targets dihydrofolate reductase (DHFR, Figure 5, dark gray, EC 1.5.1.3), an enzyme that reduces dihydrofolic acid to tetrahydrofolate, which is a coenzyme in many reactions, including metabolism of amino acids and nucleic acids.

TMP-SMX is the most common anti-PCP medication, used both for treatment (49, 50) and prophylaxis (51, 52). However, there is growing genetic evidence that the pathogen is evolving mutations in the target gene that could render it resistant to the SMX component (53–57). Recent report provides structural insights in the mechanism of catalytic activity by DHPS, the mode of inhibitory action of SMX, and the resistance development (58). Moreover, using recombinant PcDHFR, it was shown that trimethoprim is a very poor inhibitor of Pneumocystis DHFR and more potent for human DHFR (59). The latter may support the concern on adverse effects of the TMP-SMX treatment reported for different populations of patients (60–63). In addition, HIV-AIDS patients have problems with tolerating the sulfonamide component of the medication (64–66).

Atovaquone and pentamidine are considered second line treatments for PCP (67–69). However, their efficacy is low (70), and treatments frequently cause adverse reactions, including nephrotoxicity, neutropenia, hypotension, hypoglycemia, and other side effects (71). Atovaquone inhibits mitochondrial cytochrome bc<sub>1</sub> complex in parasites at much lower concentrations than the respective mammalian complex (10). However, evolving resistance to atovaquone corresponding to mutations in the Pneumocystis cytochrome b has been observed (9). Pentamidine has a broad antimicrobial action with no specific target known.

Alternative treatments for PCP may emerge from the repurposing of approved drugs used for other diseases but targeting the same pathways as known antifungal agents, or other pathways which are also essential in Pneumocystis life cycle and metabolism. In this regard, Therapeutic Targets Database (TTD) is a valuable resource that provides access to therapeutic targets (proteins and nucleic acids) along with the pathway and disease information, and corresponding drugs (72). For example, the sterol biosynthesis pathway represents a viable target in the search for alternative treatments of PCP. As indicated above, metabolic sterols are essential for Pneumocystis viability (18), and a significant portion of the pathway is active during growth within the mammalian lung (13), refer to the Azoles section for more details. Table 2 summarizes known drugs that target the sterol (and its...
precursors) biosynthesis pathways but have not been used for the treatment of fungal infections.

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (TTDS00195) is the rate-limiting enzyme in sterol biosynthesis that catalyses the synthesis of mevalonic acid, a precursor of cholesterol (and ergosterol) as well as non-sterol isoprenoids. Previous studies have shown that *P. carinii* exhibited the lovastatin-sensitive HMG-CoA reductase activity (20). Furthermore, incorporation of radiolabeled mevalonic acid and squalene into *P. carinii*-specific sterols (21, 22) indicated that farnesyl diphosphate synthetase (TTDS00372) and diphosphomevalonate decarboxylase (TTDR00413) may be potential targets in the search of new therapeutics for PCP treatment. Lanosterol synthase (TTDR00866) represents another prospective target. Lanosterol synthase (Erg7) is an essential enzyme responsible for the conversion of (S)-2,3-oxidosqualene, the last acyclic sterol precursor, into lanosterol, the first sterol intermediate of the mammalian and fungal sterol biosynthetic pathways. The inhibition of the enzyme has been shown to have less adverse effects in hamsters, squirrel monkeys and minipigs, as well as in the human liver cell line HepG2, compared to statins, presumably because Erg7 inhibitors act downstream of sterol biosynthesis pathway and do not affect the synthesis of non-sterol isoprenoids and coenzyme Q production (73). Previous studies showed that *Pneumocystis* Erg7 gene is functional and can complement a yeast deletion strain (ΔErg7 S. cerevisiae mutant) (24). Known Erg7 inhibitors were able to reduce *P. carinii* viability in an *in vitro* assay (25).

New lead compounds and drug candidates for anti-PCP treatment may also emerge from the Seattle Structural Genomics Center for Infectious Disease (SSGCID) consortium that aims to facilitate access to potential drug targets by solving new protein structures and making them publicly available (74). For example, when SSGCID consortium resolves the 3D structure of *Pneumocystis* 14α-lanosterol demethylase, it will be possible to refine existingazole-based fungicides to target specifically PCP infection. At the time of writing this review, the enzyme was in the process by SSGCID with the status “Expressed”.

**Conclusion**

*Pneumocystis* species are unusual fungi exhibiting a number of unique features making them distinct from other fungal species. A commensal-like life style and dependence on the host environment appears to result in the lack of ergosterol (a fungal sterol) production, while having the overall pathway for sterol biosynthesis functional and active. At the same time, these fungi utilize cholesterol, a sterol associated with mammals. Lanosterol 14-alpha-demethylase (ERG11), the primary target in the pathway for the most commonly used antifungal agents (azoles), in *Pneumocystis* possesses an intrinsic resistance to drugs due to amino acid mutations impeding the binding of azole-based inhibitors. Therefore, currently available azoles cannot be used for PCP treatment.

Furthermore, *Pneumocystis* shows unique, life cycle-dependent regulation of another drug target, 1,3-β-glucan synthase (GSC1). The enzyme is actively produced by the cystic forms of the pathogen only, but not in trophic forms. This limits the applicability of echinocandins to *Pneumocystis*, controlling the cysts formation only, but not eradicating the PCP infection.

Other therapies for *Pneumocystis* pneumonia exert decreased efficacy with serious side effects and low tolerability by certain groups of patients. Moreover, there is growing evidence of emerging resistance to these antimicrobial agents due to acquisition of mutations in the drug targets that subsequently abolish the inhibitor’s action.
Given the recent expansion of PCP infection to new groups of patients with underlying chronic diseases and debilitated immune system, there is an urgent need in developing new therapeutic treatments against PCP infection. The problem may be approached by utilizing the progress in the Pneumocystis genome sequencing as part of Pneumocystis Genome Project (http://pgp.cchmc.org/). For example, new targets in Pneumocystis may be found among sequenced orthologs to the known drug targets exploited for treating other diseases. The sequenced genome provides insights into metabolic pathways of the fungus and enhances the identification of vital proteins as new potential targets. Moreover, sequenced Pneumocystis genes may facilitate efforts for resolving the protein structures that could be used in rational drug design.

Acknowledgments

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List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>IC50</td>
<td>50% inhibitory concentration</td>
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<tr>
<td>EC</td>
<td>Enzyme commission number</td>
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References


Curr Drug Targets. Author manuscript; available in PMC 2014 January 30.


_Curr Drug Targets_. Author manuscript; available in PMC 2014 January 30.


Figure 1.
Steroid biosynthesis pathway as provided by KEGG Pathways. Highlighted enzymes were found in the *P. carinii* genome. Dark gray is Erg11 (EC 1.14.13.70), light gray are other genes sequenced in the genome: Erg2, Erg4, Erg6, Erg7 (EC 5.4.99.7), Erg24 (EC 1.3.1.70), Erg25 (EC 1.14.13.72), Erg26 (EC 1.1.1.170), and Erg27 (EC 1.1.1.270). The original image was altered for clarity by truncating a branch to the phytosterol biosynthesis as being irrelevant.
Figure 2.
Representative structures of polyene antifungal agents: (A) amphotericin B; (B) natamycin; and (C) nystatin. Structures are based on the ChemSpider database entries with the following IDs: 10237579, 21242908, and 23078586, respectively.
Figure 3.
Sesquiterpenoid and triterpenoid biosynthesis pathway as provided by KEGG Pathways. Highlighted enzymes were found in the *P. carinii* genome. Dark gray is squalene monooxygenase (ERG1, EC 1.14.13.132), light gray is farnesyl-diphosphate farnesyltransferase (FDFT1, EC 2.5.1.21).
Figure 4.
Structures of echinocandins: (A) anidulafungin; (B) aminocandin; (C) caspofungin; and (D) micafungin. Structures are based on the ChemSpider database entries with the following IDs: 145752, N/A, 411774, and 419105, respectively.
Figure 5.
Folate biosynthesis pathway as provided by KEGG Pathways. Highlighted enzymes were found in the *P. carinii* genome. Dark gray are the targets of TMP-SMX (FOL1, EC 2.5.1.15 and DHFR, EC 1.5.1.3), light gray are folE (EC 3.5.4.16), FAS2 (EC 6.3.2.12), MET7 (EC 6.3.2.17), ABZ1 (EC 2.6.1.85), and MOCS1.
Figure 6.
Structures of atovaquone (A) and pentamidine (B). Structures are based on the ChemSpider database entries with the following IDs: 10482034 and 4573, respectively.
Table 1

*Pneumocystis carinii* genomic sequences (assembled contigs) homologous to genes in biological pathways targeted by antifungal agents. Sequences are available via *Pneumocystis* Genome Project (PGP, http://pgp.cchmc.org/).

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<th>PGP sequence ID</th>
<th>GenBank ID Gene/Protein</th>
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### Table 2

Other targets (clinical or research) in the sterol biosynthesis pathway. Data derived from the Therapeutic Targets Database (TTD).

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<td>Atherosclerosis, Coronary heart disease, Myocardial infarction, Cervical cancer, Dyslipidemia, Head and neck squamous cell carcinomas, Hypercholesterolemia, Hypertriglyceridemia</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A reductase Terpenoid backbone biosynthesis as precursor of sterol biosynthesis pathway</td>
<td>JA2010_JC_01349_length_2782</td>
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<td>Chagas' disease, Hypercholesterolemia, Leishmania infections, Myeloma disease, Skeletal disorders, Toxoplasma infections, Trypanosomatid infections</td>
<td>Farnesyl diphosphate synthetase Sesquiterpenoid and triterpenoid biosynthesis</td>
<td>JA2010_NC_01555_length_2213, JA2010_OC_01061_length_549, JA2010_OC_01799_length_1304, JA2010_JC_01428_length_8440, JA2010_NC_00506_length_2941</td>
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<td>Hypercholesterolemia</td>
<td>Diphosphomevalonate decarboxylase Terpenoid backbone biosynthesis as precursor of sterol biosynthesis pathway</td>
<td>JA2010_JC_01157_length_4198, JA2010_NC_03517_length_1921</td>
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<td>Lanosterol synthase Sterol biosynthesis pathway</td>
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