Summary:
Advances in sequencing the Pneumocystis carinii genome have helped identify potential metabolic pathways operative in the organism. Also, data from characterizing the biochemical and physiological nature of these organisms allow elucidation of metabolic pathways as well as pose new challenges and questions that require additional experiments. These experiments are being performed despite the difficulty in doing experiments directly on this pathogen that has yet to be subcultured indefinitely and produce mass numbers of cells in vitro. This article reviews biochemical approaches that have provided insights into several Pneumocystis metabolic pathways. It focuses on 1) Sadenosyl-L-methionine (AdoMet; SAM), which is a ubiquitous participant in numerous cellular reactions; 2) sterols: focusing on oxidosqualene cyclase that forms lanosterol in P. carinii; SAM:sterol C-24 methyltransferase that adds methyl groups at the C-24 position of the sterol side chain; and sterol 14a-demethylase that removes a methyl group at the C-14 position of the sterol nucleus; and 3) synthesis of ubiquinone homologs, which play a pivotal role in mitochondrial inner membrane and other cellular membrane electron transport.

KEY WORDS: Sadenosyl-L-methionine, atovaquone, buparvaquone, chloroquine, erg6, erg7, erg11, sam1, genes, lanosterol synthase, lung, microsomes, mitochondria, nicotine, hydroxynonenzoate, pneumocystosterol, shikimate, sterol 14a-demethylase, sterol C-24-methyltransferase, stigmatellin, triazoles, ubiquinones (Coenzyme Q, CoQ).

Data on the biochemical nature and metabolic pathways of Pneumocystis comes by labor-intensive and costly research by dedicated investigators. Today, there are valuable data on several aspects of Pneumocystis metabolism and the biosynthesis of compounds such as the antigenic major surface glycoprotein. However, this review will be restricted to three pathways that are interconnected: 1) S-adenosylmethionine (SAM, AdoMet); 2) sterols; and 3) ubiquinones.

S-ADENOSYLMETHIONINE (SAM, ADOMET)

S-adenosylmethionine is essential as it participates in numerous reactions in the cell, which number only next to those involving ATP. SAM is synthesized by the methionine:ATP transferase (MAT; SAM synthetase) reaction then it serves as a methyl donor to various substrates that are modified by specific methyltransferase enzymes. A MAT gene (Sam1) was detected in Pneumocystis carinii and when expressed in Escherichia coli enzymatic activity was demonstrated (Kutty et al., 2008). On the other hand, MAT activity was not detected in P. carinii organisms isolated from rat lungs and radiolabeled SAM was efficiently taken up from an axenic culture medium by the organism. Additional studies led to the hypothesis that P. carinii scavenges SAM...
S-Adenosylmethionine (SAM, AdoMet)

R-(+)-Nicotine Effects:

- SAM in lungs: The R-(+)-isomer of nicotine upregulates lung spermine/spermidine acetyltransferase (SSAT) and increases utilization of SAM toward polyamine metabolism in vivo in the lung →

- Pneumocystis proliferation ↓

- Subsequent administration in vivo of SAM →

- P. carinii proliferation ↑

Fig. 1. – Proposed utilization and metabolism of S-adenosylmethionine (SAM, AdoMet) in the P. carinii-infected rat lung according to Merali & Clarkson.

from the lung and organism proliferation is enhanced and reduces blood SAM (Fig. 1) (Merali & Clarkson, 2004). Also, these workers tested the effects on infected rats of the R(+) isomer of nicotine (which is not the predominant isomer in tobacco) and reported that it up-regulated spermine:spermidine acetyltransferase (SSAT) and hence increased the flux of SAM to polyamine metabolism and reduced available SAM for P. carinii proliferation. Administration of SAM to the infected rat reversed the inhibitory effects of nicotine on P. carinii proliferation (Fig. 1). As it was also reported that sera from untreated Pneumocystis pneumonia (PcP) patients have reduced levels of blood SAM, this measurement can be a useful rapid method augmenting other diagnostic procedures for PcP (Moncada et al., 2008). It remains unclear whether the P. carinii strains used by different laboratories differ in expression of SSAT, or if expression of the Sam1 gene in P. carinii organisms in vivo is blocked or inhibited. More studies should be performed and verified in different laboratories to clarify current information available and resolving possible conflicting data on P. carinii SAM biosynthesis and metabolism.

STEROLS

As a methyl donor SAM donates methyl groups to the C-24 position of the sterol side chain of Pneumocystis sterols giving rise to 24-alkylergosterols. Thus, SAM:sterol methyltransferase (SAM:SMT) is an attractive drug target since drugs targeting this enzyme might be developed with little side effects on the mammalian host. In general, mammals synthesize cholesterol, higher fungi synthesize ergosterol, a C28 24-alkylergosterol and plants can synthesize C28 and C29 24-alkylergosterols. These sterols have, for example, a Δ5 double bond in the sterol nucleus but there is no evidence that Pneumocystis can form a Δ5 double bond (Kaneshiro et al., 1994; Urbina et al., 1997; Giner et al., 2002). Although Pneumocystis is a fungus it does not contain ergosterol but synthesizes unique 24-alkylergosterols with a single double bond in the sterol nucleus, which is at the Δ7 position (Kaneshiro et al., 1994; Urbina et al., 1997; Giner et al., 2002). As is common in other animal parasites, there is abundant cholesterol in Pneumocystis, which is synthesized by the host and scavenged by the pathogen (Worsham et al., 2003). Plant sterols originating from vegetable material in laboratory rat chow are taken up by the rat and scavenged by P. carinii (Giner et al., 2002; Zhou et al., 2002). Furthermore, compounds such as desmosterol, the direct precursor of cholesterol in mammals, can be taken up by Pneumocystis and modified using its own enzymes such as SAM:SMT producing Δ5 24-alkylergosterols (Giner et al., 2002; Worsham et al., 2003).

RECOMBINANT PNEUMOCYSTIS STEROL BIOSYNTHESIS ENZYMES

Due to the current labor-intensive, costly means of obtaining sufficient P. carinii organisms for performing metabolism studies and characterization of potential unique properties of its enzymes, several groups have employed recombinant protein technologies. Three such recombinant enzymes are the P. carinii oxidosqualene cyclase (lanosterol synthase) coded by the erg7 gene; SAM:sterol C-24 methyltransferase (SAM:SMT) coded by the erg6 gene; and sterol 14α-demethylase (14DM) coded by the erg11 gene.

- Oxidosqualene cyclase
  Studies of the recombinant P. carinii oxidosqualene cyclase expressed in Saccharomyces cerevisiae erg7 deletion mutant showed that cyclization of squalene results in lanosterol and not cycloartenol (as occurs in some plants) (Milla et al., 2002; Hinson et al., 2003; Oliaro-Bosso et al., 2004; Joffrion et al., 2010). The transformed yeast cells have also been used to test a variety of compounds targeting this enzyme (Hinson et al., 2003; Oliaro-Bosso et al., 2004).

- SAM.SMT
  Recombinant P. carinii SAM:SMT has been expressed in Escherichia coli (Kaneshiro et al., 2002) and Tetrahymena thermophila (Johnston L., Cassidy-Hanley D., Clark T. & Kaneshiro E.S. unpublished). These
organisms are useful as expression systems since they do not synthesize sterols and would not have their own endogenous sterol biosynthesis enzymes allowing for performing enzymatic assays on transformed cell homogenates. The SAM:SMT measured in whole cell homogenates of transformed *E. coli* expressing the *P. carinii* enzyme has some unusual properties. Its substrate preference is for lanosterol (K_m, 11 mM) and 24-methylenelanosterol (K_m, 19 mM) over zymosterol (K_m, 41 mM). Zymosterol is the preferred substrate for 24-alkylation in most fungi producing ergosterol (Kaneshiro et al. 2002). The enzyme transfers both the first and the second methyl group to the C-24 position of the sterol side chain, unlike the situation in some plants that have separate enzymes for each of the methyltransferase reactions. Purification of the *P. carinii* recombinant SAM:SMT protein and characterization of its enzyme activity is required to help design more effective and specific drugs against this unique SAM:SMT.

- **14DM**

Sterol 14α-demethylase removes a methyl group from the C-14 position of the sterol nucleus. The *P. carinii* 14DM expressed in *S. cerevisiae erg11* null mutant has been produced in two independent laboratories (Morales *et al.*, 2003; Nkinin *et al.*, 2006). The cDNA from *P. carinii* characterized in the two laboratories differed by a single nucleotide and predicted a lysine residue in one (Pc14DMMa) and a glutamic acid in the other (Pc14DMUC). However, this occurs at a site that does not include any sterol binding motifs. Both of these two *P. carinii* 14DM complemented sterol biosynthesis in the yeast *erg11* deletion mutant. This was demonstrated by the identification of the same sterols in wild type yeast, *S. cerevisiae erg11* deletion mutant expressing either *P. carinii* 14DM, and the mutant yeast control transformed with the *S. cerevisiae erg11* gene (Nkinin S.W., Stringer J.R., Keely S.P., Setchell K.D.R., Giner J.L. & Kaneshiro E.S. unpublished). In these studies, definitive structural and quantitative analyses of the sterols present also indicated that 4α-desmethyl lanosterol (only one methyl group at C-4 position of the sterol nucleus) and 24-methylergosta-8,24(28)-dien-3β-ol (only one methyl group at C-4) were substrates preferred over lanosterol (with two methyl groups at C-4). These data indicate that 14DM prefers substrates with only one methyl group at C-4 and that the presence of two methyl group at C-4 hinders access of the 14DM enzyme to the C-14 methyl group. Hence, the enzyme is more accurately referred to as sterol 14α-demethylase rather than lanosterol 14α-demethylase.
Triazoles that are most commonly used to clear fungal infections are ineffective in clearing PcP. Isolated \textit{P. carinii} were shown to be insensitive to the azoles, fluconazole and two triazole derivatives as determined by a viability assay based on the ATP content of cells (Kaneshiro et al., 2000). Also, yeast cells expressing \textit{P. carinii} 14DM\textsuperscript{NAA} were tested for the effects of fluconazole, voriconazole and itraconazole on culture proliferation. Growth of yeast expressing the \textit{S. cerevisiae} 14DM were inhibited by these triazoles. In contrast, growth of the yeast null mutant expressing the \textit{P. carinii} 14DM\textsuperscript{NAA} was less sensitive to fluconazole and voriconazole. The possibility that the \textit{P. carinii} erg11 gene is characterized by relatively low proportions of C\textsubscript{31} and C\textsubscript{32} sterols, and another with high C\textsubscript{31} and C\textsubscript{32} sterols, was shown (Kaneshiro et al., 1999a; Giner et al., 2004). There appears to be two distinct \textit{P. jirovecii} populations (strains) that differ in their sterol profiles. One branch leads to squalene and to sterols and another branch contributes polypropyl chains for formation of various ubiquinone (Coenzyme Q, CoQ) homologs (Fig. 2). CoQ homologs are designated by the number of isoprene units present; (e.g., CoQ\textsubscript{10} has 10 isoprene units with 50 carbons). The ring moiety of CoQ originates from a different pathway, the shikimic acid pathway, which is absent in mammals. The product of the shikimate pathway is chorismate, which gives rise to a number of other pathways leading to products such as folic acid, aromatic amino acids, prenylated proteins and \textit{p}-hydroxybenzoic acid (PHBA), the direct precursor of the CoQ ring structure. That \textit{P. carinii} synthesizes de novo ubiquinones was indicated by the presence of the pentafucntional \textit{aroM} gene (Banerjee et al. 1993) and the reduction of viability and short-term culture growth by glyphosate, which inhibits 5-enolpyruvlshikimate-3-phosphate synthase with exquisite specificity (Chin et al., 1999; Kaneshiro et al., 2006). CoQ is assembled by PHBA-polyprenyltransferase activity. It is not clear whether \textit{Pneumocystis} has different enzymes with specificity for substrates of different polypropyl chain lengths or a single enzyme with broad substrate specificity. However, it is known that \textit{P. carinii} synthesizes CoQ\textsubscript{7}, CoQ\textsubscript{8}, CoQ\textsubscript{9}, and CoQ\textsubscript{10} but not CoQ\textsubscript{6} (Ellis et al., 1996; Sul & Kaneshiro, 2001). The longer homologs are found in the highest proportions. There has been controversy on whether or not CoQ synthesis occurs only in the mitochondria or in both the mitochondrion and the endoplasmic reticulum-Golgi system. In some cell types, it appears that synthesis de novo occurs in the mitochondria and modifications occur outside the mitochondria after exportation from this organelle (Fernández-Ayala et al., 2005). However, there is growing evidence that CoQ synthesis may occur in both cellular compartments in some organisms (Swiezeswka et al., 1993; Jun et al., 2004). In the first subcellular fractionation of cytoplasmic organelles accomplished for \textit{Pneumocystis}, synthesis of CoQ was found to take place in both the mitochondrial and the microsomal fractions (Basselin et al., 2005). Furthermore, the incorporation of radiolabeled PHBA into CoQ in the microsomal fraction was inhibited by the CoQ analogs atovaquone and buparvaquone (Kaneshiro, 2004; Basselin et al., 2005; Kaneshiro et al., 2006). In contrast, CoQ synthesis in the mitochondrial fraction regulators event that block the production of certain enzymes.

**UBIQUINONES**

The multi-branched isoprenoid pathway produces polypropyl diphosphate chains that are made up of five-carbon monounsaturated isoprene units. One branch leads to squalene and to sterols and another branch contributes polypropyl chains for formation of various ubiquinone (Coenzyme Q, CoQ) homologs (Fig. 2). CoQ homologs are designated by the number of isoprene units present; (e.g., CoQ\textsubscript{10} has 10 isoprene units with 50 carbons). The ring moiety of CoQ originates from a different pathway, the shikimic acid pathway, which is absent in mammals. The product of the shikimate pathway is chorismate, which gives rise to a number of other pathways leading to products such as folic acid, aromatic amino acids, prenylated proteins and \textit{p}-hydroxybenzoic acid (PHBA), the direct precursor of the CoQ ring structure. That \textit{P. carinii} synthesizes de novo ubiquinones was indicated by the presence of the pentafucntional \textit{aroM} gene (Banerjee et al. 1993) and the reduction of viability and short-term culture growth by glyphosate, which inhibits 5-enolpyruvlshikimate-3-phosphate synthase with exquisite specificity (Chin et al., 1999; Kaneshiro et al., 2006). CoQ is assembled by PHBA-polyprenyltransferase activity. It is not clear whether \textit{Pneumocystis} has different enzymes with specificity for substrates of different polypropyl chain lengths or a single enzyme with broad substrate specificity. However, it is known that \textit{P. carinii} synthesizes CoQ\textsubscript{7}, CoQ\textsubscript{8}, CoQ\textsubscript{9}, and CoQ\textsubscript{10} but not CoQ\textsubscript{6} (Ellis et al., 1996; Sul & Kaneshiro, 2001). The longer homologs are found in the highest proportions. There has been controversy on whether or not CoQ synthesis occurs only in the mitochondria or in both the mitochondrion and the endoplasmic reticulum-Golgi system. In some cell types, it appears that synthesis de novo occurs in the mitochondria and modifications occur outside the mitochondria after exportation from this organelle (Fernández-Ayala et al., 2005). However, there is growing evidence that CoQ synthesis may occur in both cellular compartments in some organisms (Swiezeswka et al., 1993; Jun et al., 2004). In the first subcellular fractionation of cytoplasmic organelles accomplished for \textit{Pneumocystis}, synthesis of CoQ was found to take place in both the mitochondrial and the microsomal fractions (Basselin et al., 2005). Furthermore, the incorporation of radiolabeled PHBA into CoQ in the microsomal fraction was inhibited by the CoQ analogs atovaquone and buparvaquone (Kaneshiro, 2004; Basselin et al., 2005; Kaneshiro et al., 2006). In contrast, CoQ synthesis in the mitochondrial fraction
was not affected (Fig. 3). The CoQ analog stigmatellin A had no effect on CoQ synthesis as shown by assays using whole *P. carinii* homogenates (Kaneshiro *et al.*, 2001).

To examine regulation of CoQ biosynthesis, experiments on *P. carinii* whole cell homogenates examining radiolabeled PHBA incorporation into CoQ homologs were measured in the presence of excess specific nonradioactive CoQ homologs. When excess CoQ₆ was present, incorporation of PHBA into all four homologs was the same as controls (Table I). When excess nonradioactive CoQ₅, was included, incorporation of PHBA into all four homologs was inhibited. Interestingly, in the presence of excess cold CoQ₈, incorporation of PHBA into CoQ₇ was not reduced but incorporation into CoQ₆, CoQ₅, and CoQ₁₀ was inhibited. Also in the presence of excess nonradioactive CoQ₉, incorporation of radioactive PHBA into CoQ₇ and CoQ₈ was not inhibited, but incorporation into CoQ₅ and CoQ₁₀ was reduced (Kaneshiro *et al.*, 2003). These results indicate control mechanisms for synthesis of CoQ homologs are operative in *P. carinii*. However, the specificity of CoQ homolog synthesis needs additional experiments to determine why excess amounts of a specific homolog does not affect the synthesis of shorter homologs whereas the synthesis of longer homologs is inhibited. Also, regulation of CoQ homolog synthesis need to be evaluated in isolated mitochondrial and microsomal subcellular fractions to better understand whether these control mechanisms occur in one or both cellular compartments.

Coenzyme Q plays a central role in electron transport at the inner mitochondria membrane and the cell surface membrane (Sun *et al.*, 1992; Santos-Ocana *et al.*, 1998; Turunen *et al.*, 2004; VanderHeyden *et al.*, 2000). Distinct from studies on CoQ biosynthesis, studies on drugs that target electron transport by analysis of respiration or *P. carinii* viability have been reported. Ubiquinone analogs are used against parasitic organisms (Gutteridge, 1991b). Atovaquone (566C80) is clinically used to treat mild to moderate PCP and the mechanism of action is believed to involve inhibition of electron transport in the mitochondria; *P. carinii* cellular respiration is sensitive to this CoQ analog (Gutteridge, 1991a). These data also indicate that atovaquone enters the mitochondria where it acts on cellular respiration (but not mitochondrial CoQ synthesis, see above). Atovaquone and buparvaquone inhibited CoQ synthesis in the microsomal fraction (Kaneshiro *et al.*, 2001; Kaneshiro, 2004; Basselin *et al.*, 2005) and these analogs were also effective in reducing viability of isolated *P. carinii* organisms (Cushion *et al.*, 2000, Kaneshiro *et al.*, 2006). The CoQ analog stigmatellin A, which did not affect *P. carinii* CoQ synthesis (see above) had only moderate effects on viability and cell proliferation in *vitro* (Kaneshiro *et al.*, 2006).

**FUTURE OF PNEUMOCYSTIS BIOCHEMISTRY AND METABOLISM RESEARCH**

Although *Pneumocystis* cannot be cultured thereby hindering direct experimentation on these organisms and precluding genetic manipulations, advances in understanding the metabolic pathways
operative in these organisms have been significant. This has happened despite much difficulty and with only a handful of investigators worldwide who do these kinds of experiments (compared to the large number of laboratories working on some other parasites such as *Plasmodium*). Also, it is true that PcP does not kill as many people worldwide compared to some other infectious diseases and there are drugs available that effectively clear the infection. With the advent of antiretroviral therapy and prophylaxis against PcP the incidence of this infection among HIV/AIDS patients has dramatically dropped. However, compared to other opportunistic infections PcP remains among the highest immediate cause of death of AIDS patients.

Continued research in understanding *Pneumocystis* biochemistry and metabolism is imperative. PcP is the paradigm of opportunistic infections of immunodeficient patients. Many of these patients are chronically immunodeficient and often become reinfected. *Pneumocystis* can cause transient infections (colonizations) in immunocompetent hosts and turns pathogenic depending on the immune status of the host. Compared to other microbial pathogens, the various *Pneumocystis* species have about the longest history on earth of co-evolution with their mammalian hosts going back for over 100 million years (Aliouat-Denis et al., 2008). It is therefore an important model system for preparing for the changing demographics of PcP and the increasing population groups being treated by drugs that diminish the strength of the body’s defenses. The number of patients undergoing therapy for cancer, solid organ transplantation, Crohn’s and other diseases are increasing and the course of the disease and severity of PcP in these immunodeficient people differs from PcP in AIDS patients (Kaneshiro & Dei-Cas, 2009). Furthermore, emerging and reemerging pathogenic eukaryotic protists are not restricted to the tropics or less industrialized countries, but are just as prevalent and endemic in the USA and Europe. Better understanding of the biochemical nature of *Pneumocystis* might provide insights into being better equipped to face the challenges of emerging opportunistic pathogenic protists.

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