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BIOCHEMICAL RESEARCH ELUCIDATING METABOLIC PATHWAYS IN *PNEUMOCYSTIS*[1]

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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 now 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) *S*-adenosyl-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 14α-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: *S*-adenosyl-L-methionine, atovaquone, buparvaquone, chorismate, *erg6*, *erg7*, *erg11*, *sam1*, genes, lanosterol synthase, lung, microsomes, mitochondria, nicotine, p-hydroxybenzoate, pneumocysterol, shikimate, sterol 14α-demethylase, sterol C-24-methyltransferase, stigmatellin, triazoles, ubiquinones (Coenzyme Q, CoQ).

Résumé : RECHERCHES BIOCHIMIQUES ÉLUCIDANT DIVERSES VOIES MÉTABOLIQUES CHEZ *PNEUMOCYSTIS*

Les avancées dans le séquençage du génome de Pneumocystis carinii *ont permis d'identifier des voies métaboliques potentiellement opérationnelles chez ce microorganisme. Ainsi, les données apportées sur sa nature biochimique et physiologique permettent aujourd'hui d'élucider des voies métaboliques et ouvrent sur de nouveaux défis et questions qui appellent de nouvelles expériences. Ces expériences sont réalisées malgré la difficulté de les faire directement sur ce pathogène qui doit être cultivé indéfiniment pour produire un nombre important de cellules* in vitro. *Cet article passe en revue des approches biochimiques qui ont éclairé plusieurs pistes métaboliques chez le* Pneumocystis. *Il se focalise sur : 1) la S-adénosyl-L-méthionine (AdoMet ; SAM), qui est un participant omniprésent dans les nombreuses réactions cellulaires ; 2) les stérols : l'oxidosqualène cyclase qui forme le lanostérol chez* P. carinii *; la SAM:stérol C-24 méthyltransférase qui ajoute des groupes méthyle à la position C-24 de la chaîne latérale du stérol ; la stérol 14α-déméthylase qui enlève un groupe méthyle à la position C-14 du noyau stérol ; et 3) la synthèse d'ubiquinones homologues qui ont un rôle majeur au niveau de la membrane mitochondriale interne et d'autres transports électroniques cellulaires de membrane.*

MOTS CLÉS : *S-adénosyl-L-méthionine, atovaquone, buparvaquone, chorismate,* erg6, erg7, erg11, sam1, *gènes, lanostérol synthase, poumon, microsomes, mitochondrie, nicotine, p-hydroxybenzoate, pneumocystérol, shikimate, stérol 14α-déméthylase, stérol C-24-méthyltransférase, 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.

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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

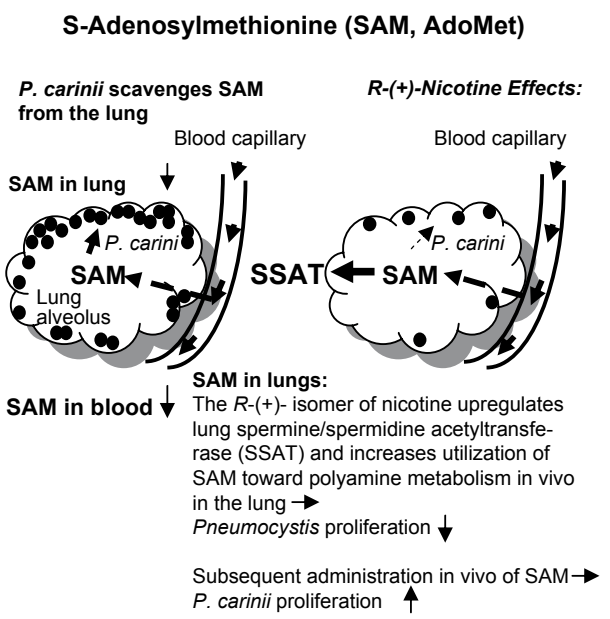

Fig. 1. – Proposed utilization and metabolism of *S*-adenosylmethionine (SAM, AdoMet) in the *P. carinii*-infected rat lung according to Merali & Clarkson.

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 MAT, 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-alkylsterols (Fig. 2). Other protists, fungi and plants synthesize these sterols, but mammals do not have enzymes that catalyze these reactions and hence lack 24-alkylsterols. 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 C_{28} 24-alkylsterol and plants can synthesize C_{28} and C_{29} 24-alkylsterols. These sterols have, for example, a Δ^5 double bond in the sterol nucleus but there is no evidence that *Pneumocystis* can form a Δ^5 double bond (Giner *et al.*, 2002). Although *Pneumocystis* is a fungus it does not contain ergosterol but synthesizes unique 24-alkylsterols with a single double bond in the sterol nucleus, which is at the Δ^7 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 Δ^5 24-alkysterols (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; Hinshaw *et al.*, 2003; Oliaro-Bosso *et al.*, 2004; Joffrion *at al.*, 2010). The transformed yeast cells have also being used to test a variety of compounds targeting this enzyme (Hinshaw *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

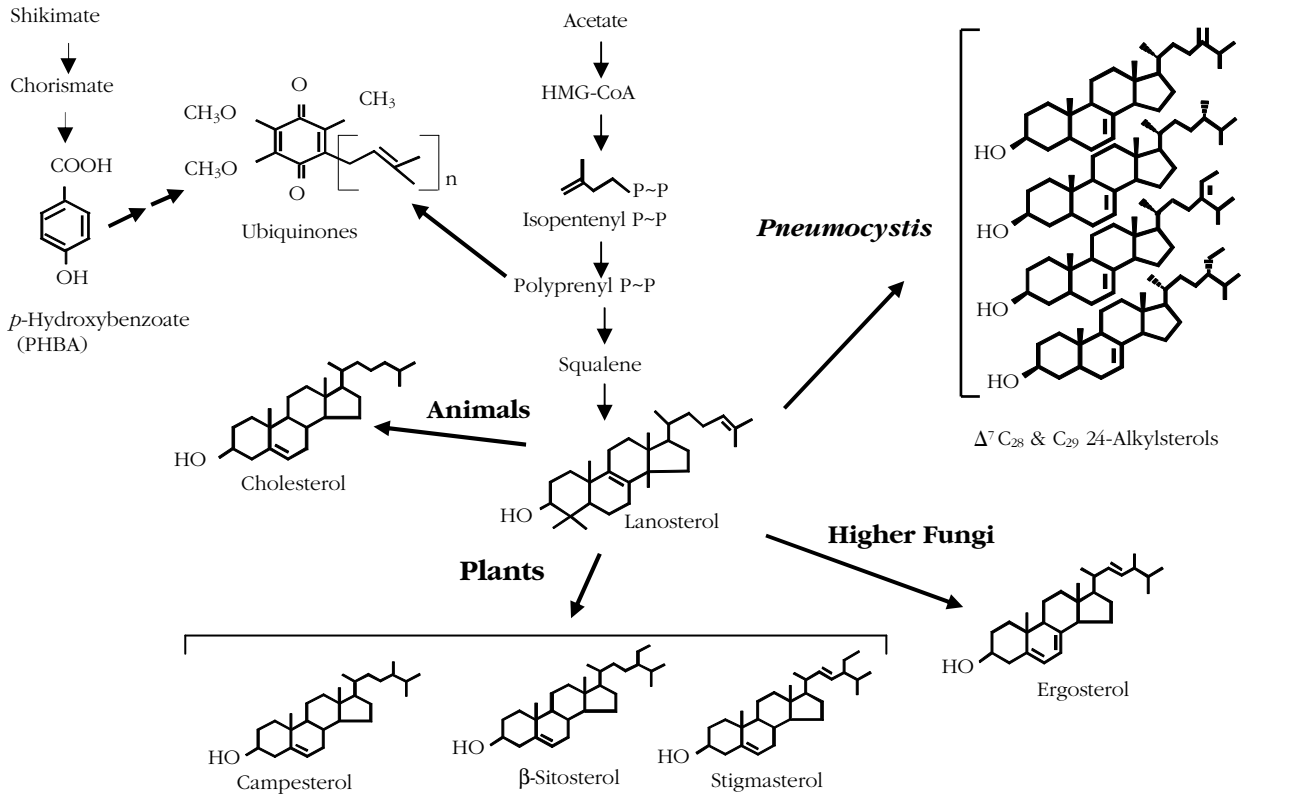

Fig. 2. – Generalized scheme of the classical acetate to 3-hydroxy-3-methyl-glutaryl-Coenzyme A (HMG-CoA) isoprenoid pathway leading to polyprenyl diphosphates. These long chain molecules are utilized in sterol biosynthesis in eukaryotes. Polyprenyl diphosphates are also transferred to *p*-hydroxybenzoate (PHBA) formed via the shikimic acid pathway and chorismate resulting in the formation of ubiquinone homologs (Coenzyme Q, CoQ).

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 ($Pc14DM^{MA}$) and a glutamic acid in the other ($Pc14DM^{UC}$). 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 demonstration 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 *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 *P. carinii* 14DM^{MA} were tested for the effects of fluconazole, voriconazole and itraconazole on culture proliferation. Growth of yeast expressing the *S. cerevisiae* 14DM were inhibited by these triazoles. In contrast, growth of the yeast null mutant expressing the *P. carinii* 14DM^{MA} was less sensitive to fluconazole and voriconazole. However, growth of cells expressing the yeast or the *P. carinii* 14DM was inhibited by itraconazole (Morales *et al.*, 2003). As with many studies on *P. carinii* enzymes, whether on the organism itself or on cells expressing a *P. carinii* enzyme, the effects of various inhibitors on the *P. carinii* 14DM enzyme activity itself remain to be tested.

The *P. jirovecii erg11* Gene and 14DM

The sterol composition of human-derived *P. jirovecii* has been reported (Kaneshiro et al., 1999a; Giner *et al.*, 2004). There appears to be two distinct *P. jirovecii* populations (strains) that differ in their sterol profiles. One is characterized by relatively low proportions of C_{31} and C_{32} sterols, and another with high C_{31} and C_{32} sterols, which are 24-alkyllanosterol derivatives. Gas chromatography data are available for characterization of *P. jirovecii* with low C_{31} and C_{32} sterols (Kaneshiro *et al.*, 1999a) but definitive structural assignments by mass spectrometry and nuclear magnetic resonance spectroscopy have yet to be reported.

In *P. jirovecii* populations with high C_{31} and C_{32} sterols, the lanosterol derivative pneumocysterol (Kaneshiro *et al.*, 1999b) (24*Z*)-ethylidenelanost-8-en-3β-ol can accumulate often comprising about half the *Pneumocystis*-specific sterols. Definitive structural analyses of sterols in a population with high C_{31} and C_{32} sterols indicated that those *P. jirovecii* organisms lacked 14DM activity (14DM$^-$) (Giner *et al.*, 2004). 24-Alkyllanosterol derivatives comprised almost 90 % of *P. jirovecii* 14DM$^-$ whereas *P. carinii* isolated from rat lungs has only 5 % of these sterols. Sterols with a Δ^7 double bond and no methyl groups at C-4 or C-14 represented less than 1 % whereas these sterols comprised approximately 44 % of *P. carinii* sterols. These data indicate that 14DM is not vital for at least these *P. jirovecii* 14DM$^-$ organisms as these organisms were the direct cause of death of PcP patients. The possibility that the *erg11* gene in *P. jirovecii* 14DM$^-$ carries a mutation giving rise to a nonfunctional 14DM remains to be investigated. It has not been ruled out that the gene is not altered but the expression of 14DM in these *P. jirovecii* strains (and perhaps MAT in *P. carinii*, see above) involves cellular regulatory events that block the production of certain enzymes.

UBIQUINONES

The multi-branched isoprenoid pathway produces polyprenyl diphosphate chains that are made up of five-carbon monounsaturated isoprene units. One branch leads to squalene and to sterols and another branch contributes polyprenyl 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_{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 *p*-hydroxybenzoic acid (PHBA), the direct precursor of the CoQ ring structure. That *P. carinii* synthesizes de novo ubiquinones was indicated by the presence of the pentafunctional *arom* gene (Banerjee et al. 1993) and the reduction of viability and short-term culture growth by glyphosate, which inhibits 5-enolpyruvylshikimate-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 *Pneumocystis* has different enzymes with specificity for substrates of different polyprenyl chain lengths or a single enzyme with broad substrate specificity. However, it is known that *P. carinii* synthesizes CoQ_7, CoQ_8, CoQ_9, and CoQ_{10} but not CoQ_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 can occur in both cellular compartments in some organisms (Swiezewska *et al.*, 1993; Jun *et al.*, 2004). In the first subcellular fractionation of cytoplasmic organelles accomplished for *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

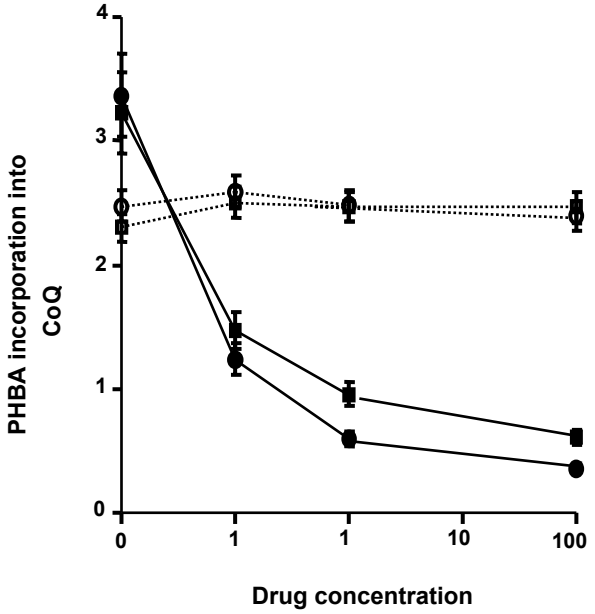

Fig. 3. – Incorporation of [U-^{14}C] *p*-hydroxybenzoate (PHBA) into total ubiquinones in both the mitochondrial and microsomal *P. carinii* subcellular fractions. The CoQ analogs atovaquone and buparvaquone inhibited microsomal but not mitochondrial CoQ synthesis. Dashed lines, mitochondrial fraction; solid lines, microsomal fraction; squares, atovaquone; circles, buparvaquone.

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 CoQ_6 was present, incorporation of PHBA into all four homologs was the same as controls (Table I). When excess nonradioactive CoQ_7 was included, incorporation of PHBA into all four homologs was inhibited. Interestingly, in the presence of excess cold CoQ_8 incorporation of PHBA into CoQ_7 was not reduced but incorporation into CoQ_8, CoQ_9 and CoQ_{10} was inhibited. Also in the presence of excess nonradioactive CoQ_9, incorporation of radioactive PHBA into CoQ_7 and CoQ_8 was not inhibited, but incorporation into CoQ_9 and CoQ_{10} 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.

Incorporation of [U-^{14}C]PHBA into CoQ homologs[a] (Percent of no addition controls)

Excess nonradioactive

homolog	CoQ_7	CoQ_8	CoQ_9	CoQ_{10}
None	100	100	100	100
CoQ_6	105	108	132	115
CoQ_7	**33**	**33**	**36**	**32**
CoQ_8	91	**31**	**42**	**43**
CoQ_9	103	99	**73**	**63**

[a] Bolded numbers indicate inhibition of [U-^{14}C]PHBA incorporation into the CoQ homolog.

Table I. – Sythesis of CoQ homologs in *P. carinii* is regulated by product feedback inhibition.

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 antiretrovirus 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 (Aliaouat-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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