XVII. Lipid metabolism of *Pneumocystis*:
toward the definition of new molecular targets

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*Keywords:* cis-9,10-Epoxy stearic acid; Sterol Δ⁸ to Δ⁷-isomerase; Lipid metabolism; Purified *Pneumocystis carinii*; C-24-alkylated sterols; Ubiquinone

1. Introduction

Drugs targeted against lipids or their biosyntheses have proved highly effective against fungi. Polyene antibiotics such as amphotericin B bind to sterols (particularly with ergosterol), the complexes aggregate, and then form pores in the organism’s membranes, thus abolishing ion gradients. Fluconazole and other related drugs inhibit specific steps in fungal sterol biosynthesis, and are effective against systemic mycoses. Parasites typically scavenge nutrients from their hosts, and metabolize them or utilize them directly for elaborating cellular structures. Thus, membranes can be formed by inserting some host-derived molecules into the bulk phase of lipid bilayers. However, it is believed that some lipids, intimately associated in domains serving important physiological functions (e.g., pumps, enzymes, signal transduction) require parasite lipids having specific three-dimensional configurations. Thus, if host lipids cannot provide the appropriate molecules, the parasite must synthesize at least a low amount of these lipids for those domains. Vital pathogen-specific lipids that are not available from the host have been described as ‘metabolic’ lipids [1,2] because they enable the organism to function properly and proliferate. These make attractive targets for elimination of the pathogen.

2. Purified organisms

Studies on *Pneumocystis carinii* lipids have been performed on organism preparations for which purity was not rigorously defined by both qualitative and quantitative criteria [3–8]. It became apparent that extensive ground work was required before credible, reproducible and interpretable lipid biochemical data were to be obtained from organisms isolated from infected animal models. The alveolar lining in which *P. carinii* proliferates is bathed in lung surfactant, which is composed primarily of lipids and lesser amounts of proteins and carbohydrates. The lipids of lung surfactant are characterized by the predominance (about half) of dipalmitoylphosphatidylcholine (disaturated PC). Lung surfactant lipids [3] and proteins [9] bind avidly to *P. carinii* surfaces which are not removed by washing with ordinary buffered salt solutions. Treatment with reagents such as the divalent cation chelator EDTA is required to remove surfactant components (e.g., surfactant protein A, SP-A) [9]. Thus the levels of host tissue and molecular contaminants

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in organism preparations had to be measured. Light microscopic monitoring of intact host cells is not sufficient for detecting contaminating host cell fragments or molecules.

A purification protocol was developed (Fig. 1) which prioritized purity over yield. The corticosteroid-immunosuppressed model [10] was adopted in which \textit{P. carinii} pneumonia (PcP) was induced by intratracheal inoculation of cryopreserved bacterial- and fungal-negative organisms into viral antigen-negative \textit{P. carinii}-free rats. Included in this isolation procedure was a mucolytic agent (sulphydryl reagent) which caused the detachment of organisms from each other and from type I pneumocytes of the lung epithelium presumably by breaking disulfide linkages. Hence, alveolar type I cells (to which organisms attach) and delicate structures, such as tubular extensions of trophozoite cell surfaces, remained intact (Fig. 2). Also, these preparations readily passed through microfilters in the final step of the purification protocol [11]. The –SH agent also aided in the removal of surfactant proteins and other host contaminants from organisms surfaces; the resultant preparations lacked SP-A and other exogenous substances (see below). Glutathione was selected as the –SH agent of choice since it is a normal component of the alveolar lining fluid, and it is milder than the other chemicals tested.

The critical aspect of developing a contaminant-free preparation was documentation that it is free of host cell fragments and molecules. Thus, the purity of the preparations was demonstrated by multiple criteria including light and electron microscopy, biochemical, and immunochemical analyses (Table 1).

(1) By light microscopy, intact host cells were not detected in stained preparations observed under bright field optics or in unstained preparations observed under phase-contrast and differential interfer-

\begin{table}
\centering
\caption{Characterization of \textit{P. carinii} carinii purified preparations}
\begin{tabular}{|l|l|l|}
\hline
Method & Observations & Conclusions \\
\hline
Light microscopy & No intact host cells & 100\% purity \\
Electron microscopy & No intact host cells & 100\% purity \\
Electron microscopy & Thick glycocalyx & >95\% purity \\
ELISA\textsuperscript{a} & SP-A & >99.5\% purity \\
GLC\textsuperscript{b} & Exogenous lipid & 100\% purity \\
Calcein AM+propidium iodide & Live, moribund, dead & 80–95\% viable \\
Luciferin/luciferase & 0.3 fmol ATP/organism & Viable \\
Light microscopy & 10^8–10^9 organisms/rat & \\
Process control & < 300 mg protein & Average recovery\textsuperscript{c} \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} \textit{Pneumocystis} was identified by its distinctive thick glycocalyx; membrane fragments with thick glycocalyces were identified as \textit{P. carinii}; those without were scored as contaminant.

\textsuperscript{b} ELISA, enzyme-linked immunosorbent assay.

\textsuperscript{c} GLC, gas-liquid chromatography.

\textsuperscript{d} Resuspending the pellet obtained after the first low spin, and pooling the supernatant from the first low spin resulted in higher recoveries.

\textsuperscript{e} Lungs from individual immunosuppressed, uninfected rats processed through the same protocol.

\textsuperscript{f} Typical \textit{P. carinii} final preparations from individual rats contained approx. 5 mg protein.
ence optics. Electron microscopy verified the absence of intact host cells. (2) Transmission electron microscopic analysis was performed on 28 separate preparations, and 2–4 blocks were prepared from each. Each block was sectioned at ≥2 levels, and 80–100 micrographs of each preparation were randomly taken at low magnification (maximal area). Quantitative analysis of the micrographs was performed by identifying organisms by their distinctive thick glycocalyx; all isolated membrane fragments that lacked a thick glycocalyx (even if they could be *P. carinii* cytoplasmic membranes) were scored as contaminant. Purity was expressed in Table 1 as the lowest (not average) estimate. (3) The lung surfactant marker SP-A was quantified using monospecific polyclonal antibodies directed against rat SP-A in an enzyme-linked immunosorbent assay (ELISA). Again, organism purity in Table 1 is expressed as

Fig. 2. Transmission electron micrograph of purified *P. carinii* preparation. Organisms are completely detached from each other, and tubular extensions (E) remain intact and hence abundant in the preparation. The preparation includes cysts/asci (C), trophic forms (T), empty cysts/asci (crescent-shaped), and some loose membrane fragments. Bar = 1 mm. (From [11].)
the lowest (not average), based on these analyses. To determine whether exogenous host lipids were adhered to organism surfaces, stigmasterol (not normally detectable in *P. carinii*) was added during the early homogenization step in the isolation procedure. Aliquots were analyzed at each step of the isolation and purification procedures; stigmasterol was not detected during the series of high- and low-speed centrifugations, midway in the protocol. Thus potential exogenous lipid contaminants were shown to be eliminated in this protocol. These quantitative analyses showed that the preparations were >95–100% free of host contamination. Several *P. carinii* lipid analyses have since been performed using this protocol [12–17].

Microscopic analysis of preparations stained by the dual (live/dead) staining procedure, using calcein acetoxymethyl ester plus propidium iodide [18,19], indicated 80–95% of the organisms were viable. In these analyses, moribund cells exhibiting dual staining, and empty cysts containing residual double-stranded nucleic acids, were scored as dead. The high levels of ATP measured in these preparations confirmed the high percentage of viable organisms [11]. Incorporation of radiolabeled precursors into *P. carinii* compounds also showed that these preparations were of metabolically active organisms [21–23]. The yield from a single heavily infected rat was $10^8$–$10^9$ organisms, typically containing approximately 5 mg protein. Process controls, in which the lungs from individual *P. carinii*-free immunosuppressed rats were subjected to the same protocol, resulted in a maximum (not average) of <300 mg protein.

3. Sterols

An early significant finding on the nature of *P. carinii* lipids was the inability to detect ergosterol, which is the major sterol of most fungi that have been analyzed [2]. This observation explained the inefficacy of the antimycotic drug amphotericin B. The C\textsubscript{27} sterol cholesterol (Fig. 3A), which is apparently scavenged from the mammalian host, was present in the highest amount in *P. carinii*. However, *P. carinii* was shown to have at least 24 sterol components, most of which were not detected in the lungs of normal rats and immunosuppressed, uninfected rats [6,8,12,13]. De novo isoprenoid biosynthesis in *P. carinii* was further demonstrated by the detection of lovastatin-inhibitable HMG-CoA reductase activity [20], the key regulatory enzyme in isoprenoid metabolism. The product of this enzyme is mevalonic acid, from which sterols and other isoprenoid compounds are synthesized. Only trace to low levels of HMG-CoA reductase activity were detected in lungs from normal rats and from immunosuppressed, uninfected rats.

The *P. carinii*-specific sterols were characterized by an alkyl group at C-24 of the sterol side chain (Fig. 3B–D), which was identified as a drug target since mammals are unable to alkylate sterol C-24 [13,22]. Another unique feature of the *P. carinii* sterols is that they had a double bond at C-7 of the sterol nucleus ($\Delta^7$). These putative ‘metabolic’ sterols are not found in mammals or in most infectious agents (e.g. *Candida*, *Aspergillus*) and are therefore also considered ‘signature’ lipids of the pathogen. Detection of these sterols in a sample would highly indicate the organism’s presence. Examples of major *P. carinii*-specific ‘signature’ and putative ‘metabolic’ sterols are fungisterol (ergost-7-en-3\beta-ol; 24\beta-methylcholest-7-en-3\beta-ol; Fig. 3B), which constituted 21% of the total, and 29% of the free non-cholesterol
sterol components) and stigmast-7-3β-ol (24 ethylcholest-7-3β-ol; Fig. 3C).

Alkylation of sterol C-24 utilizes transferase enzymes and S-adenosylmethionine (SAM) as the methyl donor. Unlike cholesterol, C28 sterols have an additional methyl or methylene group at C-24, and C29 sterols have an ethyl or ethylene group with two carbons resulting from sequential additions of two methyl groups transferred from SAM. Two inhibitors of C-24 alkylation, 22,26-azasterol and 24,25-epiiminolanosterol, were shown to inhibit rat-derived P. carinii proliferation in a monoxenic culture system and to alter its sterol composition [8]. The drugs caused a relative increase in the total percentage of C27 sterols with a concomitant decrease in the percentage of C28 and C29 sterols. The C27 sterols that accumulated in drug-treated organisms were not detectable, or present in only trace amounts, in untreated controls. The effects of sterol analogues with side groups linked by a direct P-C bond were also tested on P. carinii and were found deleterious to the organism [23].

Since the P. carinii-specific signature sterols include those with Δ7 unsaturation, the formation of this double bond may be vital for the organism [6,8,13]. Thus, structural characterizations of P. carinii-specific sterols have also identified Δ8 to Δ7-isomerase of the pathogen as a potential chemotherapeutic drug target.

Recently, very unusual and rare sterols were found in lung and bronchoalveolar lavage samples containing P. carinii hominis. They were identified as a C31 (euphorbol) and a C32 sterol, which was given the trivial name pneumocysterol [25] (Fig. 3D). Pneumocysterol may be present in trace amounts in P. carinii carinii isolated from rat lungs, but it can accumulate in P. carinii hominis in very high concentrations. The C31 and C32 sterols were identified as C-24-alkylated lanosterol molecules, previously reported in some plants. In many cells, lanosterol (C30 sterol with a double bond at C-8 and three methyl groups attached to the sterol nucleus) is an intermediate compound. Three methyl groups on the sterol nucleus are usually removed during processing of the compound to the final accumulated products (e.g. in the biosynthesis of C27 cholesterol). Identification of the C31 and C32 sterols further indicate that C-24 alkylation is important and highly active in P. carinii, and that the Δ24(25) and Δ24(28) sterol methyltransferases responsible for C-24 alkylation are excellent drug targets.

4. Ubiquinone (coenzyme Q, CoQ)

In addition to sterols, isoprenoid biosynthesis also involves branch pathways leading to the formation of a number of products, including the polyprenyl chain of ubiquinone. Polyprenyl chains are formed from isopentenyl groups (C5), and the number of these units in the ubiquinone polyprenyl chain designates CoQ homologs. For example, the CoQ9 po-
lyprenyl chain contains nine C5 isopentenyl units (45 carbons total). A completed chain, the length of which depends on the organism and tissue, is added to p-hydroxybenzoate (benzoquinone ring of CoQ) by chain length-specific polyprenyl transferases (Fig. 4). The hydrophobic polyprenyl chain allows insertion into the inner mitochondrial membrane bilayer where CoQ plays a central role in respiration, mediating the transfer of electrons from a number of dehydrogenases to the cytochrome electron transport chain or the alternative oxidase system (Fig. 5) [26]. The cytochrome bc1 complex contains ubiquinone (CoQ) which is reduced in reactions at the matrix side of the inner mitochondrial membrane (Qi site), whereas ubiquinol (CoQH2) oxidation occurs at the side facing the cytoplasm (Qo site). High levels of CoQ are also found in other cytomembranes such as those of the Golgi apparatus and the cell surface where CoQ apparently participates in electron transfer [27].

Since CoQ has a pivotal metabolic function, it is an attractive chemotherapeutic target in parasites. Differences in the cyt b gene nucleotide sequence of humans and a number of parasitic protists suggest Qi or Qo sites as attractive drug targets [26]. Several analogs of ubiquinone (e.g., the hydroxynaphthoquinone atovaquone) and 8-aminoquinolones (e.g., primaquine) are effective against malaria and also exhibit anti-Plasmodium activity [25,27,28]. 8-Aminoquinolone metabolites include compounds similar to hydroxynaphthoquinones, hence the mechanism of action of these two groups of drugs may be similar [26]. The antimalarial activity of atovaquone results from blocking electron transport to CoQ from dihydroorotate dehydrogenase (DHOD), an important enzyme in pyrimidine biosynthesis. Since Plasmodium spp. cannot scavenge host pyrimidines, inhibition of de novo pyrimidine synthesis has cidal consequences for these organisms. Unlike Plasmodium, Pneumocystis DHOD activity is relatively insensitive to atovaquone [29]. The anti-Plasmodium activity of atovaquone and other hydroxynaphthoquinones may occur by inhibition of electron transport and cellular respiration [30] resulting from blocking CoQ synthesis and/or displacing CoQ from the membrane.

Fig. 5. The central role of CoQ in electron transport. Ubiquinone in the inner mitochondrial membrane accepts electrons from dehydrogenases in the membrane and transfers them to cytochromes or the alternative oxidase system. PFOR, pyruvate:ferredoxin oxidoreductase; SD, succinate dehydrogenase; GD, glutamate dehydrogenase; GPD, glycerophosphate dehydrogenase; NADH OH, NADH dehydrogenase; DHOD, dihydroorotate dehydrogenase.
The CoQ molecule is comprised of two parts. In most microbes and plants, the benzoquinone moiety is synthesized de novo from chorismate, a key product of the shikimic acid pathway (Fig. 5). From chorismate, several pathways diverge and lead to the syntheses of vital compounds, including the aromatic amino acids, folic acid, and vitamin K (Fig. 6). Since mammals, including humans, lack the shikimic acid pathway, the products formed from chorismate are essential, or dietary requirements for mammals. Mammals can produce ubiquinone by incorporating dietary aromatic amino acids (tyrosine or phenylalanine) into the molecule. Since humans lack the shikimic acid and most post-chorismate pathways, reactions in the pathogen for synthesizing these products are excellent drug targets. For example, folic acid synthesis in *P. carinii* is an attractive drug target, and a number of anti-folates are active against *P. carinii* viability and proliferation [31]. One pathway from chorismate leads to the formation of *p*-hydroxybenzoic acid, the direct precursor of the CoQ benzoquinone moiety.

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**5. cis-9,10-Epoxy stearic acid**

Thus far, the only fatty acid in *P. carinii* that was not detected in lung controls is cis-9,10-epoxy octadecanoic acid [16] (Fig. 7). This epoxy fatty acid is synthesized from oleic acid (E.S. Kaneshiro, unpublished) which has a single double bond in the middle of the chain. Most known lipoxygenases use fatty acid substrates with two or more conjugated *cis* methylene-interrupted double bonds (e.g., arachidonic acid). It is likely that this rare fatty acid is essential for *P. carinii* viability, and that the lipoxygenase enzyme responsible for the formation of the epoxide ring in 18:1 is unique to the pathogen. The structure of the parasite enzyme catalyzing epoxide fatty acid formation from oleate would be expected to differ from the host enzymes that use substrates with conjugated *cis* methylene-interrupted double bonds. Thus, the enzyme in *P. carinii* that catalyzes the biosynthesis of the signature lipid, cis-9,10-epoxy stearic acid, has been identified as a potential molecular target for the elimination of this pathogen.

![Fig. 7. The structure of the unique *P. carinii* fatty acid, cis-9,10-epoxy octadecanoic acid (cis-9,10-stearic acid).](https://academic.oup.com/femspd/article-abstract/22/1-2/135/461828)
6. Conclusions

Characterizations of *P. carinii* lipids and those of lungs from normal rats and corticosteroid-immunosuppressed rats have proved productive in identifying several organism lipids that are not present or synthesized by the host. Reliable data using organism preparations shown to be of high purity by several independent quantitative analyses enabled the distinction between molecules of *P. carinii* and those of the host’s lung. Thus far, several unique Δ⁷ C-24-alkylated sterols, ubiquinone, and *cis*-9,10-epoxy stearic acid (and the reactions in their biosyntheses) have been identified as molecular targets for PcP. Other targets may be identified after other lipid classes and lipid species present in low concentrations have been characterized in greater detail.

Acknowledgments

Supported by grants from the National Institute for Allergy and Infectious Diseases (RO1 AI38758 and RO1 AI29316).

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