Pneumocystis S-Adenosylmethionine Transport
A Potential Drug Target

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***Pneumocystis* pneumonia (PCP) is a life-threatening condition in immunosuppressed patients. Current treatments are inadequate, and new drug leads are needed. This fungus depends on its host for S-adenosylmethionine (AdoMet), a critical metabolic intermediate ordinarily synthesized by individual cells as needed. *Pneumocystis* contains a gene coding for the AdoMet-synthesizing enzyme methionine ATP transferase (MAT), and the protein is expressed. However, the fungus lacks MAT activity, and infection causes the depletion of host plasma AdoMet. The uptake of *Pneumocystis* AdoMet was shown to be exquisitely specific, which suggests the transport of AdoMet as a potential drug target. Here we report on the discovery of *PcPET8*, a *Pneumocystis* gene with homology to mitochondrial AdoMet transporters. When expressed by *Saccharomyces cerevisiae*, it locates properly to the mitochondrion and complements a strain of *S. cerevisiae* lacking its native mitochondrial AdoMet transporter. The importance of AdoMet transport is demonstrated by the ability of the AdoMet analogue sinefungin to block the uptake of *Pneumocystis* AdoMet and inhibit growth in culture. Because *PcPET8* is likely critical for *Pneumocystis*, the yeast construct has potential as a surrogate for testing compounds against *Pneumocystis*.**

Keywords: *Pneumocystis*; S-adenosylmethionine; mitochondrial transporter; PET8; *PcPET8*; PCP pneumonia

The genus *Pneumocystis* contains genetically distinct but morphologically similar species, each limited to a single mammalian host species (1, 2). A species of *Pneumocystis* has been found in every mammalian species where it has been sought. Although the prevalence of colonization is high in humans and other mammals, *Pneumocystis* pneumonia (PCP) occurs only when the host is immunosuppressed (3). PCP in humans is associated with advanced HIV disease, severe malnourishment in children, and treatments for cancer, rheumatic disease, and the prevention of organ transplant rejection (3). It is frequently fatal if untreated (4). The antifolate combination of trimethoprim with sulfamethoxazole has been the mainstay of treatment, but is unsatisfactory because of significant adverse drug effects (5), especially for patients with advanced HIV, and treatment failures exceed 20%. Genetic data suggest increasing drug resistance (6). Alternative drugs exist, but all have drawbacks that include lower efficacy or greater toxicity. The mortality rate for severe infections has not changed appreciably for 30 years (1, 7). New drugs and new drug leads are urgently needed.

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CLINICAL RELEVANCE

The *Pneumocystis* mitochondrial S-adenosylmethionine (AdoMet) transporter may be of assistance in discovering new drugs by serving as a surrogate for testing the ability of compounds to block the acquisition and use of AdoMet by *Pneumocystis*.

Among the unusual properties of *Pneumocystis* is the dependence on its host for S-adenosylmethionine (AdoMet), a key metabolic intermediate with a wide range of functions, including protein and nucleic acid methylation, phospholipid synthesis, polyamine synthesis, folate metabolism, and the production of glutathione, and it also exists as a bound prosthetic group for a class of enzymes (8). The condensation of methionine and ATP is catalyzed by methionine ATP transferase (MAT), yielding AdoMet, phosphate, and pyrophosphate. With the exceptions of *Pneumocystis*, some species of *Rickettsia*, and to some extent *Leishmania*, all cells examined were found capable of producing the AdoMet they require (9). Whereas AdoMet-requiring *Rickettsia* lacks a gene for MAT, *Pneumocystis* and *Leishmania* contain genes that code for functional MAT enzymes, and both are expressed (10, 11). Cultured *Leishmania* expresses MAT activity when in the log phase of growth, but activity ceases during the stationary phase, despite the continued expression of MAT, and AdoMet is scavenged from the culture medium. *Leishmania* and *Pneumocystis* both possess high-affinity, highly selective AdoMet transporters. Although *Pneumocystis* may express MAT activity at some stage of its life cycle, this activity has been undetectable in *Pneumocystis* isolated from infected animals, even when sensitive assays and procedures to eliminate possible interference are used (9). The dependence of *Pneumocystis* on host AdoMet is supported by evidence from experimental animals: PCP reduces pulmonary AdoMet and depletes plasma AdoMet, the selective reduction of pulmonary AdoMet attenuates the infection, and an infusion of AdoMet exacerbates the disease (9, 12, 13). For humans with PCP, plasma AdoMet is depleted and recovers rapidly upon the initiation of treatment (12, 14, 15).

This requirement of exogenous AdoMet suggests transport as a drug target, a suggestion supported by our previous demonstration that rat-specific *P. carinii* possesses a high-affinity, high-specificity AdoMet transporter, and close AdoMet analogues such as S-adenosylhomocysteine and S-adenosylethionine exert no effect on the uptake of *P. carinii* AdoMet, despite inhibiting uptake by *Saccharomyces cerevisiae* (9, 16). To exploit this finding, we searched for potential *Pneumocystis* AdoMet transporter genes with the expectation of improving our understanding of *Pneumocystis* AdoMet metabolism and producing a surrogate test system for drug development. Using the limited *Pneumocystis* genomic data available, we identified a sequence predicting a protein with similarity to the *S. cerevisiae* miochondrial AdoMet transporter (PET8). Here we describe the identification

of the *P. carinii* PET8 gene (*PcPET8*), demonstrate transcription by *P. carinii*, show the functionality of the *PcPET8* gene product by the complementation of an *S. cerevisiae PET8* knockout strain, and demonstrate that sinefungin, a PET8 inhibitor, blocks the uptake of *Pneumocystis* AdoMet in a dose-dependent fashion.

MATERIALS AND METHODS

cDNA Synthesis

P. carinii was isolated from lungs that had been removed from infected rats and stored frozen, as previously described (12, 17). RNA was isolated from *P. carinii* cells, using the TRIZOL reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), and was then treated with RQ1 RNase-free DNase (Promega, San Luis Obispo, CA) to destroy any trace of genomic DNA. cDNA was synthesized using 1.0 μg of extracted RNA and the Superscript III One-Step RT-PCR system (Invitrogen) in a 50-μl reaction mix with the forward primer ATG GAT TTG AAA CTA ATT TAT G and the reverse primer AAT GCA CGT ATA CCT TCT TC. cDNA synthesis proceeded with the reverse transcription reaction at 56°C for 30 minutes, followed by 40 PCR amplification cycles (15 seconds at 94°C, 30 seconds at 56°C, and 60 seconds at 68°C), with a final extension for 5 minutes at 68°C. As a negative control to ensure a lack of contamination with genomic DNA, pseudo-cDNA synthesis was performed using Platinum Taq (Invitrogen) without reverse transcriptase and 1 μg of DNase-treated RNA.

Expression of Green Fluorescent Protein–Tagged PcPET8 in *S. cerevisiae*

To produce *PcPET8* tagged at the C-terminus with green fluorescent protein (GFP) for cellular localization studies, we used the shuttle vector PKT128 (18), designed to produce such fusion proteins in yeast. Detailed methods are described in the online supplement.

S. cerevisiae PET8 Knockout (PET8Δ) Complementation by *PcPET8*

The *S. cerevisiae* PET8Δ strain was obtained from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/). *PcPET8* was cloned into pGREG506, to allow for expression in *S. cerevisiae* without extra amino acids. Detailed methods are described in the online supplement.

Measurement of AdoMet Transport

AdoMet transport was measured as previously described (9) and summarized here. *P. carinii* cells cultured for 4–8 days after isolation from rat lungs were washed three times with Eagle's minimum essential medium and suspended in PBS. Assays included 45 μl of *P. carinii* cell suspension and 5 μl of PBS buffer containing S-[methyl-^{14}C]AdoMet (52.7 mCi/mmol; Moravek Biochemicals, Inc., Brea, CA) and sinefungin, to yield 33 μM of AdoMet and the indicated sinefungin concentrations. After mixing, assays were incubated for 30 seconds at 37°C, and then cells were washed by microfuging through 100 μl of dibutyl phthalate and mineral oil (1:1), to remove unincorporated AdoMet. The supernatants were discarded, and the pellets were counted by liquid scintillation.

RESULTS

Identification of *PcPET8*

A basic local alignment search tool (BLAST) query of the *Pneumocystis* Genome Project (http://pgp.cchmc.org) based on PET8 identified a 1,391-base pair (bp) sequence in the contig database that contained an open reading frame (ORF) of 663 bp, coding for a 220 amino acid polypeptide of 24.99-kD molecular mass and a predicted isoelectric point of 8.9. Alignment of the theoretical protein with the amino acid sequence of *S. cerevisiae* PET8 (19) showed a 24% identity and 39% homology (Figure 1). Signal prediction did not reveal a signal peptide, but did identify two mitochondrial carrier domains between amino acids 61–150 and 170–198 (MotifScan; http://myhits.isb-sib.ch/cgi-bin/motif_scan), as well as two transmembrane regions between amino acids 59–79 and 172–192 (TopPred; available at http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) (20) (Figure 2). To confirm the sequence, we cloned the gene *de novo*. PCR amplification from *P. carinii* DNA, using primers based on the already mentioned 663-bp ORF, produced a single product of approximately 663 bp, which we cloned into the vector pET161/GW/D-TOPO. Sequencing demonstrated the new clone sequence to be identical to that of the *Pneumocystis* Genome Project contig sequence. Following the nomenclature guidelines for *P. carinii* (21), we named the sequence *PcPet8* (GenBank accession number FJ769260).

Transcription of *PcPET8* by *P. carinii*

To demonstrate the presence of *PcPET8*-coded RNA in *P. carinii*, we cloned and sequenced the product of a one-step RT-PCR reaction, using primers based on *PcPET8* and 1 μg of DNase-treated RNA extracted from *P. carinii* isolated from rat lungs (Figure 3). To ensure that the RT-PCR product did not result from genomic DNA contaminating the RNA preparation, a control reaction omitted the reverse transcriptase. The sequence of the RT-PCR product did not deviate from the genomic sequence, a result showing that the gene is transcribed and that it contains no introns.

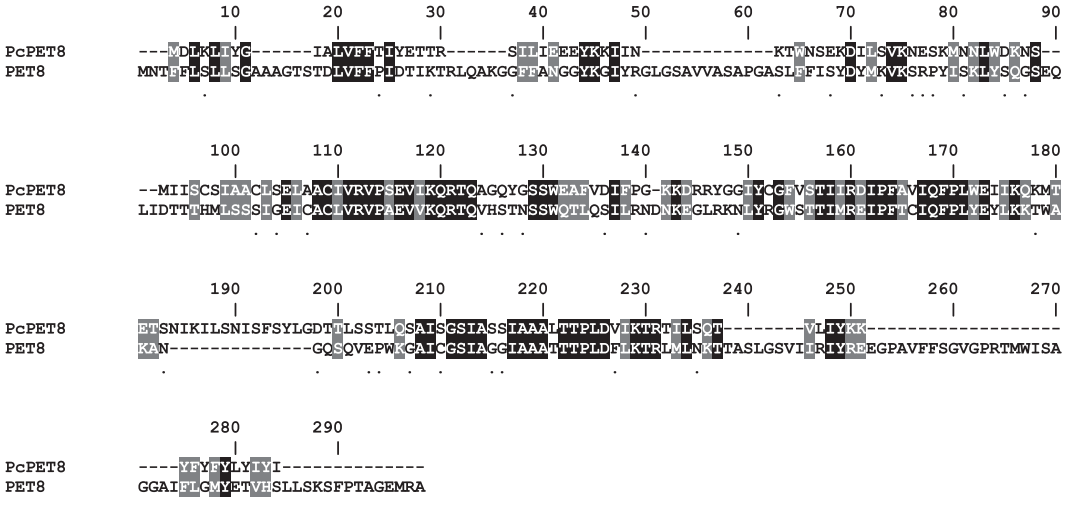

Figure 1. Alignment of the *Pneumocystis carinii* PET8 gene (*PcPET8*) and the *S. cerevisiae* miochondrial AdoMet transporter (PET8). The predicted *PcPET8* protein sequence is aligned with PET8 of *Saccharomyces cerevisiae* (*S. cerevisiae* accession number NP_014395.1). *Dark shading,* identical amino acid; *lighter shading,* strongly similar amino acid; *weakly similar amino acid.

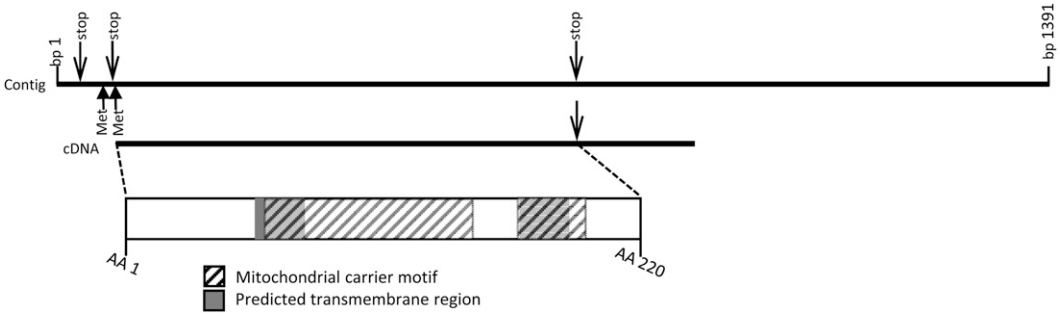

Figure 2. Structural characteristics of *PcPET8*. Scale diagram shows structural features of *PcPET8*, including positions of the putative mitochondrial carrier motif and transmembrane regions, as indicated.

Expression of *PcPET8* in *S. cerevisiae* and Intracellular Localization

PcPET8 was inserted into the yeast expression vector PKT128 (18) to produce a fusion protein with GFP, linked to the C-terminus of *PcPET8*. To allow for the simultaneous detection of yeast mitochondria and this fusion protein, transformed yeast cells were grown in liquid medium with MitoTracker Red 580 (Invitrogen), a fluorescent stain specific for mitochondria. Confocal images (Figure 4) show the colocalization of GFP and MitoTracker Red 580, indicating a mitochondrial localization for the recombinant protein.

PcPET8 Complementation of an *S. cerevisiae PET8* Knockout

We tested the function of *PcPET8* according to its expression in a *PET8* knockout strain of *S. cerevisiae* (PET8Δ). Because the conversion of desthiobiotin to biotin occurs in mitochondria, which requires that AdoMet be transported from the cytosol, PET8Δ is a biotin auxotroph (22). Therefore, if recombinant *PcPET8* can function as a mitochondrial AdoMet transporter in *S. cerevisiae*, the expression of *PcPET8* by PET8Δ should relieve the requirement for biotin. PET8Δ was transformed using pGREG506 with a *PcPET8* insert (PET8Δ–PcPET8). For positive and negative controls, respectively, the *PET8*-competent strain BY4741 and PET8Δ were transformed with an empty vector. After selection with uracil dropout medium, all three transformed types grew well when supplemented with biotin, but only BY4741 and PET8Δ–PcPET8 grew on plates containing desthiobiotin but lacking biotin (Figure 5). This successful complementation demonstrates that the product of *PcPet8* functions as a mitochondrial AdoMet transporter in *S. cerevisiae*.

Sinefungin Inhibition of *Pneumocystis* AdoMet Transport

We measured the ability of the *PET8* inhibitor sinefungin to block the uptake of AdoMet by isolated *P. carinii*. The data presented in Figure 6 show that 50 μM of sinefungin inhibited uptake by approximately 50%, and 100 μM inhibited uptake by approximately 90%.

DISCUSSION

We identified and confirmed by independent cloning the sequence of a contig in the *Pneumocystis* Genome Project database with similarity to the *S. cerevisiae* mitochondrial AdoMet transporter gene *PET8*. We named this gene *PcPET8*, in accordance with the suggested guidelines for nomenclature. The production of *PcPET8* cDNA shows that the gene is transcribed, and the identification of cDNA and genomic sequences indicates

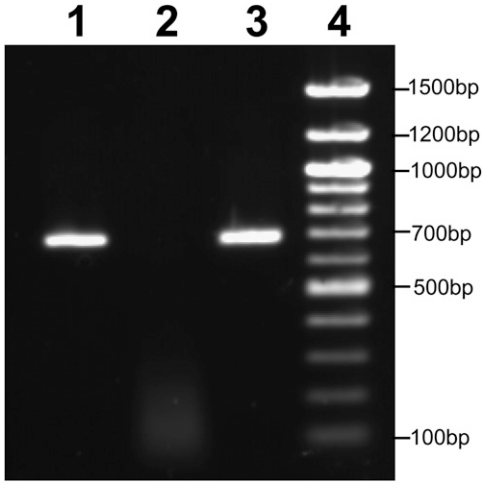

Figure 3. Transcription of *PcPET8* by *P. carinii*. A "one-step" RT-PCR reaction was performed using total RNA isolated from *P. carinii* and primers, based on the *PcPET8* sequence. *Lane 1*, DNase-treated total RNA template. The product demonstrates the presence of *PcPET8* transcript in RNA. *Lane 2*, DNase-treated total RNA template, with omission of reverse transcriptase. The lack of product demonstrates a lack of genomic DNA contamination in the DNase-treated RNA template. *Lane 3*, Genomic DNA template, with omission of reverse transcriptase. The correct size of the product shows primer specificity, and confirms the lack of introns.

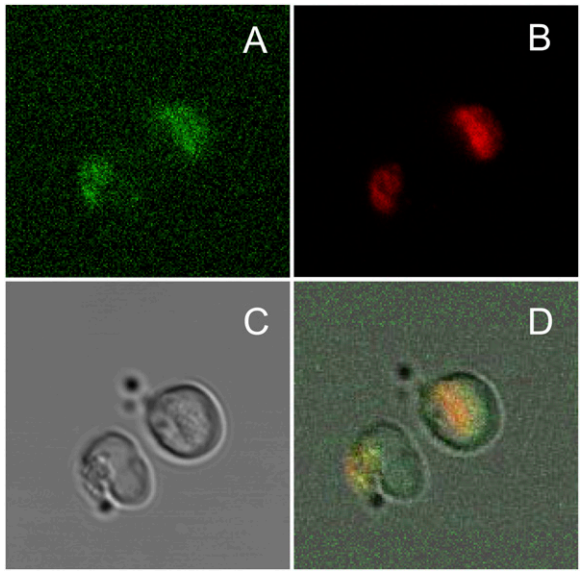

Figure 4. Cellular localization of *PcPET8* expressed by *S. cerevisiae*. A wild-type *S. cerevisiae* cell expresses *PcPET8* tagged with green fluorescent protein (GFP) at the C-terminus, and grown in the presence of a mitochondrial marker. (*A*) PcPET8–GFP. (*B*) MitoTracker Red 580. (*C*) Phase contrast. (*D*) Merged images show the colocalization of GFP and MitoTracker Red 580.

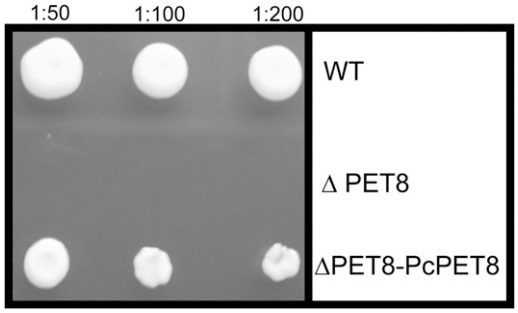

Figure 5. Functional assay of *PcPET8*. Expression of *PcPET8* allows the *S. cerevisiae PET8* knockout strain (PET8Δ) to grow in a medium lacking biotin but containing the biotin precursor desthiobiotin, a molecule that needs S-adenosylmethionine in the mitochondria to be transformed in biotin. Cells were plated in three serial dilutions. WT, wild type.

a lack of introns. When expressed by *S. cerevisiae*, the gene product localizes to the mitochondrion, and complements a strain of *S. cerevisiae* lacking *PET8*.

PET8Δ–PcPET8 does not grow quite as well as the wild type (Figure 5). Perhaps less PcPET8 protein is produced by the transfectant than the amount of PET8 produced by the wild type, or else *S. cerevisiae* and *P. carinii* are sufficiently different that the exotic mitochondrial AdoMet transporter functions less well than the native protein. Regardless, the ability to complement PET8Δ and allow growth without biotin clearly demonstrates that *PcPET8* functions as a mitochondrial AdoMet transporter in *S. cerevisiae*. Despite the short 5′ untranslated region and an ORF coding for 220 amino acids versus the 298 of *PET8*, several points indicate that the coding sequence of *PcPET8* is complete. Alignment of the predicted protein with *PET8* (Figure 1) shows gaps interspersed throughout, yet *PcPET8* codes for a functional protein. The N-terminus of *PcPET8* is shorter by only three amino acids, suggesting the completeness of the 5′ end, an interpretation reinforced by the presence of two in-frame stop codons 5′ to the putative initiating met codon, with no intervening met codons. Although we did not find a canonical eukaryote ribosome–binding (Kozak) sequence surrounding the initiating met codon, the invariant characteristic of a G following that codon is present, despite the absence of the characteristic A/G, located three bases 5′ to the met codon. The lack of a recognizable pattern is of limited significance because Kozak sequences are variable, have been little studied in yeast, and have not been studied at all in *Pneumocystis*. The completeness of the 3′ end is supported by the sequence identity of cDNA, including the stop codon 660 bp after the initiating met codon.

Although *PcPET8* is a mitochondrial AdoMet transporter, it could theoretically contribute to the cellular uptake of AdoMet. Our data show that *PcPET8* localizes to *S. cerevisiae* mitochondria and substitutes for the native transporter, but we have no data for its localization in *P. carinii*, and therefore we cannot rule out the possibility that PcPET8 locates to the cell membrane, where it mediates the uptake of AdoMet. Another possibility is that the *Pneumocystis* cell membrane lacks an active AdoMet transporter, but has an AdoMet permease. If that were the case, then mitochondrial uptake could create an AdoMet concentration gradient across the cell membrane, thereby promoting cellular uptake. Regardless of whether *PcPET8* is responsible for cellular uptake, mitochondrial uptake would be a viable drug target if it can be selectively blocked in *Pneumocystis*.

The antibiotic sinefungin is active against *P. carinii* in culture (12), is known to compete with AdoMet for *S. cerevisiae* PET8 transport (23), and is the only compound we found able to block the uptake of AdoMet by *Pneumocystis*. Kinetic data for the inhibition of AdoMet transport by sinefungin are lacking, but because the observed inhibition occurred after 30 seconds and sinefungin is known to compete with the PET8-mediated transport of AdoMet (22), we are confident that sinefungin directly inhibits the uptake of *Pneumocystis* AdoMet by blocking transport rather than indirectly, though interference with AdoMet-dependent reactions. Our data showing that 100 μM of sinefungin block the uptake of AdoMet by 90% confirm our earlier result using that concentration (12). More importantly, this concentration also completely blocks growth in culture, a finding that supports AdoMet transport as a drug target. The fact that *P. carinii* AdoMet uptake is more sensitive to sinefungin than *S. cerevisiae* suggests that *P. carinii* AdoMet transport may be a sensitive drug target. Although adverse reactions make sinefungin an unlikely drug candidate, the ability of this compound to inhibit the growth of *S. cerevisiae* dependent on *PcPET8* suggests that this construct may prove useful as a tool to identify other compounds that block *Pneumocystis* AdoMet transport, and may prove helpful in identifying viable drug candidates.

Furthermore, the ability of sinefungin to inhibit the growth of *S. cerevisiae* dependent on *PcPET8* suggests that this construct

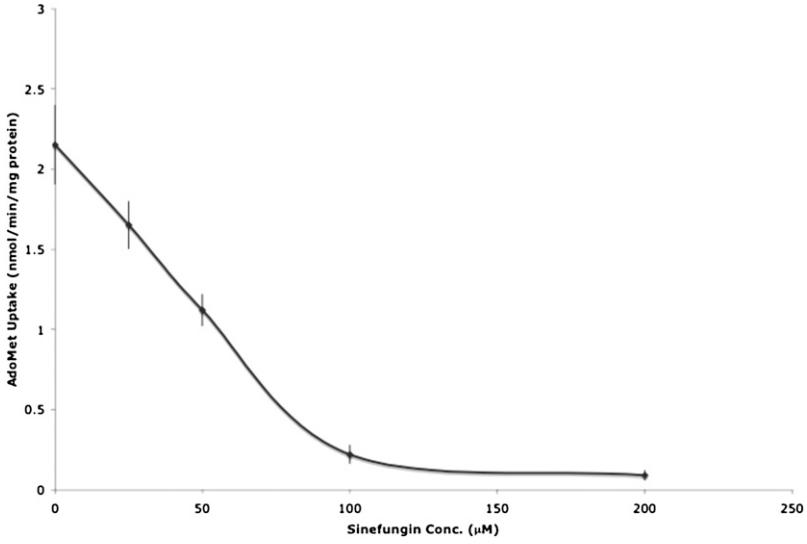

Figure 6. Dose-dependent inhibition of S-adenosylmethionine (AdoMet) transport by sinefungin. Uptake measurement used [^{14}CH3]AdoMet at 33 μM. See MATERIALS AND METHODS for details. Conc., concentration.

may prove useful as surrogate when screening compounds for anti-*Pneumocystis* activity.

In conclusion, the newly discovered *PcPET8* gene shows homology with the *S. cerevisiae* mitochondrial AdoMet transporter, is transcribed by *Pneumocystis*, and lacks introns. When expressed by *S. cerevisiae*, it locates to the mitochondrion and is able to complement a strain of *S. cerevisiae* lacking its native mitochondrial AdoMet transporter. Although this gene may or may not be involved in the uptake of AdoMet by *Pneumocystis*, it likely serves a critical function. Furthermore, sinefungin, which is known to inhibit *S. cerevisiae* PET8, also inhibited the uptake of *Pneumocystis* AdoMet in dose-dependent manner. *PcPET8*-dependent *S. cerevisiae* has potential as a surrogate when testing compounds for the ability of to block the acquisition or use of AdoMet by *Pneumocystis*. Compounds able to block *PcPET8*-dependent AdoMet transport may have further utility because BLAST searches of genomic databases of other human pathogens such as *Plasmodium* spp., *Leishmania major*, and *Trypanosoma brucei* suggest the presence of similar transporters in those parasites.

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