

Characterization of a Lanosterol 14 α -Demethylase from *Pneumocystis carinii*

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Pneumocystis carinii (PC) causes severe pneumonia in immunocompromised patients. PC is intrinsically resistant to treatment with azole antifungal medications. The enzyme lanosterol 14 α -demethylase (Erg11) is the target for azole antifungals. We cloned *PCERG11* and compared its sequence to Erg11 proteins present in azole-resistant organisms, and performed chromosomal and Northern blot analysis for *PCERG11*. Of 13 potential sites which could confer resistance to azoles, two were identical to azole-resistant *Candida*. By site-directed mutagenesis we changed these two sites in *PCERG11* to those present in azole-sensitive *Candida* to generate *PCERG11-SDM* (E113D, T125K). We tested the susceptibility of *ERG11* deletion strains of *Saccharomyces cerevisiae* (SC) expressing *PCERG11*, *PCERG11-SDM*, and wild-type *SCERG11* to three azole antifungals: fluconazole, itraconazole, and voriconazole. *PCERG11* required a 2.2-fold higher dose of voriconazole and 3.5-fold higher dose of fluconazole than *SCERG11* for a 50% reduction in growth. No difference was observed in the sensitivity to itraconazole. *PCERG11-SDM* has increased sensitivity to fluconazole and voriconazole, but not itraconazole. We believe that the molecular structure of the lanosterol 14 α -demethylase encoded by *PCERG11* confers inherent resistance to azole antifungals and plays an integral part in the overall resistance of this PC to azole therapy.

Pneumocystis carinii is a fungus that causes pneumonia in immunocompromised hosts. Humans infected with the human immunodeficiency virus (HIV), or those receiving chemotherapy or high dose corticosteroids, are at high risk for developing *P. carinii* pneumonia (PCP) (1, 2). Trimethoprim-sulfamethoxazole is the drug of choice for prophylaxis and treatment of PCP, whereas the combination of clindamycin and primaquine is one alternative for patients who are allergic to sulfa-containing medications. Despite the fungal nature of *P. carinii*, azole antifungals have been found to be ineffective against this pathogen in short-term cultures and in immunocompromised animals infected with *P. carinii* (3). Indeed, azole medications such as fluconazole, ketoconazole, or itraconazole are not clinically useful for treating patients with PCP. Newer azole antifungal agents,

such as voriconazole, are in clinical use for treating invasive aspergillosis (4). Because the ability to culture *P. carinii* outside of the infected host has not yet been achieved, it is impossible to use standard microbiological methods to determine whether a drug has a fungistatic or fungicidal effect on *P. carinii*. The immunocompromised animal model of PCP is the standard method of obtaining organisms for study, and is either achieved by steroid treatment, selective depletion of helper T lymphocytes, or infection in severe combined immunodeficiency animals (5–8).

Azoles inhibit the enzyme lanosterol 14 α -demethylase (Erg11), which is encoded by the gene *ERG11* (9, 10). Lanosterol 14 α -demethylase proteins have been found in many organisms, including humans and other mammals, plants, and fungi. This enzyme is essential in the initial steps of sterol biosynthesis, removing a 14 α -methyl group, which is necessary for subsequent sterol processing (11). In humans and other mammals, the end-product of sterol biosynthesis is cholesterol, whereas in plants it is stigmasterol and sitosterol, and in most fungi it is ergosterol. Ergosterol is incorporated into the fungal cell wall as a structural compound, where it provides necessary stability for the cell wall. Azole antifungal compounds prevent the accumulation of ergosterol in the fungal cell wall that subsequently leads to cell lysis. Ergosterol has not been found among the sterols in several analyses of *P. carinii* lipids. Instead, *P. carinii* appears to contain a high amount of cholesterol, as well as unique “signature” sterols described by Kaneshiro and colleagues (12). A detailed understanding of the steps involved in the synthesis of *P. carinii* sterols is unknown, and *P. carinii* might have a unique way of processing sterol products. It is also controversial whether *P. carinii* scavenges lipids from the host lung during infection (13). Recently, an S-adenosyl-L-methionine:sterol C-24 methyl transferase, encoded by *ERG6*, was identified in *P. carinii*, which appears to be capable of transferring both the first and the second methyl groups to produce several 24-alkylsterols (14). Interestingly, this enzyme had higher affinity for both lanosterol and 24-methylenelanosterol than for zymosterol, highlighting another unique feature of *P. carinii* biology.

Various mechanisms have been described for the development of fungal resistance to azole compounds, including an increased production of the lanosterol 14 α -demethylase enzyme, the development of mutations in the form of amino acid substitutions in Erg11p that alter the substrate-specific binding of the enzyme, and the active cellular efflux of these azole compounds by multidrug-resistant transporter proteins (9, 10, 15). In an example of the first mechanism, a global upregulation of genes in the sterol biosynthesis

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Abbreviations: enzyme lanosterol 14 α -demethylase, Erg11; *Pneumocystis carinii*, PC; *P. carinii* pneumonia, PCP; polymerase chain reaction, PCR; *Saccharomyces cerevisiae*, SC; substrate recognition site-1, SRS-1.

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pathway in response to azole treatment has been described in *Candida albicans*, with *ERG11* transcription increasing up to 12-fold (16). Numerous amino-acid substitutions in Erg11p proteins have been found in fungi that are resistant to azole antifungal medications. Many of these amino-acid substitutions occur in "hot spot" regions that likely affect binding of the azole to the enzyme, thereby preventing inhibition of the enzyme activity. Two such regions have been described, clustered in amino acids 105 to 165, and from 266 to 287 (17). Additional single amino acid mutations have been reported to have a significant effect on azole resistance, such as the R467K mutation in *C. albicans* (15).

We sought to determine if *P. carinii* contains a lanosterol 14 α -demethylase enzyme as part of the sterol biosynthesis pathway. Here we show the identification of the *PCERG11* gene and describe nucleotide substitutions in *PCERG11* that are also present in azole-resistant fungi and might contribute to azole resistance in *P. carinii*. We mutated two amino acids in *PCERG11* to those found in azole-sensitive *Candida* to generate *PCERG11*-SDM (E113D, T125K). We examined the sensitivity of *PCERG11* and *PCERG11*-SDM to azole compounds by using a growth inhibition assay in a heterologous fungal system as described by Ma and coworkers (18).

Materials and Methods

Materials

All reagents were from Sigma Chemical (St. Louis, MO) unless otherwise specified. Restriction endonucleases, *Taq* and *Pfx* polymerases were from Invitrogen (Carlsbad, CA).

Preparation of *P. carinii* Organisms

P. carinii pneumonia was induced in Harlan Sprague-Dawley rats by immunosuppression with dexamethasone, as previously reported (19–23). Lungs from moribund rats were minced in Hanks' balanced salt solution and homogenized in a Tissue Blender (Stomacher; Tekmar, Cincinnati, OH) for 10 min. *P. carinii* were purified from host lung cells by filtration through a 10- μ m filter (Millipore, Billerica, MA). *P. carinii* organisms were confirmed by Wright-Giemsa staining, and samples containing contaminating bacterial or fungal organisms were discarded.

Cloning of *PCERG11*

A degenerate polymerase chain reaction (PCR)-based cloning strategy was used as previously described (19, 20, 23, 24). We designed degenerate oligonucleotide PCR primers after aligning the amino acid sequences of fungal lanosterol 14 α -demethylase enzymes. PCR was performed in a 50- μ l reaction using 1 μ M of each degenerate primer (5'-HLTPVFG, 5'-CAT(T/C)T(A/T/C/G)AC(A/T)AC(A/T)CC(A/T)GT(A/T)TT(T/C)G-3', and 3'-GRHRCIGE, 5'-TC(A/T)CC(A/T)ATACA(A/T/C/G)C(G/T)ATG(A/T/C/G)C(G/T)(A/T)CC-3' with 200 ng of purified *P. carinii* DNA as the template. An initial hot start at 94°C for 5 min was followed by 30 cycles of 94°C for 30 s, 56°C for 60 s, 72°C for 60 s, with a final 72°C extension for 15 min. A single 1,100-bp amplicon was identified by electrophoresis on 1% agarose after ethidium bromide staining, and was subcloned into the pGEM T-Easy vector (Promega) and sequenced. Rapid amplification of cDNA ends (RACE) was used to clone the 5' and 3' ends of the gene using

the GeneRacer Kit (Invitrogen). Briefly, 5 μ g of *P. carinii* total RNA was isolated with Trizol Reagent (Invitrogen) as described, capped at the 5' end with an oligonucleotide following the manufacturer's instructions, and reverse-transcribed to cDNA with an oligonucleotide linked to the 3' end of the gene. From the sequence of the original degenerate PCR product we generated a *P. carinii*-specific 5' RACE primer (5'-CGCCAGGTGTCAGCTCAGTTGATG-3') and the 3' RACE primer (5'-ATCCTTTCCAAAAATCGGGCTAGTTTC-3'). RACE reactions in the 5' and 3' directions were performed in separate tubes using 1 μ l of the above cDNA as a template with 0.2 μ M of GeneRacer primers and *PCERG11* primers. A modified touchdown PCR technique was used to increase specificity. After an initial hot start at 94°C for 5 min, the following cycles were performed: 5 cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 60 s; 5 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 60 s; 20 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s; and a final 72°C extension for 15 min. A single 700-bp amplicon from the 5' RACE reaction and a single 500-bp amplicon from the 3' RACE reaction were identified by electrophoresis on 2% agarose after ethidium bromide staining, and both amplicons were subcloned into the pGEM T-Easy vector (Promega) and sequenced. From these sequences we generated PCR primers for the open-reading frame for *PCERG11* and used PCR with the proofreading polymerase *Pfx* to amplify the entire gene from *P. carinii* cDNA. PCR using 0.2 μ M of each of the sense primer (5'-ATGGGATTTTTAAAGACTATTCTT-3') and the antisense primer (5'-TCTTTTTTGTCTTCTTCTCCATTC-3') was performed for 25 cycles, followed by the addition of 1 U of *Taq* polymerase and subsequent ligation into pYES2.1 TOPO TA (Invitrogen). The pYES2.1/*PCERG11* clone was completely sequenced to verify the correct orientation of *PCERG11* to allow expression from the yeast *GALI* promoter and to ensure that PCR errors did not occur. *S. cerevisiae* *ERG11* was cloned by PCR from *S. cerevisiae* DNA using *Pfx* and was ligated into pYES2.1 TOPO TA using PCR primers sense (5'-ATGCTGCTACCAAGTCAATCGTTGGA-3') and antisense (5'-GATCTTTTGTCTGGATTTCTCTTTT-3').

Site-Directed Mutagenesis

To create *PCERG11*-SMD, we mutated E113 to D and T125 to K using a PCR-based method, the QuikChange Multi Site-Directed Mutagenesis (Stratagene, La Jolla, CA). First, we generated a *PCERG11* sense primer 5'-ATCAGCAGAAGATGCATATACTCATCTGACAACACCCGTTTTTGGTAAAG-ATGTCGTTT-3' and an antisense primer 5'-AAACGACATCTTTACCAAAAACGGGTGTTG-TCAGATGAGTATATGCATCTTCTGCTGAT-3' to create the desired dual mutations. These oligonucleotides were annealed to *PCERG11* in the pYES2.1 plasmid and the mutations were generated by PCR following the instructions by the manufacturer. After mutagenesis, the entire plasmid was sequenced to verify that the correct mutations were generated.

Chromosomal and Northern Hybridizations

To verify that the PCR product amplified from *P. carinii* nucleic acids was of *P. carinii* origin and was not a product of rat or other microorganism contamination, the 1,100-bp PCR amplicon was hybridized to a blot containing *P. carinii* chromosomes separated by contour-clamped homogenous field electrophoresis (CHEF) (22). The 1,100-bp PCR amplicon was labeled with (α -³²P)dATP by the random primer method (Rediprimer System, Amersham Pharmacia Biotech, Piscataway, NJ) and hybridized to the nitrocellulose membrane under high stringency conditions. Hybridization was examined by autoradiography. Expression of *PCERG11* was

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60
ATGGGATTTTTAAAGACTATTCTTAAAGTATTAGGAATAGAAAATTTTCACTCAGTTTT
M G F L K T I L K V L G I E N F T L S F
120
GTAATAATCATCTTAACATTTTTTTTCTCTACATATTTTCCCTCAATTATACCAAAAA
V I I I L T F F F L L H I F P Q L Y Q K
180
GATTCGTCAAACCTCCTGTAGTATTCATTGGCTTCCTTTATTGGATCAACTATTCAA
D S S K P P V V F H W L P F I G S T I Q
240
TATGGCATGGACCCGTATAAGTTTTTCAAACAACAAAAAACAATGGAAATATTTTT
Y G M D P Y K F F Q K Q K K K H G N I F
300
ACTTTTATTCTACTTGGAAAAAATGACAGTAGCATTAGGACCTAAAGGCAATGATATA
T F I L L G K K M T V A L G P K G N D I
360
CTTTTAAATGAAAAATTATCAAGTTTATCAGCAGAAGAAGCATATACTCATCTGACAACA
L F N G K L S S L S A E E A Y T H L T T
420
CCCGTTTTGGTACAGATGCTGTTTATGATGTTTCCTAATCATGTTAATGGAACAGAAA
P V F G T D V V Y D V P N H V L M E Q K
480
AAATTGTAAAACTGGTTTTACTATAGAAACATTTAGAGCATATGTTCCATTGATTTAT
K F V K T G F T I E T F R A Y V P L I I
540
GAAGAAGTAAAAACGTAAGTACTAGAACTAGCCGATTTTGGAAAGGATAAACTCTCTG
E E V K T Y L E T S P I F G K D K L S G
600
GTTTCATCTTAAATGAAAGCTTTCCTGAAATAACAATTTTACTGCATCTCGTACTCTT
V S S L M K A L P E I T I F T A S R T L
660
CAAGGAAAAGAAGTACCGAGTAATTTGACGCATCATTTGCAAAGCTTTATCATGACTTA
Q G K E V R S N F D A S F A K L Y H D L
720
GATGGGGATTACTCCAATTAACCTTCTAGCTCCTGGTTACCACTTCCAAAAATCGT
D G G F T P I N F L A P W L P L P K N R
780
CTACGTGATGCTCTCAAAAAAATGGCACAATTTATATGAATATATAAGCAAAGA
L R D A A Q K K M A Q I Y M N I I K Q R
840
AGAAAAACATGCCAATGAAGAAAAGGATATGATTGGAATCTTATGAATCAACATTAC
R K T C Q H E E K D M I W N L M N Q H Y
900
AAAGATGGCCGTAATTAACAGATAAAGAAATGCTCATTAAATGATTGCTATATTAATG
K D G R K L T D K M A Q I Y M N I I A I L M
960
GCTGGCCAGCATACCAGTGCAGCGACAGGATGTTGGGCTTTTATACATCTGCAGAAAAG
A G Q H T S A A T G C W A L L H L A E K
1020
CCAGAGTATATTAAGCTATGCTTGGAGAACAAAAAGAGTATTGGAGACAACCTTAGAC
P E Y I K L L L E E Q K R V F G D N L D
1080
GATCTAATATGACAATCTTAAGGATATGGAATTTGTTCTTATGTAATAAAAGAACT
D L T Y D N L K D M E L L S Y V I K E T
1140
CTAAGATTACATCTCTCTCATAGTATTATAAGAAAAGTCAAATCACCGATTCTTAATA
L R L H P P L H S I I R K V K S P I L I
1200
GAAACTCACCTCATTGTTCTCAAAAATCATTATTTGTTAGCAGCGCCAGGTGTCAGC
E N S P Y I V P K N H Y L L A A P G V S
1260
TCAGTTGATGAAGAATACTTTGAAAATGCATTAGAATTTATACCTGAAAGATGGAATGC
S V D E E Y F E N A I E H L M I P E R W K C
1320
GAAAAAATACAGAAAGATCCGATAAAAATGATTACGGATATGGTTTACTACTAAAGGA
E K N T E D S D K I D Y G Y G L V T K G
1380
GCATTTCCGCCCTATTTGCCATTTGGTCAGGAGACATAGATGTTGGGAGAGCAATTT
A F L P F G A G R H R C I G E Q F
1440
GCATACATGCAACTTGGTACAATTATTACAATATTTGTTTCATGAATAGAATGGACTTTA
A Y M Q L G T I I T I F V H E L E W T L
1500
CCAAAAATCAAATTACTATTCCAAAGCCTGACTACACTAGCATGGTTGATTACAGAA
P K N Q I T I P K P D Y T S M V V L P E
1545
AGACCTTCTAACATTGAATGGAGAAGAAGACAAAAAGATAA
R P S N I E W R R R Q K R STOP
    
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Figure 1. Sequence and cDNA translation of *PCERG11*. *PCERG11* cDNA has an open-reading frame of 1,542 bp. Translation of the cDNA yields a protein of 59.2 kD comprised of 514 amino acids. GenBank Accession number AY228706.

verified by Northern analysis. RNA was extracted from *P. carinii* or uninfected rat lung with Trizol Reagent, and 5.0 µg was separated by electrophoresis through 1.2% agarose in the presence of 2.2 M formaldehyde and transferred to nitrocellulose. The *PCERG11* probe was hybridized to the membrane and visualized by autoradiography. The membrane was re-probed with *P. carinii* actin to confirm equal RNA loading (22).

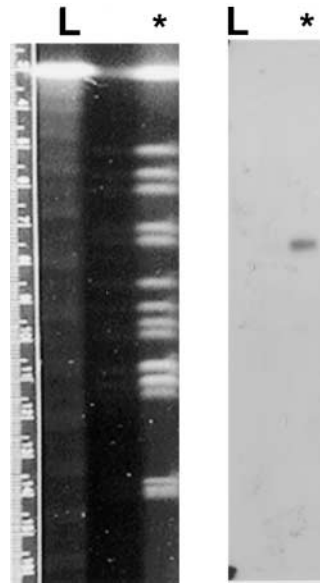


Figure 2. Chromosomal location of *PCERG11*. The *PCERG11* gene was labeled and hybridized to a blot containing *P. carinii* chromosomes separated by contour-clamped homogenous field electrophoreses (CHEF). L indicates the MW ladder, and asterisk indicates the chromosomal location of *PCERG11*.

Yeast Strains

The diploid *S. cerevisiae* yeast *MATa*/α *his3-1/his3-1*, *leu2-0/leu2-0*, *ura3-0/ura3-0*, *lys2-0/LYS2*, *MET15/met15-0*, *ERG11::kanMX4/ERG11* used in this study was obtained from ATCC. These yeast were grown overnight in YEPD with 200 mg/L G418 at 30°C to an OD₆₀₀ of 1.0 and were transformed with the plasmids pYES2.1/*PCERG11*, pYES2.1/*PCERG11-SDM*, or pYES2.1/*SCERG11* using the lithium acetate method (25-27). Diploid transformants were grown on glucose-containing minimal media lacking uracil at 30°C. Segregation of the *MATa* haploid yeast (which lack the *S. cerevisiae* *ERG11* and contain the pYES2.1/*PCERG11*, pYES2.1/*PCERG11-SDM*, or pYES2.1/*SCERG11*) was performed by sporulation and random spore analysis. Briefly, yeast were grown to saturation (OD₆₀₀ > 2.5) at 30°C, pelleted, washed, and resuspended in sporulation media (1% potassium acetate, 0.1% yeast extract, 0.05% galactose). These cultures were grown for 3 d at 30°C, pelleted and washed, and treated with zymolyase 20T (ICN). This suspension was sonicated on ice to separate spores, then plated on galactose-containing minimal media lacking uracil and methionine. We confirmed the correct haplotype of the yeast (*MATa*) by multiplex PCR (28, 29). The *MATa* haplotype segregates with the *SCERG11* deletion. PCR confirmed that the wild-type *SCERG11* is not present in any of the clones tested. Yeast colonies were dissolved in standard PCR buffer and 1 µM of primers SCIVS (5'-AGTCACATCAAGATCGTTTATGG-3'), SC α (5'-GCACGGAATATGGGACTACTTCG-3'), and SCa (5'-ACTCCACTTCAAGTAAGAGTTTG-3') were added. After an initial hot start at 94°C for 5 min, 30 cycles were performed at 94°C for 15 s, 58°C for 15 s, 72°C for 30 s, and a final 72°C extension for 15 min. Reactions were identified on a 2% agarose gel with ethidium bromide staining. We selected those colonies that gave a single 544-bp PCR product (haploid *MATa*) for further growth and analysis. *MATa* generates a single 404-bp product, and *MATa*/*MATα* diploids generate both products (28, 29).

Expression of Recombinant Proteins and Immunoblotting

The *MATa* haplotype of *S. cerevisiae* yeast containing pYES2.1/*PCERG11*, pYES2.1/*PCERG11-SDM*, or pYES2.1/*SCERG11* were grown in 2% galactose minimal media lacking uracil and methio-

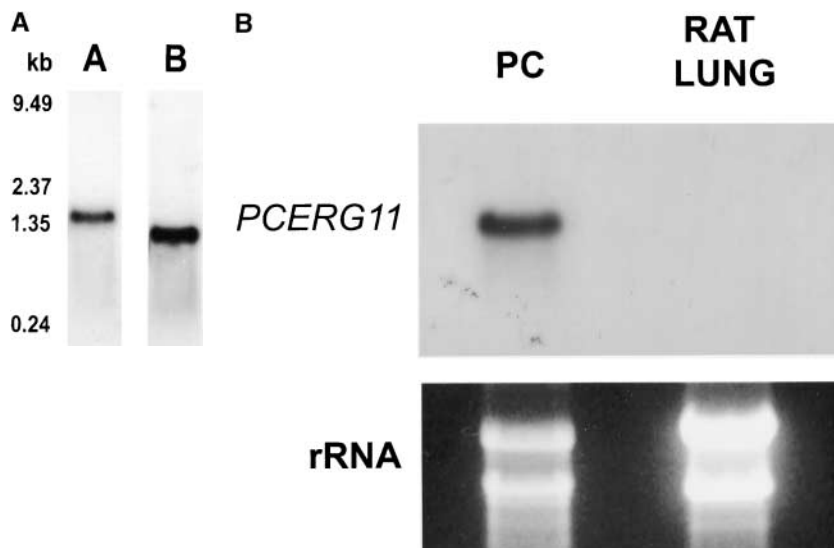


Figure 3. mRNA expression of *PCERG11*. As shown in A, lane A, *PCERG11* hybridizes to RNA from *P. carinii* organisms. The observed transcript size is 1,600 bp. Lane B is the same blot which has been re-probed with *P. carinii* actin as a control. (B) *PCERG11* specifically hybridizes to *P. carinii* RNA and not to rat lung RNA (5 μ g total RNA each), confirming that the observed transcript is from *P. carinii*.

nine at 30°C. The cells were harvested at mid-log phase and lysed in YPER reagent (Pierce, Rockford, IL) containing an inhibitor cocktail (1 μ g/ml each of leupeptin, aprotinin, and pepstatin; 1 mM each of phenylmethylsulfonyl fluoride and sodium orthovanadate; and 50 mM sodium fluoride) for 20 min at room temperature. The suspension was clarified by centrifugation at 13,000 \times g for 10 min, and the protein concentration of the lysates was determined spectrophotometrically using the BCA method (Pierce). The protein lysates were used for immunoblotting. Proteins were separated by SDS-PAGE, and transferred to nitrocellulose membranes. Non-specific binding sites were blocked with Tris-buffered saline containing 5% milk and 0.05% Tween, then the membranes were incubated with anti-V5 horseradish peroxidase-conjugated antibody (Invitrogen) for 1 h and immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Growth Inhibition Assays to Azole Compounds

Following the procedure described by Ma, Jia, and Kovacs (18), 5 \times 10⁵ yeast cells (*S. cerevisiae* *MATa* haplotypes expressing pYES2.1/*PCERG11*, pYES2.1/*PCERG11-SDM*, or pYES2.1/*SCERG11*) were inoculated into 15-ml polypropylene tubes con-

taining dilutions of azoles and were grown at 35°C (18) in 1 ml galactose minimal media at 230 rpm. Growth inhibition was quantified by spectrophotometric analysis for each yeast suspension tube by measuring the OD₆₀₀. Control tubes (same yeast culture without the azole) were grown alongside the suspensions, which received azoles and were processed identically at 48 h. Experiments for each strain and azole dilution were performed in quadruplicate from four separate yeast transformants with appropriate controls. The minimal dose of azole compound that produced at least a 50% reduction in yeast growth compared with its control tube suspension was designated inhibitory growth of 50%, or IG₅₀ (18).

Results

Pneumocystis carinii* Contains a Gene with Homology to Lanosterol C14 Demethylase, *PCERG11

We cloned the *PCERG11* gene using a combined method of degenerate PCR and RACE. *PCERG11* cDNA has a

TABLE 1
Sequence comparison of the lanosterol 14 α -demethylases in *C. albicans*, fluconazole resistant (FR) *C. albicans*, and *P. carinii*

Amino Acid Number	<i>C. albicans</i>	FR <i>C. albicans</i>	<i>P. carinii</i>
116	D	E	E
128	K	T	T
129	G	A	D
149	A	V	G
153	D	E	E
266	E	D	K
267	R	H	T
278	D	E	W
279	S	F	N
405	S	F	A
437	V	I	K
449	F	L	Y
465	G	S	A

Amino acid substitutions in bold are exact matches between FR *C. albicans* and *P. carinii*. Amino acid numbering corresponds to *Candida*.

<i>A. fumigatis</i>	AE EV YSPLTTPVFG R HVVYD
<i>C. albicans</i>	AEDAYKHLTTPVFGKGVYD
<i>C. albicans</i> FR	AEE AY KHLTTPVFG T GVYD
<i>C. glabrata</i>	AEAAYSHLTPVFGKGVYD
Human	AEDVYSRLTTPVFGKGVAYD
<i>P. carinii</i>	AEE AY THLTPVFG T DVVYD
Rat	AEE EV YGRLLTPVFGKGVAYD
<i>S. cerevisiae</i>	AEAAYAHLLTPVFGKGVYD
<i>S. pombe</i>	AEE AY SHLTPVFGKDVVYD

Figure 4. The dual mutations, D116E and K128T (**bold**), have been found to be important for fluconazole resistance in *C. albicans*. *P. carinii* Erg11p has the identical two mutations. The region shown is the substrate recognition site one (SRS-1). FR indicates fluconazole resistant. These two amino acids were mutated back to those found in azole-sensitive *Candida* to generate *PCERG11-SDM* (E113D, T125K).

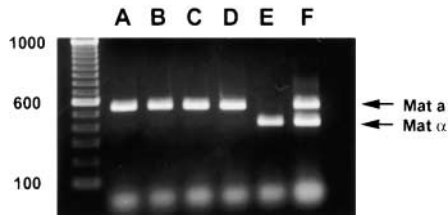


Figure 5. Multiplex PCR of yeast mutants containing *PCERG11*, *PCERG11-SDM*, or *SCERG11*. Segregation of the correct haplotype (*MATa*) was verified by multiplex PCR. Lane A (*PCERG11*), lane B (*SCERG11*), and lane C (*PCERG11-SDM*) have a single 544-bp product indicating the *MATa* locus. Lane D is the *MATa* control yeast. Lane E is a *MATα* control yeast, which generates a 404-bp product, and lane F is the original diploid yeast, which generates both bands (*MATa*/*MATα*).

single open-reading frame of 1542-bp (GenBank Accession number AY228706). The translated open-reading frame of *PCERG11* is a 59.2-kD protein with 514 amino acids, a size typical of other Erg11 proteins (Figure 1). We confirmed that *PCERG11* is part of the *P. carinii* genome by hybridizing it to separated *P. carinii* chromosomes (Figure 2). Expression of *PCERG11* was detected as a 1,600-bp transcript by northern analysis (Figure 3).

***PCERG11* Contains Regions that Are Also Present in Azole-Resistant Fungi and Might Contribute to Azole Resistance**

Comparison of the translated open-reading frame of *PCERG11* with fungal Erg11p proteins revealed the presence of dual amino acids thought to confer resistance to azole medications (Figure 4). In the region of the Erg11 protein called substrate recognition site-1 (SRS-1), the D116E and K128T mutations confer fluconazole resistance in *Candida albicans* (30, 31). *P. carinii* Erg11 has both of these identical amino acid substitutions in position 113 and 125. The T315A mutation, which is also important in *Candida* for fluconazole resistance (32), is not present in the *P. carinii* Erg11. In an analysis of potential sites of amino acids substitutions in *Candida* Erg11, thirty sites were found in fluconazole-resistant *Candida* strains, but not in fluconazole-sensitive strains (17).

When we align *P. carinii* Erg11 with both *Candida*-sensitive and -resistant strains as reported by Marichal and coworkers, we find that *P. carinii* Erg11 has 13 amino acids which are potential candidates

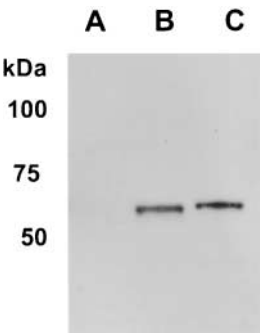


Figure 6. Detection of recombinant proteins from yeast containing *PCERG11* or wild-type *SCERG11*. Following confirmation of the correct haplotype segregation (*MATa*), we detected proper protein production of these yeast after induction with galactose. Lane B (*PCERG11*) and lane C (*SCERG11*) were cloned in frame with a C-terminal V5 epitope tag, which is detected by enhanced chemiluminescence (dilution 1:10,000). The tag adds ~5 kD to the predicted weight, therefore the tagged PC

Erg11 is 64.2 kD and SC Erg11 is 66 kD. Lane A is the yeast strain without either plasmid used as a control for the V5 antibody.

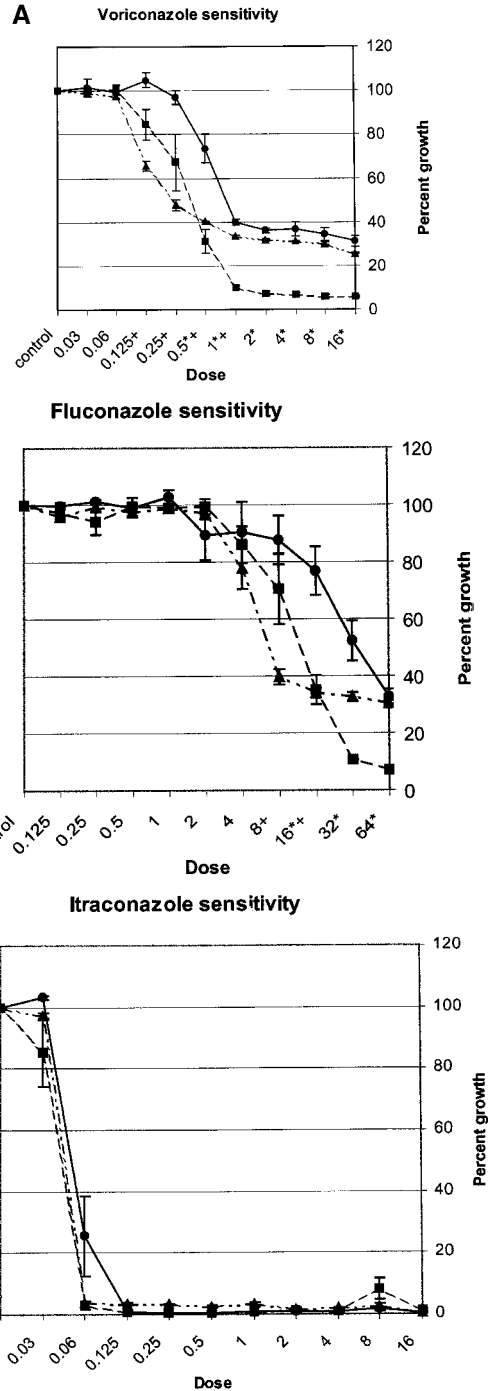


Figure 7. Growth inhibition curves of *S. cerevisiae* expressing *PCERG11* (circles), *PCERG11-SDM* (triangles), or wild-type *SCERG11* (squares) with various doses of voriconazole (A), fluconazole (B), and itraconazole (C). Drug concentrations are in μg/ml. Each data point represents the mean of four individual cultures. Error bars represent standard deviation. **P* < 0.05, PC versus SC; +*P* < 0.05, PC versus PC SDM.

TABLE 2
IG₅₀ values for three azole antifungals in *S. cerevisiae* mutants expressing PCERG11, PCERG11-SDM, or wild-type SCERG11

Antifungal	PC ERG11	SC ERG11	PC ERG11-SDM	P Value	
				PC versus SC	PC versus PC-SDM
Voriconazole	1, 1, 1, 1 (1)	0.5, 0.5, 0.25, 0.5 (0.44 \pm 0.13)	0.5, 0.5, 0.25, 0.25 (0.375 \pm 0.1)	0.008	0.003
Fluconazole	64, 32, 32, 64 (42.7 \pm 18.5)	16, 16, 8, 8 (12 \pm 4.6)	8, 8, 8, 8 (8)	0.032	0.023
Itraconazole	0.06, 0.06, 0.06, 0.125 (0.08 \pm 0.03)	0.06, 0.06, 0.06, 0.06 (0.06)	0.06, 0.06, 0.06, 0.06 (0.06)	0.391	0.391

Parentheses show mean values \pm SD. *PCERG11* sensitivity to voriconazole and fluconazole is statistically significantly reduced compared to *SCERG11* (bold values), however sensitivity to itraconazole was identical. *PCERG11-SDM* has statistically significantly increased sensitivity to voriconazole and fluconazole, but not itraconazole.

for contributing to azole resistance (Table 1). Three amino acids are identical in these positions (D116E, K128T, and D153E) and may be more significant. The others may represent polymorphisms between the two different fungal organisms.

PCERG11 Confers Resistance to Fluconazole and Voriconazole, but Sensitivity to Itraconazole

After transforming the *S. cerevisiae* *SCERG11* deletion strains with *PCERG11*, *PCERG11-SDM*, and wild-type *SCERG11*, we confirmed that we had segregated the correct haplotype by growth on selection media (lacking uracil and methionine) and by multiplex PCR (Figure 5). These yeast have their intrinsic *ERG11* gene deleted and contain *PCERG11*, *PCERG11-SDM*, or wild-type *SCERG11* on the yeast expression plasmid pYES2.1. Additionally, we left off the stop codons for each gene to allow a C-terminal fusion with the V5 epitope tag. We then confirmed protein expression in these strains by immunoblotting each with the epitope-tagged V5 antibody after each strain was grown to identical cell numbers (OD₆₀₀ = 0.8) in galactose minimal media (Figures 6B and 6C). These yeast were used in the subsequent growth inhibition assays. We also used the *S. cerevisiae* *SCERG11* deletion strain without any plasmid as a control for the western blot (Figure 6A). *S. cerevisiae* *SCERG11* deletion strains with *PCERG11* required a higher dose of voriconazole (1.0 μ g/ml versus 0.44 \pm 0.13 μ g/ml, P = 0.0028) and fluconazole (42.7 \pm 18.5 μ g/ml versus 12.0 \pm 4.6 μ g/ml, P = 0.0324) to inhibit their growth by 50% compared with *S. cerevisiae* *SC ERG11* deletion strains expressing wild-type *SC ERG11* (Figure 7 and Table 2). No difference was seen in their respective susceptibility patterns to itraconazole (0.08 \pm 0.03 μ g/ml versus 0.06 μ g/ml, respectively, P = 0.39). The *PCERG11-SDM*, containing E113D, T125K, were generated by PCR to revert these amino acids in *PCERG11* to those found in azole-sensitive *Candida*. *PCERG11-SDM* has increased sensitivity to voriconazole and fluconazole, but caused no difference in sensitivity to itraconazole compared with both wild-type *PCERG11* or *SCERG11*.

Discussion

We have identified and cloned a gene from *P. carinii* which has significant homology to fungal Erg11 proteins. Greatest

homology is to *S. pombe* sterol 14 α -demethylase (59%). It has an open-reading frame of 1,542 bp. *PCERG11* mRNA was detected in *P. carinii* as a single 1,600-bp transcript. Comparison of the translated cDNA to fungal Erg11 proteins demonstrates common amino acid sequences that have been shown in resistant fungi to be important for azole resistance. In an area described as SRS-1, the dual mutations, D116E and K128T, have been shown to confer resistance to fluconazole in *C. albicans* (30, 31). *P. carinii* Erg11 has the same two amino acid substitutions in positions 113 and 125 (Figure 4). The *PCERG11-SDM*, where the mutations E113D and T125K were made to revert these amino acids in *PCERG11* to those found in azole-sensitive *Candida*, resulted in increases sensitivity to voriconazole and fluconazole. This finding highly supports the molecular structure of *PCERG11* in conferring resistance to fluconazole and voriconazole. In some strains of fluconazole-resistant *Candida*, the T315A mutation has been noted to cause fluconazole resistance by reduced affinity to the enzyme (32). The *P. carinii* Erg11 has the threonine in this position. *A. fumigatis* has a T315I mutation, which is postulated to lead to the intrinsic fluconazole resistance of this fungus (33). Multiple other areas of *P. carinii* Erg11 align to amino acid substitutions in fluconazole-resistant *Candida*, but only three are identical (Table 1). The others may represent variability attributed to the difference among fungi.

The enzymatic pathways of sterol synthesis in *P. carinii* remain to be elucidated. There might be alternative pathways to final sterol products even if Erg11 is completely inhibited. Nakayama and colleagues reported that in a *C. glabrata* mutant in which *ERG11* expression was placed under the control of a tetracycline-regulatable promoter, the colony-forming units of these mutants were lowered by only 1/10 with doxycycline treatment (34). Other authors have described that strains of *C. albicans* that are resistant to azole antifungals might shunt lanosterol through a 24-transmethylation pathway before the 14-demethylation step (30). It has also been reported that strains of *C. glabrata* can rescue a defect in sterol biosynthesis by incorporating cholesterol from serum (35). This is an interesting concept, because it is believed that *P. carinii* scavenges cholesterol from the host during lung infection (13). Also, inactivation of *ERG3*, an enzyme that acts above 14-demethylase in the

ergosterol pathway, results in an altered sterol biosynthetic pathway with increased fluconazole resistance (36–38).

Other factors could also be important for resistance of *P. carinii* to azoles. Induction of multidrug resistance pumps occurs in fungi receiving azole medication. In patients with AIDS, it has been noted that under the influence of therapy with fluconazole, resistant isolates of *Candida* frequently emerge. These isolates have been shown to have an overexpression of multidrug resistance genes (10). Additionally, upregulation of *ERG* genes has also been reported as a mechanism of azole resistance (16). Any of these mechanisms could contribute an important role in the overall resistance to azoles and other antifungals in *P. carinii*. Clearly further investigations into the sterol pathway in *P. carinii* might lead to a better understanding of how this organism metabolizes these products, and might lead to the development of medications which would be highly effective against *P. carinii*.

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