

Immunosuppressive and Nonimmunosuppressive Cyclosporine Analogs Are Toxic to the Opportunistic Fungal Pathogen *Cryptococcus neoformans* via Cyclophilin-Dependent Inhibition of Calcineurin

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Received 24 June 1999/Returned for modification 18 August 1999/Accepted 25 October 1999

Cyclosporine (CsA) is an immunosuppressive and antimicrobial drug which, in complex with cyclophilin A, inhibits the protein phosphatase calcineurin. We recently found that *Cryptococcus neoformans* growth is resistant to CsA at 24°C but sensitive at 37°C and that calcineurin is required for growth at 37°C and pathogenicity. Here CsA analogs were screened for toxicity against *C. neoformans* in vitro. In most cases, antifungal activity was correlated with cyclophilin A binding in vitro and inhibition of the mixed-lymphocyte reaction and interleukin 2 production in cell culture. Two unusual nonimmunosuppressive CsA derivatives, (γ -OH) MeLeu⁴-Cs (211-810) and D-Sar (α -SMe)³ Val²-DH-Cs (209-825), which are also toxic to *C. neoformans* were identified. These CsA analogs inhibit *C. neoformans* via fungal cyclophilin A and calcineurin homologs. Our findings identify calcineurin as a novel antifungal drug target and suggest nonimmunosuppressive CsA analogs warrant investigation as antifungal agents.

Cryptococcus neoformans is a basidiomycetous opportunistic fungal pathogen that causes systemic mycosis in patients with immunosuppression as a result of chemotherapy, immune system dysfunction, solid-organ transplantation, or infection with the human immunodeficiency virus. Primary infections begin in the lung following inhalation of the infectious propagule and then spread hematogenously to the brain where severe meningoencephalitis develops (10, 12, 35, 40). Two primary antifungal therapies are available, amphotericin B and fluconazole, which target the fungal membrane sterol ergosterol. However, amphotericin B has a number of adverse serious side effects, fluconazole is fungistatic, and drug-resistant mutants are arising in *Candida* species and in *C. neoformans* (3, 49, 61, 62). Therefore, there is a need to find new antifungal agents that are more fungicidal and less toxic for the treatment of cryptococcal meningitis and that have different mechanisms of action for use in combination drug therapies.

Cyclosporine (CsA) is an immunosuppressant that inhibits signal transduction events required for T-cell activation following antigen presentation (reviewed in references 8, 9, 23, and 55). CsA enters the cell by diffusion and associates with an intracellular receptor, cyclophilin A, which belongs to a family of proteins that catalyze *cis-trans* peptidyl-prolyl isomerization, a rate-limiting step in protein folding (for reviews, see references 17, 23, and 53). CsA binds to the active site of cyclophilin and potently inhibits prolyl isomerase activity. However, immunosuppression is not related to the inhibition of this enzyme activity. The target of the cyclophilin A-CsA complex is a Ca²⁺-calmodulin-dependent serine-threonine-specific protein

phosphatase, calcineurin (27, 36, 37). In T cells responding to antigen presentation, an increase in intracellular Ca²⁺ activates calcineurin, which subsequently dephosphorylates a transcription factor, NF-AT, allowing nuclear import and expression of T-cell activation genes (11, 18, 30, 45, 46, 56).

CsA is a natural product of a soil fungus and exhibits potent antimicrobial activities (reviewed in reference 6). Previous studies in the yeast *Saccharomyces cerevisiae* reveal that the mechanisms of CsA immunosuppressive and antifungal action are essentially identical (2, 7, 19, 24–26, 43). CsA binds to *S. cerevisiae* cyclophilin A (21), which shares 65% identity with the human homolog, to form a protein-drug complex that inhibits the *S. cerevisiae* calcineurin homolog (2, 5, 7, 13–15, 20, 26, 43, 58).

It has been suggested that the antimicrobial activities of CsA might have clinical applicability (28). For instance, CsA is toxic to the pathogenic fungus *Coccidioides immitis* (34), to *Neurospora crassa* (60), and to *Aspergillus niger* (34). However, in contrast to *S. cerevisiae*, little is known about the mechanism(s) of drug action in these other fungi. We have previously reported that CsA is markedly toxic to the opportunistic fungal pathogen *C. neoformans* at 37°C but not at 24°C in vitro (47). By gene disruption, we demonstrated that calcineurin, the target of the cyclophilin A-CsA complex, is required for growth at 37°C and virulence of *C. neoformans* (47).

These observations suggest that drugs which inhibit calcineurin should similarly prevent *C. neoformans* infections in vivo. Indeed, CsA can protect mice from cryptococcal pneumonia (41). However, because in previous studies CsA also exacerbated cryptococcal meningitis in mice and rabbits because of potent immunosuppressive activity (42, 50), we sought to identify nonimmunosuppressive CsA analogs that retain antifungal activity. We report here an analysis of a collection of CsA analogs with alterations in the effector domain of the

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drug, which interacts with calcineurin. Antifungal activity is generally correlated with binding to human cyclophilin A in vitro and to immunosuppressive activity in vivo, supporting a model in which the cyclophilin A-CsA complex is toxic to *C. neoformans* by inhibiting calcineurin. More importantly, we identify two nonimmunosuppressive CsA analogs that retain antifungal activity in vitro. Our studies reveal that these analogs inhibit *C. neoformans* growth via cyclophilin A-dependent inhibition of calcineurin. We suggest that these drugs take advantage of structural differences between host and fungal cyclophilin A and calcineurin to inhibit fungal growth but spare immune system function. Our studies suggest further examination of CsA analogs as potential novel antifungal agents is warranted.

MATERIALS AND METHODS

Strains and compounds. The pathogenic serotype A strain H99 has been previously described (59). *C. neoformans* T1 and 89-610 are azole-resistant strains and were kindly provided by Mahmoud Ghannoum (University of California at Los Angeles-Harbor) and John Graybill (University of Texas Health Science Center at San Antonio), respectively. CsA and analogs were prepared at Novartis (Basel, Switzerland). Compounds were dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml and stored frozen at -20°C .

In vitro susceptibility testing. Experiments for determination of MICs were performed by the broth macrodilution method following the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) (44). The only difference from the standardized method was the choice of drug dilutions, which ranged from 100 to 0.09 $\mu\text{g/ml}$. The fungal growth inhibition assay was performed with drug concentrations of 0.01, 0.1, 1, and 10 $\mu\text{g/ml}$. Drug dilutions and inoculum preparation were done, using the NCCLS criteria (44). Optical density at 600 nm (OD_{600}) was measured with a Beckman spectrophotometer following incubation for 72 h at 24, 30, and 37°C . The MIC was defined as the lowest drug concentration in which a visual turbidity less than or equal to 80% inhibition compared to that produced by the growth control tube was observed. The minimum fungicidal concentration (MFC) was determined as previously described (16). Briefly, 10- μl aliquots from tubes with growth inhibition were plated onto Sabouraud agar plates. The lowest drug concentration that yielded three or fewer *C. neoformans* colonies was recorded as the MFC.

Preparation of protein extracts and [^3H]CsA LH-20 drug binding assays. Protein extracts were prepared from 100 OD_{600} units of cell pellet by glass bead homogenization in 1 ml of lysis buffer (150 mM Tris [pH 7.5], 10 mM MgCl_2 , 1 mM dithiothreitol, 10% [vol/vol] glycerol, 1 mM phenylmethylsulfonyl fluoride, 3 μg of benzamide per ml, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml) with eight 1-min bursts with cooling on ice. Extracts were centrifuged at 12,000 rpm for 10 min at 4°C in a Sorvall Microfuge MC12, and the supernatants were transferred to fresh tubes. Protein content was quantified by the Bradford assay, and extracts were stored frozen at -80°C .

LH-20 drug binding assays were performed as previously described (22). His₆-tagged *S. cerevisiae* cyclophilin A protein was overexpressed and affinity purified on Ni^{2+} -nitriloacetic acid-resin (Qiagen) as previously described (4). [^3H]CsA was obtained from Amersham (specific activity, 7.0 Ci/mmol). LH-20 assays were performed with ~ 5 μg of purified *S. cerevisiae* cyclophilin A protein and with 180 μg of *C. neoformans* total protein.

Enzyme immunoassays. Human recombinant cyclophilin A protein (CYP) was expressed in *Escherichia coli*, purified, and biotinylated as previously described (54). The solid-phase enzyme immunoassays (enzyme-linked immunosorbent assay) were performed as previously described (52, 54). Briefly, a D-Lys-CsA derivative was coupled to bovine serum albumin (BSA) and used to coat the wells of polyvinyl microtiter plates (2 $\mu\text{g/ml}$ in phosphate-buffered saline [PBS] [pH 7.4] for 2 h at 37°C). After saturation of the plate with PBS containing 2% BSA (1 h at 37°C) and washing once with PBS containing 0.05% Tween 20 and three times with PBS, CYP-biotin was incubated overnight at 4°C (in PBS containing 1% BSA). Bound CYP-biotin was detected with streptavidin coupled to alkaline phosphatase (Jackson Immunoresearch Labs, Inc.) (1:6,000 in PBS containing 1% BSA for 2 h at 37°C). The absorbance at 405 nm was measured after hydrolysis of *p*-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer [pH 9.6] for 1 to 2 h at 37°C). In the competitive assays, the CsA derivatives (1 mg/ml in ethanol) were immediately added to the CYP solutions (at 1:100 dilution, 0.83×10^{-5} M) and further 10-fold dilutions were made directly in the microtiter plate. After incubation overnight at 4°C , unbound CYP was removed and the assay was calculated as the percent inhibition of the control reaction between CYP and coated CsA in the absence of inhibitor (10 replicates per plate). The 50% inhibitory concentrations for the CsA derivatives were compared with the 50% inhibitory concentration for CsA run in triplicate on each microtiter plate.

Mouse MLR. The mixed-lymphocyte reaction (MLR) was performed essentially as previously described (38, 57). Equal amounts of spleen cells from CBA and BALB/c mice were mixed and incubated (2×10^5 cells per well) with appropriate serial dilutions of compounds in 200 μl of serum-free CG medium

(Bioreba, Basel, Switzerland) in flat-bottom tissue culture microtiter plates at 37°C in 5% CO_2 . After 4 days, 1 μCi of [^3H]thymidine (2 Ci/mmol) was added to each well and the plates were subsequently incubated for an additional 16 h. Cells were harvested on filter paper and counted in a β -counter. Background values (low control, proliferation of BALB/c cells alone) were subtracted from all values. Proliferation of mixed cells without any compound was taken as 100% proliferation.

IL-2 reporter gene assay. The interleukin 2 (IL-2) reporter gene assay was performed as previously described (1). Briefly, the *E. coli* gene *lacZ* (reporter gene), which encodes the enzyme β -galactosidase, was placed under the transcriptional control of the human IL-2 promoter and stably transfected into the human T-cell line Jurkat. Assay plates (96-well microtiter plates) were prepared containing the compounds at appropriate dilutions. Transfected Jurkat cells (5×10^4 per well) which were stimulated with phorbol myristate acetate and phytohemagglutinin were added. The plates were incubated for 16 h at 37°C in 5% CO_2 . The IL-2 promoter-driven β -galactosidase expression, which was quantified by measuring the fluorescence of its cleaved substrate, correlates with IL-2 expression and is a direct readout of IL-2 gene transcription.

Cloning, expression, purification, and binding of intein-cryptococcal cyclophilin A to chitin beads. The cloned cryptococcal *CPA1* gene encoding the cyclophilin A homolog (P. Wang and J. Heitman, unpublished data) was PCR amplified with two flanking oligonucleotides, 5'-GGGAATCCATATGTCCCAA GTTACTTTGACATTGCCATTAAC (1189) and 5'-CAGTCAGCTCTTCC GCAGACAGTGCCGGAGGAGCATGGTATCTTGGC, cleaved with *NdeI* and *SapI*, cloned into the IMPACT I (for intein-mediated purification with affinity chitin-binding tag) system expression vector pCYB1 (New England Biolabs) and confirmed by DNA sequence analysis. Cells expressing the intein-tagged cryptococcal cyclophilin A protein were grown at 37°C overnight in a 3-ml culture of FB medium (63) supplemented with 200 μg of ampicillin per ml. A portion (1 ml) of this culture was pelleted and resuspended in 700 μl FB medium with ampicillin and grown at 37°C to an OD_{600} of 0.6. At this point, 2 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the culture and incubation continued for 4 h at 37°C . Cells were collected by centrifugation at 10,000 rpm for 10 min in a Sorvall GS-3 rotor and resuspended in 20 ml of ice-cold lysis buffer (20 mM Tris [pH 7.5], 0.1 mM EDTA, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride) and 500 mM KCl. Cells were lysed by sonication eight times for 1 min each time, with periods of cooling between. The cell lysate was clarified by centrifugation at 40,000 rpm for 45 min in a Beckman Ti-70 rotor. Chitin beads (1 ml) (preequilibrated in lysis buffer) were added to the cell lysates, and the suspension was stirred gently for 1 h at 4°C . The bead-cell extract mix was loaded onto an econocolumn (Bio-Rad) and washed three times, first with 150 ml of lysis buffer containing 500 mM NaCl, second with 50 ml of lysis buffer containing 1 M NaCl, and third with 50 ml of lysis buffer (without Triton X-100) with 100 mM KCl. Intein-cyclophilin A-chitin beads were resuspended in 1 ml of lysis buffer (without Triton X-100) and stored at 4°C in 0.2% NaN_3 .

Cryptococcal cyclophilin A-calcineurin interactions in vitro. Cyclophilin A bound to chitin beads described above was used for cyclophilin-calcineurin binding assays. Incubation mixtures contained 800 μl of cryptococcal cell extract (4 mg of protein) and 40 μl of cyclophilin A-chitin beads (50% [vol/vol] suspension). CsA and CsA analogs D-Sar (α -SMe)³ Val²-DH-Cs (209-825) and (γ -OH) MeLeu²-Cs (211-810) were added where indicated to a final concentration of 100 μM . The binding mixtures were incubated at 4°C on a nutator shaker for 2 h. The chitin beads were collected by centrifugation for 10 s and washed four times with lysis buffer (described above). Bound proteins were eluted from the beads by boiling for 4 min in 30 μl of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer, fractionated on SDS-12.5% polyacrylamide gels, and transferred to a nitrocellulose membrane. The membrane was blocked overnight in block-wash buffer (10 mM imidazole [pH 7.3], 100 mM KCl, 5 mM CaCl_2 , 5% BSA, 0.05% Tween 20, 0.02% NaN_3), transferred to fresh buffer containing 10^6 cpm of [^{125}I]-calmodulin, and incubated at room temperature for 2 h with gentle agitation. The membrane was washed twice in block-wash buffer, air dried, and exposed to film overnight at -80°C .

Site-directed mutagenesis of *C. neoformans* calcineurin A. Two *C. neoformans* calcineurin mutant strains were created by transformation of the wild-type serotype A strain H99 with mutant alleles of the calcineurin A *CNA1* gene. The *C. neoformans* *CNA1-1* (Val344Arg) and *CNA1-2* (Val344Lys) alleles were created by PCR overlap mutagenesis (29), using genomic DNA as the template. The first round of PCR was performed, using primer 5'-GTTAGAGTATCGATCAAGG TAGTTAGGTG (1006) with flanking primer 5'-GTGATTTCACCTATATCC TGCATC (1003) and 5'-CTACCTTGATCGATACTCTAACAAAGGCCGCTG (1005) with flanking primer 5'-GAGTTAGCGACCAATGGAGTGTGACG (1004) for the *CNA1-1* (Val344Arg) allele (mutations in boldface). To construct the *CNA1-2* (Val344Lys) allele, PCR was performed, using primer 5'-GTTAG AGTACTTATCAAGGTAGTTAGGTG (1008) with flanking primer 1003 and 5'-CTACCTTGATAAGTACTCTAACAAAGGCCGCTG (1007) with flanking primer 1004. First-round PCR overlap products were gel purified and used as template for the second-round PCR with flanking primers 1003 and 1004. The PCR protocol was as follows: an initial step of 10 min at 96°C ; 35 cycles of PCR, with 1 cycle consisting of 30 s at 95°C , 30 s at 55°C , and 2 min at 72°C ; and a final step of 5 min at 72°C . The resulting PCR product was cloned into the TA vector (Invitrogen) and confirmed by sequencing. The second-round-confirmed PCR fragments were used to transform wild-type strain H99 by the biolistic method

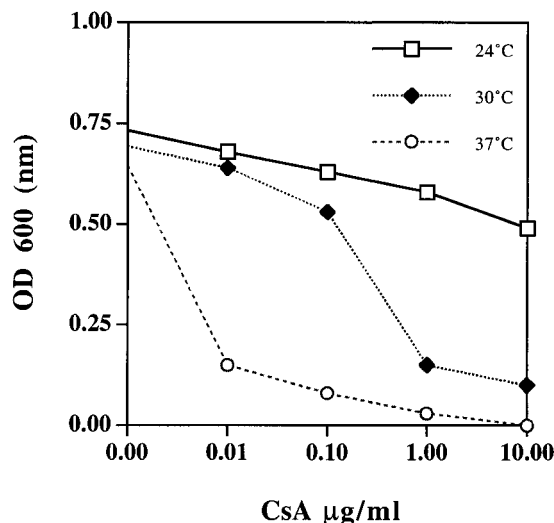


FIG. 1. CsA is toxic to *C. neoformans* at 37°C but not at 24°C. *C. neoformans* H99 was grown in RPMI 1640 medium with the indicated concentrations of CsA for 72 h at 24, 30, and 37°C. Growth was assayed by determining the OD₆₀₀, and three independent experiments were done.

(59). Transformants were first grown on plates containing 1 M sorbitol for 8 h to allow phenotypic expression, and CsA-resistant colonies were selected on YPD medium containing CsA (100 µg/ml) at 37°C. Colonies that were CsA resistant and FK506 sensitive contained the *CNA1-1* (MCC11 strain) or *CNA1-2* (MCC12 strain) allele and could be readily distinguished from spontaneous CsA-resistant mutants, which were all cross-resistant to FK506 (1 µg/ml) at 37°C.

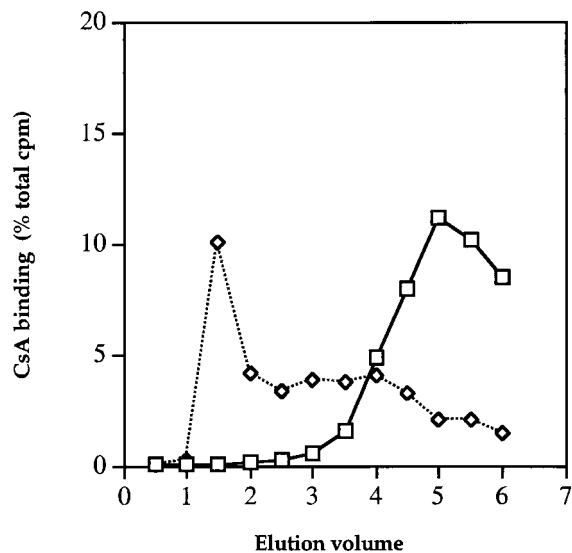
RESULTS

CsA is toxic to *C. neoformans* at 37°C but not at 24°C. We previously reported that the immunosuppressive drugs CsA and FK506 are toxic to *C. neoformans* when grown at 37°C but not at 24°C in vitro (47). In these experiments, CsA and FK506 toxicity was determined by growth on solid YPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar) containing these compounds. We extended these observations, using the NCCLS standardized growth inhibition criteria (44) for assessing antifungal activity in *C. neoformans*. In this assay, cells of *C. neoformans* were cultured in RPMI 1640 medium for 72 h with increasing concentrations of CsA and growth was assessed by measuring OD₆₀₀. Representative CsA growth inhibition curves for the pathogenic *C. neoformans* serotype A strain H99 are presented in Fig. 1 for cultures incubated at 24, 30, and 37°C. CsA toxicity was also readily detectable in the standardized in vitro assay, with MICs of 0.39 µg/ml at 37°C and 12.5 µg/ml at 30°C and MFCs of 0.78 µg/ml at 37°C and >100 µg/ml at 30°C. In contrast, no growth inhibition was observed at 24°C (data not shown), even at drug concentrations up to 1,000-fold greater than the MIC at 37°C. These observations are in accord with our previous findings and confirm that CsA is toxic to *C. neoformans* in vitro at elevated growth temperatures.

***C. neoformans* expresses CsA binding activity.** Previous studies with the yeast *S. cerevisiae* and with T lymphocytes have established that CsA binds to the CsA-binding protein cyclophilin A to form an active drug-protein complex. To determine whether CsA could exert its toxic effects in *C. neoformans* via a similar mechanism, protein extracts were prepared from *C. neoformans* H99 and assayed for CsA-binding protein. We employed the standard LH-20 drug binding assay, in which [³H]CsA is mixed with protein extract and then chromatographed through Sephadex LH-20, a hydrophobic matrix that

retards the mobility of free CsA compared to that of the cyclophilin-CsA complexes (22). As shown in Fig. 2A, whereas free [³H]CsA elutes as a late peak in the LH-20 drug binding assay, [³H]CsA incubated with purified *S. cerevisiae* cyclophilin A protein eluted primarily as an early peak attributable to a cyclophilin A-CsA complex, with some free [³H]CsA eluting as a less-pronounced later peak. Similarly, [³H]CsA incubated with *C. neoformans* protein extract eluted as both an early cyclophilin-³H]CsA complex and a late peak of free [³H]CsA

A



B

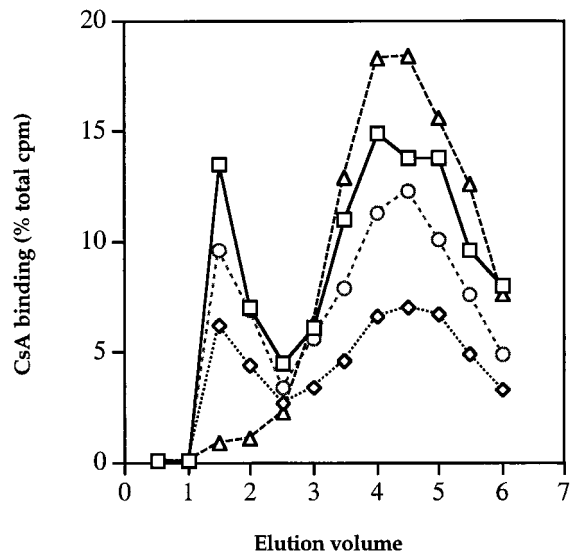


FIG. 2. *C. neoformans* expresses [³H]CsA binding activities. CsA-binding protein activity was measured by the LH-20 drug binding assay. (A) Tritiated CsA alone (squares) or incubated with purified *S. cerevisiae* cyclophilin A protein (diamonds) was subjected to Sephadex LH-20 chromatography. Fractions were analyzed by scintillation counting, and the percentages of total counts per minute were plotted. (B) Protein extracts from the *C. neoformans* wild-type *CPA1 CPA2* strain H99 (squares), *cpa1* mutant strain PW67 (diamonds), *cpa2* mutant strain PW71 (circles), and *cpa1 cpa2* mutant strain PW62 (triangles) were subjected to Sephadex LH-20 chromatography. Fractions were analyzed by scintillation counting, and the percentages of total counts per minute were plotted. Elution volume is given in milliliters.

TABLE 1. Cyclophilin A binding and inhibition of MLR and IL-2 production by CsA analogs^a

CsA derivative ^b	MLR	IL-2	CYP A binding
CsA	100	100	100
(γ -OH) MeLeu ⁴ -Cs (211-810)	0.8	0.9	100
D-Sar (α -SMe) ³ Val ² -DH-Cs (209-825)	10	55	200
Allo-Thr-2-Cs	9	11	20
Norvaline-2-Cs	48	37	20
D-Ala(3-acetylamino)-8-Cs	55	33	125
Thr-2-Cs	59	100	71
D-MeSer-3-Cs	77	42	333
D-Ser(O-CH ₂ CH ₂ -OH)-8-Cs	100	100	200
D-Ser-8-Cs	139	167	167

^a The ability of a series of CsA analogs to inhibit an MLR assay, IL-2 production, or cyclophilin A (CYP A) binding was compared to that of CsA and is expressed here as percentage of activity relative to CsA activity.

^b In CsA derivatives, CsA is shown as Cs.

in the LH-20 drug binding assay (Fig. 2B). Extracts were also analyzed for isogenic mutant strains lacking the cyclophilin A protein CPA1 (*cpa1*), CPA2 (*cpa2*), or both (*cpa1 cpa2*). CsA binding was reduced to ~50% of the wild-type level in extracts containing CPA2 and lacking CPA1, to ~80% of the wild-type level in extracts containing CPA1 and lacking CPA2, and to ~9% in extracts lacking both CPA1 and CPA2 (Fig. 2B). These observations provide evidence that *C. neoformans* expresses two CsA-binding forms of the cyclophilin A protein and that the CPA1 protein contributes more of the total CsA binding activity than the CPA2 form of cyclophilin A. CsA toxicity in *C. neoformans* could be mediated by these cyclophilin-CsA complexes, as is the case in other organisms.

Nonimmunosuppressive CsA analogs have altered binding to human cyclophilin A and reduced ability to inhibit calcineurin in T cells. In previous studies, CsA was found to exacerbate *C. neoformans* infections in the immunosuppressed rabbit model of cryptococcal meningitis (50). Thus, the potent immunosuppressive effects of CsA outweigh any antifungal effect in this animal model of central nervous system infection. We therefore assessed the activity of a series of CsA analogs that are modified on the cyclophilin binding and calcineurin effector surfaces of CsA. The activities of these CsA analogs were compared to that of CsA in binding to human cyclophilin A and in two bioassays: inhibition of IL-2 gene expression (1) and inhibition of the MLR (38, 57). These bioassays measure cyclophilin-dependent inhibition of calcineurin function by CsA. Several CsA analogs have increased or reduced binding to cyclophilin A and reduced immunosuppressive activity as a result of these structural modifications (Table 1). Interestingly, one derivative [(γ -OH) MeLeu⁴-Cs (211-810)] exhibited markedly reduced (~100-fold) immunosuppressive activity. The structure of CsA is depicted in Fig. 3. The upper portion of the CsA ring interacts with cyclophilin A, whereas the lower portion of the ring is responsible for calcineurin binding. In the 211-810 analog, a hydroxyl group has been introduced onto the surface of CsA that interacts with calcineurin.

Nonimmunosuppressive CsA analogs are toxic to *C. neoformans*. We next assessed the antifungal activity of these CsA analogs in the serotype A *C. neoformans* H99 strain, using the NCCLS standardized criteria. A number of immunosuppressive CsA analogs potentially inhibited the growth of strain H99 at 37°C (Table 2) but not at 30°C (data not shown). In general, the immunosuppressive activity correlated well with antifungal activity, indicating that the ability to interact with cyclophilin A and effectively inhibit calcineurin likely accounts for the antifungal activity of these CsA analogs. Importantly, two CsA

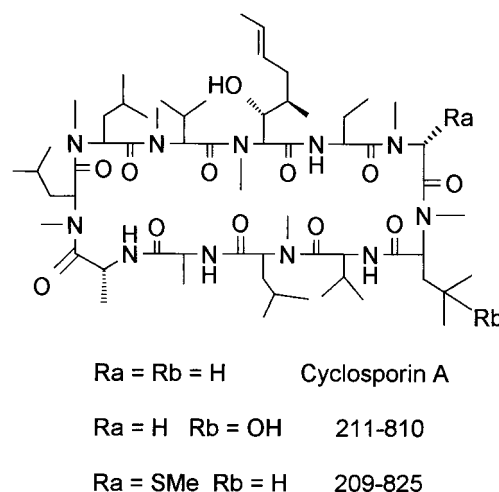


FIG. 3. Structures of CsA and CsA analogs 211-810 and 209-825. The structures of CsA and two nonimmunosuppressive CsA analogs are depicted. The upper half of the CsA ring binds cyclophilin A, and the effector surface of CsA that is exposed to the solvent in the cyclophilin A-CsA complex and which interacts with calcineurin is the lower half of the ring.

analog whose immunosuppressive activity is reduced by 10- to 100-fold compared to that of CsA in the MLR assay retained potent antifungal activity against *C. neoformans*. These two nonimmunosuppressive CsA analogs were toxic to *C. neoformans* at 37°C but not at 30°C (Tables 2 and 3), suggesting that calcineurin is their common target. These analogs, (γ -OH) MeLeu⁴-Cs (211-810) and D-Sar (α -SMe)³ Val²-DH-Cs (209-825), have substitutions on the effector surface of CsA (Fig. 3), which is exposed to the solvent in the cyclophilin A-CsA complex and interacts with calcineurin (31-33, 39, 51). Alterations of this effector surface are known to reduce the affinity of the cyclophilin A-CsA complex for mammalian calcineurin, resulting in reduced or abolished immunosuppressive activity (36).

Calcineurin A mutations confer resistance to CsA and nonimmunosuppressive CsA analogs. By site-directed PCR overlap mutagenesis, we introduced mutations into the *CNA1* gene encoding the calcineurin A catalytic subunit in the region proposed to contain the cyclophilin A-CsA binding site. In

TABLE 2. CsA and CsA analogs are toxic to fluconazole-sensitive and resistant *C. neoformans* isolates^a

CsA derivative ^b or drug	<i>C. neoformans</i> H99		<i>C. neoformans</i> T1		<i>C. neoformans</i> 89-610	
	MIC	MFC	MIC	MFC	MIC	MFC
CsA	0.39	3.12	0.78	25	0.78	12.5
(γ -OH) MeLeu ⁴ -Cs (211-810)	0.78	1.56	0.39	6.25	0.78	6.25
D-Sar (α -SMe) ³ Val ² -DH-Cs (209-825)	0.78	0.78	0.19	1.56	0.39	0.78
aThr-2-Cs	1.56	25	≤0.09	25	0.78	12.5
Nva-2-Cs	0.39	25	1.56	>100	0.39	>12.5
D-Ala(3-acetylamino)-8-Cs	0.78	6.25	≤0.09	12.5	0.39	6.25
Thr-2-Cs	0.78	1.56	≤0.09	>12.5	0.39	1.56
D-MeSer-3-Cs	0.78	1.56	0.78	3.12	0.39	3.12
D-Ser(O-CH ₂ CH ₂ -OH)-8-Cs	0.78	3.12	<0.09	25	0.39	6.25
D-Ser-8-Cs	0.78	1.56	0.39	>12.5	0.78	6.25
Fluconazole	1	8	4	64	32	>64

^a All MICs and MFCs are given in micrograms per milliliter.

^b In CsA derivatives, CsA is shown as Cs.

TABLE 3. Calcineurin and cyclophilin A mutations confer resistance to CsA and CsA analogs^a

Strain	Genotype	CsA				209-825				211-810			
		30°C		37°C		30°C		37°C		30°C		37°C	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
H99	<i>CNA1 CPA1 CPA2</i>	12.5	>100	0.39	0.78	>100	–	0.78	1.56	>100	–	1.56	25
PW26	<i>CPA1 cpa1::ADE2</i>	>100	–	50	>100	>100	–	50	>100	>100	–	1.56	>50
PW47	<i>cpa1 cpa2</i>	>100	–	>100	–	>100	–	>100	–	>100	–	>100	–
PW48	<i>cpa1 cpa2</i>	>100	–	>100	–	>100	–	>100	–	>100	–	>100	–
MCC11	<i>CNA1-1</i>	>100	–	>100	–	>100	–	>100	–	>100	–	>100	–
MCC12	<i>CNA1-2</i>	>100	–	>100	–	>100	–	>100	–	>100	–	>100	–

^a All MICs and MFCs given in micrograms per milliliter. –, not determined.

previous studies, these mutations were found to confer CsA resistance in the yeast *S. cerevisiae* by preventing cyclophilin A-CsA binding to calcineurin (7). We found that *C. neoformans* MCC11 and MCC12, which express the Val344Arg and Val344Lys calcineurin A mutant proteins, respectively, were resistant to CsA (Table 3), but not to FK506 (data not shown). In addition, these calcineurin A mutant strains are also resistant to the nonimmunosuppressive CsA analogs D-Sar (α -SMe)³ Val²-DH-Cs (209-825) and (γ -OH) MeLeu⁴-Cs (211-810), demonstrating that these CsA analogs inhibit the fungal calcineurin homolog by a mechanism similar to that of CsA (Table 3).

Cyclophilin A mediates antifungal activity of CsA and non-immunosuppressive CsA analogs. In recent studies we have identified the genes encoding cyclophilin A homologs in *C. neoformans* and generated mutants with reduced or no cyclophilin A expression (P. Wang and J. Heitman, unpublished data). Because cyclophilin A mediates CsA action in *S. cerevisiae* and T lymphocytes, we tested whether cyclophilin A also mediates CsA antifungal effects in *C. neoformans*. The MIC of CsA was increased from 0.78 μ g/ml at 37°C in the cyclophilin A-expressing wild-type strain H99 to >50 μ g/ml at 37°C in strain PW26 in which cyclophilin A expression is reduced but not abolished as a consequence of transgene-induced gene repression (Wang and Heitman, unpublished) (Table 3). In addition, strain PW26 was resistant to the toxic effects of the CsA analog D-Sar (α -SMe)³ Val²-DH-Cs (209-825) but, most interestingly, was not resistant to the CsA analog (γ -OH) MeLeu⁴-Cs (211-810). These observations confirm that cyclophilin A mediates the action of both CsA and the CsA analog D-Sar (α -SMe)³ Val²-DH-Cs (209-825) in *C. neoformans* and suggest that the CsA analog (γ -OH) MeLeu⁴-Cs (211-810) has an unusual mechanism of action or can still inhibit calcineurin when the levels of cyclophilin A are reduced (see below).

Recently we have obtained mutant strains PW47 and PW48 in which two genes encoding cyclophilin A homologs (*CPA1* and *CPA2*) have been disrupted by homologous recombination. Both of these mutants lack all cyclophilin A protein (Fig. 2 and data not shown) and are completely resistant to the toxic effects of CsA and both the 209-825 and 211-810 CsA analogs (Table 3). These findings demonstrate that cyclophilin A mediates the toxic effects of both CsA and the nonimmunosuppressive CsA analogs.

Nonimmunosuppressive CsA analogs promote cryptococcal cyclophilin-calcineurin complexes in vitro. We used affinity chromatography to test whether CsA and the two CsA analogs with reduced immunosuppressive activity mediate cryptococcal cyclophilin A-calcineurin interactions in vitro. Purified cryptococcal cyclophilin A bound to chitin beads was incubated with cryptococcal cell extract in the presence or absence of CsA and the D-Sar (α -SMe)³ Val²-DH-Cs (209-825) and (γ -OH) Me-

Leu⁴-Cs (211-810) analogs. Proteins associated with the cyclophilin A-drug complex were eluted, fractionated on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The calcineurin A catalytic subunit was detected by its ability to associate with ¹²⁵I-calmodulin in an overlay blot. Because cell extracts used for the affinity chromatography contained equal amounts of calcineurin, we were able to compare the affinity of each cyclophilin A-drug complex for cryptococcal calcineurin.

As expected, CsA promoted cyclophilin A binding to cryptococcal calcineurin (Fig. 4). In comparison, the cyclophilin A-calcineurin interaction was stimulated to an even greater extent by the CsA analog (γ -OH) MeLeu⁴-Cs (211-810) (Fig. 4). This finding provides a molecular explanation as to why the (γ -OH) MeLeu⁴-Cs (211-810) analog retains antifungal activity even in strains with reduced expression of cyclophilin A: namely, that the increased affinity of the cyclophilin A-analog complex for calcineurin mitigates the effects of reduced cyclophilin A levels in mutant strain PW26. The CsA analog D-Sar (α -SMe)³ Val²-DH-Cs (209-825) also promoted binding to calcineurin A (Fig. 4A). Similar results were obtained when serotype D strains were used as the source of protein extracts used for the affinity chromatography (Fig. 4B). Taken together, these findings indicate CsA and nonimmunosuppressive CsA analogs promote cyclophilin A binding to calcineurin.

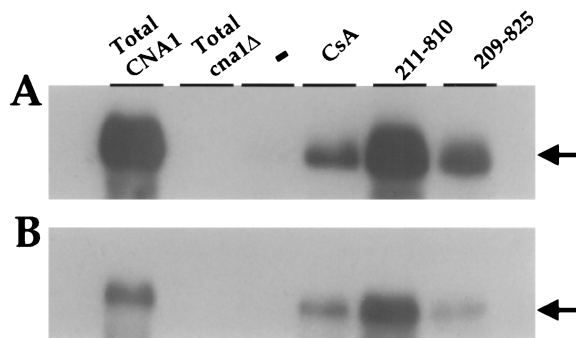


FIG. 4. CsA and CsA analogs promote cryptococcal cyclophilin A binding to calcineurin. Binding assays with purified cryptococcal cyclophilin A immobilized on chitin beads were performed with equal amounts of protein extract from serotype A strain H99 (A) and serotype D strain JEC21 (B) in the absence (–) or presence of 100 μ M CsA and CsA analogs (γ -OH) MeLeu⁴-Cs (211-810) and D-Sar (α -SMe)³ Val²-DH-Cs (209-825). Bound proteins were eluted and fractionated by SDS-polyacrylamide gel electrophoresis, and the calcineurin A catalytic subunit was detected by an overlay blot with ¹²⁵I-calmodulin. The migration position of the CNA1 protein is indicated by an arrow. Total protein extracts from serotype A and D *CNA1* wild-type and *cna1* mutant strains served as controls for the identity and electrophoretic mobility of the calcineurin A catalytic subunit CNA1.

Fluconazole-resistant *C. neoformans* clinical isolates are sensitive to immunosuppressive and nonimmunosuppressive CsA analogs. We assessed the action of CsA and CsA analogs against two fluconazole-resistant isolates of *C. neoformans* obtained from AIDS patients. As shown in Table 2, both CsA and several CsA analogs exhibited antifungal activity against fluconazole-resistant clinical isolates T1 and 89-610. These observations confirm that CsA and its analogs retain activity against *C. neoformans* clinical isolates which have decreased susceptibility to azole compounds.

DISCUSSION

C. neoformans is a fungal pathogen of increasing importance, given its worldwide distribution, common occurrence in AIDS and other immunosuppressed patients, and the appearance of azole-resistant strains (10). Because current antifungal agents have a number of undesirable adverse side effects and poor fungicidal properties and because drug-resistant strains are emerging, the identification of novel antifungal drug targets and inhibitors is of significant clinical importance and may allow us to further exploit the use of combination antifungal therapy in the future.

We previously discovered that two immunosuppressive compounds, CsA and FK506, are toxic to *C. neoformans* at 37°C but not at 24°C in vitro (47). In addition, we found that *C. neoformans* mutants lacking the target of CsA and FK506, calcineurin, exhibited temperature-sensitive growth in vitro and were nonpathogenic in vivo. These observations identify calcineurin as a novel antifungal drug target.

In previous studies, Mody and coworkers found that CsA protected mice from extraneural cryptococcal infections and reported that CsA was toxic to *C. neoformans* under some in vitro conditions (41, 42). In contrast, Perfect and Durack found no evidence for CsA toxicity in vitro (50). Our findings that CsA toxicity is temperature dependent resolved the earlier discrepancies over CsA toxicity against *C. neoformans* in vitro, as the studies of Mody et al. were performed at 35°C and those of Perfect and Durack were conducted at 30°C. In both mice and rabbits, CsA exacerbated cryptococcal meningitis (42, 50), so the immunosuppressive activity of CsA outweighs any antifungal effect. In addition, CsA does not cross the blood-brain barrier, which likely also diminishes effectiveness against cryptococcal meningitis. Although complicated by steroid use, organ transplant recipients receiving CsA are similarly at risk for development of cryptococcal infection. These observations prompted us to identify and study nonimmunosuppressive CsA analogs to determine whether there was a difference in drug interactions between the mammalian and fungal target proteins which could be exploited to develop antifungal compounds.

Here we have assessed the antifungal activities of CsA analogs with diminished or absent immunosuppressive activities. In general, the immunosuppressive activity of this series of analogs correlated well with antifungal action, providing additional support for our model that CsA toxicity results from calcineurin inhibition by a cyclophilin A-CsA complex. In addition, we found two CsA analogs, (γ -OH) MeLeu⁴-Cs (211-810) and D-Sar (α -SMe)³ Val²-DH-Cs (209-825), that have dramatically decreased immunosuppressive activity but retain potent antifungal activity against *C. neoformans*. In related studies, we have also identified a nonimmunosuppressive FK506 analog, L-685,818, which similarly retains antifungal activity (48). Here we establish that CsA and the nonimmunosuppressive analogs are toxic to *C. neoformans* via cyclophilin A-dependent inhibition of calcineurin. First, we show that the compounds are toxic at 37°C and not 30°C, which correlates

with the temperature-dependent growth of calcineurin mutant strains. Second, we show that mutations on the cyclophilin A-CsA binding surface of calcineurin (Val344Arg and Val344Lys) confer resistance to CsA and CsA analogs. Third, reduction in the level of the cyclophilin A protein confers resistance to CsA and the D-Sar (α -SMe)³ Val²-DH-Cs (209-825) analog. Fourth, two mutant strains completely lacking the cyclophilin A protein are resistant to CsA and both CsA analogs (Table 3). Finally, we present biochemical evidence that CsA and the CsA analogs D-Sar (α -SMe)³ Val²-DH-Cs (209-825) and (γ -OH) MeLeu⁴-Cs (211-810) promote cyclophilin A binding to fungal calcineurin. Most interestingly, the affinity of the cyclophilin A-(γ -OH) MeLeu⁴-Cs (211-810) analog complex for calcineurin is increased relative to that of cyclophilin A-CsA, and this analog is toxic to *C. neoformans*. These findings suggest that the γ -hydroxy substitution on the leucine 4 residue on the effector surface of CsA prevents binding to mammalian calcineurin but increases binding to the fungal calcineurin homolog.

We suggest that these CsA nonimmunosuppressive analogs take advantage of subtle inherent structural differences between host and fungal cyclophilin A and possibly also the calcineurin A catalytic and calcineurin B regulatory subunits, such that host immune function is spared but fungal growth is inhibited. Elucidation of the X-ray structure of the cyclophilin A-CsA-calcineurin complex and cloning and sequencing of the fungal calcineurin B subunit should allow further structural insights into these mechanisms of drug and drug analog action. It remains to be tested whether these nonimmunosuppressive CsA analogs will have therapeutic effects in animal models of cryptococcal meningitis, possibly in conjunction with agents that permeabilize the blood-brain barrier, but we suggest that these and other calcineurin inhibitors warrant consideration as novel antifungal agents in further studies.

ACKNOWLEDGMENTS

We thank Tamara Breuder, Dena Toffaletti, and Lora Cavallo for technical assistance. We are indebted to Tony Means and Elizabeth MacDougall for generously providing ¹²⁵I-calmodulin.

This work was supported in part by RO1 grants AI39115, AI41937, and AI42159 from NIAID and PO1 grant AI44975 from NIAID to the Duke University Mycology Research Unit. Joseph Heitman is an associate investigator of the Howard Hughes Medical Institute and a Burroughs Wellcome Scholar in Molecular Pathogenic Mycology.

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