A Calcineurin-Independent Mechanism of Angiogenesis Inhibition by a Nonimmunosuppressive Cyclosporin A Analog[§]

Benjamin A. Nacev, Woon-Kai Low,¹ Zhennian Huang, Tina T. Su,² Zhuang Su, Hisham Alkuraya, Dan Kasuga, Woong Sun, Mario Träger, Manfred Braun, Gunter Fischer, Kang Zhang, and Jun O. Liu

Department of Pharmacology and Molecular Sciences (B.A.N., W.-K.L., J.O.L.), Medical Scientist Training Program (B.A.N.), and Department of Oncology (J.O.L.), The Johns Hopkins University School of Medicine, Baltimore, Maryland; S & T Global Inc., Woburn, Massachusetts (Z.H., T.T.S., Z.S.); Shiley Eye Center and Institute for Genomic Medicine, University of California at San Diego, La Jolla, California (H.A., D.K., W.S., K.Z.); Max Planck Research Unit for Enzymology of Protein Folding, Saale, Germany (M.T., G.F.); and Department of Chemistry, University of Dusseldorf, Dusseldorf, Germany (M.B.)

Received February 21, 2011; accepted May 5, 2011

ABSTRACT

Cyclosporin A (CsA) is a widely used immunosuppressant drug. Its immunosuppressive activity occurs through the inhibition of the protein phosphatase calcineurin via formation of a ternary complex with cyclophilin A (CypA). CsA also inhibits endothelial cell proliferation and angiogenesis. This has been thought to occur through calcineurin inhibition as well. However, CsA is also a potent inhibitor of cyclophilins, a class of prolyl isomerases. Because calcineurin inhibition requires binding, and therefore inhibition of CypA, the relative contributions of calcineurin and cyclophilin inhibition in antiangiogenesis have not been addressed. We have taken a chemical biology approach

to explore this question by dissociating the two activities of CsA at the molecular level. We have identified a nonimmunosuppressive analog of CsA that does not inhibit calcineurin but maintains inhibition of endothelial cell proliferation and in vivo angiogenesis. The same analog also maintains inhibition of all cyclophilin isoforms tested. We also show that a second, structurally distinct, cyclophilin inhibitor is sufficient to block endothelial cell proliferation. These results suggest that the inhibition of cyclophilins may play a larger role in the antiangiogenic activity of CsA than previously believed, and that cyclophilins may be potential antiangiogenic drug targets.

Introduction

Cyclosporin A (CsA) is a powerful immunosuppressant drug prescribed in the context of solid organ transplantation.

This work was supported in part by the National Institutes of Health National Cancer Institute [Grant R01-CA122814]; the National Institutes of Health National Center for Research Resources [Grant UL1-RR025005]; the Flight Attendant Medical Research Institute Fund; the Keck Foundation; the Walsh Prostate Cancer Fund; the Commonwealth Foundation; the National Institutes of Health Roadmap for Medical Research (to J.O.L.); the National Institutes of Health Medical Scientist Training Program [Grant T32GM07309] (to B.A.N.); Deutsche Forschungsgemeinschaft [Grants SFB 610, GRK 1026] (to G.F.); the National Eye Institute; a VA Merit Award; and the Burroughs Wellcome Fund (K.Z.).

¹ Current affiliation: Department of Pharmaceutical Sciences, College of Pharmacy, St. John's University, Queens, New York.

² Current affiliation: Philips Academy, Andover, Massachusetts.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

doi:10.1124/jpet.111.180851.

[§] The online version of this article (available at <http://jpet.aspetjournals.org>) contains supplemental material.

Originally isolated from the fungus *Cylindrocarpon lucidum*, CsA is an undecapeptide that potently inhibits T cell activation (Borel et al., 1976). It has been recognized for two decades that, in addition to this well characterized and clinically relevant immunosuppressive property, CsA possesses a second potentially useful, but largely unexplored activity, inhibition of angiogenesis (Sharpe et al., 1989; Benelli et al., 1997). Based on CsA's well characterized activity in immunosuppression, this antiangiogenic activity has been largely ascribed to a similar mode of action through inhibition of calcineurin. However, CsA also targets a large class of prolyl isomerases, the cyclophilins. The role and relative contribution of cyclophilin inhibition in antiangiogenesis by CsA has not been fully addressed.

Angiogenesis is the process of new blood vessel growth and development, which requires the proliferation and migration of vascular endothelial cells. Pathological angiogenesis has

ABBREVIATIONS: CsA, cyclosporin A; HUVEC, human umbilical vein endothelial cells; HFF, human foreskin fibroblasts; *N*-MeVal-4-CsA, *N*-methylvaline-4-cyclosporin A; Io, ionomycin; NFAT, nuclear factor of activated T cells; CyP, cyclophilin; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; CNV, choroidal neovascularization; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; DAPI, 4,6-diamidino-2-phenylindole; IL, interleukin; TBS-T, 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20; DMEM, Dulbecco's modified Eagle's medium; RT, room temperature; ROI, region of interest; MAS, Masson.

been implicated in a number of prevalent human diseases including cancer and macular degeneration (Carmeliet, 2003). The first generation of clinical angiogenesis inhibitors has shown modest, but definitive, efficacy in the treatment of some of these conditions, thereby validating angiogenesis as a clinical target. However, there is a need for more effective antiangiogenic drugs (Jain et al., 2006; Ivy et al., 2009).

As an immunosuppressant, CsA inhibits the activation of T cells by blocking T cell receptor-mediated signaling. Activated T cell receptors induce a rise in intracellular calcium, which activates the cytoplasmic phosphatase calcineurin in a calmodulin-dependent manner (Crabtree and Clipstone, 1994; Rao et al., 1997; Liu, 2009). In turn, calcineurin dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT). This allows NFAT to translocate to the nucleus and activate the transcription of cytokines necessary for T cell proliferation, including IL-2. CsA disrupts this process by binding to a member of the immunophilin family, cyclophilin A (CyPA), a peptidyl prolyl *cis-trans* isomerase, to form a binary CsA-CyPA complex. By gain of function, this complex subsequently binds to and inhibits calcineurin (Liu et al., 1991). It is noteworthy that CsA requires cyclophilin binding to affect the phosphatase activity of calcineurin (Liu et al., 1991). In contrast, formation of the CsA-CyPA complex abolishes the enzymatic activity of CyPA independently of calcineurin binding (Fischer et al., 1989).

The antiangiogenic property of CsA has also been thought to depend on inhibition of calcineurin (Armesilla et al., 1999; Hernández et al., 2001; Rafiee et al., 2004). However, the IC_{50} dose for calcineurin inhibition by CsA in T cells is 100- to 1000-fold lower than the IC_{50} for endothelial cell proliferation, which suggests a possible mechanistic difference. In addition, NFAT, via calcineurin, is dephosphorylated in endothelial cells in a CsA-sensitive manner upon calcium influx induced by either a calcium ionophore or acute exposure to vascular endothelial growth factor (VEGF), but this activation is transient, returning to baseline within 2 to 4 h of stimulation (Armesilla et al., 1999; Rafiee et al., 2004). Because proliferation occurs on a long time scale, this transient calcineurin activity may play a more minor role in the endothelium than appreciated previously. Together, these observations suggest an as-yet-unexplored mode of action for CsA in the endothelium that may not be apparent or significant during immune suppression.

Thus, we sought to determine whether the mechanisms of immunosuppression and angiogenesis inhibition by CsA were in fact identical and to determine the extent of a role for cyclophilins in angiogenesis. A standard approach would be to knock out calcineurin in endothelial cells and then to assess the impact on cellular growth and angiogenesis. However, because of the limitations of genetic manipulations in primary cells we have used a chemical biology approach to instead "knock out" function in CsA to address this fundamental question. We have identified a nonimmunosuppressive CsA analog that does not affect the phosphatase activity of calcineurin but is comparable in potency to CsA for inhibition of endothelial cell proliferation. This nonimmunosuppressive analog also retained potency against a panel of eight cyclophilins and was therefore used as a tool to assess the role for CyPs in human umbilical vein endothelial cell (HUVEC) proliferation and in two models of in vivo angio-

genesis in which it retained activity. In addition, we showed that in proliferating endothelial cells calcineurin was inactive and that when exogenously stimulated the IC_{50} for calcineurin inhibition by CsA was much lower than that for proliferation inhibition. Together, these results suggest that cyclophilins may be a more relevant target for the antiangiogenic activity of CsA than recognized previously.

Materials and Methods

Materials and Equipment. Bovine serum albumin (BSA), recombinant cyclophilin A, and 10% neutral buffered formalin were purchased from Sigma-Aldrich (St. Louis, MO). Triton X-100 was purchased from Thermo Fisher Scientific (Waltham, MA). Immumount was purchased from Thermo Fisher Scientific. [3H]thymidine (1 mCi/ml in aqueous buffer) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Anti-poly(ADP-ribose) polymerase antibody was purchased from Cell Signaling Technology (Danvers, MA). Antitubulin, anti-NFAT2, anti-NFAT3, anti-NFAT4, and anti-glyceraldehyde-3-phosphate dehydrogenase antibodies and anti-mouse and anti-goat horseradish peroxidase-conjugated IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antilamin A/C antibody was purchased from Novocastra (Wetzlar, Germany). Anti-rabbit IgG was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Donkey anti-mouse Alexa Fluor 488 was purchased from Invitrogen (Carlsbad, CA). Carrier-free recombinant mouse basic fibroblast growth factor (bFGF), recombinant mouse VEGF₁₆₄, and recombinant human VEGF₁₆₅ was purchased from R&D Systems (Minneapolis, MN). High-concentration Matrigel and phenol-red free Matrigel was purchased from BD Biosciences (San Jose, CA). Pooled HUVEC and endothelial growth medium-2 bullet kits were purchased from Lonza (Basel, Switzerland). DMEM, RPMI 1640 media, fetal bovine serum (FBS), trypsin, and penicillin/streptomycin were purchased from Invitrogen. Cyclosporin A and ionomycin (Io) were purchased from LC Laboratories (Woburn, MA).

Cell Culture. Cells were incubated at 37°C in a humidified environment with 5% CO₂ present. HUVEC were grown in endothelial growth medium-2 bullet kit media. All experiments were conducted with HUVEC between passages 3 and 8. HeLa cells were grown in low-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Jurkat T cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 media supplemented with 10% FBS and 1% penicillin/streptomycin (complete RPMI 1640) unless otherwise noted, and human foreskin fibroblasts (HFF) were grown in high-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

Electroporation. Jurkat T cells from cultures grown at a density between 0.5×10^6 and 2×10^6 cells/ml were collected by centrifugation at 500g and washed three times with serum-free RPMI 1640 media. Cells were resuspended in serum-free RPMI 1640 media at a density of 10^7 cells/360 μ l. Fifteen micrograms of the minimal IL-2 luciferase reporter plasmid (Youn et al., 1999) was added per 10^7 cells and incubated at room temperature (RT), 22°C, for 10 min before transferring the suspension in 360- μ l aliquots to 0.4-cm gap gene pulser cuvettes (Bio-Rad Laboratories, Hercules, CA). Cells were electroporated using a Gene Pulser II and Capacitance Extender II (Bio-Rad Laboratories) at the high capacitance setting, 0.250 kV, and 0.950 μ F. After resting for 10 min in the cuvette, cells were transferred to complete RPMI 1640 media at a density of 10^6 cells/ml and allowed to recover for 24 h under normal growth conditions.

IL-2 Reporter Assay. After a 24-h recovery, cells electroporated with the IL-2 reporter construct were collected by 500g centrifugation and resuspended at a density of 10^6 cells/ml in complete RPMI 1640 media. Eight hundred microliters of this suspension was aliquoted per well of a 24-well plate. Drugs were serially diluted in

DMSO as 1000 $\times$ stocks, which were subsequently diluted to 40 $\times$ in serum-free RPMI 1640 media before addition to the 24-well plate. Vehicle only was used as a negative control. Cells were incubated with drugs for 30 min under normal culture conditions before the addition of phorbol 12-myristate 13-acetate (40 nM) and ionomycin (1 μ M), both from 1000 $\times$ stocks in DMSO that were diluted to 40 $\times$ in PBS before addition to the culture media at the final 1 $\times$ concentration. Vehicle only was used as a negative control for stimulation. After a 6-h incubation, cells were transferred to microcentrifuge tubes on ice, pelleted by centrifugation (500g at 4°C), and washed once with ice-cold PBS. Pellets were flash-frozen in a dry-ice ethanol bath, transferred to wet ice, and resuspended in 100 μ l of lysis buffer by pipetting and vortexing briefly. Seventy five microliters of lysate was transferred to an opaque 96-well plate (Nalge Nunc International, Rochester, NY), and luciferase activity of this sample was determined over a 5-s interval after a 5-s delay after automated injection of 100 μ l of luciferase substrate buffer using a 1450 Micro-beta apparatus (PerkinElmer Life and Analytical Sciences). Both the lysis buffer and luciferase buffer were prepared as described previously, except that the concentration of luciferin was 121 μ M (Dyer et al., 2000). The luciferase activity in each sample was normalized to the total protein level in the lysates (determined by Bio-Rad protein assay).

Cell Proliferation Assay. Cells were seeded at 2000 cells/well in a 96-well plate (Costar, Cambridge, MA) in 199 μ l of media and allowed to recover overnight. Drugs were added from 200 $\times$ stocks in DMSO. After a 24-h incubation, cells were pulsed with 0.9 μ Ci of [3 H]thymidine for 6 h, washed once with PBS, trypsinized, and transferred to filtermats (PerkinElmer Life and Analytical Sciences) using a Mach III M Harvester 96 (Tomtec, Hamden, CT). After drying overnight, the amount of [3 H]thymidine retained on the membrane was determined by scintillation counting using a 1450 Micro-beta apparatus (PerkinElmer Life and Analytical Sciences). At least three independent experiments with multiple technical replicates for each drug concentration were performed. All counts were normalized to vehicle only-treated cells. Prism (version 4.03) software (Graph-Pad Software Inc., San Diego, CA) was used to determine IC₅₀ values using a four-parameter logistic regression.

Cell Viability Assay. Cells were plated at 2000 cells/well in a 96-well plate in 199 μ l of media. Drugs were added from 200 $\times$ stocks in DMSO, and cells were incubated for 30 h under normal growth conditions. The cells were then washed with 200 μ l of PBS and incubated for 30 min at 37°C with 100 μ l of 1 μ M Calcein AM (Invitrogen) diluted from a 1-mM stock in DMSO. The excess dye was then aspirated and replaced with 100 μ l of PBS before analysis using a fluorescence plate reader with gain adjusted to 100 μ l of 1 μ M hydrolyzed Calcein AM (to hydrolyze the dye a 1-mM solution was incubated 1:1 with 100 mM NaOH overnight and then diluted to 100 μ M with 1 M Tris, pH 8.0) (Fluostar Optima; GE Healthcare). The fluorescence of drug-treated samples was normalized to DMSO controls. Five independent experiments with duplicate technical replicates for each drug concentration were performed.

Immunofluorescence. HUVEC were seeded on glass coverslips at 50 to 60% confluence and allowed to recover overnight. Cells were pretreated with drugs from 200 $\times$ stocks in DMSO before stimulation with ionomycin from a 40 $\times$ stock in PBS (diluted from a 2000 $\times$ DMSO stock) or with VEGF₁₆₅ diluted from a 2000 $\times$ stock in 0.1% BSA in PBS. Mock stimulations were carried out by treating similarly but with vehicle only. After stimulation, media were aspirated and cells were fixed with ~4% paraformaldehyde in PBS for 20 min. Coverslips were washed three times with PBS, and the cells were permeabilized with 0.1% Triton X-100 in PBS for 1 to 3 min. After a PBS wash, the cells were blocked in 4% BSA in PBS for 1 to 1.5 h and then incubated with anti-NFAT2 or anti-NFAT4 (1:100 dilution in 1% BSA in PBS) for 1 h at RT, 22°C, or 6 h at 4°C. Coverslips were washed three times in PBS and incubated for 30 min in the dark with donkey anti-mouse Alexa Fluor 488 (1:800 dilution) and DAPI (0.1 μ g/ml) in 1% BSA in PBS. After a 3 $\times$ PBS wash, coverslips were

mounted using Immu-mount and allowed to dry at RT in the dark overnight. Confocal imaging was performed using a Zeiss Axiovert 200 microscope with the 510-Meta confocal module and LSM 10 Meta software (Carl Zeiss Inc., Thornwood, NY). The same software was used to generate two ROIs for each cell, one outlining the nucleus as defined by the DAPI signal and the other outlining the entire cell as defined by the maximum extent of the Alexa Fluor 488 signal. For each ROI, the sum intensity of the Alexa Fluor 488 signal was calculated from the product of the mean intensity and area, and the sum intensity of the nuclear ROI was subtracted from that of the total cell ROI to give the cytoplasmic sum intensity. The ratio of the cytoplasmic sum intensity to the nuclear sum intensity was then calculated.

Jurkat T cell immunofluorescence experiments were carried out essentially as above except that cells were grown in suspension until just before treatment with ionomycin (1 μ M) or vehicle control for 15 min during which time they were incubated on poly-lysine (Sigma-Aldrich)-coated glass-bottom dishes (MatTek, Ashland, MA). In order not to disrupt cell adhesion, all aspirations were performed with a pipette instead of the house vacuum.

Calcineurin Phosphatase Activity Assay. This assay was performed using the Calcineurin Colorimetric Drug Discovery Kit (Enzo Life Sciences, Inc., Farmingdale, NY) according to the manufacturer's instructions with modifications for cyclophilin-drug complex formation. The cyclophilin A-drug complexes were allowed to form in assay buffer for 60 min at RT by combining 45 pmol cyclophilin A (from a 78- μ M stock) with 225 pmol drug (from a 450- μ M stock in DMSO) in a 10- μ l volume. The complex was then added to recombinant calmodulin (625 pmol) and recombinant calcineurin (0.15 pmol) and allowed to incubate at RT for 30 min before the addition of the RII phosphopeptide substrate (16.4 μ g). After a 15-min reaction at 30°C, 100 μ l of developing reagent was added. After a 16-min incubation at RT the A₆₂₀ of the samples was measured in a half-volume 96-well plate.

Enzyme Purification. Recombinant human CyPA, CyPB, CyPC, CyPD, and USA-CyP were purified as described previously (Daum et al., 2009). To obtain human Cyp40 (PPID) and Cyp33 (PPIE) the genes were polymerase chain reaction-amplified using gene-specific primers from an open reading frame encoding human PPID and PPIE (imaGenes, Berlin, Germany) and cloned into pET28a. After overexpression in *Escherichia coli* BL21 cells, purification of His-tag fusion proteins were performed using affinity chromatography on nickel-nitrilotriacetic acid resin followed by size exclusion chromatography in 10 mM HEPES, pH 7.8, 6 mM KCl, 1.5 mM MgCl₂, and 1 mM dithiothreitol.

The expression vector CypJ-pTXB1 for human CypJ was a kind gift from Long Yu, Fudan University, Shanghai. CypJ was expressed with an intein tag and purified by chitin affinity chromatography using the IMPACT-CN system (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions.

Cyclophilin Inhibition Assay. K_i values were determined with the protease-free PPIase assay as described previously (Janowski et al., 1997) in 35 mM HEPES buffer, pH 7.8 (4 nM bovine serum albumin) at 283 K, using Suc-Ala-Ala-Pro-Phe-pNA (64 μ M) as substrate. K_i values were calculated from 8 to 11 different inhibitor concentrations; standard deviations for first-order rate constants of the *cis-trans* isomerizations of the assay substrate were not larger than 10% of the mean.

Matrigel Plug Assay. These assays were performed as reported previously with minor modifications (Passaniti et al., 1992). Animals were treated according to a protocol approved by the Johns Hopkins Animal Care and Use Committee. Male athymic nude mice, 4 to 6 weeks old, weighing an average of 25 g, were purchased from NCI Frederick (Frederick, MD). Mice were treated once daily for 12 days with 25 mg/kg drug or vehicle (4.5% DMSO in sterile olive oil; Sigma-Aldrich) by intraperitoneal injection of a 100- μ l suspension in the lower right quadrant. After day 2 of treatment, 250 μ l of high concentration Matrigel combined with freshly reconstituted 100 ng/ml recombinant mouse VEGF₁₆₄ and 150 ng/ml recombinant

mouse bFGF was injected subcutaneously in the abdominal skin at approximately the level of the iliac crest, lateral to the midline. After 12 days of treatment, the mice were sacrificed, and the Matrigel plugs were excised and fixed in 10% neutral buffered formalin before processing at the Johns Hopkins University School of Medicine histology core facility. Two sections of each plug separated by 10 steps were mounted and stained with MAS trichrome. The slides were coded and a blinded observer determined the number of erythrocyte filled vessels per 200 $\times$ field within one field depth of the surface of the plug. The entire perimeter of each plug was analyzed. The average number of erythrocyte-filled vessels per field across both sections was calculated.

Laser-Induced Choroidal Neovascularization. Six-week-old C57BL/6 mice were anesthetized with a mixture of ketamine (150 mg/kg) and xylazine (10 mg/kg), and the pupils were dilated with a single drop of 1% tropicamide. Argon laser photocoagulation (75- μ m spot size, 100-ms duration, 100-mW power) (IRIDEX, Mountain View, CA) was used to generate four laser spots in each eye surrounding the optic nerve by using a hand-held coverslip as a contact lens. Immediately after, intravitreal injection of 1 μ l of *N*-methylvaline-4-cyclosporin A (*N*-MeVal-4-CsA) (10 μ M) was performed on the right eye. Left eyes received a sham injection with saline solution. Choroidal flatmounts were prepared in the standard technique. The laser-treated areas were evaluated for the presence of choroidal neovascularization (CNV) on day 10 after laser treatment, using isolectin staining and confocal microscopy (Jones et al., 2008).

Statistical Analysis. The data from the Matrigel plug assays were skewed toward near-zero values. As a result, it was necessary to log-transform the raw data to better approximate a normal distribution before performing a parametric analysis. All hypothesis testing was performed using a two-sample, two-tailed *t* test in R (version 2.8.1, <http://www.r-project.org/>) or Excel (Microsoft, Redmond, WA).

Cell Cycle Analysis. HUVEC were seeded at 5×10^5 cells/15-cm dish, allowed to recover overnight, and subsequently treated with drugs or vehicle control for 24 h. Media were then collected and set aside. Cells were washed with PBS, trypsinized, combined with set-aside media, pelleted at 500g, and then washed with 10 ml of PBS followed by another 500g spin. The pellet was resuspended in 0.5 ml of PBS and added dropwise using a Pasteur pipette to 2 ml of 75% ethanol in a 5-ml polystyrene tube being slowly agitated by a vortex. The cells were stored at 4°C until staining. To do so, cells were pelleted at 500g, resuspended in 5 ml of PBS, rested 60 s, pelleted again, and washed in 5 ml of PBS. The cell pellet was then resuspended in 0.5 ml of staining solution (0.1% Triton X-100, 0.2 mg/ml DNase-free RNase A, and 0.02 mg/ml propidium iodide; Sigma-Aldrich). Cells were allowed to stain for 30 min to 1 h before analysis. Propidium iodide incorporation was measured using a FACSCalibur (BD Biosciences). The percentage of cells in each cell cycle stage was determined with FlowJo (version 7.5.5; Tree Star, Inc., Ashland, OR) using a Watson analysis (Watson et al., 1987).

Cell Fractionation. A total of 1.5×10^6 HUVEC were seeded on a 15-cm dish (Falcon; BD Biosciences Discovery Labware, Bedford, MA), allowed to recover overnight, and then treated with either ionomycin (1 μ M) from a 40 $\times$ stock in PBS (diluted from a 2000 $\times$ DMSO stock) or vehicle for 15 min. The cells were then washed once with PBS and incubated with prewarmed (37°C) trypsin-EDTA (Invitrogen) for 1 min at 37°C. The cells were then collected in 10 ml of ice-cold media, spun at 1800g for 5 min at 4°C, resuspended in 10 ml of ice-cold PBS, and spun again at 1800g for 5 min at 4°C. The cell pellet was resuspended in 300 μ l of hypotonic buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 10 mM β -glycerophosphate, 10 mM sodium pyrophosphate, 10 mM NaF, 0.5 mM NaVO₄, and 0.5 mM dithiothreitol (added fresh), and 1 $\times$ protease inhibitor cocktail (added fresh); Roche Diagnostics (Indianapolis, IN)], spun at 1800g for 5 min at 4°C, resuspended in 300 μ l of fresh hypotonic buffer, and rested on ice for 20 min. The cells were then transferred to a 2-ml Dounce homogenizer and lysed by 20 strokes of a type B glass pestle.

The lysate was spun at 3300g for 15 min at 4°C, and the supernatant (cytoplasmic fraction) was transferred to a new tube. The cell pellet was resuspended in a volume of hypotonic buffer equal to that of cytoplasmic fraction. Four times SDS loading buffer (240 mM Tris, pH 6.8, 7.7% SDS, 38.4% glycerol, 0.0024% bromphenol blue, 50 μ l/ml β -mercapto-ethanol) was immediately added to each fraction to give a 1 $\times$ final concentration, and the samples were boiled for 10 min before separation by SDS-PAGE.

Apoptosis Drug Treatments. A total of 5×10^4 cells were seeded in 3 ml of media in each well of a six-well plate and allowed to recover overnight. The media were then replaced with 3 ml of fresh media, and cells were treated with DMSO (0.5% final concentration) or $\sim 2 \times$ the IC₅₀ dose of CsA (5 μ M) or *N*-MeVal-4-CsA (8 μ M) for 24 h or 200 nM staurosporine for 5 h. The media were then aspirated, and 80 μ l of 2 $\times$ SDS loading buffer was added to each well and collected after a 20-min incubation on ice. The lysate was then boiled for 10 min, and proteins were separated by SDS-PAGE.

Western Blotting. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific), which were then blocked for 15 to 30 min in 5% blotto (Santa Cruz Biotechnology, Inc.) in TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for NFAT blots or 5% BSA (Sigma-Aldrich) in TBS-T for all other blots. Membranes were incubated overnight with primary antibody (1:500 dilution in 1% blotto for NFAT antibodies, 1:3000 in 1% BSA Lamin A/C antibody, and 1:1000 in 1% BSA for all others). After washing three times with TBS-T, horseradish peroxidase-conjugated secondary antibody was added in the same type of incubation buffer as the primary antibody at a 1:5000 dilution and incubated for 30 min to 1 h. Membranes were washed three times with TBS-T and incubated for 1 to 5 min with ECL substrate (Millipore Corporation, Billerica, MA). Bands were visualized with a Kodak Image Station 440 CF (Eastman Kodak, Rochester, NY).

Results

Identification of a CsA Analog That Potently Inhibits Endothelial Cell Proliferation but Not an IL-2 Reporter. Because CsA possess two distinct intrinsic properties, binding and inhibition of cyclophilins, and inhibition of calcineurin by the CsA-cyclophilin complex, we sought to dissociate the two binding activities in a series of analogs of CsA. CsA was derivatized at the third or fourth position to create a series of analogs (Fig. 1A), which were then screened in assays for inhibition of HUVEC proliferation and calcineurin-dependent activation of an IL-2 reporter gene in Jurkat T cells (Table 1). Vascular endothelial cells, such as HUVEC, make up the inner lining of blood vessels and must proliferate as part of the angiogenic process. Jurkat T cells, like primary T cells, undergo a program of activation, leading to the production of IL-2 and other cytokines as part of an immune response. Thus, by assaying the ability of CsA analogs to inhibit HUVEC proliferation and IL-2 reporter activation in Jurkat T cells, we were able to assess in vitro the antiangiogenic and immunosuppressive potential of the analogs and compare these with CsA itself.

Among the CsA analogs tested was *N*-MeVal-4-CsA, which has been reported previously to suffer from a dramatic loss of relative immunosuppressive activity in a mixed-lymphocyte reaction and in an IL-2 reporter assay compared with CsA (Papageorgiou et al., 1994). This analog was approximately 10,000-fold less potent than CsA in the IL-2 reporter assay, which is 4-fold less potent than reported previously (Table 1 and Fig. 1C). In contrast, *N*-MeVal-4-CsA was similar in potency to CsA against HUVEC proliferation, in which the

A

cyclosporin A: X,Y=H; Z=iBu

N-MeVal-4-CsA: X,Y=H; Z=iPr

compound 1: X=H; Y=-S(CH₂)₂-NH-CH₂CMe₃; Z=iBucompound 2: X=-S(CH₂)₂-NH-CH₂CMe₃; Y=H; Z=iBucompound 3: X=H; Y=-S(CH₂)₂NMe₂; Z=iBucompound 4: X=H; Y=-S(CH₂)₂NEt₂; Z=iBucompound 5: X=-[O(CH₂)₂]₃-OH; Y=H; Z=iBucompound 6: X=H; Y=-[O(CH₂)₂]₃-OH; Z=iBu

B

C

D

E

TABLE 1

Potency of CsA analogs against the IL-2 reporter assay and HUVEC proliferation

For CsA and N-MeVal-4-CsA, $n = 3$ for proliferation and $n = 4$ and 5 , respectively, for the IL-2 reporter. The parenthetical values are 95% confidence intervals. For all other analogs, experiments were conducted in duplicate and parenthetical values are S.E.M.

Compound	IL-2 Reporter IC ₅₀	HUVEC Proliferation IC ₅₀
	nM	μM
CsA	1.22 (0.37, 4.03)	2.62 (1.87, 3.65)
N-MeVal-4-CsA	>10,000	3.95 (2.97, 5.26)
1	53.9 (22.9)	1.17 (0.24)
2	107 (12.1)	3.38 (3.17)
3	40.4 (26.3)	3.34 (0.94)
4	12.9 (6.51)	2.71 (1.42)
5	34.2 (23.2)	7.28 (0.92)
6	116 (86.6)	5.49 (3.08)

IC₅₀ was 2.6 μM for CsA and 4.0 μM for N-MeVal-4-CsA (Table 1 and Fig. 1B).

Given the micromolar IC₅₀ concentrations for HUVEC proliferation, HFF growth was assayed in the presence of CsA and N-MeVal-4-CsA to determine whether the inhibition of HUVEC proliferation was caused by nonspecific cytotoxicity.

Fig. 1. Structural modification of CsA leads to the separation of its immunosuppressive and antiangiogenic properties. A, molecular structures of CsA and its analogs. B, HUVEC proliferation assay in which cells were treated for 24 h with drug before incubation with [³H]thymidine for 6 h. Bars indicate S.E.M. ($n = 3$). C, IL-2 reporter assay in which Jurkat T cells harboring the murine IL-2-luciferase reporter plasmid were incubated for 30 min with drug before stimulation with ionomycin (1 μM) and phorbol 12-myristate 13-acetate (40 nM). After 6 h, cells were lysed and the luciferase activity in the lysates was measured. Bars indicate S.E.M. ($n = 5$ for N-MeVal-4-CsA; $n = 4$ for CsA). D and E, the viability of HFF and HUVEC after treatment with CsA (D) and N-MeVal-4-CsA (E) was measured by staining with Calcein AM. Bars indicate S.E.M. ($n = 5$).

HFF in particular were chosen for comparison because, like HUVEC, they are primary cells and are therefore potentially more sensitive to a generalized toxic insult than immortalized cancer cell lines. Neither drug caused a loss of viability in HFF at doses sufficient to completely inhibit HUVEC proliferation (Fig. 1, D and E). In contrast, both drugs affected the viability of HUVEC at or above the IC₅₀, suggesting a cell type-specific effect. It is noteworthy that N-MeVal-4-CsA caused a greater effect at high concentrations than CsA itself. Although both drugs affected HeLa proliferation, they did so less potently than in HUVEC (Supplemental Fig. 1). HeLa were used in addition to HFF in toxicity testing because as an epithelial adenocarcinoma line they are of a different origin from both HUVEC and HFF. In addition to the loss of viability in HUVEC, at doses 2-fold above their respective IC₅₀ doses, CsA and N-MeVal-4-CsA caused a minor G₁-specific delay in HUVEC and did not induce apoptosis (Supplemental Fig. 1).

N-MeVal-4-CsA Does Not Inhibit Calcineurin in HUVEC. Given that N-MeVal-4-CsA did not significantly inhibit the IL-2 reporter assay, we next investigated the direct effects of N-MeVal-4-CsA on calcineurin activity in an

in vitro assay for calcineurin phosphatase activity in the presence of CyPA. As expected, calcineurin activity was not affected by either CsA, *N*-MeVal-4-CsA, or CyPA alone but was strongly inhibited by CsA and CyPA in combination (Fig. 2A) (Liu et al., 1991). In contrast, the combination of *N*-MeVal-4-CsA and CyPA had no effect on calcineurin activity.

In a cellular context, activation of calcineurin activity leads to dephosphorylation of NFAT and its translocation from the cytosol to the nucleus, with resulting transcriptional activation of NFAT-dependent genes (Rao et al., 1997). In the absence of an appropriate calcium signal, NFAT remains in the cytosol and is incapable of activating gene expression in the nucleus. As a readout of cellular calcineurin activity, we determined the effect of *N*-MeVal-4-CsA treatment on the subcellular localization of NFAT2 in HUVEC in comparison with CsA treatment. Of several NFAT isoforms, we chose to examine NFAT2 because it is expressed in multiple types of vascular endothelium, in which it translocates to the nucleus in response to rising intracellular calcium (Johnson et al., 2003; Rinne et al., 2009). Using confocal microscopy and immunofluorescence of endogenous NFAT2, we found that whereas 5 μ M CsA blocked ionomycin-induced NFAT2 nuclear translocation in HUVEC, 10 μ M *N*-MeVal-4-CsA had no effect (Fig. 2B). CsA, but not *N*-MeVal-4-CsA, was also able to block NFAT2 translocation after the acute addition of VEGF₁₆₅, a physiologic stimulus that activates calcineurin in endothelial cells (Supplemental Figs. 4 and 5A) (Armesilla et al., 1999; Rafiee et al., 2004).

CsA and *N*-MeVal-4-CsA Inhibit Proliferation in Endothelial Cells in Which Calcineurin Is Inactive. The inability of the *N*-MeVal-4-CsA analog to block calcineurin activity in vitro or NFAT translocation in HUVEC suggested that calcineurin is not the relevant target of *N*-MeVal-4-CsA or CsA for their antiproliferative effects on endothelial cells. However, several studies have shown that the nuclear translocation of NFAT in endothelial cells is induced by the addition of VEGF and inhibited by CsA, suggesting that calcineurin may be crucial for endothelial cell proliferation (Armesilla et al., 1999; Rafiee et al., 2004). In these studies the translocation of NFAT was transient, returning to baseline within 2 to 4 h after acute stimulation with VEGF. In contrast, we

cultured HUVEC in the constant presence of VEGF and other growth factors before and during drug treatment.

Therefore, we sought to determine how calcineurin behaved under our culture conditions. NFAT2 translocation from the cytosol to the nucleus was used as a readout for cellular calcineurin activity. In HUVEC grown under the conditions used to assess the potency of CsA and *N*-MeVal-4-CsA (Fig. 1), nearly all the NFAT2 was localized to the cytoplasm (Fig. 3A). Although we did observe that a small fraction of NFAT2 signal colocalized with the DAPI signal (a nuclear marker) in the absence of ionomycin, this fraction was not significantly perturbed ($p = 0.27$) by incubation with CsA (5 μ M) for 30 min (Fig. 3, A and B). Treatment with 10 μ M *N*-MeVal-4-CsA in the absence of ionomycin stimulation also had no effect on the distribution of NFAT2 (Supplemental Fig. 3). Nuclear export of NFAT2 was sufficiently rapid in HUVEC to allow NFAT2 that was potentially in the nucleus before the addition of drug to be trapped in the cytosol by CsA treatment (Supplemental Fig. 2). Taken together, these experiments indicate that calcineurin activity leading to the translocation of NFAT2 to the nucleus is not crucial for HUVEC proliferation under the conventional culture conditions in which we evaluated CsA and its nonimmunosuppressive analog.

Likewise, NFAT3 and NFAT4, both of which have been proposed to function in vascular development, were solely located in the cytoplasm in proliferating HUVEC and, surprisingly, did not translocate to the nucleus after ionomycin treatment (Supplemental Fig. 2) (Graef et al., 2001). This is consistent with previous findings in other endothelial cell lines; unlike NFAT2, NFAT4 is not activated in response to stimulation with high-dose VEGF (Johnson et al., 2003).

The Potencies of CsA as a Calcineurin Inhibitor and Proliferation Inhibitor Do Not Correlate in HUVEC. In Jurkat T cells stimulated with ionomycin and phorbol myristate acetate to mimic the effects of T cell receptor ligation, CsA inhibits NFAT-dependent IL-2 reporter activation with an IC₅₀ in the single-digit nanomolar range (Fig. 1C). In contrast, CsA inhibits HUVEC proliferation with an IC₅₀ of 2.6 μ M (Fig. 1B). This approximately 1000-fold difference in potency may be due to a number of possibilities including that in the endothelium there are fundamental differences in

Fig. 2. *N*-MeVal-4-CsA does not inhibit calcineurin in vitro or in vivo. **A**, the in vitro phosphatase activity of calcineurin was determined after preincubation with CyPA, drug, or drug-CyPA complexes as indicated. Data were normalized to the activity in control samples lacking both drugs and CyPA. Bars indicate S.E.M. ($n = 3$). **B**, representative micrographs of confocal images of NFAT2 immunofluorescence and DAPI staining in HUVEC treated with CsA (5 μ M), *N*-MeVal-4-CsA (10 μ M), or the aryl 1-indanylketone cyclophilin inhibitor (20 μ M) for 30 min followed by a 15-min treatment with ionomycin (1 μ M). Scale bar, 20 μ m.

Fig. 3. The calcineurin-NFAT pathway is not active in proliferating HUVEC. **A**, representative micrographs of confocal images of NFAT2 immunofluorescence and DAPI staining in HUVEC treated with DMSO for 30 min followed by a 15-min treatment with ionomycin (1 μM) (Io column), or CsA (5 μM) or DMSO for 30 min followed by a mock stimulation. Scale bars, 20 μm. **B**, quantitation of the cytoplasmic (CYT) to nuclear (NUC) ratio of NFAT2 in **A**. Cells from at least two independent experiments were analyzed. Bars indicate S.E.M. (*n* = 8 for CsA and Io; *n* = 9 for DMSO). n.s. = not significant for α = 0.05. **C**, representative micrographs of confocal images of NFAT2 immunofluorescence and DAPI staining in HUVEC preincubated for 30 min with the indicated doses of CsA before stimulation for 15 min with ionomycin (1 μM). Scale bars, 20 μm.

the ability of CsA-CyPA to inhibit calcineurin, differences in calcineurin abundance or isoform expression, or alternatively, that calcineurin is not the relevant target for the inhibition of endothelial cell proliferation. Thus, to better understand this discrepancy we directly compared the potencies of CsA against ionomycin- and VEGF-stimulated calcineurin activity in HUVEC and HUVEC proliferation.

Again using confocal microscopy, we determined the ability of CsA to inhibit ionomycin-induced NFAT2 nuclear translocation in HUVEC as a readout for calcineurin activity. Cells were stimulated for 15 min with 1 μM ionomycin after a 30-min pretreatment with either vehicle or varying concentrations of CsA (Fig. 3C). We observed that CsA blocked ionomycin-induced nuclear NFAT2 localization in HUVEC with an IC₅₀ near 10 nM. In HUVEC treated with 1 to 50 ng/ml VEGF₁₆₅, a physiologic stimulus used in place of ionomycin, CsA had a similar potency against NFAT2 nuclear translocation (Supplemental Fig. 5B). At 10 nM, CsA had no effect on HUVEC proliferation (Fig. 1B), suggesting that inhibition of calcineurin is not linked to its effects on endothelial proliferation.

***N*-MeVal-4-CsA Is a Potent Inhibitor of the Prolyl Isomerase Activity of Multiple Cyclophilins.** CsA has two known protein targets, calcineurin and the cyclophilins. Because *N*-MeVal-4-CsA has no effect on calcineurin activity (Fig. 2) but inhibits HUVEC proliferation, the next logical functionality that might affect cell proliferation would be the inhibition of cyclophilins by the analog. Thus, we determined the effect of *N*-MeVal-4-CsA on the enzymatic activity of purified human cyclophilins (Table 2). For two of the eight human cyclophilins tested, CyPA and CyPB, both CsA and *N*-MeVal-4-CsA were equipotent with IC₅₀ values in the low nanomolar concentration range. *N*-MeVal-4-CsA lost approximately 3- to 6-fold of potency against CyPC, CyPD, and USA-CyP but for CyP40 it exhibited a more than 4-fold increase in potency over CsA. Inhibition of CyPA by *N*-MeVal-4-CsA served as an internal control as this activity has been established (Papageorgiou et al., 1994; Nicolli et al., 1996).

To further examine the potential role of cyclophilins in endothelial cell proliferation, we measured the proliferation of HUVEC in the presence of an aryl 1-indanylketone that is structurally distinct from CsA but inhibits CyPA with a *K_i* of 300 nM and less potently, CyPB and CyPD (Supplemental Fig. 6) (Daum et al., 2009). The aryl 1-indanylketone had an IC₅₀ value of 5.4 μM (3.2, 8.9, 95% confidence interval) for HUVEC proliferation, but did not inhibit ionomycin-induced NFAT2 translocation, indicating that it has no activity against calcineurin (Fig. 2B and Supplemental Fig. 5). The potency of the cyclophilin inhibitor was remarkably similar to the IC₅₀ values of 2.6 and 4.0 μM for CsA and *N*-MeVal-4-CsA, respectively. These results suggest that inhibition of cyclophilins is sufficient to prevent HUVEC proliferation and support a model in which cyclophilins may be more important to CsA's antiangiogenic effects than appreciated previously.

***N*-MeVal-4-CsA Inhibits Angiogenesis In Vivo.** Because HUVEC proliferation is only an in vitro proxy for angiogenesis, we tested the potency of *N*-MeVal-4-CsA in vivo using the Matrigel plug assay and a mouse model for wet age-related macular degeneration. Matrigel, a recombinant extracellular matrix, was supplemented with recombinant murine bFGF and VEGF₁₆₄ and injected subcutaneously into male athymic mice that had been receiving daily intraperitoneal injections of CsA (25 mg/kg), *N*-MeVal-4-CsA (25 mg/kg), or vehicle beginning 2 days before the implantation of the plugs. After the daily drug dosing was continued for 10 additional days, the mice were sacrificed, and the plugs were

TABLE 2
Comparison of the *K_i* for CsA and *N*-MeVal-4-CsA against eight human cyclophilins

Enzyme	Alias/Gene Name	<i>K_i</i> <i>N</i> -MeVal-4-CsA	<i>K_i</i> CsA ^a
<i>nM</i>			
CypA	Cyp18/PPIA	7.2 ± 2.0	2.9
CypB	Cyp23/PPIB	9.7 ± 1.8	8.4
CypC	Cyp23a/PPIC	25.1 ± 8.8	7.7
Cyp40	PPID	72.7 ± 13.7	319 ± 76
CypD	Cyp22/PPIF	39.6 ± 7.5	6.7
USA-Cyp	Cyp19.2/PPIH	293 ± 40	91
CypJ	Cyp18.1/PPIL3	900 ± 800	N.D.
Cyp33	PPIE	147.0 ± 21	N.D.

N.D., not determined.
^a *K_i* values for inhibition by CsA of CypA, CypB, CypC, PPIL1, and USA-Cyp were taken from Daum et al. (2009).

excised, fixed, and stained with MAS trichrome. The number of erythrocyte-filled vessels per field was counted in a blinded fashion and averaged (Fig. 4A). Compared with the control group, angiogenesis in plugs from *N*-MeVal-4-CsA-treated mice was reduced by 46.9% ($p = 0.034$) and in CsA-treated mice by 46.1% ($p = 0.052$). Microscopically, the plugs from the drug-treated mice appeared largely acellular with the exception of a narrow band of infiltrating cellular material near the plug surface (Fig. 4B).

To further test whether *N*-MeVal-4-CsA might reduce pathologic angiogenesis in vivo, we turned to a mouse model for neovascular (wet) age-related macular degeneration, a major cause of vision loss in humans. Vision loss in wet age-related macular degeneration is caused by the abnormal angiogenesis and invasion of choroidal vessels through a disrupted Bruch's membrane into the subretinal space. This process of choroidal neovascularization (CNV) is modeled in animals by disrupting Bruch's membrane with a laser (laser-induced CNV) (Jones et al., 2008). Intravitreal administration of *N*-MeVal-4-CsA showed a significant reduction in the size of CNV lesions by 53% compared with eyes treated with a control saline solution (Fig. 5; $p < 0.0065$). The extent of inhibition of CNV lesions by *N*-MeVal-4-CsA is comparable with some of the most recent published results in this model (Jones et al., 2008).

Discussion

In this study, we assessed the relative contributions of calcineurin and cyclophilin activity in endothelial cell prolif-

Fig. 4. *N*-MeVal-4-CsA inhibits in vivo angiogenesis in Matrigel plugs. A, mice with subcutaneous Matrigel plugs containing recombinant murine bFGF and VEGF₁₆₄ were treated with either vehicle, *N*-MeVal-4-CsA (25 mg/kg), or CsA (25 mg/kg). The average number of erythrocyte-filled vessels per 200 $\times$ field within one field depth of the plug surface around the entire perimeter was determined by a blinded observer and plotted. Each data point represents a single mouse. Bars represent the group mean ($n = 7$ for vehicle; $n = 8$ for *N*-MeVal-4-CsA and CsA). P values are shown. B, representative microscopic images of MAS trichrome-stained Matrigel plugs with the surface positioned in the upper left-hand corner. Arrowheads indicate erythrocyte-filled vessels. Scale bars, 100 μ m.

Fig. 5. Efficacy of *N*-MeVal-4-CsA in an animal model of laser-induced CNV. A, bar graph shows the average size of choroidal neovascularization. Error bars indicate S.E.M. ($P < 0.0065$; $n = 24$). B and C, isolectin staining (green) of choroidal flatmount showing an area of CNV in *N*-MeVal-4-CsA (B) and vehicle-treated eyes (C). Note the marked reduction in CNV size with the *N*-MeVal-4-CsA treatment. Scale bar, 100 μ m.

eration and angiogenesis using *N*-MeVal-4-CsA, an analog of CsA in which the calcineurin inhibitory activity was knocked out but cyclophilin inhibition activity was retained. *N*-MeVal-4-CsA was 1000 to 10,000 times more potent against HUVEC proliferation than in an IL-2 reporter assay (Fig. 1, B and C) and possessed no activity against calcineurin in either biochemical or cellular assays (Fig. 2 and Supplemental Figs. 3 and 5). Despite the loss of calcineurin inhibition, *N*-MeVal-4-CsA inhibited in vivo angiogenesis in two independent animal models (Figs. 4 and 5). Although the existence of nonimmunosuppressive CsA analogs including *N*-MeVal-4-CsA and the antiangiogenic properties of CsA have been known for some time, that a nonimmunosuppressive CsA analog inhibits angiogenesis has not been demonstrated (Papageorgiou et al., 1994; Rosenwirth et al., 1994).

These findings suggested that the immunosuppressive and antiangiogenic activities of CsA may be mechanistically distinct. Further supporting this hypothesis were the results that the potency of CsA against ionomycin- and VEGF₁₆₅-induced nuclear translocation of NFAT2 in HUVEC was several hundredfold greater than that for inhibition of HUVEC proliferation and doses of CsA that completely block calcineurin activation have no effect on HUVEC proliferation (Figs. 1B and 3C and Supplemental Fig. 5A). Although the dose of CsA required to block nuclear translocation of NFAT in HUVEC was known to be lower than the dose required to block proliferation, to our knowledge ours is the first study to systematically determine the ionomycin- and VEGF₁₆₅-induced IC₅₀ values for inhibition of calcineurin activation in endothelial cells (Armesilla et al., 1999; Hernández et al., 2001). It is noteworthy that the 10 nM IC₅₀ value for NFAT

translocation in HUVEC corresponds well to the IC₅₀ value for the inhibition of calcineurin activation in Jurkat T cells, suggesting that there are no gross differences between calcineurin-mediated NFAT translocation in HUVEC and Jurkat T cells. If inhibition of HUVEC proliferation and calcineurin were mechanistically linked, one would expect each to occur at a similar dose of CsA. However, 10 nM CsA has no effect on HUVEC proliferation. This, together with the finding that the calcineurin-NFAT pathway was not active under our proliferation conditions, suggests that the inhibition of calcineurin activity is not likely to be the major mechanism contributing to CsA-mediated inhibition of HUVEC proliferation. This suggests that, although the role of the calcineurin-NFAT pathway is irrefutable in the case of vascular development, this pathway seems far less important in the case of endothelial proliferation and pathological angiogenesis (Graef et al., 2001). Thus, the role of this pathway in endothelial cells seems to be limited to a subset of environments and cellular contexts, not unlike its role in the immune system.

Whereas others had speculated previously that differences in the potency of CsA against calcineurin and HUVEC proliferation suggested an additional target of CsA in endothelial cells, ours is to our knowledge the first study to evaluate the other known target of CsA, namely the cyclophilins (Hernández et al., 2001). We were able to do so by taking advantage of the unique properties of *N*-MeVal-4-CsA. Despite the loss of calcineurin inhibitory activity, *N*-MeVal-4-CsA retained the ability to inhibit all eight cyclophilins tested. Thus, our findings suggest that the different potencies observed for T cell activation inhibition and antiangiogenic activity may be explained by the effects of CsA on cyclophilins without the need to implicate new binding targets for CsA in endothelial cells. That the aryl 1-indanylketone cyclophilin ligand also inhibited HUVEC proliferation, but not cellular calcineurin (Fig. 2A and Supplemental Fig. 6), lends further support to the notion that inhibition of a cyclophilin isoform may be responsible for the antiangiogenic activity of CsA. Thus, although the cyclophilins play the role of scaffold in the immunosuppressive action of CsA, they may be the primary targets in the antiangiogenic mechanism of the drug. The in vivo antiangiogenic activity of *N*-MeVal-4-CsA further suggests that cyclophilins play an essential role in our models of pathological angiogenesis. It is noteworthy that compared with CsA in the Matrigel model *N*-MeVal-4-CsA was equipotent (Fig. 4), which indicates that the calcineurin inhibitory activity of CsA does not contribute additional potency above what is conferred by cyclophilin inhibition alone.

The existence of a least 20 isoforms of cyclophilin in the human proteome makes it difficult to pinpoint the specific isoforms that may mediate the antiangiogenic activity of CsA and other cyclophilin inhibitors (Daum et al., 2009). The sheer number of candidate cyclophilins is further complicated by significant redundancy within the cyclophilin family. This is evident even in the case of ternary complex formation, in which CyPA, CyPB, and CyPC all are capable of ligating CsA to inhibit calcineurin (Swanson et al., 1992; Bram et al., 1993). In addition, mice lacking CyPA or CyPD, two potential targets for the antiangiogenic activity of CsA, have not been noted to have defects in angiogenesis (Colgan et al., 2004; Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). This implies that either

these cyclophilins are probably not relevant targets for the antiangiogenic properties CsA or that redundancy among the cyclophilins also extends to their role in angiogenesis.

Nonetheless, inhibition of cyclophilin activity by CsA is a plausible molecular mechanism for CsA's antiangiogenic activity. Indeed, cyclophilins are important for a number of biological processes. For instance, the enzymatic activity of CyPD is necessary for formation of the mitochondrial permeability transition pore, and CyP40 forms a complex with Hsp90 and the glucocorticoid receptor as part of a steroid signaling pathway (Owens-Grillo et al., 1995; Tanveer et al., 1996). Mitochondrial permeability transition pore formation promotes cell death and is prevented by CsA and nonimmunosuppressive analogs, presumably through CyPD inhibition (Kim et al., 2003). The potential of CyPD inhibition by nonimmunosuppressive CsA analogs is being pursued as a therapeutic strategy in a number of diseases including muscular dystrophy (Tiepolo et al., 2009; Wissing et al., 2010). It is possible that CyP inhibition in antiangiogenesis may develop as a similarly viable target. That we have shown that *N*-MeVal-4-CsA is capable of inhibiting angiogenesis in vivo in two independent animal models buoys this potential. In addition, although a major complication of CsA treatment is dose-limiting nephrotoxicity, this correlates with calcineurin inhibition not cyclophilin binding, which raises the possibility that high-dose treatment with nonimmunosuppressive analogs such as *N*-MeVal-4-CsA may be adapted safely as part of an antiangiogenic drug regimen (Sigal et al., 1991).

Acknowledgments

We thank Professor Wade Gibson (The Johns Hopkins University) for the kind gift of HFFs; members of the Liu laboratory for helpful advice, stimulating discussions, and technical assistance; Denis Titov and Dr. Noy Bassik for careful reading of this article; and Dr. Matthias Weiwad for a sample of Cyp33.

Authorship Contributions

Participated in research design: Nacev, Low, Fischer, Zhang, and Liu.

Conducted experiments: Nacev, Low, Huang, T. T. Su, Alkuraya, Kasuga, Sun, and Träger.

Contributed new reagents or analytic tools: Z. Su and Braun.

Performed data analysis: Nacev, Zhang, and Liu.

Wrote or contributed to the writing of the manuscript: Nacev, Low, Fischer, and Liu.

References

- Armesilla AL, Lorenzo E, Gómez del Arco P, Martínez-Martínez S, Alfranca A, and Redondo JM (1999) Vascular endothelial growth factor activates nuclear factor of activated T cells in human endothelial cells: a role for tissue factor gene expression. *Mol Cell Biol* 19:2032–2043.
- Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, et al. (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 434:658–662.
- Basso E, Fante L, Fowlkes J, Petronilli V, Forte MA, and Bernardi P (2005) Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. *J Biol Chem* 280:18558–18561.
- Benelli U, Ross JR, Nardi M, and Klintworth GK (1997) Corneal neovascularization induced by xenografts or chemical cautery. Inhibition by cyclosporin A. *Invest Ophthalmol Vis Sci* 38:274–282.
- Borel JF, Feurer C, Gubler HU, and Stähelin H (1976) Biological effects of cyclosporin A: a new antilymphocytic agent. *Agents Actions* 6:468–475.
- Bram RJ, Hung DT, Martin PK, Schreiber SL, and Crabtree GR (1993) Identification of the immunophilins capable of mediating inhibition of signal transduction by cyclosporin A and FK506: roles of calcineurin binding and cellular location. *Mol Cell Biol* 13:4760–4769.
- Carmeliet P (2003) Angiogenesis in health and disease. *Nat Med* 9:653–660.
- Colgan J, Asmal M, Neagu M, Yu B, Schneidkraut J, Lee Y, Sokolskaja E, Andreotti A, and Luban J (2004) Cyclophilin A regulates TCR signal strength in CD4+ T cells via a proline-directed conformational switch in Itk. *Immunity* 21:189–201.

- Crabtree GR and Clipstone NA (1994) Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Annu Rev Biochem* **63**:1045–1083.
- Daum S, Schumann M, Mathea S, Aumüller T, Balsley MA, Constant SL, de Lacroix BF, Kruska F, Braun M, and Schiene-Fischer C (2009) Isoform-specific inhibition of cyclophilins. *Biochemistry* **48**:6268–6277.
- Dyer BW, Ferrer FA, Klinedinst DK, and Rodriguez R (2000) A noncommercial dual luciferase enzyme assay system for reporter gene analysis. *Anal Biochem* **282**: 158–161.
- Fischer G, Wittmann-Liebold B, Lang K, Kieffhaber T, and Schmid FX (1989) Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. *Nature* **337**:476–478.
- Graef IA, Chen F, Chen L, Kuo A, and Crabtree GR (2001) Signals transduced by Ca^{2+} /calcineurin and NFATc3/c4 pattern the developing vasculature. *Cell* **105**: 863–875.
- Hernández GL, Volpert OV, Iñiguez MA, Lorenzo E, Martínez-Martínez S, Grau R, Fresno M, and Redondo JM (2001) Selective inhibition of vascular endothelial growth factor-mediated angiogenesis by cyclosporin A: roles of the nuclear factor of activated T cells and cyclooxygenase 2. *J Exp Med* **193**:607–620.
- Ivy SP, Wick JY, and Kaufman BM (2009) An overview of small-molecule inhibitors of VEGFR signaling. *Nat Rev Clin Oncol* **6**:569–579.
- Jain RK, Duda DG, Clark JW, and Loeffler JS (2006) Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nat Clin Pract Oncol* **3**:24–40.
- Janowski B, Wöllner S, Schutkowski M, and Fischer G (1997) A protease-free assay for peptidyl prolyl *cis/trans* isomerases using standard peptide substrates. *Anal Biochem* **252**:299–307.
- Johnson EN, Lee YM, Sander TL, Rabkin E, Schoen FJ, Kaushal S, and Bischoff J (2003) NFATc1 mediates vascular endothelial growth factor-induced proliferation of human pulmonary valve endothelial cells. *J Biol Chem* **278**:1686–1692.
- Jones CA, London NR, Chen H, Park KW, Sauvaget D, Stockton RA, Wythe JD, Suh W, Larrieu-Lahargue F, Mukoyama YS, et al. (2008) Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability. *Nat Med* **14**:448–453.
- Kim JS, He L, and Lemasters JJ (2003) Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. *Biochem Biophys Res Commun* **304**: 463–470.
- Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, and Schreiber SL (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**:807–815.
- Liu JO (2009) Calmodulin-dependent phosphatase, kinases, and transcriptional corepressors involved in T-cell activation. *Immunol Rev* **228**:184–198.
- Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, and Tsujimoto Y (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* **434**:652–658.
- Nicolli A, Basso E, Petronilli V, Wenger RM, and Bernardi P (1996) Interactions of cyclophilin with the mitochondrial inner membrane and regulation of the permeability transition pore, and cyclosporin A-sensitive channel. *J Biol Chem* **271**: 2185–2192.
- Owens-Grillo JK, Hoffmann K, Hutchison KA, Yem AW, Deibel MR Jr, Handschumacher RE, and Pratt WB (1995) The cyclosporin A-binding immunophilin CyP-40 and the FK506-binding immunophilin hsp56 bind to a common site on hsp90 and exist in independent cytosolic heterocomplexes with the untransformed glucocorticoid receptor. *J Biol Chem* **270**:20479–20484.
- Papageorgiou C, Borer X, and French RR (1994) Calcineurin has a very tight-binding pocket for the side chain of residue 4 of cyclosporin. *Bioorg Med Chem Lett* **4**:267–272.
- Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA, Pauly RR, Grant DS, and Martin GR (1992) A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest* **67**:519–528.
- Rafiee P, Heidemann J, Ogawa H, Johnson NA, Fisher PJ, Li MS, Otterson MF, Johnson CP, and Binion DG (2004) Cyclosporin A differentially inhibits multiple steps in VEGF induced angiogenesis in human microvascular endothelial cells through altered intracellular signaling. *Cell Commun Signal* **2**:3.
- Rao A, Luo C, and Hogan PG (1997) Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* **15**:707–747.
- Rinne A, Banach K, and Blatter LA (2009) Regulation of nuclear factor of activated T cells (NFAT) in vascular endothelial cells. *J Mol Cell Cardiol* **47**:400–410.
- Rosenwirth B, Billich A, Datema R, Donatsch P, Hammerschmid F, Harrison R, Hiestand P, Jaksche H, Mayer P, and Peichl P (1994) Inhibition of human immunodeficiency virus type 1 replication by SDZ NIM 811, a nonimmunosuppressive cyclosporine analog. *Antimicrob Agents Chemother* **38**:1763–1772.
- Schinz AC, Takeuchi O, Huang Z, Fisher JK, Zhou Z, Rubens J, Hetz C, Danial NN, Moskowitz MA, and Korsmeyer SJ (2005) Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. *Proc Natl Acad Sci USA* **102**:12005–12010.
- Sharpe RJ, Arndt KA, Bauer SI, and Maione TE (1989) Cyclosporine inhibits basic fibroblast growth factor-driven proliferation of human endothelial cells and keratinocytes. *Arch Dermatol* **125**:1359–1362.
- Sigal NH, Dumont F, Durette P, Siekierka JJ, Peterson L, Rich DH, Dunlap BE, Staruch MJ, Melino MR, and Koprak SL (1991) Is cyclophilin involved in the immunosuppressive and nephrotoxic mechanism of action of cyclosporin A? *J Exp Med* **173**:619–628.
- Swanson SK, Born T, Zydowsky LD, Cho H, Chang HY, Walsh CT, and Rusnak F (1992) Cyclosporin-mediated inhibition of bovine calcineurin by cyclophilins A and B. *Proc Natl Acad Sci USA* **89**:3741–3745.
- Tanveer A, Virji S, Andreeva L, Totty NF, Hsuan JJ, Ward JM, and Crompton M (1996) Involvement of cyclophilin D in the activation of a mitochondrial pore by Ca^{2+} and oxidant stress. *Eur J Biochem* **238**:166–172.
- Tiepolo T, Angelin A, Palma E, Sabatelli P, Merlini L, Nicolosi L, Finetti F, Braghetta P, Vuagniaux G, Dumont JM, et al. (2009) The cyclophilin inhibitor Debio 025 normalizes mitochondrial function, muscle apoptosis and ultrastructural defects in Col6a1^{-/-} myopathic mice. *Br J Pharmacol* **157**:1045–1052.
- Watson JV, Chambers SH, and Smith PJ (1987) A pragmatic approach to the analysis of DNA histograms with a definable G1 peak. *Cytometry* **8**:1–8.
- Wissing ER, Millay DP, Vuagniaux G, and Molkentin JD (2010) Debio-025 is more effective than prednisone in reducing muscular pathology in *mdx* mice. *Neuromuscul Disord* **20**:753–760.
- Youn HD, Sun L, Prywes R, and Liu JO (1999) Apoptosis of T cells mediated by Ca^{2+} -induced release of the transcription factor MEF2. *Science* **286**:790–793.

Address correspondence to: Jun O. Liu, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Hunterian 516, Baltimore, MD 21205. E-mail: joliu@jhu.edu