A calcineurin-independent mechanism of angiogenesis inhibition by a non-immunosuppressive Cyclosporin A analog

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

Department of Pharmacology and Molecular Sciences (B.A.N., W.L., J.O.L), Medical Scientist Training Program (B.A.N.), and Department of Oncology (J.O.L), Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA; S & T Global Inc. (Z.H., T.T.S, Z.S.), Woburn, MA 01801, USA; Shiley Eye Center and Institute for Genomic Medicine (H.A., D.K., W.S., K.Z.), University of California San Diego, La Jolla, CA 92037, USA; Max Planck Research Unit for Enzymology of Protein Folding (M.T., G.F.), D-06120 Halle (Saale), Germany; Department of Chemistry (M.B.), University of Dusseldorf, D-40225 Dusseldorf, Germany.
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Address Correspondence to: Jun O. Liu; 725 N. Wolfe St., Hunterian 516, Baltimore, MD 21210. Phone: 410-955-4619  Fax: 410-955-4620. Email: joliu@jhu.edu

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Nonstandard Abbreviations: HUVEC, human umbilical vein endothelial cells; HFF, Human Foreskin Fibroblasts; CsA, cyclosporin A; N-MeVal-4-CsA, N-Methylvaline-4-cyclosporin A; Io, ionomycin; NFAT, Nuclear Factor of Activated T cells; CyP, cyclophilin; CyPA, cyclophilin A; VEGF, Vascular Endothelial Growth Factor; bFGF, basic fibroblast growth factor.

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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 non-immunosuppressive analog of CsA which 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 anti-angiogenic activity of CsA than previously believed, and that cyclophilins may be potential anti-angiogenic drug targets.
Introduction

Cyclosporin A (CsA) is a powerful immunosuppressant drug prescribed in the context of solid organ transplantation. 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 (Benelli et al., 1997; Sharpe et al., 1989). Based on its well characterized activity in immunosuppression, this anti-angiogenic 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 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 anti-angiogenic drugs (Ivy et al., 2009; Jain et al., 2006).

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; Liu, 2009; Rao et al., 1997). In turn, calcineurin dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT). This allows NFAT to translocate to the nucleus and to 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). Importantly, 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 anti-angiogenic property of CsA has also been thought to depend on inhibition of calcineurin (Armesilla et al., 1999; Hernandez 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 while 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), this activation is transient, returning to baseline within two to four hours 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 previously appreciated. 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, due to the limitations of genetic manipulations in primary cells we have employed a chemical biology approach to instead “knock-out” function in CsA to address this fundamental question. We have identified a non-immunosuppressive 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 non-immunosuppressive analog also retained potency against a panel of eight cyclophilins, and was therefore used as a tool to assess the role for CyPs in HUVEC proliferation and in two models of in vivo angiogenesis in which it retained activity. In addition, we also showed that in proliferating endothelial cells calcineurin was inactive and that when exogenously stimulated, the IC₅₀ for calcineurin inhibition by CsA was much lower than that for proliferation inhibition. Together, these results suggest that cyclophilin(s) may be a more relevant target for the anti-angiogenic activity of CsA than previously recognized.
Materials and Methods

Materials and Equipment

Bovine serum albumin (BSA), recombinant cyclophilin A, and 10% neutral buffered formalin were purchased from Sigma (St. Louis, MO). Triton-X-100 was purchased from Fischer (Waltham, MA). Immu-mount was purchased from Thermo Scientific (Waltham, MA). [3H]-thymidine (1 mCi/ml in aqueous buffer) was purchased from PerkinElmer (Waltham, MA). Anti-PARP (9542) antibody was purchased from Cell Signaling (Danvers, MA). Anti-tubulin (B7, sc-5286), anti-NFAT2 (7A6, sc-7294), anti-NFAT3 (H74, sc-130306), anti-NFAT4 (M75, sc-8321) and anti-GAPDH (V18, sc-20357) antibodies and anti-mouse (sc-2031) and anti-goat (sc-2033) HRP conjugated IgG (sc-2031) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-lamin A/C antibody (NCL-LAM-A/C) was purchased from Novocastra (Wetzlar, Germany). Anti-rabbit IgG (NA394V) was purchased from GE Healthcare (Chalfont St. Giles, UK). Donkey anti-mouse Alexa Fluor 488 was purchased from Invitrogen (A11008; Carlsbad, CA). Carrier free recombinant mouse basic FGF, 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. Pooled HUVEC and EGM-2 bullet kits were purchased from Lonza (Basel, Switzerland). DMEM media, RPMI 1640 media, fetal bovine serum (FBS), trypsin, and penicillin/streptomycin were purchased from Gibco (Carlsbad, CA). Cyclosporin A and ionomycin were purchased from LC labs (Woburn, MA).
Cell Culture

Cells were incubated at 37°C in a humidified environment with 5% CO₂ present. HUVEC were grown in EGM-2 bullet kit media. All experiments were conducted with HUVEC between passage 3 and 8. HeLa cells were grown in low glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Jurkat T cells (E6.1, ATCC; 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 0.5x10⁶ and 2x10⁶ cells/mL were collected by centrifugation at 500xg and washed 3x with serum free RPMI 1640 media. Cells were resuspended in serum free RPMI 1640 media at a density of 10⁷ cells/360 μL. 15 μg of the mIL-2 luciferase reporter plasmid (Youn et al., 1999) was added per 10⁷ cells and incubated at room temperature (RT), 22°C, for 10 minutes before transferring the suspension in 360 μL aliquots to 0.4 cm gap gene pulser cuvettes (BioRad; Hercules, CA). Cells were electroporated using a Gene Pulser II and Capacitance Extender II (BioRad) at the high capacitance setting, 0.250 kV, and 0.950 μF. After resting for 10 minutes in the cuvette, cells were transferred to complete RPMI 1640 media at a density of 10⁶ cells/mL and allowed to recover for 24 h under normal growth conditions.

IL-2 reporter assay
After a 24h recovery, cells electroporated with the IL-2 reporter construct were collected by 500xg centrifugation and resuspended at a density of $10^6$ cells/mL in complete RPMI 1640 media. 800 μL of this suspension was aliquoted per well of a 24-well plate. Drugs were serially diluted in DMSO as 1000X stocks which were subsequently diluted to 40X in serum free RPMI 1640 media prior to addition to the 24-well plate. Vehicle only was used as a negative control. Cells were incubated with drug for 30 minutes under normal culture conditions prior to the addition of PMA (40 nM) and ionomycin (1 μM), both from 1000X stocks in DMSO that were diluted to 40X in PBS prior to addition to the culture media at the final 1X 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 (500xg at 4°C) and washed 1X with ice cold PBS. Pellets were flash frozen in a dry ice EtOH bath, transferred to wet ice and resuspended in 100 μL lysis buffer by pipetting and vortexing briefly. 75 μL of lysate was transferred to an opaque 96-well plate (Nunc) and luciferase activity of this sample was determined over a 5 second interval following a 5 second delay after automated injection of 100 μL of luciferase substrate buffer using a 1450 Microbeta apparatus (Wallac). Both the lysis buffer and luciferase buffer were prepared as previously described, except that the concentration of luciferin was 121 μM (Dyer et al., 2000). The luciferase activity in each samples was normalized to the total protein level in the lysates (determined by Bio-Rad protein assay, cat # 500-0006).

**Cell proliferation assay**

Cells were seeded at 2000 cells/well in a 96-well plate (Costar) in 199 μL media and allowed to recover overnight. Drugs were added from 200X stocks in DMSO. After a 24 h incubation,
cells were pulsed with 0.9 μCi of [3H]-thymidine for 6 h, washed once with PBS, trypsinized and transferred to filtermats (Wallac; Waltham, MA) using a Mach III M Harvester 96 (Tomtec; Hamden, CT). After drying overnight, the amount of [3H]-thymidine retained on the membrane was determined by scintillation counting using a 1450 Microbeta apparatus (Wallac). At least three independent experiments with multiple technical replicates for each drug concentration were performed. All counts were normalized to vehicle only treated cells. GraphPad Prism (v4.03) software (GraphPad Software; La Jolla, 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 μL of media. Drugs were added from 200X stocks in DMSO and cells were incubated for 30 hours under normal growth conditions. The cells were then washed with 200 μL of PBS and incubated for 30 minutes at 37°C with 100 μL of 1 μM Calcein AM (Invitrogen, C1430) diluted from a 1 mM stock in DMSO. The excess dye was then aspirated and replaced with 100 μL PBS prior to analysis using a fluorescence plate reader with gain adjusted to 100 μL of 1 μM 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 μM with 1M 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 preformed.

**Immunofluorescence**
HUVEC were seeded on glass coverslips at 50-60% confluency and allowed to recover overnight. Cells were pre-treated with drugs from 200X stocks in DMSO prior to stimulation with ionomycin from a 40X stock in PBS (diluted from a 2000X DMSO stock) or with VEGF<sub>165</sub> diluted from a 2000X stock in 0.1% BSA in PBS. Mock stimulations were carried out by treating similarly but with vehicle only. After stimulation, media was aspirated and cells were fixed with ~4% paraformaldehyde in PBS for 20 minutes. Coverslips were washed 3X with PBS, and the cells were permeabilized with 0.1% triton-X-100 in PBS for 1-3 minutes. After a PBS wash, the cells were blocked in 4% BSA in PBS for 1-1.5 hours 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 hours at 4°C. Coverslips were washed 3X in PBS and incubated for 30 minutes in the dark with donkey anti-mouse Alexa Fluor 488 (1:800 dilution) and DAPI (0.1 μg/mL) in 1% BSA in PBS. After a 3X PBS wash, coverslips were mounted using Immuno-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. 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 prior to treatment with ionomycin (1 μM) or vehicle...
control for 15 minutes during which time they were incubated on poly-lysine (Sigma, P8920) coated glass bottom dishes (Mat Tek, P35G-0-14-C; 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 Calceinurin Colorimetric Drug Discovery Kit (Biomol, AK-804; Plymouth Meeting, PA) 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 minutes at RT by combining 45 pmol cyclophilin A (from a 78 μM stock) with 225 pmol drug (from a 450 μM stock in DMSO) in a 10 μL 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 minutes prior to the addition of the RII phosphopeptide substrate (16.4 μg). After a 15 minute reaction at 30°C, 100 μL of developing reagent was added. Following a 16 minute incubation at RT the A$_{620}$ 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 previously described (Daum et al., 2009). To obtain human Cyp40 (PPID) and Cyp33 (PPIE) the genes was PCR-amplified using gene-specific primers from an ORF encoding human PPID and PPIE (imaGenes, Berlin) and cloned into pET28a. After overexpression in *E.coli* BL21 cells, purification of His-Tag fusion proteins were performed using affinity chromatography on Ni-
NTA resin followed by size exclusion chromatography in 10 mM HEPES, pH 7.8, 6 mM KCl, 1.5 mM MgCl₂, 1 mM DTT.

The expression vector CypJ-pTXB1 for human CypJ was a kind gift of Long Yu, 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**

*Kᵢ* values were determined with the protease-free PPIase assay as previously described (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 µM) as substrate. *Kᵢ*-values were calculated from 8-11 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 per a protocol approved by the Johns Hopkins Animal Care and Use Committee. Male athymic nude mice, 4-6 weeks old, weighing an average of 25 g were purchased from NCI Frederick (Frederick, MD). Mice were treated once daily for twelve days with 25 mg/kg drug or vehicle (4.5% DMSO in sterile olive oil [Sigma]) by intra-peritoneal injection of a 100 µL suspension in the lower right quadrant. After day two of treatment, 250 µL of high concentration Matrigel combined with freshly reconstituted 100 ng/mL recombinant mouse VEGF₁₆₄ and 150 ng/mL recombinant mouse basic FGF was injected subcutaneously in
the abdominal skin at roughly the level of the iliac crest, lateral to the midline. After twelve days of treatment, the mice were sacrificed and the Matrigel plugs were excised and fixed in 10% neutral buffered formalin prior to processing by the Johns Hopkins University School of Medicine histology core facility. Two sections of each plug separated by ten steps were mounted and stained with MAS trichrome. The slides were coded and a blinded observer determined the number of erythrocyte filled vessels per 200X 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 µm spot size, 100 millisecond 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 µl of N-MeVal-4-CsA (10 µM) 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 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 was skewed towards near-zero values. As a result, it was necessary to log transform the raw data to better approximate a normal distribution prior to
performing a parametric analysis. All hypothesis testing was performed using a two-sample, two-tailed t-test in R (version 2.8.1) or Microsoft Excel.

**Cell Cycle Analysis**

HUVEC were seeded at 5 x 10⁵ cells / 15 cm dish, allowed to recover overnight and subsequently treated with drugs or vehicle control for 24 hours. Media was then collected and set aside. Cells were washed with PBS, trypsinized, combined with set aside media, pelleted at 500xg and then washed with 10 mL PBS followed by another 500xg spin. The pellet was resuspended in 0.5 mL PBS and added dropwise using a Pasteur pipet to 2 mL 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 500xg, resuspended in 5 mL PBS, rested 60 seconds, pelleted again and washed in 5 mL PBS. The cell pellet was then resuspended in 0.5 mL staining solution (0.1% Triton-X-100, 0.2 mg/mL DNase free RNase A, and 0.02 mg/mL propidium iodide [O1514, Sigma]). Cells were allowed to stain for 30 minutes – 1 hour prior to analysis. Propidium iodide incorporation was measured using using a FACSCalibur (Becton Dickinson). The percentage of cells in each cell cycle stage was determined with FlowJo (v7.5.5) using a Watson analysis (Watson et al., 1987).

**Cell Fractionation**

1.5 x 10⁶ HUVEC were seeded on a 15 cm dish (Falcon; Bedford, MA)), allowed to recover overnight, and then treated with either ionomycin (1 μM) from a 40X stock in PBS (diluted from a 2000X DMSO stock) or vehicle for 15 minutes. The cells were then washed once with PBS, incubated with pre-warmed (37 °C) trypsin-EDTA (Gibco) for one minute at 37 °C. The cells
were then collected in 10 mL ice cold media, spun at 1800xg for 5 minutes at 4 °C, resuspended in 10 mL ice cold PBS and spun again at 1800xg for 5 minutes at 4 °C. The cell pellet was resuspended in 300 μL 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, 0.5 mM DTT [added fresh], and 1X protease inhibitor cocktail [added fresh] [11872580001, Roche;]), spun at 1800xg for 5 minutes at 4 °C, resuspended in 300 μL fresh hypotonic buffer and rested on ice for 20 minutes. 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 3300xg for 15 minutes 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. 4X SDS loading buffer (240 mM Tris pH 6.8, 7.7 % sodium dodecyl sulfate, 38.4 % glycerol, 0.0024 % bromophenol blue, 50 μL/mL β-mercapto-ethanol) was immediately added to each fraction to give a 1X final concentration and the samples were boiled for 10 minutes prior to separation by SDS-PAGE.

**Apoptosis drug treatments**

5 x 10⁴ cells were seeded in 3 mL media in each well of a 6-well plate and allowed to recover overnight. The media was then replaced with 3 mL fresh media and cells were treated with DMSO (0.5% final concentration) or ~2X the IC₅₀ dose of CsA (5 μM) or N-MeVal-4-CsA (8 μM) for 24 hours or 200 nM staurosporine for 5 hours. The media was then aspirated and 80 μL of 2X SDS loading buffer was added to each well and collected after a 20 minute incubation on ice. The lysates was then boiled for 10 minutes and proteins were separated by SDS-PAGE.
Western blotting

After SDS-PAGE, proteins were transferred to PVDF membranes (88518, Thermo) which were then blocked for 15 – 30 minutes in 5 % blotto (sc-2325, Santa Cruz) in TBS-T (10 mM Tris pH 8.0, 150 mM NaCl, 0.05 % Tween 20) for NFAT blots or 5 % BSA (A9647, Sigma) 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 3X with TBS-T, HRP-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 minutes to 1 hour. Membranes were washed 3X with TBS-T and incubated for 1 – 5 minutes with ECL substrate (WBKLS0500, Millipore; Billerica, MA) and bands were visualized with a Kodak Image Station 440 CF.
Results

Identification of a CsA Analog Which 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 3rd or 4th position to create a series of analogs (Figure 1A) which were then screened in assays for inhibition of human umbilical vein endothelial cell (HUVEC) proliferation and for 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 to CsA itself.

Among the CsA analogs tested was N-Methylvaline-4-cyclosporin A (N-MeVal-4-CsA), which has been previously reported to suffer from a dramatic loss of relative immunosuppressive activity in a mixed-lymphocyte reaction (MLR) and in an IL-2 reporter assay when compared to CsA (Papageorgiou et al., 1994). This analog was roughly 10,000-fold less potent than CsA in the IL-2 reporter assay, which is four fold less potent than previously reported (Table 1, Figure
In contrast, N-MeVal-4-CsA was similar in potency to CsA against HUVEC proliferation, in which the IC$_{50}$ was 2.6 $\mu$M for CsA and 4.0 $\mu$M for N-MeVal-4-CsA (Table 1, Figure 1B).

Given the micromolar IC$_{50}$ concentrations for HUVEC proliferation, human foreskin fibroblasts (HFF) growth was assayed in the presence of CsA and N-MeVal-4-CsA to determine if the inhibition of HUVEC proliferation was due to non-specific cytotoxicity. 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 (Figure 1D and 1E). In contrast, both drugs affected the viability of HUVEC at or above the IC$_{50}$, suggesting a cell type-specific effect. Notably, N-MeVal-4-CsA caused a greater effect at high concentrations than CsA itself. While both drugs affected HeLa proliferation, they did so less potently than in HUVEC (Supplemental Figure 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 IC$_{50}$ doses, CsA and N-MeVal-4-CsA caused a minor G1-specific delay in HUVEC and did not induce apoptosis (Supplemental Figure 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 the CsA
and CyPA in combination (Figure 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. Out 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 while 5 μM CsA blocked ionomycin-induced NFAT2 nuclear translocation in HUVEC, 10 μM N-MeVal-4-CsA had no effect (Figure 2B). CsA, but not N-MeVal-4-CsA, was also able to block NFAT2 translocation after the acute addition of VEGF165, a physiologic stimulus which activates calcineurin in endothelial cells (Supplemental Figures 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-4CsA analog to block calcineurin activity in vitro, or NFAT translocation in HUVEC suggested that the 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 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 two to four hours 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 (Figure 1), nearly all the NFAT2 was localized to the cytoplasm (Figure 3A). While we did observe that a small fraction of NFAT2 signal colocalized with the DAPI (a nuclear marker) in absence of ionomycin, this fraction was not significantly perturbed (p = 0.27) by incubation with CsA (5 μM) for 30 minutes (Figure 3A and 3B). Treatment with 10 μM N-MeVal-4-CsA in the absence of ionomycin stimulation also had no effect on the distribution of NFAT2 (Supplemental Figure 3). Nuclear export of NFAT2 was sufficiently rapid in HUVEC to allow NFAT2 that was potentially in the nucleus prior to the addition of drug to be trapped in the cytosol by CsA treatment (Supplemental Figure 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 non-immunosuppressive analog.
Similarly, 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 Figure 2) (Graef et al., 2001). This is consistent with previous findings in other endothelial cell lines that 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 IC$_{50}$ in the single-digit nanomolar range (Figure 1C). In contrast, CsA inhibits HUVEC proliferation with an IC$_{50}$ of 2.6 µM (Figure 1B). This roughly 1000-fold difference in potency may be due to a number of possibilities including that in the endothelium there are fundamental differences in 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 minutes with 1 µM ionomycin following a 30 minute pre-treatment with
either vehicle or varying concentrations of CsA (Figure 3C). We observed that CsA blocked ionomycin-induced nuclear NFAT2 localization in HUVEC with an IC$_{50}$ near 10 nM. In HUVEC treated with 1 to 50 ng/mL VEGF$_{165}$, a physiologic stimulus used in place of ionomycin, CsA has a similar potency against NFAT2 nuclear translocation (Supplemental Figure 5B). At 10 nM, CsA had no effect on HUVEC proliferation (Figure 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. As N-MeVal-4-CsA has no effect on calcineurin activity (see Figure 2) but inhibits HUVEC proliferation, the next logical functionality that might impact 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 eight human cyclophilins tested, CyPA and CyPB, both CsA and N-MeVal-4-CsA were equipotent with IC$_{50}$ values in the low nanomolar concentration range. N-MeVal-4-CsA lost approximately 3-6 fold of potency against CyPC, CyPD, and USA-CyP but for CyP40 exhibited greater than a four-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 (Nicolli et al., 1996; Papageorgiou et al., 1994).

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 which is structurally distinct from CsA but inhibits CyPA with a K$_i$ of 300 nM, and less potently, CyPB.
and CyPD (Supplemental Figure 6) (Daum et al., 2009). The aryl 1-indanylketone had an IC\textsubscript{50} value of 5.4 μM (3.2, 8.9 [95% CI]) for HUVEC proliferation, but did not inhibit ionomycin-induced NFAT2 translocation, indicating that it has no activity against calcineurin (Figures 2B and Supplemental Figure 5). The potency of the cyclophilin inhibitor was remarkably similar to the IC\textsubscript{50} 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 supports a model in which cyclophilins may be more important to CsA’s anti-angiogenic effects than previously appreciated.

**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 VEGF\textsubscript{164} and injected subcutaneously into male athymic mice which had been receiving daily intra-peritoneal injections of CsA (25 mg/kg), N-MeVal-4-CsA (25 mg/kg), or vehicle beginning two days prior to the implantation of the plugs. After the daily drug dosing was continued for ten additional days, the mice were sacrificed and the plugs were excised, fixed, and stained with MAS trichrome. The number of erythrocyte filled vessels per field was counted in a blinded fashion and averaged (Figure 4A). Compared to 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 (Figure 4B).
To further test whether N-MeVal-4-CsA might reduce pathologic angiogenesis in vivo, we turned to a mouse model for neovascular age-related macular degeneration (wet AMD), a major cause of vision loss in humans. Vision loss in wet AMD is due to the abnormal angiogenesis and invasion of choroidal vessels through a disrupted Bruch’s membrane into the subretinal space. This process of choroidal neovascularization 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 to eyes treated with a control saline solution (Figure 5; p < 0.0065). The extent of inhibition of CNV lesions by N-MeVal-4-CsA is comparable to 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 proliferation 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 1,000-10,000 times more potent against HUVEC proliferation than an in an IL-2 reporter assay (see Figure 1B and 1C), and possessed no activity against calcineurin in either biochemical or cellular assays (see Figures 2, Supplemental Figures 3 and 5). Despite the loss of calcineurin inhibition, N-MeVal-4-CsA inhibited in vivo angiogenesis in two independent animal models (see Figures 4 and 5). While the existence of non-immunosuppressive CsA analogs including N-MeVal-4-CsA and the anti-angiogenic properties of CsA have been known for some time, that a non-immunosuppressive CsA analog inhibits angiogenesis has not been demonstrated (Papageorgiou et al., 1994; Rosenwirth et al., 1994).

These findings suggested that the immunosuppressive and anti-angiogenic 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 hundred fold greater than that for inhibition of HUVEC proliferation and that doses of CsA which completely block calcineurin activation have no effect on HUVEC proliferation (see Figures 1B, 3C, and Supplemental Figure 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 for inhibition of calcineurin activation in endothelial cells (Armesilla et al., 1999; Hernandez et al., 2001). Notably, the 10 nM IC50 for NFAT
translocation in HUVEC corresponds well to the IC$_{50}$ for 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 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 appears far less important 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.

While 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 (Hernandez et al., 2001). We were able to do so by taking advantage of the unique properties of N-Me-Val-4-CsA. Despite the loss of calcineurin inhibitory activity, N-Me-Val-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 anti-angiogenic 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 calceinurin (see Figure 2A and Supplemental
Figure 6), lends further support to the notion that inhibition of a cyclophilin isoform(s) may be responsible for the anti-angiogenic activity of CsA. Thus, while the cyclophilins play the role of scaffold in the immunosuppressive action of CsA, they may be the primary targets in the anti-angiogenic 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. Importantly, when compared to CsA in the Matrigel model, N-MeVal-4-CsA was equipotent (see Figure 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 twenty isoforms of cyclophilin in the human proteome makes it difficult to pinpoint the specific isoform(s) that may mediate the anti-angiogenic 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 are all capable of ligating CsA to inhibit calcineurin (Bram et al., 1993; Swanson et al., 1992). In addition, mice lacking CyPA or CyPD, two potential targets for the anti-angiogenic activity of CsA, have not been noted to have defects in angiogenesis (Baines et al., 2005; Basso et al., 2005; Colgan et al., 2004; Nakagawa et al., 2005; Schinzel et al., 2005). This implies that either these cyclophilins are likely not relevant targets for the anti-angiogenic 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 anti-angiogenic 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 (MPT) 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). MTP pore formation promotes cell death and is prevented by CsA and non-immunosuppressive analogs, presumably through CyPD inhibition (Kim et al., 2003). The potential of CyPD inhibition by non-immunosuppressive 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. Additionally, while 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 non-immunosuppressive analogs like N-MeVal-4-CsA may be adapted safely as part of antiangiogenic drug regimen (Sigal et al., 1991).
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We wish to thank Professor Wade Gibson (JHU) for the kind gift of HFFs and the members of the Liu lab for helpful advice, stimulating discussions, and technical assistance. We are also grateful to Mr. Denis Titov and Dr. Noy Bassik for the careful reading of this manuscript. We thank Dr. Matthias Weiwad for a sample of Cyp33.
Authorship Contributions

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

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

*Contributed new reagents or analytic tools:* Z.Su, Braun.

*Performed data analysis:* Nacev, Zhang, Liu.

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


Footnotes

W.L. Current address: Department of Pharmaceutical Sciences, College of Pharmacy, St. John’s University, Queens, NY 11439, USA

T.T.S Current address: Philips Academy, Andover, MA 01810, USA

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Legends for Figures

Figure 1. Structural Modification Of CsA Leads to the Separation of Its Immunosuppressive and Anti-Angiogenic Properties.

(A) Molecular structures of CsA and its analogs.

(B) HUVEC proliferation assay in which cells were treated for 24 h with drug prior to incubation with $[^3]$H-thymidine for 6 h. Bars indicate SEM (n=3).

(C) IL-2 reporter assay in which Jurkat T cells harboring the mIL-2-luciferase reporter plasmid were incubated for 30 minutes with drug prior to stimulation with ionomycin (1 μM) and PMA (40 nM). After 6 h, cells were lysed and the luciferase activity in the lysates was measured. Bars indicate SEM (n=5 for N-MeVal-4-CsA; n=4 for CsA).

(D) The viability of HFF and HUVEC after treatment with CsA and with (E) N-MeVal-4-CsA was measured by staining with calcein AM. Bars indicate SEM (n=5).

Figure 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 pre-incubation 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 SEM (n=3).

(B) Representative micrographs of confocal images of NFAT2 immunofluorescence and DAPI staining in HUVEC treated with CsA (5 μM), N-MeVal-4-CsA (10 μM), or the aryl 1-indanylketone cyclophilin inhibitor (20 μM) for 30 minutes followed by a 15 minute treatment with ionomycin (1 μM). Scale bar, 20 μm.
Figure 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 minutes followed by a 15 minute treatment with ionomycin (1 μM) (Io column), or CsA (5 μM) or DMSO for 30 minutes followed by a mock stimulation. Scale bar, 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 standard error of the mean (SEM) (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 pre-incubated for 30 minutes with the indicated doses of CsA prior to stimulation for 15 minutes with ionomycin (1 μM). Scale bar, 20 μm.

Figure 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 200X 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 bar, 100 μm.
Figure 5. Efficacy of N-MeVal-4-CsA in an Animal Model of Laser Induced Choroidal Neovascularization (CNV). (A) Bar graph shows the average size of choroidal neovascularization. Error bars indicate SEM (P < 0.0065; n = 24). 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 μm.
### Tables

**Table 1. Potency of CsA Analogs Against the IL-2 Reporter Assay and HUVEC Proliferation**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IL-2 reporter IC$_{50}^{a}$ (nM)</th>
<th>HUVEC Proliferation IC$_{50}^{a}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporin A</td>
<td>1.22 (0.37, 4.03)</td>
<td>2.62 (1.87, 3.65)</td>
</tr>
<tr>
<td>N-MeVal-4-cyclosporin A</td>
<td>&gt;10,000</td>
<td>3.95 (2.97, 5.26)</td>
</tr>
<tr>
<td>1</td>
<td>53.9 (22.9)</td>
<td>1.17 (0.24)</td>
</tr>
<tr>
<td>2</td>
<td>107 (12.1)</td>
<td>3.38 (3.17)</td>
</tr>
<tr>
<td>3</td>
<td>40.4 (26.3)</td>
<td>3.34 (0.94)</td>
</tr>
<tr>
<td>4</td>
<td>12.9 (6.51)</td>
<td>2.71 (1.42)</td>
</tr>
<tr>
<td>5</td>
<td>34.2 (23.2)</td>
<td>7.28 (0.92)</td>
</tr>
<tr>
<td>6</td>
<td>116 (86.6)</td>
<td>5.49 (3.08)</td>
</tr>
</tbody>
</table>

*For Cyclosporin A and N-Me-Val-4-cyclosporin A, 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 SEM.*
Table 2. Comparison of the $K_i$ for CsA and N-MeVal-4-CsA Against Eight Human Cyclophilins

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>alias/gene name</th>
<th>$K_i$ N-MeVal-4-CsA (nM)</th>
<th>$K_i$ CsA$^a$ (nM)</th>
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</thead>
<tbody>
<tr>
<td>CypA</td>
<td>Cyp18/PPIA</td>
<td>7.2±2.0</td>
<td>2.9</td>
</tr>
<tr>
<td>CypB</td>
<td>Cyp23/PPIB</td>
<td>9.7±1.8</td>
<td>8.4</td>
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<tr>
<td>CypC</td>
<td>Cyp23a/PPIC</td>
<td>25.1±8.8</td>
<td>7.7</td>
</tr>
<tr>
<td>Cyp40</td>
<td>PPID</td>
<td>72.7±13.7</td>
<td>319±76</td>
</tr>
<tr>
<td>CypD</td>
<td>Cyp22/PPIF</td>
<td>39.6±7.5</td>
<td>6.7</td>
</tr>
<tr>
<td>USA-Cyp</td>
<td>Cyp19.2/PPIH</td>
<td>293±40</td>
<td>91</td>
</tr>
<tr>
<td>CypJ</td>
<td>Cyp18.1/PPIL3</td>
<td>900±800</td>
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<tr>
<td>Cyp33</td>
<td>PPIE</td>
<td>147.0±21</td>
<td>n.d.</td>
</tr>
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</table>

$^aK_i$ values for inhibition by CsA of CypA, CypB, CypC, PPIL1, and USA-Cyp were taken from (Daum et al., 2009).
**Figure 1**

**A**

Cyclosporin A: \( X,Y=H; \) \( Z=\text{iBu} \)
N-MeVal-4-CsA: \( X,Y=H; \) \( Z=\text{iPr} \)
compound 1: \( X=H; \) \( Y=-\text{S(CH}_2)_2\text{NH-CH}_2\text{CMe}_3; \) \( Z=\text{iBu} \)
compound 2: \( X=-\text{S(CH}_2)_2\text{NH-CH}_2\text{CMe}_3; \) \( Y=H, \) \( Z=\text{iBu} \)
compound 3: \( X=H; \) \( Y=-\text{S(CH}_2)_2\text{NMe}_2; \) \( Z=\text{iBu} \)
compound 4: \( X=H; \) \( Y=-\text{S(CH}_2)_2\text{NEt}_2; \) \( Z=\text{iBu} \)
compound 5: \( X=\text{[O(CH}_2)_3\text{OH}; \) \( Y=H; \) \( Z=\text{iBu} \)
compound 6: \( X=H; \) \( Y=\text{[O(CH}_2)_3\text{OH}; \) \( Z=\text{iBu} \)

**B**

**HUVEC Proliferation**

<table>
<thead>
<tr>
<th>Drug Concentration (M)</th>
<th>CsA</th>
<th>N-MeVal-4-CsA</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-7}</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>10^{-5}</td>
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**C**

**IL-2 Reporter**

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**D**

**CsA**

<table>
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<td>10^{-5}</td>
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**E**

**N-MeVal-4-CsA**

<table>
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Figure 3

A  

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B  

NFAT2 Localization

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<th>CYT/NUC Ratio</th>
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<tr>
<td>DMSO</td>
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<tr>
<td>CsA</td>
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C  

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<td>1nM</td>
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Figure 4

A

Matrigel Plug Model

Erythrocyte Filled Vessels (avg/field)

Vehicle

N-MeVal-4-CsA

CsA

p = 0.052

p = 0.034

B

Vehicle

N-MeVal-4-CsA

CsA
Figure 5

A

Choroidal Neovascularization

CNV lesion size (μm²)

0 5000 10000 15000 20000 25000 30000

N-MeVal-4-CsA  CsA

B

C

Bar scale

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