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

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Introduction

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

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 isomerasases. 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.

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 isomerasases, 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 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 100-1000-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 angiogenesis 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). Immunohistostaining 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) poly- merase 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). Anti-VEGF 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 VEGF_{165} and recombinant human VEGF_{165} were 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% CO_{2} 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 μl. Fifteen micrometers 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-μl 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 μF. 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 1000x stocks, which were subsequently diluted to 40x 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 μM), both from 1000x stocks in DMSO that were diluted to 40x in PBS before 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 (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 μl of lysis buffer by pipetting and vortexing briefly. Seventy five microliters of lyseate was transferred to an opaque 96-well plate (Nalg 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 μl of luciferase substrate buffer using a 1450 Microbeta 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 μM (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 0.199 μl of 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 (PerkinElmer Life and Analytical Sciences) using a Mach III M Harvester 96 (Tomtec, Hamden, CT). After drying overnight, the amount of [3H]thymidine retained on the membrane was determined over a 5-s interval after a 5-s delay after automated addition of 100 μl of ScintiVerse (PerkinElmer Life and Analytical Sciences). The cells were then washed with 200 μl of PBS and 50 μl of 1% BSA in PBS (diluted from a 1000x stock in PBS). The excess dye was allowed to recover overnight. Drugs were added from 200x stocks in DMSO in a 10-μl volume. The complex was then added to recombinant human CypA (25 pmol) and recombinant calmodulin (25 pmol) and allowed to incubate at RT for 30 min before the addition of the ITIO phosphopeptide substrate (16.4 μg). After a 15-min reaction at 30°C, 100 μl 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.

**Gene Expression Assay.** Recombinant human CypA and CypB were purified as described previously (Dyer et al., 2000). To obtain human Cyp40 (PPIP) and Cyp33 (PPIE) the genes were polymerase chain reaction-amplified using gene-specific primers from an open reading frame encoding human PPIP 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-pXBX1 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.** Kᵢ values were determined with the protease-free PPlase assay as described previously (Janowski et al., 1997) in 35 mM HEPES buffer, pH 7.8 (4 mM bovine serum albumin) at 283 K, using Suc-Ala-Ala-Pro-Phe-pNA (64 μM) as substrate. Kᵢ 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 Plugs 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 25g, 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-μl suspension in the lower right quadrant. After day 2 of treatment, 250 μl of high concentration Matrigel combined with freshly reconstituted 100 ng/ml recombinant mouse VEGF₁₆₅ and 150 ng/ml recombinant matrigel 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.
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 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 photoagulation (75-μm 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 mg/kg of IC50 dose of CsA (5 μM) or N-MeVal-4-CsA (8 μM) for 24 h or 200 nM staurosporine for 5 h. The media were then aspirated, and 80 μl of 2× SDS loading buffer was added to each well and collected after a 20-min incubation on ice. The lysates were 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 possesses 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 make up the inner lining of blood vessels and must proliferate 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 angiogenic 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...
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. 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).

**TABLE 1**

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

For CsA and N-Me-Val-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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IL-2 Reporter IC50</th>
<th>HUVEC Proliferation IC50</th>
</tr>
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<tbody>
<tr>
<td>CsA</td>
<td>1.22 (0.37, 4.03)</td>
<td>2.62 (1.87, 3.65)</td>
</tr>
<tr>
<td>N-Me-Val-4-CsA</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>
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</table>

**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-Me-Val-4-CsA; n = 4 for CsA). D and E, the viability of HFF and HUVEC after treatment with CsA (D) and N-Me-Val-4-CsA (E) was measured by staining with Calcein AM. Bars indicate S.E.M. (n = 5).
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 μM CsA blocked ionomycin-induced NFAT2 nuclear translocation in HUVEC, 10 μM 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 VEGF, 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 μM) for 30 min (Fig. 3, A and B). Treatment with 10 μM 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 μM (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 μM), N-MeVal-4-CsA (10 μM), or the aryl 1-indanylketone cyclophilin inhibitor (20 μM) for 30 min followed by a 15-min treatment with ionomycin (1 μM). Scale bar, 20 μm.](image-url)
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-induced NFAT2 translocation, indicating that it has no activity against calcineurin (Fig. 2B and Supplemental Fig. 5). The potency of the calcineurin inhibitor was remarkably similar to the IC_{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 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 VEGF_{164} 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 harvested for analysis.

### 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} (nM)</th>
<th>K_{i} CsA* (nM)</th>
</tr>
</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>
</tr>
<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/PPF</td>
<td>39.8 ± 7.5</td>
<td>6.7</td>
</tr>
<tr>
<td>USA-Cyp</td>
<td>Cyp19/2PPIH</td>
<td>293 ± 40</td>
<td>91</td>
</tr>
<tr>
<td>CypJ</td>
<td>Cyp18.1/PPIL3</td>
<td>900 ± 800</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cyp33</td>
<td>PPIE</td>
<td>147.0 ± 21</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

* K_{i} values for inhibition by CsA of CypA, CypB, CypC, PPI1, 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 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 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 VEGF_{165} 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 VEGF_{165}-induced IC_{50} values for inhibition of calcineurin activation in endothelial cells (Armésilla et al., 1999; Hernández et al., 2001). It is noteworthy that the 10 nM IC_{50} value for NFAT...
translocation in HUVEC corresponds well to the IC<sub>50</sub> 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 endothelial proliferation and pathological angiogenesis (Graef et al., 2001), 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 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 ary1-indanylketo 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 CyP 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 angiogenesis may develop as a similarly viable target. That we have shown that N-Me-Val-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 but 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).

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