Selective Modulation of Nuclear Factor of Activated T-Cell Function in Restenosis by a Potent Bipartite Peptide Inhibitor

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Rationale: Nuclear factor of activated T-cells (NFAT) is importantly implicated in pathological cardiac remodeling and vascular lesion formation. NFAT functionality is mainly regulated by calcineurin, a $\text{Ca}^{2+}$-dependent multi-effector phosphatase. Calcineurin inhibitors such as cyclosporine A (CsA) were shown to be effective in the treatment of restenosis and vascular inflammation but with adverse side effects.

Objective: This prompted the design of more selective inhibitors such as VIVIT and inhibitors of NFAT-calcineurin association, which unfortunately have a poor potency precluding clinical use.

Methods and Results: Here, we describe the rational design of a potent bipartite inhibitor of NFAT–calcineurin interaction, MCV1, which targets two separate calcineurin docking motifs. Modeling, site-directed mutagenesis, and functional studies demonstrated that MCV1 acts by allosteric modulation of calcineurin. Comparable to CsA, MCV1 prevents NFAT activation at nanomolar potency without impairing calcineurin phosphatase activity, nuclear factor-$\kappa B$ nuclear import, and general cell signaling. In contrast, CsA but not MCV1-activated basal level extracellular signal-regulated kinases activity and prevented nuclear import of calcineurin, independent of NFAT activation. In vivo MCV1 abrogated NFAT-mediated T-cell activation in a model of PMA-elicited peritonitis, whereas topical application of MCV1 markedly reduced neointima formation in a mouse model of restenosis.

Conclusions: We designed a bipartite NFAT inhibitor that is more potent than VIVIT and more selective than CsA. MCV1 constitutes not only a powerful tool to unravel NFAT function but also a potential drug candidate for the treatment of diseases implicating NFAT activation. (Circ Res. 2012;110:00-00.)

Key Words: calcineurin • cardiovascular disease • peptide inhibitor • restenosis

Transcription factors of the nuclear factor of activated T-cells (NFAT; C1–C5) family are expressed in T-cells, B-cells, NK-cells, and mast cells, as well as in all major nonimmune cell types relevant to cardiovascular diseases, including cardiomyocytes, vascular smooth muscle cells (VSMCs), endothelial cells, and macrophages. NFAT functionality is tightly regulated by calcineurin, a calmodulin-dependent calcium-activated phosphatase. On activation, calcineurin binds and dephosphorylates cytoplasmic NFAT, which then translocates to the nucleus of activated cells to induce cytokine expression. Except for its key role in immunity, NFAT has been importantly implicated in osteoclast differentiation, muscle fiber-type specialization, cardiac valve development, myocardial hypertrophy, heart failure, and restenosis.1–9

Given the key role of calcineurin–NFAT signaling in various physiological and immunologic processes, its inhibition has long been considered a powerful therapeutic modality in the treatment of graft transplant rejection, autoimmune diseases, and cardiovascular disorders. The traditional immunosuppressants cyclosporine A (CsA) and FK506 disrupt calcineurin phosphatase activity to inhibit all of its downstream effectors, including NFAT. However, their application was associated with adverse side effects such as nephrotoxicity, hypertension, and malignancy.10–12 In search of more selective and less toxic NFAT inhibitors, recent attention has been given to calcineurin–NFAT docking inhibitors that block calcineurin–NFAT interactions rather than phosphatase activity of calcineurin. These efforts were given further impetus to the identification of VIVIT peptide

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The inhibitory potential of synthetic leads, termed inhibitors of NFAT–calcineurin (INCA),16 however, the low potency of VIVIT analog creating a potent bipartite compound, which simultaneously targets two calcineurin docking sites and selectively inhibits NFAT at low nanomolar potency without affecting calcineurin phosphatase activity. Given its favorable features, we propose that this conjugate has great potential in the treatment of immune-related and cardiovascular disorders such as restenosis, cardiac hypertrophy, and transplant rejection.

Materials and Methods

Detailed Methods are provided online.

Transient Transfection and Dual Luciferase Assay

Cells were seeded in 24-well plates. After 24 hours, cells were cotransfected with pNFAT-Luc reporter and pRL-CMV plasmid with FuGene 6 transfection reagent according to the manufacturer’s instructions.

Antibody Array

Custom-designed murine cytokine antibody arrays were performed according to the manufacturer’s instructions (RayBiotech, UK).

Mouse Models

C57BL/6 mice were obtained from Charles River Laboratories, Maastricht, the Netherlands. To measure T-cell activation in vivo, mice were first injected intraperitoneally with either phosphate-buffered saline control, MCV1, or CsA (both 50 μmol, n=5). After 30 minutes, the mice were injected intraperitoneal with PMA (100 ng) or phosphate-buffered saline and, after 2 hours, blood and peritoneal leukocytes were collected.

To induce neointimal lesions, 9-week-old apolipoprotein E−/− mice were fed a western-type diet ad libitum for 1 week before injury and throughout the experiment. Transluminal wire injury of the left common carotid artery was performed as described previously.32 Immediately after denudation, a 25% F-127 pluronic gel (Sigma-Aldrich, Zwijndrecht, the Netherlands) containing phosphate-buffered saline, MCV1 (100 μmol/L), or CsA (100 μmol/L) was applied to the adventitia of the left common carotid artery (10 μL/mouse). After 1 or 4 weeks, the mice were anesthetized, in situ fixed through the left cardiac ventricle, and the carotid arteries were isolated.

Results

Identify the Essential Motif of VIVIT for NFAT Inhibition

Because both SPRIEIT and truncated VIVIT peptides were able to inhibit NFAT activation,13,16,19 it is conceivable that not all of the amino acids of VIVIT are essential for the blockade of calcineurin–NFAT docking. We first sought to define the minimal essential motif of VIVIT via alanine scanning and stepwise truncation. A NFAT1 (also known as NFATc2 or NFATp) reporter gene assay in murine macrophage RAW cells or proliferation assay in murine VSMCs was used to assess NFAT inhibition by the synthetic peptides.20 Stepwise truncation and alanine scan studies confirmed the critical importance of the GxHPVIVIx core motif, where “x” is any amino acid (Figure 1A, B). Second, our lead peptides HPVIVI, HPVIVIT, GHPVIVI, and GHPVIVIT were synthesized, which showed similar inhibition of NFAT transcriptional activation in RAW cells at 100 μmol/L (data not shown). We therefore examined their effect on the inhibition of PDGF-BB–mediated VSMC proliferation with titrated concentration. HPVIVIT and GHPVIVIT were much more potent than HPVIVI or GHPVIVI, suggesting that threonine in the PxxIIT motif is strictly required. HPVIVIT was 10-fold more potent than GHPVIVIT and was thus chosen as the minimal essential motif of VIVIT (Figure 1C).

Bipartite Conjugates of INCA and HPVIVIT Are More Potent Than VIVIT in NFAT Inhibition

We next took advantage of recent findings that INCA compounds inhibit NFAT–calcineurin interactions by reaction to sulfhydryl groups near the putative VIVIT binding cleft.18 We conjugated several INCA mimetics to the N-terminal amino group of HPVIVIT (Figure 2A), arguing that bipartite antagonists of NFAT should bind more avidly to calcineurin by simultaneously interacting with the VIVIT and the INCA binding clefts. Flexible 8- to 27.6-Å linker arms were introduced to stretch the estimated 15-Å gap between the two binding sites. Two of these maleimido-conjugated VIVIT motifs, MCV1 and MCV2, were able to inhibit NFAT activation to basal levels at 100 nmol/L, whereas HPVIVIT or INCA12 were completely ineffective at this concentration. Although MCV3 and MCV4 appeared to be more potent than HPVIVIT, they were unable to totally prevent ionomycin/PMA-stimulated NFAT activation (Online Figure IA). The IC50 values of MCV1 and MCV2 were 61.7 nmol/L and 127.7 nmol/L, respectively (Figure 2B), which is a 1000-fold...
more potent than the parent peptide VIVIT (IC₅₀ = 30–100 μmol/L).¹³,²⁰ MCV1 is at least equally potent as CsA (IC₅₀ = 29.3 nmol/L) and was used as the lead peptide.

We next tested the ability of MCV1 to compete with purified GST-NFATc2 for binding to calcineurin in vitro in a pull-down assay (Figure 2C). MCV1 was partly effective at 100 nmol/L and completely disrupted calcineurin–NFAT interactions at 10 μmol/L, showing similar inhibitory effect to VIVIT in calcineurin binding to GST-NFAT. In keeping, pull-down assay with full-length calcineurin showed that MCV1 partially inhibited calcineurin–NFAT interaction at 1 μmol/L and completely disrupted the binding at 10 μmol/L (Online Figure II). Moreover, MCV1 completely inhibited NFAT dephosphorylation at 1 μmol/L, whereas INCA12, the VIVIT parent peptide, and HPVIVIT were completely ineffective at this concentration (Figure 2D). Quantitative analyses of Western blots are given in Figures 2E and 2F, respectively (n = 3). We next addressed NFAT inhibition by MCV1 in different cells relevant to vasculopathies such as restenosis. MCV1 but not VIVIT at 1 μmol/L could significantly inhibit NFAT transcriptional activation not only in macrophages as shown in Figure 2B but also in endothelial cells, VSMCs, and cardiomyocytes (Online Figure III). Furthermore, MCV1 at 100 nmol/L was seen to abrogate nuclear translocation of NFAT1-GFP after ionomycin stimulation, whereas HPVIVIT showed only weak inhibition even at a 100-fold higher concentration (Figure 2G, 2H). Nuclear distribution of GFP protein was confirmed with DAPI counterstain (Online Figure IV). Remarkably, MCV2 showed potent inhibition of NFAT nuclear import at 1 μmol/L but lost its inhibitory capacity at 10 μmol/L. The mechanism underlying this biphasic activity is unclear.

The inhibitory effect of MCV1 was persistent (Online Figure VA), with an estimated half-life of approximately 36 hours. In addition, MCV1 did not show overt signs of cytotoxicity in murine VSMCs cells as assessed by Trypan blue exclusion assay at concentrations of up to 10 μmol/L (Online Figure VB). Flow cytometry analysis of VSMC apoptosis by Annexin V–FITC/propidium iodine showed that both MCV1 and CsA did not induce VSMC apoptosis or necrosis. (Online Figure VI).

**MCV1 Is More Selective Than CsA in NFAT Inhibition**

The currently available inhibitors of calcineurin–NFAT signaling such as CsA generally cause quite severe side effects, such as renal dysfunction and hypertension. Of note, the toxicity of CsA not only is attributable to disruption of calcineurin–NFAT signaling but also may do so by perturbing other signaling pathways, such as MAP kinase or nuclear factor κB signaling, or by activating endothelial functionality through activation of endothelin-1 (ET-1) or nitric oxide signaling. We first addressed the selectivity of MCV1 over CsA in calcineurin–NFAT signaling. MCV1 at concentrations of up to 10 μmol/L did not inhibit calcineurin phosphatase activity, indicating that MCV1 does not target the calcineurin catalytic site, despite effectively inhibiting calcineurin–NFAT interaction and NFAT dephosphorylation. In contrast, 1 μmol/L CsA inhibited calcineurin phosphatase activity.

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**Figure 1. Identification of the minimal motif in VIVIT essential for effective nuclear factor of activated T-cells (NFAT) inhibition.** A dual-luciferase NFAT reporter assay was used as readout to assess effects of stepwise truncation (A) and alanine scan (B) of the VIVIT parent peptide on NFAT inhibition in murine RAW 264.7 macrophages. Cells were transiently transfected with pNFAT-luc and pRL-CMV and stimulated for 12 hours with PMA (200 nmol/L) and ionomycin (500 nmol/L). Peptides (30 μmol/L) were added 1 hour before stimulation. VIVIT (100 μmol/L) was used as a positive control. Cell lysates were assayed for firefly luciferase activity and values were normalized for that of renilla luciferase. C, Growth-arrested vascular smooth muscle cells (VSMC) were treated for 4 hours with or without PDGF-BB (20 ng/mL) in the presence or absence of peptides at the indicated concentrations. [³H] thymidine (1 μCi/ mL) was added and DNA incorporation was measured after 20 hours. Values represent means±SD of three individual experiments (P<0.05, **P<0.01 vs PDGF-BB–treated group).
Figure 2. MCV1 displays a dramatically enhanced potency compared with VIVIT to inhibit nuclear factor of activated T-cells (NFAT). A, Schematic (left) illustrating MCV structures (pentagon, maleimide; open box, two tandem aminoheptanoic [Ahx] linkers; black box, HPVIVIT peptide backbone). The estimated maximal reach of the maleimido group relative to the histidine of HPVIVIT peptide.
by approximately 90% (Figure 3A). Similar results were observed after PMA/ionomycin stimulation in the presence of MCV1 or CsA (Online Figure VII). Because CsA-induced hypertension and kidney dysfunction were seen to proceed through ET-1–dependent vasoconstriction,13–16 we next examined the release of ET-1 in murine endothelial EOMA cells treated with CsA or MCV1 by enzyme-linked immunosorbent assay, in which CsA but not MCV1 at 10 μmol/L showed a significant increase in ET-1 secretion (Figure 3B).

In addition to NFAT family members, calcineurin also binds endogenous regulators such as A-kinase anchoring protein 79 (AKAP 79) and calcineurin-binding protein–1/ calcium in inhibitor (Cabin-1/Cain), which carry motifs with similarity to the PxIxEIT consensus motif of NFAT.21,22 (Online Figure IB). As shown by Figure 3C, binding of calcineurin to either AKAP 79 or Cabin-1 was not affected by MCV1 at concentrations that suffice for effective disruption of calcineurin–NFAT interaction. We also examined YFP-p65 protein nuclear translocation because nuclear factor κB is another downstream effector of calcineurin (Figure 3D). CsA completely disrupted p65 activation and prevented its nuclear import to basal levels, whereas MCV1 showed no significant effect (Figure 3F).

Calcineurin was seen to translocate to the nucleus together with NFAT as a complex.23–26 We observed a partial nuclear co-import of endogenous calcineurin with NFAT1-GFP on ionomycin stimulation (Figure 3E). CsA at 1 μmol/L completely blocked the nuclear co-import of calcineurin and NFAT. Surprisingly, MCV1 only blunted nuclear import of NFAT (Figure 3G) but not of calcineurin. Therefore, Ca2+-mediated nuclear import of calcineurin is involved in but not directly linked to NFAT activation. Furthermore, we tested if MCV1 impaired general cellular signaling over extracellular signal-regulated kinases (ERK) activation (Figure 3H). Pretreatment with either MCV1 or CsA did not alter PMA-induced ERK activation, and pretreatment with MCV1 alone had no effect on endogenous ERK activity. Interestingly, although CsA did not affect PMA-stimulated ERK activity, it markedly increased phospho-ERK levels in nonstimulated cells at concentrations as low as 0.1 μmol/L. Thus, CsA not only prevented dephosphorylation of NFAT but also facilitated its phosphorylation via activation of the NFAT pathway in an unknown mechanism. This dual mode of action of CsA may be instrumental in the high potency of CsA and its off-target immunotoxicity. CsA was shown to be able to dose-dependently activate ERK, reaching a maximal plateau of ERK phosphorylation under 1 to 10 μmol/L of CsA stimulation (Online Figure VIII).

Molecular modeling studies of MCV1 interaction with calcineurin were performed to provide a mechanistic basis for the profound gain in affinity of MCV1 versus the separate building blocks HPVIVIT and MPB. The most favorable configuration with the lowest energy (Figure 4A) shows that MCV1 has a distinct binding site from the catalytic site of calcineurin and should not impair the calcineurin phosphatase activity. The hydrophobic side chains of MCV1 are in close contact with the β11 loop, whereas the maleimido group is accommodated in the groove formed by the β11–β12 and β13–β14 turns. No solutions in the 20 runs with low energies were found to support a direct contact between the maleimido unit and Cys266, which actually was partially protected by the β11–β12 loop. The minimal binding energy of MCV1 was −12.59 kcal/mol, which is comparable to that reported for PVIVIT (−13 kcal/mol).27 To assess the hypothesis that there is a covalent reaction between maleimido group of MCV1 and sulfhydryl group, NFAT translocation inhibitory capacity of MCV1 was compared to that of MCV1 that had been preincubated with DTT. Pretreatment with DTT totally blunted the inhibitory effect of MCV1 (Figure 4B). Likewise, preincubation cells with sulfhydryl-modifying reagents IAM or INCA12 dose-dependently prevented the MCV1-induced inhibition of NFAT translocation (Figure 4C). To firmly establish that MCV1 covalently binds to calcineurin, purified calcineurin protein was incubated in vitro with MCV1 in the presence or absence of DTT. After native polyacrylamide gel electrophoresis (10%; none denaturing), binding characteristics were analyzed by Western blotting against calcineurin. A progressive portion of full-length calcineurin, size 81 kDa, appeared to migrate faster after incubation with increasing concentration of MCV1 than untreated protein, possibly because of MCV1-induced conformational changes in calcineurin or changes in surface-exposed charge. Importantly, preincubation with equimolar of DTT negated the quicker migration pattern of calcineurin caused by MCV1 at 10 μmol/L, pointing to covalent binding between calcineurin and MCV1 (Online Figure IV). Furthermore, site-directed mutation of Cys266 of calcineurin catalytic unit led to impaired displacement of NFAT by MCV1 but not VIVIT (Figure 4D), supporting a covalent interaction between MCV1 and calcineurin. On the basis of these studies, we propose the model for MCV1-mediated NFAT inhibition depicted in Figure 4E.

Figure 2. (Continued). is given for each of the MCV leads. Spacer arm data were provided by Pierce. Chemical structures of inhibitors of NFAT–calcineurin association (INCA12, MPB, and MBA (right)). B, Inhibition curves of NFAT by MCV1/MCV2 and CsA determined by dual luciferase assay in RAW cells (n=6). C, Lysates from HEK-293 cells expressing FLAG-tagged CnA proteins were analyzed by affinity binding to GST (lane 1) and GST-NFATc2 (residues 4–385) in the presence of VIVIT (lane 3–6) or MCV1 (lane 7–10) at the indicated concentrations. Bound CnA protein was evaluated by Western blotting against FLAG tag. Ponceau S staining is provided as a loading control. D, Western blotting for anti-HA staining demonstrating multiple bands of phosphorylated and faster migrating dephosphorylated NFATc2 proteins. HA-NFAT1–transfected COS-1 cells were stimulated for 15 minutes with ionomycin (1 μmol/L) in the presence of cyclosporine A (CsA), MCV1, INCA12, VIVIT, or HPVIVIT at 1 μmol/L. Ponceau S staining is provided as a loading control. Quantitative analysis of Western blot was performed with Visionworks LS software (UVP, Cambridge, UK) and shown in (E) and (F), respectively, expressed as arbitrary units of relative protein concentration. G, Cellular localization of NFAT1 proteins as visualized by GFP fluorescence. COS-1 cells were transfected with pNFAT1-GFP and 24 hours later were stimulated for 15 minutes with 1 μmol/L ionomycin in the presence or absence of 1 μmol/L of CsA or MCV1. Scale bar=10 μm. H, Quantification of nuclear localization of NFAT1-GFP in the presence or absence of CsA, INCA12, VIVIT, MCV1, or MCV2 at the indicated concentrations (in each well, randomly selected microscopic fields were scored containing ~100 GFP+ cells; n=3).
MCV1 Abrogates NFAT-Mediated T-Cell Activation, VSMC Proliferation, and Reduces Neointima Formation in a Mouse Model of Restenosis

To define the therapeutic potential of MCV1 in a PMA-elicited peritonitis model in vivo, we inoculated mice with MCV1 or CsA. Intraperitoneal pretreatment with both MCV1 and CsA inhibited PMA-elicited CD4\(^+\) T-cell activation as demonstrated by expression of CD69, CD40, and CD86. No effect was observed on the CD8\(^+\) T-cell activation for both compounds (Figure 5A). Total blood cell numbers and pattern remained unaffected (data not shown). ANOVA analysis of creatinin/aspartate amino transferase/alanine amino transferase/lactate dehydrogenase concentrations in PMA stimu-
lated plasma under CsA/MCV1 treatment did not reveal any statistically significant differences between groups (Online Figure X). All other liver toxicity data between groups were negative, which suggests that MCV1 should not have an unexpected side effect. However, unlike MCV1, CsA was seen to stimulate IL-6 secretion, pointing to increased NFκB activation, in accordance with previous findings in Figure 3 (Figure 5B).

NFAT has been shown to regulate myocardial hypertrophy and neointimal formation after vascular injury.17 Previously, we showed that FK506 blocked VSMC hyperplasia and plaque development in atherosclerotic lesions.28 We therefore exam-
ined whether selective NFAT inhibition was equally potent as CsA in preventing VSMC proliferation in vitro and neointimal formation in vivo. MCV1 at 10 μmol/L showed similar potency to CsA (Online Figure XI). We have examined the antihyperplastic effect of MCV1 on neointimal formation in a mouse model of carotid denudation at 4 weeks after surgery. Interestingly, after 4 weeks of lesion development, MCV1 and CsA treatment resulted in a significant 50% reduction in neointima formation in denuded carotid arteries of apolipoprotein E−/− mice compared to a mock-treated control (P=0.02; Figure 6A). Whereas no significant changes in macrophage and collagen content were noted, α-smooth muscle actin-positive VSMCs were significantly increased both in the MCV1 and the CsA-treated groups, indicating a distinct regulation of VSMC proliferation and differentiation by calcineurin–NFAT signaling (Figure 6B). To determine whether either MCV1 or CsA affected the initial process after vascular injury, we also analyzed lesion formation at 1 week after surgery. Topical MCV1 and CsA treatment did not affect reendothelialization, as judged by CD31 staining (Online Figure XII) and by quantitative analysis of Evans Blue-stained vessel area (Online Figure XIV). Adequate carotid endothelial denudation in our mouse model was demonstrated by performing Evans Blue staining at day 1 after injury, in which more than 80% of injured left common carotid artery showed positive staining (Online Figure XV). Macrophage content also did not differ between the groups (Online Figure XIII). The vessel areas of media plus intima and lesion cellularity were unaltered at that time point as well as examined by hematoxyline and eosin staining. Similarly, as in the 4-week study, smooth muscle α-actin-positive VSMC content was increased, particularly in the MCV1-treated mice. Thus, MCV1 seems to preferentially inhibit injury-induced smooth muscle cell differentiation from a contractile to a proliferative phenotype. No significant differences were observed in the morphology of the contralateral right common carotid artery either 1 or 4 weeks after surgery (Online Figure XVI).

Discussion

NFAT is deemed to play a pivotal role in the treatment of transplant rejection, autoimmune diseases, and cardiovascular disorders such as restenosis and cardiac hypertrophy. As primary and the most thoroughly characterized substrate of calcineurin, NFAT is regarded as the best candidate target for therapy. Previously, we and others have shown that selective NFAT inhibition by the peptide antagonist VIVIT is an effective strategy to coordinately target inflammatory and VSMC hyperplastic responses that underlie restenosis.17 However, the suboptimal pharmacological features of VIVIT and particularly its low micromolar potency will hamper its direct clinical application. Recently identified small organic molecule inhibitors, INCAs, could selectively inhibit calcineurin–NFAT interaction at micromolar potency16 in vitro. These quinine-based or quinoneimine-based compounds were, however, found to be rather cytotoxic, disqualifying them for therapeutic use.18 Therefore, small molecule inhibitors of NFAT with improved potency are eagerly awaited. Previous attempts to improve cellular entry and thus potency of VIVIT by conjugating a cell-permeable peptide tag to the N-terminal end of VIVIT did display an increased potency, but the gain in affinity was only limited.29 We therefore sought to optimize the peptide by modifying the terminal ends of the central motif with a maleimido entity that was previously shown to allosterically interfere with VIVIT binding to calcineurin, aiming at the generation of a bipartite inhibitor. By stepwise stripping, we identified the shortest motif of VIVIT, HPVIVIT, as an effective inhibitor of NFAT-dependent transcriptional activation and VSMC proliferation. Subsequent conjugation of HPVIVIT at its N-terminal end to a maleimide unit led to the successful design of a maleimido-conjugated peptide with low nanomo-
lar potency for NFAT inhibition. In fact, the potency of the resultant conjugate, MCV1, was almost 1000-fold higher than that of the parental peptide VIVIT. In comparison with INCAs, MCV1 showed no overt cytotoxicity both in vivo and in vitro. We also show that MCV1 does not display the NFAT-independent effects associated with CsA and establishes its efficacy in vivo in a model of peritonitis as well as of restenosis.

Conceivably, the remarkably high affinity of MCV1 results from the simultaneous targeting of two separate NFAT docking sites of calcineurin. However, the binding energy for MCV1 and for PVIVIT are rather similar and combined with the observed DTT-induced loss in affinity, this led us to hypothesize that the high affinity of MCV1 is likely attributable to covalent interaction with calcineurin. The binding model presented in this study may thus reflect the initial step in MCV1 binding to calcineurin, inducing a reconfiguration of the β11-β12 loop and rendering the Cys266 more accessible to the C-terminal maleimide. A second beneficial factor may be that in contrast to the highly hydrophilic characteristic of VIVIT, the MCV analogues are rather lipophilic, favoring cellular uptake. Furthermore, MCV1 contains the central HPVIVIT motif of VIVIT, which was shown capable of inhibiting all four members of the cytoplasmic NFAT family (NFATc1-c4). Combined with the fact that NFATc1, NFATc2, and NFATc3 are coexpressed both in immune cells and in VSMCs, the observed potent NFAT inhibition by MCV1 in various cell types suggests that the conjugate targets all cytosolic isoforms of NFAT.

Nuclear co-import of calcineurin and NFAT may potentially help to maintain a certain level of NFAT activation. Nuclear calcineurin could be detected in more than 90% of cardiomyocytes after chronic stimulation by angiotensin II for 6 hours or longer. In contrast, fewer cells with calcineurin nuclear import were seen within 2 hours of treatment. Interestingly, in the same study inhibition of nuclear import of calcineurin was seen to blunt NFAT activation rather than to prevent NFAT dephosphorylation, indicating that the former is regulated by calcineurin nuclear co-import. In agreement, we observed partial (15%–20%) nuclear colocalization of calcineurin and NFAT after acute stimulation by ionomycin for 30 minutes. Here, we extend these findings by demonstrating that calcineurin nuclear co-import is probably independent of NFAT activation because MCV1 specifi-
ically interferes with calcineurin docking to NFAT, leaving calcineurin phosphates activity unchanged.

Calcineurin was shown to be a multifunctional regulator of various downstream signaling pathways. Except for its major substrate NFAT, calcineurin is able to activate nuclear factor κB (NF-κB) and dephosphorylate BAD. Thus, CsA or FK506 treatment will simultaneously inhibit NFAT activity and that of other downstream substrates of calcineurin. The clinical application of CsA and FK506 is accompanied by undesirable side effects such as nephrotoxicity, hypertension, cancer, and renal dysfunction. So far, it is still unclear to what extent their toxicity is associated with disruption of calcineurin targets other than NFAT, with calcineurin-independent activation of transforming growth factor-β or synergistic activation of multiple signaling pathways. A selective NFAT inhibitor MCV1 may shed light on this issue and constitute a novel drug candidate and may be a possibly less toxic alternative to CsA in the treatment of NFAT-mediated diseases. With the help of MCV1, we were able to more selectively examine the downstream pathway of calcineurin and to compare its inhibitory pattern with CsA. As summarized in Figure 4E, CsA is able not only to quench calcineurin phosphatase activity but also to facilitate MAP kinase-mediated NFAT rephosphorylation in VSMCs. The observed dual mode of action of CsA could at least partly explain the off-target effects of this immunosuppressant. Further study will be needed to elucidate the location and underlying pathways of CsA-induced NFAT hyperphosphorylation.

In conclusion, we identify MCV1 as a synthetic peptide inhibitor of NFAT with nanomolar potency that is not only more potent than VIVIT but also more selective than CsA in inhibiting NFAT signaling. MCV1 represents a novel tool for probing NFAT function in vitro and in vivo in various cell types, and it may assist in clarifying the role of NFAT in these diseases. Its high and selective immunosuppressant capacity in vivo indicates that MCV1 holds promise for clinical use in the treatment of transplant rejection, autoimmune disorders, and cardiovascular diseases implicating NFAT activation, such as restenosis and potentially cardiac hypertropy.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Nuclear factor of activated T-cells (NFAT) is known to play a pivotal role in autoimmune diseases and cardiovascular disorders.
- Calcineurin phosphatase inhibitor cyclosporin A (CsA) is a rather nonspecific inhibitor of NFAT activation and its use is associated with adverse side effects.
- Recently designed NFAT inhibitory peptides (VIVIT) or imidazole-based small molecules are more selective but are toxic or of low potency.

What New Information Does This Article Contribute?

- We have developed a potent VIVIT-based bipartite inhibitor of NFAT–calcineurin interaction, MCV1, which targets two separate calcineurin docking motifs.
- MCV1 is shown to be more potent than VIVIT peptide and more selective than CsA in NFAT inhibition.
- MCV1 inhibits vascular smooth muscle cell proliferation and neointima formation in a carotid injury mouse model.

NFAT is importantly implicated in autoimmune diseases, transplant rejection, and cardiovascular disorders. Calcineurin inhibitors such as CsA generally lack NFAT specificity and cause adverse side effects, whereas selective NFAT inhibitors such as VIVIT and inhibitors of NFAT–calcineurin association (INCA) have poor potency, precluding clinical use. We describe the rational design of a potent bipartite inhibitor of NFAT–calcineurin interaction, MCV1, which targets two separate calcineurin docking motifs. Functional and modeling studies demonstrated that MCV1 acts by allosteric modulation of calcineurin. MCV1 prevents NFAT activation at nanomolar potency, without impairing calcineurin phosphatase activity, nuclear factor-κB nuclear import, and general cell signaling. In contrast, CsA, but not MCV1, activated basal level extracellular signal-regulated kinase nuclear import, and general cell signaling. Thus, the designed bipartite compound is more potent than VIVIT and more selective than CsA in NFAT inhibition. In vivo, MCV1 abrogated NFAT-mediated T-cell activation in a model of PMA-elicited peritonitis, whereas topical application of MCV1 markedly reduced neointima formation in a mouse model of restenosis. Thus, the designed bipartite compound is more potent than VIVIT and more selective than CsA in NFAT inhibition. We envisage that MCV1 constitutes not only a powerful tool to unravel NFAT function but also a potential drug candidate for the treatment of diseases implicating NFAT activation.
Selective Modulation of Nuclear Factor of Activated T-Cell Function in Restenosis by a Potent Bipartite Peptide Inhibitor

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Online Methods

Reagents
N-(9-fluorenyl)methoxy-carbonyl (Fmoc)-protected amino acids, 1-hydroxybenzotriazole (HOBT), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylenonium tetrafluoroborate (TBTU), Fmoc-Thr(Bu)-Wang-resin were purchased from Nova Biochem (Läufelingen, Switzerland). Trifluoroacetic acid (TFA), N,N-diisopropylethylamine (Dipea), dichloromethane (DCM), dichloroethane (DCE), N,N-dimethylformamide (DMF), and piperidine were of peptide grade or higher and purchased from Biosolve (Valkenswaard, The Netherlands). FK506 was obtained from Fujisawa GmbH, Munchen, Germany; cyclosporin A (CsA), phorbol 12-myristate 13-acetate (PMA) and ionomycin were from Sigma (St. Louis, MO, USA); FuGENE-6 transfection reagent was purchased from Roche Applied Science (Basel, Switzerland). The dual luciferase assay kit was from Promega (Madison, WI, USA); PDGF-BB from Biosource International Inc (Camarillo, CA, USA). 4-maleimido-benzoic acid (INCA12) was obtained from ChemBridge Europe, UK. mN-Maleimidobenzoic acid-OSu (3-MBA-OSu) was purchased from Bachem AG, Germany. Succinimidyl 4-[p-maleimidophenyl]butyrate (SMPB) was purchased from Pierce Biotechnology, USA. Pan-calcineurin A antibody, p44/p42 and phospho-p44/p42 MAP Kinase (Thr202/Tyr204) antibodies were from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-mouse-HRP and swine anti-rabbit-HRP antibody were from DAKO (Denmark). Anti-FLAG (M5) monoclonal and rabbit anti-actin antibodies were from Sigma (St. Louis, MO, USA). Mouse anti-HA-Tag monoclonal antibody (clone 12CA5) was from Roche Applied Science (Basel, Switzerland)

Cell culture
Murine RAW 264.7 macrophages, African green monkey kidney (COS-1), human embryonic kidney (HEK293) cells and murine vascular smooth muscle cells (vSMC), isolated from thoracic aortas of male C57BL/6 mice as described, were grown in Dulbecco’s modified Eagles’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. Jurkat cells were cultured in RPMI 1640 Medium. Cultures were maintained at 37 ºC in humidified 95% air/5% CO2. Unless otherwise stated, vSMC were growth-arrested prior to the experiments by incubating in DMEM containing 0.1% FBS for 72 h.

Plasmids and constructs
The NFAT reporter plasmid (pNFAT-luc) was a kind gift from Dr. De Windt, Hubrech Laboratory, Interuniversity Cardiology Institute Netherlands; pRL-CMV was from Promega (Madison, WI, USA); NFAT1-GFP plasmid was provided by Dr. Anjana Rao, Harvard Medical School, Boston, USA. Human AKAP79 (238-380) sequences were amplified from cDNA of HEK293 cells by PCR, employing the following primers: 5’gcggatccaaaaaacagatgcatcaaccccaagcag3’ (forward hAKAP79), 5’cggattcgaaccataaggacccatcgtgc3’ (reverse hAKAP79). The PCR products of AKAP79 (238-380) were cloned into EcoRI-NotI and BamHI-EcoRI digested pGEX-4T3 plasmid (Amersham). Human pGEX-NFAT2C(4-385), pGEX-Cabin-1(2143-2220) and FLAG-tagged human calcineurin Aα subunit (pEF-FLAG-hCnAα 2-389) encoding a constitutive active calcineurin plasmids were kindly provided by Dr. Juan M Redondon. Site-directed C266S mutagenesis of CnAα was generated following the manufacturer’s protocol (Stratagene).

Solid Phase Peptide Synthesis (SPPS)
Full-length MAGPHPVIVITGPHCEE (VIVIT), truncated and alanine-scanned VIVIT peptides, and penetratin-VIVIT were synthesized by Fmoc solid-phase peptide synthesis on a
Multisynotech Syro Multiple Peptide Synthesizer. For MCV peptides, core sequence FmocHN-His(Ttrt)-Pro-Val-Ile-Val-Ile-Thr(tBu)-Wang-resin was manually synthesized by standard Fmoc chemistry. The N-terminal Fmoc group was removed by 20% piperidine in DMF. After washing with 1-Methyl-2-pyrrolidinone (NMP), 3-MBA-OSu (4eq) or SMPB (4eq), DIPEA (8eq) was added and progression of the reaction was monitored by Kaiser’s Test until a negative signal was obtained. The resin was washed with DMF, iso-propanol, methanol and DCM and dried. After removal of the solvent, the peptide conjugates were cleaved from the resin with a trifluoroacetic acid, trisopropylsilane, and water mixture (95:2.5:2.5, v/v/v). Crude peptides were purified on a preparative C18 RP-HPLC column (Alltech) using a BIOCAD VISION automated purification system. Purified peptides were characterized by LC-MS at Leiden Institute of Chemistry, the Netherlands.

**Transient transfection and dual luciferase assay**

RAW cells were seeded in 24-well plates at a density of 5 \times 10^4 cells/well and grown in DMEM supplemented with 10% (v/v) FBS, 100 units/ml penicillin and 100 \mu g/ml streptomycin. After 24 h, cells were co-transfected with pNFAT-Luc (encoding Firefly luciferase) reporter and pRL-CMV reference plasmid (encoding Renilla luciferase) with FuGene 6 transfection reagent according to the manufacturer’s instructions. One day after transfection, cells were treated with or without PMA (200 nmol/L)/ionomycin (500 nmol/L) in the presence or absence of CsA or peptide inhibitor for 12 h. Cell lysates were prepared and simultaneously assayed for firefly and renilla luciferase activity by Dual Luciferase Assay System (Promega) and Turner Luminometer.

**SMC proliferation assay**

Growth-arrested vSMC were treated with 20 ng/ml PDGF-BB in the presence or absence of pretreatment with inhibitors. Four hours later, 1 \mu Ci/ml of \[^{3}H\]-thymidine was added to vSMCs and incubated for 20 h. Cells were washed three times with 1ml ice-cold phosphate buffered saline (PBS) and lysed in 500 \mu l 0.1M NaOH. Cell lysates were transferred to a liquid scintillation vial and 4.5 ml Emulsifier-Safe was added (Packard-Biosciences, Groningen, the Netherlands), Radioactivity was measured in a liquid scintillation counter.

**Western immunoblotting**

Cells were lysed in 1x Protein loading buffer, lysates boiled for 5 min and separated by electrophoresis by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes and incubated in blocking solution (5% (w/v) milk/0.1% Tween 20 in PBS (PBST)) for 60 min at room temperature. To detect ERK activation, membranes were probed with anti-phospho-p44/p42 antibody (0.1% (v/v)) in 5% BSA/PBST for 1h at room temperature followed by incubation for 1 h with horse radish peroxidase-conjugated (HRP) secondary swine anti-rabbit antibody (0.05% (v/v)) in 5% (w/v) milk/PBST. To detect NFAT dephosphorylation, membranes were incubated for 1 h with mouse monoclonal anti-HA antibody 12CA5 (0.05% (v/v)) in 5% (w/v) milk/PBST followed by 1h incubation with rabbit anti-mouse IgG-HRP (0.05% (v/v)) in 5% (w/v) milk in PBST. To detect FLAG-tagged proteins, membranes were incubated for 30 min with mouse monoclonal M5 anti-FLAG antibody (0.02% (v/v)) and incubated for 30 min with rabbit anti-mouse IgG-HRP (0.05% (v/v)) in 5% (w/v) milk in PBST. After extensive washing in PBST and PBS, proteins were visualized by ECL-plus detection system according to the manufacturer’s instructions (GE Life Sciences). Equal loading was verified by Ponceau S staining.

**Glutathione s-transferase (GST) pull-down assay**

All GST fusion proteins were expressed in Escherichia coli strain BL21 (DE3) pLys (Novagen). An overnight culture was diluted 1:100 and grown until the A_{600 nm}=0.8. Production of fusion protein was induced by adding 1 mM isopropyl \beta-D-thiogalactopyranoside (IPTG) for 3 h at 37°C, and cell pellets collected and suspended in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 1 full protease inhibitor tablet (Roche), 1.5 mg/ml lysozyme, 0.02% benzonase). Soluble proteins were
purified with Sepharose 4B beads (Amersham Biosciences) and purified GST protein content confirmed by Coomassie staining on SDS-PAGE. GST fusion proteins were employed as baits in pull-down experiments. Briefly GST-containing beads were washed with lysis buffer and incubated for 30 min under gentle shaking at 4 °C with 60 µl of lysates of HEK 293 cells transfected with FLAG-tagged hCnAα constructs. Beads were then washed five times with 1 ml of lysis buffer, and bound protein eluted by boiling samples for 5 min in the presence of 1x Laemmli buffer. Samples were loaded onto an SDS-PAGE gel, transferred to PVDF membranes, and detected by Western blotting.

Immunofluorescence Analysis

COS-1 cells cultured on coverslips were transfected with pNFAT1-GFP or pYFP-p65 using FuGENE6 (Roche). 24 h after transfection, cells were incubated for 1 h at 37°C with or without CsA or peptide inhibitor, and then stimulated for 20 min with 1 µmol/L of ionomycin and examined on a Leica fluorescence microscope. To study calcineurin-NFAT co-translocation, COS-1 cells were cultured on cover slips and transfected with pNFAT1-GFP as described above. 24 h after transfection, cells were incubated for 1 h at 37°C with or without MCV1 or CsA, and cells then stimulated for 20 min with 1 µmol/L ionomycin. Cells were then fixed with 4% formaldehyde in PBS for 15 min at room temperature, washed three times in PBS and permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature. Permeabilized cells were incubated in blocking buffer (5% normal goat serum in PBS/0.3% Triton X-100/PBS/Triton) for 60 min, followed by 1 h incubation with a 1:500 dilution of pan-calcineurin A antibody (CST) in PBS/Triton. The subcellular localizations of NFATc2 and endogenous CnA were visualized after incubation for 1 h with 1:1000 diluted goat anti-rabbit IgG Alexa 555 (Molecular Probes). Cells were incubated with DAPI working solution (Molecular Probes, Oregon, USA) for 30 min at room temperature before mounting the coverslips. Nuclear localization was quantified by scoring 10 randomly selected microscopic fields containing approximately 100 cells of three different cell cultures.

Modeling of MCV1 docking to calcineurin

Docking simulation was performed essentially as described previously. The structure coordinates of calcineurin A (green) and calcineurin B (blue) were obtained from the 1TCO (PDB entry). The structure of the ligand, MCV1, was built using Cambridge chemDRAW. AutoDock 3.0 was used to perform the docking. The docking space was 120 Å×100 Å × 80 Å in volume and with a grid of 3.75 Å. For the Lamarck genetic algorithm, the numbers of energy evaluations and populations were set at 10 million and 50, respectively. A total of 50 simulation runs was carried out. The number of active torsions for the ligand was 30.

Mouse models

C57BL/6 mice were obtained from Charles River laboratories, Maastricht, The Netherlands. Female ApoE−/− mice were obtained from the local animal breeding facility (Leiden, the Netherlands). All animal work was approved by the regulatory authorities of Leiden and complied with the Dutch government guidelines. To measure T-cell activation in vivo, mice were first injected intraperitonealy (i.p.) with either PBS control, MCV1 or CsA (both 50 µmol, n=5). After 30 minutes, the mice were injected i.p. with PMA (100 ng) or PBS and after 2 h, blood and peritoneal leukocytes were collected. To assess T-cell activation levels, peritoneal leukocyte suspensions were incubated with 1% mouse serum in PBS and stained for surface markers (0.25 µg/200,000 cells, Immunosource, Halle-Zoersel, Belgium) for 45 min, after which surface marker expression was analyzed by FACS analysis (FACScalibur, BD Biosciences).

To induce neointimal lesions, nine week old ApoE−/− mice were fed a western-type diet ad libitum for 1 week prior to injury and throughout the experiment. Transluminal wire injury of the left common carotid artery was performed as described previously. In short, a flexible 0.014 inch angioplasty guide-wire was advanced into the common carotid artery via the left
external carotid artery and endothelial denudation was achieved by three rotational passes. Immediately after denudation, a 25% F-127 pluronic gel (Sigma-Aldrich, Zwijndrecht, The Netherlands), containing either PBS, MCV-1 (100 µM) or CsA (100 µM) was applied to the adventitia of the left common carotid artery (10 µL/mouse). After 1 (PBS control: n=9, MCV1: n=9, CsA: n=9) or 4 (PBS control: n=6, MCV1: n=6, CsA: n=7) weeks, the mice were anaesthetized, *in situ* fixed through the left cardiac ventricle and the left common carotid arteries were isolated.

**Re-endothelialization analysis by Evans blue staining**

To determine whether MCV-1 or CsA affected re-endothelialization of the vessel wall after denudation, we performed the denudation protocol as described above (Control n=6; MCV-1, 100 µM, n=5; CsA, 100 µM, n=5). After 7 days, re-endothelialization was assessed as previously described1, 2. In short, mice were intravenously injected with 50 µL Evans Blue (5% in saline). After 10 minutes, fixation through the left cardiac was performed and the left common carotid arteries were isolated. Evans Blue positive area was measured as percentage of total vessel area.

**Histology**

Starting at the bifurcation, serial tissue cryosections (5 µm) were obtained from left common carotid arteries after which morphometric analysis (Leica Qwin image analysis software) was performed on 4 hematoxylin-eosin stained sections (each 150 µm apart). The number of nuclei was scored manually. Vascular smooth muscle cells were stained using an alpha smooth muscle cell actin monoclonal antibody (clone 1A4, dilution 1:500; Sigma-Aldrich). Macrophage content of the lesions was assessed using a rat monoclonal MOMA-2 antibody (dilution 1:50, Serotec, Kidlington, Oxford, UK), while collagen was visualized using a Masson’s Trichrome kit (Sigma-Aldrich). CD31 was stained with a rabbit α-CD31 antibody (1:50, Thermo Fisher Scientific, Fremon, CA, USA). All morphometric analyses were performed by blinded independent operators (I.B.).

**Calcineurin phosphatase activity assay**

Jurkat cells (1x10⁶ cells) in complete RPMI 1640 medium were incubated with indicated concentrations of MCV1 and CsA at 37°C for 15 min. After washing three times with 1 ml cold Tris-buffer saline (TBS), cells were collected by centrifugation at 1500 rpm for 5 min and lysed in 100 µl lysis buffer (Biomol). The effect of indicated peptide and CsA treatment on calcineurin enzymatic activity was analyzed with Calcineurin Cellular Assay Kit (Biomol) according to the manufacturer’s instructions, n=5.

**Antibody array and IL-6 ELISA**

Custom-designed murine cytokine antibody arrays were performed according to the manufacturer’s instructions (RayBiotech, UK). In brief, membranes were incubated with blocking buffer for 1 h, washed and incubated with conditioned medium derived from equal numbers of BMCs or VSMCs for 1 h. After washing, membranes were incubated in parallel with biotin-conjugated anti-cytokine primary antibodies for 1 h. After washing, membranes were incubated for 2 h with HRP-conjugated streptavidin, and exposed together for varying times to detect the signal. Following scanning densitometry, quantification was performed using Visionworks LS software (UVP, Cambridge, UK). IL-6 ELISA was performed according to the manufacturer’s instructions, n=5.

**Endothelin-1 ELISA**

Murine EOMA endothelial cells were cultured in complete DMEM at a density of 10⁵ cells/well in 24-well plates. Cells were treated with medium control or CsA/MCV1 at
indicated concentrations for 24 h. The ET-1 level in the conditioned medium was then determined by an ELISA (Endothelin Immunoassay, Catalog number QET00B, R&D Systems Inc. Minneapolis MN, USA) according to manufacturer’s instructions.

**Statistical Analysis**

Data are expressed as mean±SEM. A 2-tailed Student’s t-test was used to compare individual groups, while multiple groups were compared with a one-way ANOVA. A level of $p<0.05$ was considered significant.

Alignment of VIVIT sequence

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<th>Sequence</th>
<th>Origin of NFAT consensus motif</th>
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<td>VIVIT peptide short motif we identified</td>
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</table>

**Online figure I** (a) Dual luciferase NFAT reporter assay showing the inhibitory activity of the MCV compounds compared to that of HPVIVIT or INCA12 at the indicated concentrations, n=3, (b) Alignment of proteins sharing the homologues sequence of PxIxIT where x is any amino acid.
Online Figure II. MCV1 inhibits calcineurin binding to NFATc2 but not to Cabin1 or AKAP79. (a) Purified calcineurin (20 nmol/L, Sigma#C1907, plus 600 nmol/L calmodulin, Sigma#C4874 and 1.5mM CaCl$_2$) was reacted in pull-down assays by affinity binding to GST (lane 1) and GST-NFATc2 (residues 4-385, lane 3-9) in the presence of VIVIT (lane 4-6) or MCV1 (lane 7-9) at the indicated concentrations. Bound calcineurin was visualized by Western blot with a monoclonal anti-CnA antibody (BD biosciences #556350). Western blotting for FLAG in lysates from HEK-293 cells expressing FLAG-tagged CnA protein analyzed by affinity binding to GST, GST-Cabin-1 (b) and GST-AKAP79 (c) in the presence of MCV1 at the indicated concentrations. Ponceau S staining is provided as a loading control.
Online Figure III. Dual luciferase NFAT reporter assay was tested in (a) murine endothelial EOMA cells, (b) rat cardiomyocytes H9C2 and (c) murine vSMCs. 100,000 cells in 24 well plates transfected with pNFAT-luc and pRL-CMV were stimulated with PMA/ionomycin for 12 h in the presence of 1 μM CsA, VIVIT or MCV1. (n=6, *** P<0.001 v.s PMA/ionomycin treated group)
**Online Figure IV**: Cellular localization of NFAT1 proteins as visualized by GFP fluorescence, COS-1 cells were transfected with pNFAT1-GFP and 24 h later stimulated for 15 min with 1 µmol/L ionomycin in the presence or absence of 1 µmol/L of CsA or MCV1. Nuclear was counter stained by DAPI.
**Online Figure V** MCV1 shows persistent NFAT inhibition and low cytotoxicity. (a) NFAT1-GFP transfected COS-1 cells were incubated with 1 µmol/L MCV1 for the indicated time. Cells were then stimulated with ionomycin (1 µmol/L) for 20 min and nuclear translocation of NFAT1-GFP was examined. (**, p<0.01 *** p<0.001 as compared with ionomycin treated only group). (b) VSMCs were incubated for 24 h with medium control, MCV1 or CsA at indicated concentrations. Cells were then trypsinized, mixed with Trypan blue and viewed under a light microscope. The calculated percentage of unstaining cells represents the percentage of viable cells, n=5.
Online Figure VI Flow cytometry analysis of VSMC death by Annexin V-FITC/Propidium Iodine (PI) after treatment by CsA or MCV1 at different concentration for 24 hours. Y axis, FL1=Annexin V-FITC, X axis FL2=PI. Lower left (LL)= live cells with negative staining; Upper left (UL)=apoptotic cells which are AV positive only; Lower right (LR)=necrotic cells, PI only; Upper right=AV/PI double positive cells which are in early phase apoptosis. A, medium control, B-D, CsA at 0.1, 1, 10 µmol/L, E-G, MCV1 at 0.1, 1, 10 µmol/L.
Online Figure VII Jurkat cells were stimulated with PMA (200 nmol/L) and ionomycin (1 μmol/L) for 1 h in the presence of MCV1 or CsA at indicated concentrations. Cellular phosphatase activity of calcineurin on the substrate RII phosphopeptide was measured with a Calcineurin Cellular Assay Kit (Biomol) according to the manufacturer's instructions. Free phosphates released during the reaction are expressed as mean ± SD, n=4 (***, P<0.001).

Online Figure VIII Growth-arrested vSMC were incubated with medium control or CsA at the indicated concentrations for 1 h. Cell lysates were subjected to protein immunoblotting and probed with anti-phospho-p42/p44 antibody. Total ERK antibody staining is shown as a loading control.
Online Figure IX Western blot analysis of covalent interaction between calcineurin and MCV1 after native gel electrophoresis. 50 nmol/L calcineurin (Sigma, #C1907) was incubated with MCV1 with or without DTT for 15 min at room temperature. 25 nmol calcineurin was loaded for 10% native gel electrophoresis. After transferred to PVDF membrane, proteins were detected by mouse anti-Calcineurin (BD biosciences #556350).
Online Figure X. Analysis of creatinin (a) /ASAT (b) /ALAT (c)/LDH (d) concentrations/units in PMA stimulated peritoneal ascites in the presence of CsA/MCV1, n=5.
Online Figure XI MCV1 inhibits NFAT mediated VSMC proliferation. [H]$^3$ thymidine incorporation of growth arrested vSMC treated for 4h with or without PDGF-BB (20 ng/mL) in the presence or absence of peptides at the indicated concentrations. DNA incorporation was measured after 20 h. Values represent means±SD of three individual experiments( * p<0.05, ** p< 0.01 versus PDGF-BB treated only group. n=3).
Online figure XI Representative photomicrographs of CD31 staining in PBS-, MCV1- or CsA-treated lesions in LCCA 7 days after surgery, Bar = 100 µm. CD31 positive stained endothelial cells present after re-endothelialisation as shown in high-powered views (arrows)
Online figure XIII Representative photomicrographs of MOMA and HE staining (a) in PBS-, MCV1- or CsA-treated lesions in LCCA 7 days after surgery, Bar = 200 μm. Quantification of the lesion area (b), cell counts (c) and macrophage contents (d) showed no significant difference between groups.
**Online figure XIV** Representative photomicrographs of Evans blue staining defines endothelial integrity (a) in PBS-, MCV1- or CsA-treated LCCA 7 days after surgery. RCCA was shown as a negative control. (b) Quantitative analysis showed no significant difference in re-endothelialization between groups.
Online figure XV (a) representative Evans Blue positive carotids 1 day after injury. Uninjured regions are resistant to dye and appear white (n=5 mice). (b) Quantification of carotid endothelium denudation by Evans blue leakage at day 1 after injury. Proximal, carotid bifurcation end; Distal, carotid end towards aortic arch.
Online figure XVI Representative photomicrographs of smooth muscle alpha actin staining (a) in PBS-, MCV1- or CsA-treated lesions in LCCA 7 days after surgery (Bar = 200 µm). (b) Representative negative SMA staining for LCCA or contralateral control RCCA, 7 or 28 days after surgery.