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Nona-D-Arginine Amide (D9R) Suppresses Corneal Cytokines In *Pseudomonas aeruginosa* Keratitis

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Abstract

Purpose—Nona-D-arginine amide (D9R) suppressed IL-1 β production during *Pseudomonas aeruginosa* corneal infection. The purpose of this study was to determine the cellular disposition of D9R and its effect on other inflammatory mediators induced by infection.

Methods—Mouse eyes received 5 μ l of either phosphate buffered saline (PBS, pH 7.4) or 100 μ M D9R hourly for 5 hours (total of 6 drops/eye) immediately after corneal wounding and infection with 1 $\times$ 10⁶ colony forming units (CFU) of *P. aeruginosa* PAO1. At 6, 12, and 24 hours post infection (PI), eyes were scored on a scale of 0 (normal eye) to +4 (corneal perforation). After scoring, mice were sacrificed and eyes enucleated. Whole eyes were used for determining viable CFU/eye. Corneas were excised for quantitation of TNF- α , IFN- γ , IL-10, and GM-CSF. The fate of D9R in cells was determined using a labeled peptide.

Results—Eyes treated with D9R had significantly lower disease scores ($P \leq 0.001$) and fewer CFU ($P \leq 0.01$) than PBS-treated eyes. No corneal cytokines were detected in any D9R-treated eyes. In contrast, beginning at 12 hours PI, increasing amounts of TNF- α , IL-10, and GM-CSF were detectable in corneas of PBS-treated eyes. Within 60 minutes, D9R accumulated in the cell nucleus and nucleolus and remained for over 24 hours.

Conclusion—D9R reduces the severity of *P. aeruginosa* ocular infection in part by reducing bacterial burden and in part by controlling a destructive pro-inflammatory response. D9R might be a useful alternative to steroids in treating other inflammation-mediated pathologies of the eye.

Keywords

nona-D-arginine; *Pseudomonas aeruginosa*; cytokine; keratitis

Introduction

Pseudomonas aeruginosa is a common cause of corneal infections, especially in users of soft, extended wear contact lenses (1). The organism can be readily cultured from the lenses themselves as well as from contact lens cases and lens care solutions (2). *P. aeruginosa* corneal ulcers are considered an ophthalmic emergency given the rapid and significant loss of corneal epithelium and stromal tissue. Even with effective antibacterial therapy, corneal perforation can still occur within 24 to 48 hours (3). Tissue destruction in *P. aeruginosa* corneal disease is due to the host responding to infection with an acute inflammatory response (a cytokine

storm accompanied by an influx of predominately polymorphonuclear leukocytes [PMN]) as well as by toxic products secreted by the bacteria.

In an earlier study (4), nona-D-arginine amide (D9R) topically applied at a concentration of 100 μ M significantly reduced corneal damage in a mouse model of *P. aeruginosa* ocular disease. When combined with ciprofloxacin, a true clinical cure was achieved, i.e., elimination of viable *P. aeruginosa* from the infected eye with minimal damage to the cornea. It also significantly reduced the pro-inflammatory cytokine interleukin 1 β (IL-1 β) response in infected corneas, an event which likely contributed to a better disease outcome.

In this study, D9R administered topically without a concomitant antibacterial drug significantly reduced corneal pathology and bacterial burden associated with an ocular *P. aeruginosa* infection. As with IL-1 β , treatment with D9R significantly reduced corneal concentrations of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and granulocyte macrophage colony stimulating factor (GM-CSF). When applied to human corneal epithelial cells and macrophages *in vitro*, labeled D9R translocated to the nucleus and nucleolus of cells where it remained for over 24 hours.

Materials and Methods

Bacteria and Mouse Model of Infection

Pseudomonas aeruginosa strain PAO1 was obtained from the *Pseudomonas* Genetic Stock Center, East Carolina University Brody School of Medicine in Greenville, North Carolina. Male and female, 6–8 week old black Swiss (BLKSW) mice were purchased from Taconic Farms, Inc. (Germantown, NY). Mice were maintained in accordance with institutional guidelines and tenants of the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research.

To prepare an inoculum, a bacterial colony was picked from a *Pseudomonas* Isolation Agar (PIA; Difco, BD Diagnostics, Sparks, MD) plate and inoculated into 10 ml of LB broth (Sigma-Aldrich, St. Louis, MO). Cultures were grown at 37° C on a rotary shaker at 160 rpm for 18 hours. The overnight cultures were then sub-cultured into 10 ml of fresh LB broth and the bacteria were grown to logarithmic phase to an optical density of 0.2 (2×10^8 CFU/ml) at 600 nm. The inoculum was centrifuged at $5,000 \times g$ for 15 minutes and re-suspended in phosphate buffered saline (PBS, pH 7.4; GIBCO Invitrogen, Grand Island, NY) to a concentration of 2×10^8 CFU/ml.

Mice were infected essentially as described by Kwon and Hazlett (5). In brief, mice were anesthetized with ketamine (200mg/kg, Vedco, St. Joseph, MO) and xylazine (10mg/kg, NLS Animal Health, Pittsburg, PA) intra-peritoneally. The corneas of right eyes were scarified by making three parallel incisions (1mm deep) with a 21G needle. These incisions did not penetrate beyond the superficial stroma. Wounded corneas were then topically inoculated with 5 μ l (1×10^6 CFU) of *P. aeruginosa* in PBS.

Treatment with Nona-D-Arginine amide (D9R)

D9R (American Peptide Co., Sunnyvale, CA) was dissolved in sterile distilled, de-ionized water at a stock concentration of 1 mM, and stored at –80° C. D9R was used at a concentration of 100 μ M by diluting the stock solution in PBS. Immediately following infection with *P. aeruginosa* PAO1, eyes received a 5 μ l drop of either PBS or 100 μ M D9R. Application of PBS or D9R continued hourly for 5 hours (total of six drops/infected eye). All eyes were assessed for disease progression at 6, 12, and 24 hours post infection (PI). At least five mice were infected per treatment group per time point and all experiments were repeated at least two times (i.e., $N \geq 15$ eyes/treatment group/time point).

Slit Lamp Examination

At 6, 12, and 24 hours PI, the ocular response to bacterial challenge and treatment was examined and photographed with a slit lamp microscope (Topcon Biomicroscope SL-D7, Japan). Disease was scored in a masked fashion. The scoring system employed was essentially one described by Hazlett *et al* (6) with some minor modification: 0= clear or normal cornea; +1= light to dense opacity at wound site; +2= dense opacity fully covering the pupil; +3= dense opacity fully covering the anterior segment; and +4= corneal perforation or phthisis bulbi (i.e. shrunken eye).

Determination of Viable Bacteria/Eye

After slit lamp examination and scoring, mice were euthanized and the eyes were enucleated. For CFU determination, individual whole eyes were homogenized in 1 ml sterile PBS. Serial 10-fold dilutions of homogenate were made (1:10 dilution) and 0.1 ml was plated onto PIA in duplicate and the plates were incubated for 24 h at 37° C. Numbers of viable bacteria in the eyes are expressed as the mean log₁₀ number of CFU of bacteria $\pm$ standard error of mean.

Quantitation of Corneal Cytokine Concentrations

Corneas were dissected from infected and uninfected enucleated eyes at 6, 12 and 24 hours PI and individually frozen at -80° C. Excised corneas were thawed on ice and individually homogenized in 60 μ L PBS with 1% Triton X 100 using sterile tissue homogenizers. Homogenates were centrifuged at 5,000 $\times$ g at 4° C for 15 minutes. Supernatants were immediately frozen at -80° C until assayed. TNF- α , interferon gamma (INF- γ), interleukin 10 (IL-10) and GM-CSF in the homogenates were quantified using a Bio-Rad Laboratories (Hercules, CA) Bio-Plex assay per the manufacturer's instructions. The sensitivity of the assay is 10 pg/ml for each of the cytokines. A value of 0 was assigned to samples that were below the range of detection. Corneal cytokine concentrations are expressed as pg/ml.

Cellular Localization of D9R

D9R labeled with the red fluorescent dye 5-carboxy-tetramethylrhodamine (TAMRA-D9R, American Peptide Co.) was dissolved in sterile distilled, deionized water at a stock concentration of 1 mM, and stored at -80° C. TAMRA-D9R was used at a concentration of 5 μ M by diluting the stock solution with PBS (GIBCO Invitrogen). Hoechst 33342 (Sigma, 10mg/ml solution in water), a cell nucleus stain, was used at a concentration of 200 μ g/ml in distilled water for cell culture assays.

Immortalized human corneal epithelial cells (HCEC) (7) were seeded in two-well Lab-Tek II chamber slides (Nunc, Rochester, NY) in serum-free keratinocyte growth medium (KGM, Lonza Biologics, Inc., Portsmouth, NH) for 24 hours at 37° C. After the cells reached ~60% confluence, 5 μ L of 5 μ M TAMRA-D9R was added to slides which were then incubated at 37° C for 1 hour. Cells were then washed with PBS to remove excess TAMRA-D9R and then fresh medium without TAMRA-D9R was added. Cells were incubated for a further 1, 3, 6, 24 or 48 hours. At the end of each time point, the medium was removed and the cells were gently washed with PBS three times. Cells were then stained with the blue nuclear dye, Hoechst 33342 (Sigma) for 45 seconds and washed three times with PBS. The localization of TAMRA-D9R in live cells was then visualized and photographed with a fluorescence microscope (Zeiss, Thornwood, NY) at a magnification of 100 $\times$.

Because macrophages play an important role in determining the pathological consequences (corneas of "susceptible" mice perforate vs. "resistant" mice that clear infection without loss of the eye) of a *P. aeruginosa* corneal infection in mice (8), a macrophage-like cell line (RAW 264.7) was also used in these experiments [117]. These cells were obtained from the American

Type Culture Collection (ATCC TIB-71, Manassas, VA) and grown in two-well Lab-Tek II chamber slides in Dulbecco's Minimal Essential Medium (GIBCO Invitrogen). The localization of TAMRA-D9R was visualized in live RAW 264.7 cells as described for HCEC.

Effect of D9R on Transcription

The localization of D9R within the nucleus and nucleolus is reminiscent of two arginine-rich viral activators of transcription: Epstein-Barr Nuclear Antigen 1 (EBNA1) and Human Immunodeficiency Virus type 1 Trans-Activator of Transcription (HIV-TAT). The arginine-rich regions of EBNA1 and HIV-TAT are found in DNA-binding motifs known as AT-hooks. These AT-hook motifs are essential for EBNA1 and HIV-TAT to function as transcriptional activators. This experiment was performed to determine if D9R could be regulating transcription of cytokines and other mediators of inflammation by preventing proteins with AT-hooks (such as EBNA1 or HIV-TAT) from binding to DNA.

Using a luciferase-based assay essentially as described by Aras et al. (9) and Singh et al. (10), the effects of D9R on transcription were examined. In brief, herpes virus-free human cervical carcinoma C33a cells were either co-transfected with an EBNA1 expression vector and a reporter plasmid with a luciferase gene under the control of a natural EBNA1 (Cp) promoter; or an HIV-TAT expression vector and a reporter plasmid with a HIV-TAR (trans-activating response region) luciferase fusion under the control of a herpes simplex thymidine kinase promoter. Thus, co-transfected cells constitutively express luciferase and produce a quantifiable amount of luminescence. Interference with binding of EBNA1 or HIV-TAR by D9R would result in less luciferase produced and a subsequent decrease in luminescence.

Six hours post-transfection, cells were split on to a 12 well plate and individual wells remained untreated (0 D9R) or were treated with 10, 30, and 100 μ M D9R. After 24 hours incubation, cells were harvested, counted twice in a Coulter counter, pelleted and lysed using reporter lysis buffer (Promega, Madison, WI) and analyzed for luciferase expression using a Zylux FB15 tube luminometer (Zylux Corp., Huntsville, AL). This experiment was repeated twice and results are expressed as the mean relative expression of luciferase $\pm$ standard deviation.

Statistical Analyses

All analyses were performed using GraphPad Prism v. 4.03 (GraphPad Software, San Diego, CA). Mean disease scores between D9R and PBS-treated groups ($N \geq 15$ /treatment group/time point) were compared using one-way analysis of variance (ANOVA). All other analyses were performed using an unpaired Student's *t*-test. A probability value of $p \leq 0.05$ was considered statistically significant.

Results

D9R reduces ocular pathology and viable CFU/eye

Mean ($\pm$ SEM) slit lamp examination scores for *P. aeruginosa* -infected eyes assessed at 6, 12 and 24 hours PI are shown in Table 1. At 6 hours PI, there were no observable signs of ocular disease and all eyes were given a score of 0. By 12 hours PI, eyes treated with PBS had mild opacities partially covering the pupil (+2) whereas eyes receiving D9R treatment did not show any signs of infection (0). At 24 hours PI, all eyes treated with PBS had dense corneal opacities covering the entire eye (+3). In contrast, D9R-treated eyes had significantly lower disease scores ($p \leq 0.0001$) with opacities limited to the wound site (+1).

The number of viable bacteria present in eyes at 6, 12 and 24 hours PI, expressed as the mean $\log_{10}$ CFU ($\pm$ SEM)/eye, is shown in Figure 1. As expected, the numbers of bacteria in infected eyes, regardless of treatment, increased over time. However, eyes that were treated with

100 μ M D9R had significantly fewer CFU/eye ($p \leq 0.01$) when compared to PBS-treated eyes at all time points examined.

D9R suppresses cytokine expression

The concentrations of corneal TNF- α , IFN γ , IL-10 and GM-CSF at 6, 12 and 24 hours PI, expressed as mean ($\pm$ SEM) picograms of cytokine/ml corneal homogenate, are shown in Table 2. No cytokines were detected in uninfected, untreated corneas. Similarly, any concentrations of TNF- α , IFN γ , IL-10 and GM-CSF were below the limits of detection (10 pg/ml) at all time points tested in corneas treated with D9R. However, by 12 hours PI, concentrations of TNF- α , IL-10 and GM-CSF were detectable in corneas treated with PBS. Concentrations of these three cytokines continued to increase by 24 hours PI, most noticeably with TNF- α . IFN γ was not detected in any of the infected corneas, regardless of the time PI or treatment with PBS or D9R.

D9R rapidly localizes in the nucleus and nucleolus of cells

TAMRA-D9R localized to the nucleus and nucleolus of HCEC and macrophage-like RAW 264.7 cells within an hour. The labeled peptide remained in the nuclei and nucleoli for over 24 hours post-incubation. The nuclear localization was confirmed by co-staining cells with Hoechst.

As shown in Figure 2, at 6 hours post incubation, both TAMRA-D9R as well as the Hoechst dye co-localized to the nucleus and appeared to concentrate in the nucleolus. Staining of RAW 264.7 cells is shown in Figure 3. By 48 hours post-incubation, cells had undergone mitosis and D9R could not be located in the nucleus (data not shown).

D9R inhibits transcription

As shown in Table 3, exposure to increasing concentrations of D9R resulted in incremental decreases in luminescence. Treatment of co-transfected C33a cells with 100 μ M D9R resulted in luminescence being decreased by approximately 50 to 65% relative to untreated cells. These findings suggest that D9R was inhibiting transcription of the reporter gene luciferase, perhaps by competing with the arginine-rich AT-hook regions of the transcriptional activators EBNA1 and HIV-TAT.

Discussion

The host immune response is critical in determining the outcome of *P. aeruginosa* keratitis. The primary immune cells involved in controlling bacterial replication in the cornea are PMNs. Rapid recruitment of PMNs into the infected cornea is essential for bacterial killing but persistence of these cells leads to stromal scarring and possible perforation (11). The recruitment of PMNs from blood vessels into the inflamed cornea is a highly orchestrated process that involves a wide array of inflammatory mediators.

Several studies have shown that over-expression of pro-inflammatory cytokines and chemokines leads to a vigorous immune response that contributes to corneal damage (3,11). Studies by Rudner et al. (12) indicate that significantly greater amounts of IL-1 β are present in infected corneas of susceptible (C57BL/6; cornea is destroyed) vs. resistant (BALB/c; cornea is scarred but otherwise intact) mice and that excess amounts of this cytokine can result in poor disease outcome. IL-1 β up-regulates MIP-2, leading to prolonged neutrophil recruitment into the cornea of susceptible mice. A timelier down-regulation of IL-1 β in resistant mice allows for bacterial clearance with much less corneal destruction. When determined using the same experimental conditions and method of assay as in this study, D9R (100 μ M) significantly

reduced the production of IL-1 β in *P. aeruginosa* infected corneas at 6, 12, and 24 hours PI (4).

Similar to the reduction of IL-1 β , treatment with 100 μ M D9R reduced the concentrations of TNF- α , IFN γ , IL-10 and GM-CSF below the limits of detection (10 pg/ml) of the Bio-Plex (Bio-Rad) assay. GM-CSF was detected in an inflamed, *P. aeruginosa*-infected cornea treated with PBS 12 hours PI and the concentration of this cytokine practically doubled by 24 hours PI. This cytokine functions as a growth factor involved in survival of PMNs. HCEC strongly express GM-CSF in response to heat-killed *P. aeruginosa* (13,14).

In this study, IL-10 was expressed at 12 hours PI in PBS-treated corneas, and this expression increased approximately 10-fold by 24 hours PI. No IL-10 was detected in any cornea treated with 100 μ M D9R. To date, only two investigators have specifically looked at the role of IL-10, a Th2 cytokine, in *P. aeruginosa* corneal disease and their results are somewhat contradictory. Discrepancies between the two studies are likely due to differences in study design. Cole et al. (14) infected corneas of IL-10 knock-out mice and their wild type C57BL/6 controls with *P. aeruginosa* strains 6206 and 6294 and observed a significant reduction of bacterial burden and in neutrophil numbers in corneas of knock-out mice compared to controls. Accompanying this reduction in bacteria and neutrophils was a significant amount of corneal neovascularization. Hazlett et al. (15) treated wild type C57BL/6 mice infected with *P. aeruginosa* strain ATCC 19660 with spantide I (a substance P antagonist) and reported enhanced corneal production of IL-10. Associated with increased IL-10 production was a significant reduction in corneal perforation and a better disease outcome. Corneal neovascularization in spantide I-treated mice was not assessed.

Blood vessel growth into the avascular cornea compromises its transparency and thus affects vision. Others have shown reduced neovascularization in corneas of IL-10 knock-out mice with herpes simplex virus-1 stromal keratitis (16) or suture-induced inflammation (17). In the current study, the lack of corneal IL-10 in 100 μ M D9R-treated mice at 24 hours PI cannot be definitively associated with a reduction in neovascularization because this pathology is not observed in *P. aeruginosa*-infected corneas until 7 days PI (4).

The last cytokine tested to be up-regulated in an infected, PBS-treated cornea, TNF- α plays an essential role in the activation and recruitment of PMNs in the cornea following infection (18).

Interestingly, there was no IFN γ detected from any of the corneas sampled. IFN γ is an important cytokine in the immuno-pathogenesis of *P. aeruginosa* corneal disease (11). It can be detected by ELISA as early as 6 hours PI (19), although most studies study IFN γ production by RT-PCR. The apparent absence of IFN γ expression in this study could be explained by differences in detection methods (Bio-Plex vs ELISA vs RT-PCR), in strains of *P. aeruginosa* employed, or in what strain of mouse was infected.

The mechanism(s) by which D9R down-regulates the pro-inflammatory cytokine response is unclear. The most potent inducer of inflammation in Gram negative bacteria is lipopolysaccharide (LPS). Cyclic cationic peptides such as polymyxin B inhibit the biological activity of LPS by binding to the lipid A moiety (20). Because D9R is positively charged, the peptide might bind to poly-anionic LPS and prevent its interaction with lipid binding protein (LBP), thereby blocking induction of pro-inflammatory cytokine production. Interaction of poly-cationic D9R with LPS might also perturb the barrier function of the outer membrane much like polymyxin B and result in the death of *P. aeruginosa*.

Another possible mechanism D9R might affect cytokine production involves binding of D9R with *P. aeruginosa* unmethylated CpG DNA. CpG DNA is a potent inducer of pro-

inflammatory cytokines through TLR-9 signaling. Huang et al. (21) have shown that injection of *P. aeruginosa* CpG DNA in C57/B6 mice led to increased expression of TLR-9, elevated pro-inflammatory cytokine production and rapid influx of PMNs into the corneas of susceptible mice. Down-regulation of TLR-9 signaling by injection of TLR-9 specific siRNA prior to infection significantly reduced pro-inflammatory cytokine induction and prevented corneal perforation in these mice. Lingnau et al. (22) demonstrated that poly-L-arginine bound to oligonucleotides containing CpG DNA and prevented the systemic production of pro-inflammatory cytokines in mice. Taken together, poly-cationic D9R might bind to anionic CpG DNA of *P. aeruginosa* and prevent its interaction with TLR-9; thereby down-regulating pro-inflammatory cytokine production and PMN persistence in infected corneas.

D9R labeled with 5-carboxy-tetramethylrhodamine (TAMRA-D9R) was used to study the peptide's localization in HCECs and RAW 264.7 cells. TAMRA-D9R was efficiently taken up by these cells within an hour and localized to the nucleus and nucleolus. These observations are in accordance with the results obtained by Futaki et al (23) who demonstrated that arginine rich peptides were capable of translocating into RAW 264.7 cells and localize to the nucleus and nucleolus after three hours of incubation. Futaki et al (23) also established that the chain length of these peptides was a major factor in deciding uptake by these cells. Only polyarginines with chain lengths at or above 6 arginine residues were efficiently translocated to the nucleus. However, increasing the chain lengths to 16 arginine residues led to their accumulation on cell membranes. Small cationic peptides that have the ability to cross lipid bilayers have been termed "protein transduction domains" (PTDs) and nona-L-arginine was found to be the most efficacious PTD to date (24).

There is a biological precedent for arginine-rich peptides to expediently translocate across cell membranes, accumulate in the nucleus, and subsequently affect gene expression. Studies (25) with HIV-TAT showed that purified TAT was efficiently taken up and directed to the nucleus of HeLa cells and was able to transactivate HIV-1 promoters. The arginine-rich basic region (amino acids 49–58) of TAT is essential for uptake and nuclear localization as mutation of arginine residues abolished uptake (26). Portions of HIV-TAT comprised only of this basic region were efficiently translocated and localized to the nucleus of cells. Likewise, the transcription factor EBNA-1 contains glycine-arginine-rich motifs that bind to AT-rich regions of DNA (27).

The arginine-rich AT-hook motifs of high mobility group (HMG) eukaryotic chromosomal proteins regulate gene expression by binding to the minor groove of AT-rich regions of DNA (28). Based on these observations, it was hypothesized that D9R might be binding AT-rich DNA similar to HMG proteins and thus regulate expression of pro-inflammatory cytokines and chemokines. D9R was able to significantly inhibit transcription of a reporter gene (luciferase) that required transactivation by EBNA-1 or HIV-TAT (two viral homologues of HMG proteins) in a concentration dependant manner. At a concentration of 100µM, D9R was able to reduce luminescence 50 to 65% of control cells (cells that were transfected with the luciferase reporter gene and incubated with culture medium alone without any exposure to D9R). The finding that D9R concentrated in the nucleolus (site of rRNA synthesis) suggests that the peptide is able to efficiently interact with other anionic targets such as RNA within the cell. Further studies are required to determine whether D9R binds to specific RNA targets.

The current study also showed that D9R was detected in HCECs and RAW 264.7 cells for up to 24 hours. By 48 hours post incubation, none of the peptide was observed in the cell. As D-peptides are resistant to naturally occurring proteases (29), the peptide may have been diluted out due to cell proliferation to a point where it could not be visualized.

In conclusion, D9R would be a useful addition to the current regimen of topical antibiotics for the treatment of *P. aeruginosa* keratitis because of its anti-inflammatory and antibacterial properties. This peptide might also be useful in treating other ocular diseases where the host inflammatory response is a significant contributor to pathology.

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Figure 1. Viable Bacteria in Mouse Eyes at 6, 12 and 24 Hours Post-Infection

Corneas were scarified with a 21G needle and topically inoculated with 1×10^6 CFU of *P. aeruginosa* PAO1 followed by treatment with PBS or 100 μ M D9R. Eyes were enucleated at 6, 12 and 24 hours post-infection (PI) and processed for determining log₁₀ CFU/eye. Eyes that were treated with 100 μ M D9R had significantly lower (* $p \leq 0.01$) CFU compared to PBS-treated eyes.

Figure 2. Localization of Nona-D-Arginine (D9R) in HCEC 6 Hours Post-Incubation

HCEC were exposed to 5-carboxy-tetramethylrhodamine (TAMRA-D9R) for 1 hour, washed with PBS, and then incubated for a further 6 hours. Cells were also briefly exposed to the blue nuclear dye, Hoechst, and immediately washed with PBS. Localization of TAMRA-D9R and Hoechst to the cell's nuclei and nucleoli were then visualized with a fluorescence microscope. A= cells labeled with TAMRA-D9R, B= cells labeled with Hoechst, C=differential interference contrast (DIC) pictures of HCEC, D= co-localization of Hoechst and TAMRA-D9R in the nucleus. Mag. =100 ×

Figure 3. Localization of Nona-D-Arginine in RAW 264.7 Cells 3 Hours Post-Incubation
RAW 264.7 cells were exposed to 5-carboxy-tetramethylrhodamine (TAMRA-D9R) for 1 hour, washed with PBS, and then incubated for a further 3 hours. Cells were briefly exposed to the blue nuclear dye, Hoechst, and immediately washed with PBS. Localization of TAMRA-D9R was visualized with a fluorescence microscope. TAMRA-D9R localized to the nuclei and nucleoli of these cells. Top image = cells labeled with TAMRA-D9R; Middle image = cells labeled with Hoechst; Bottom image = Co-localization of Hoechst and TAMRA-D9R in the nucleus. Mag. = 63×

Table 1

Mean slit lamp examination (SLE) scores $\pm$ standard error of the mean (SEM) of eyes treated with phosphate buffered saline (PBS) or 100 mM nona-D-arginine (D9R-100 μ M) at 6, 12 and 24 hours post-infection (PI).

Hours PI	SLE $\pm$ SEM scores	
	PBS	D9R-100 μ M
6	0 $\pm$ 0	0 $\pm$ 0
12	1.1 $\pm$ 0.1	0 $\pm$ 0
24	2.90 $\pm$ 0.10	0.95 $\pm$ 0.05

Table 2

Corneal concentrations of granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), and interleukin 10 (IL-10) at 6, 12, and 24 hours post-infection (PI). Cytokines are expressed as mean picograms cytokine/ml $\pm$ standard error of the mean. Concentrations below the limit of detection (10 pg/ml) were assigned a value of 0.

Hours PI	GM-CSF		TNF- α		IFN- γ		IL-10	
	PBS	D9R	PBS	D9R	PBS	D9R	PBS	D9R
6	0	0	0	0	0	0	0	0
12	138.5 $\pm$ 49.4	0	195.8 $\pm$ 47.5	0	0	0	12.05 $\pm$ 1	0
24	216.9 $\pm$ 44.7	0	1,448 $\pm$ 405	0	0	0	148.7 $\pm$ 44	0

Table 3

Mean relative expression of luciferase $\pm$ standard deviation by C33a cells co-transfected with Epstein-Barr Nuclear Antigen 1 (EBNA1) or Human Immunodeficiency Virus type 1 Trans-Activator of Transcription (HIV-TAT) and treated with nona-D-arginine.

D9R (μ M)	EBNA1	HIV-TAT
0	100	100
10	89.4 $\pm$ 15.8	70.4 $\pm$ 15.9
30	80.3 $\pm$ 9.4	61.3 $\pm$ 1.4
100	33.9 $\pm$ 3.1	48.7 $\pm$ 8.1