An Anti-inflammatory Role of VEGFR2/Src Kinase Inhibitor in HSV-1 Induced Immunopathology

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Abstract

Corneal neovascularization represents a key step in the blinding inflammatory stromal keratitis (SK) lesion caused by ocular infection with Herpes simplex virus (HSV). In this report we describe a novel approach to limit the angiogenesis caused by HSV infection of the mouse eye. We show that topical or systemic administration of the src kinase inhibitor (TG100572) that inhibits downstream molecules involved in the VEGF signaling pathway, resulted in markedly diminished levels of HSV induced angiogenesis, and significantly reduced the severity of SK lesions. Multiple mechanisms were involved in the inhibitory effects. These included blockade of IL-8/CXCL1 involved in inflammatory cells recruitment that are a source of VEGF, diminished cellular infiltration in the cornea, and reduced proliferation and migration of CD4+ T cells into the corneas. As multiple angiogenic factors (VEGF, bFGF) play a role in promoting angiogenesis during SK and since src kinases are involved in signaling by many of them, the use of src kinase inhibition represents a promising way of limiting the severity of SK lesions the most common cause of infectious blindness in the Western world.
Introduction

Ocular Herpes Simplex virus (HSV) infection can result in blinding immuno-inflammatory lesions in the cornea termed stromal keratitis (SK) (3, 24). A critical step in the pathogenesis in SK is neovascularization of the normally avascular cornea, but such vessels are leaky and permit the escape of cells and inflammatory molecules into stromal tissues, events that impair vision. Preventing, or limiting, neovascularization was shown in animal models of SK to be a useful means to control the severity of lesions (15, 28, 29). Many molecules may participate in causing neovascularization in the HSV infected eye, but VEGF-A is the principal angiogenic factor involved (28). The VEGF-A can derive from multiple sources, that include endogenous production of VEGF-A whose angiogenic function is blocked by being bound to a soluble form of one of its receptors (2). HSV infection results in the breakdown of this inhibitory interaction (Suryawanshi et al, Manuscript accepted for publication). Additional VEGF-A supplies come from newly synthesized protein by infected or cytokine stimulated cells, as well as from VEGF-A being transported to the eye by inflammatory cells (8). Whatever the source, VEGF-A mediates ocular angiogenesis by signaling mainly through the VEGFR2 receptor that in turn sets off a sequence of intracellular events that involve src kinases (6, 7, 27).

Recent studies have shown that src family of tyrosine kinases are responsible for VEGF mediated vascular permeability and angiogenesis in several systems (6, 10, 23). Accordingly, using inhibitors of src kinases represents a logical approach for therapy against pathological angiogenesis such as occurs in
SK. Approaches tested to date to inhibit angiogenesis in the SK system have either targeted VEGF or one of its receptors, but inhibiting biochemical events set off by VEGF signaling such as src kinase activation has not been evaluated. This approach could have advantages over others since src kinase are also responsible for mediating vascular permeability and may also be involved in signaling by other angiogenic factors, such as fibroblast growth factors (23). The later are known to be involved in pathological angiogenesis caused by ocular HSV infection (9, 28).

Drugs have recently become available that effectively inhibit one or more src kinases and which can function to inhibit new blood vessel development and function (5, 18, 23). One such example is the drug TG100572, shown recently to be effective at inhibiting VEGF mediated events involved in a non infectious vascular disease of the retina (23). A compound of particular interest is the pro-drug src kinase inhibitor TG100801, since upon topical ocular administration to the eye it converts to the active src kinase inhibitor molecule TG100572 that inhibits VEGF signaling (23). In the present report, we demonstrate that TG100801 given topically is an effective means of inhibiting neovascularization and the subsequent severity of SK in the HSV infected eye. The use of src kinase inhibitors could add to the arsenal of therapeutics useful for the clinical management of SK, an important cause of impaired vision in humans.
Materials and methods

Mice and virus:

Female 5-6-week-old C57BL/6 mice and Balb/c mice were obtained from Harlan Sprague–Dawly (Indianapolis IN). The animals were housed in the animal facility at the University of Tennessee. All manipulations were done in a laminar flow hood. All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. HSV-1 RE was propagated and titrated on vero cells (American type culture collection CCL81) using standard protocols. The virus was stored in aliquots at -80°C until use.

Corneal HSV-1 infection and clinical observations:

Corneal infections of C57BL/6 mice were conducted under deep anesthesia. Mice were scarified on their corneas with 27 gauge needles and a 3 μl drop containing the required viral dose (10⁴ PFU of HSV RE) was applied to the eye. The eyes were examined on different time points post infection with a slit lamp biomicroscope (KOWA), and the clinical severity of keratitis and angiogenesis of individually scored mice was recorded. The scoring system was as follows: 0, normal eye; 1, mild corneal haze; 2, moderate corneal opacity, iris visible; 3, severe corneal opacity, iris visible; 4, opaque cornea, ulcer formation; and 5, necrotizing SK. Similarly, the angiogenic scoring system was based on quantifying the degree of neovessel formation based on three primary parameters: 1) the circumferential extent of neovessels (as the angiogenic
response is not uniformly circumferential in all cases). 2) The centripetal growth of the longest vessel in each quadrant of the circle; and 3) the longest neovessels in each quadrant was identified and graded between 0 (no neovessels) and 4) (neovessels in corneal centre) in increments of nearly 0.4mm (radius of the cornea is 1.5mm). According to this system, a grade of 4 for a given quadrant of the circle represents the centripetal growth of 1.5mm towards the corneal centre. The score of four quadrants of the eye were then be summed up to derive neovessel index (range, 0-16) for each eye at a given time point.

**Antibodies and reagents:**

For flow cytometry measurement of the infiltrating cells, 6 corneas per group were collected at the indicated time points by dissecting the corneal buttons above the limbus by a scalpel. Corneas were digested in liberase (Roche diagnostics) for 45 minutes at 37°C. Single cell suspension was prepared as described elsewhere (28). The Fc receptors were blocked with unconjugated anti CD16/32 (BD pharminogen) for 30 min. Samples were incubated with CD4-APC (RM4-5), CD11b-PerCP (M1/70), F4/80–FITC (BM8), Gr1-PE (RB6-8C5), CD45-APC (LCA-Ly5, 30-F11), CD31-PE(MEC 13.3) ,CD49d (MFR4.B) CD8-PerCP, IFNγ-APC, CD44-FITC and CD62L-FITC purchased from BD pharmingen (San Diego, CA) for 30 min. All samples were collected on a FACS scan (BD biosciences) and data were analyzed using Flowjo software.
Immunofluorescent staining for PECAM-1 in vascular endothelium was also performed on the corneal flat mounts. Corneas of Balb/c mice infected with $5 \times 10^5$ PFU of HSV-1 RE were dissected under stereomicroscope (Leica, Wetzlar, Germany) and corneal flat mounts were rinsed in PBS for 30 min and flattened on a glass slide under stereo microscope. Corneal flat mounts were dried and fixed in 100% acetone (Sigma St. Louis, MO) for 10 min at -20°C. Non specific binding was blocked with 10% goat serum (Sigma G 9023) for 24 hours at 4°C. Invitrogen). The PECAM-1 was detected by an antibody directly labeled with conjugate (PE anti mouse CD-31 MEC 13.3; BD pharmingen). Each step was followed by three washing with PBS. Stained corneal flat mounts were mounted with prolong gold antifade mounting reagent (invitrogen P36934) and visualized with Nikon Ti fluorescent microscope using the software Nikon elements.

TG100801 - a topically applied prodrug form of a src kinase inhibitor was obtained from Sanofi-Aventis (Paris)/ Targegen Inc., San Diego, California. The active compound, TG100572 (also obtained from Targegen for in-vitro use and intra-peritoneal injections), is an ATP competitive multi targeted tyrosine kinase inhibitor whereas the prodrug TG100801 is devoid of kinase inhibitory activity.

**Treatment of animals with Src kinase inhibitor TG100801/TG100572:**

Female 5-6-week-old C57 BL/6 mice were used. Corneal infection were conducted under deep anesthesia induced by i.p. injection of Avertin (Sigma Aldrich). The mice were scarified on their corneas with a 27 gauge needle and infected with $10^4$ PFU of HSV-1 RE per eye and divided randomly into groups. In
some groups src kinase inhibitor TG100801 (0.6%, 0.3%; Targegen Inc.) was applied topically (5μl eye drop, twice daily) and in other group TG100572 (0.5, 1.5 and 5mg/kg body weight dissolved in DMSO was administered intra peritoneally) starting from either day 1 to day 14 post infection or day 6 to day 14 p.i. Animals in the control group received liposomal vehicle (25% phospholipon vehicle 90 G) in the case of topical drug administration and DMSO in the intra peritoneal treatment modality following the same regimen. Mice were observed for the development and the progression of HSK lesions and angiogenesis from day 6 until day 15 as described previously. Most of the experiments were repeated at least 3 times unless stated otherwise.

*Treatment of animals with anti VEGF antibody, Bevacizumab (Avastin):*

Anti VEGF antibody Avastin was obtained from Genentech. Female 5-6-wk-old C57 BL/6 mice were used. Corneal infection were conducted under deep anesthesia induced by i.p. injection of Avertin (Sigma Aldrich). The mice were scarified on their corneas with a 27 gauge needle and infected with 10^4 PFU of HSV-1 RE per eye and divided randomly into groups. In one group src kinase inhibitor TG100572 (5mg/kg, Targegen Inc.) was administered intra-peritoneally, daily and in the other group Avastin (5mg/kg body weight at day 3, 6, 9 and 12p.i.) was given intra-peritoneally. Animals in the control group received mock treatment. Mice were observed for the development and the progression of HSK lesions and angiogenesis from day 6 until day 15 as described previously in
materials and methods. Most of the experiments were repeated at least 3 times unless stated otherwise.

**Virus specific CD8+ IFNγ staining**

To determine the immune response generated in the controls and src kinase treated groups, intracellular cytokine staining was performed as previously described (12). Single cell suspension of infected DLN was prepared and 10⁶ cells per well were cultured in 96 well U bottom plates. Cells were left untreated/stimulated with gB peptide (1 μg/ml) for 5 hrs at 37°C in 5% CO₂. Brefeldin A (10μg/ml) was added to the culture for the intracellular cytokine accumulation. Cell surface marker and intracellular cytokine staining for IFN-γ was performed using a cytofix /cytoperm kit (BD Pharmingen). All samples were collected with a FACSCAN and were analyzed by FLOWJO.

**Thymidine incorporation assay**

Lymphocytes from the draining cervical draining lymph nodes were obtained at day 15 p.i. and enriched for CD4+T cell population by Miltenyi biotech CD4+T cell isolation kit. The cells were plated at the density of 5x10⁵ in 96 well round bottom tissue culture plate in a total volume of 200 ul of RPMI (GIBCO). The cells were stimulated with anti-CD3 (1μg/well) anti-CD28 (1μg/well). 16 hours before harvest, [³H] thymidine (1mCi/well; 1Ci=37GBq) was added. The cells were harvested onto-UniFilter (PerKinElmer). [³H] Thymidine incorporation was
measured in a scintillation counter and the results were expressed as mean CPM from triplicate cultures.

**Western blotting:**
For the detection of FAK phosphorylation, 3 corneas per group were dissected at the indicated time points in 300 ul of RIPA buffer containing protease inhibitor cocktail (aprotinin, PMSF and sodium orthovanadate), cell debris was removed by centrifugation and the samples stored at -80°C till used for SDS-PAGE. In brief, after 2 hrs of blocking with 3% nonfat milk in TBS, membranes were incubated with 1:1000 dilution of rabbit polyclonal phosphor-specific anti FAK861 antibody and membranes were then incubated for 1 hr with secondary antibody coupled to horse raddish peroxidase. Specific bands were detected with Immobilon™ western; Millipore). Membranes were stripped and then reprobed to detect Total FAK and and β actin antibody.

**Histopathology:**
For histopathological analysis, eye balls from different groups of mice were extirpated at the indicated time point post infection in 10% formalin. In brief, the samples were put overnight in tissue tek (Sakura) which virtually removes all the moisture content from the samples and embeds it in paraffin. Tissue tek was automatically programmed, that treated the samples sequentially with alcohol (100%), xylene (100%), and paraffin. 5μm sections were then cut using microtome and stained with hematotoxylin and eosin.
**Cytokine ELISA:**

Six corneas per group were collected at indicated time points. The corneas were sonicated and the levels of CXCL1/KC/mouse homologue of IL-8 were measured in the supernatants using Qantikine kit as per manufacturer’s protocol. (R&D systems).

**Quantitative PCR (QPCR):** Total mRNA was isolated from corneal cells using TRlzol LS reagent (Invitrogen). The cDNA prepared using 1 μg of RNA was used for subsequent analysis. QPCR was done using SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA) with iQ5 real-time PCR detection system. (Bio Rad, Hercules, CA). The expression levels of cytokines were normalized to β-actin with ΔCt method and relative quantification between control and infected mice was performed using the \(2^{\Delta\Delta Ct}\) formula. The primers used were as follows:

- **IL-1β**
  - Forward primer: CATCAACAAGAGCTTCAGGC
  - Reverse primer: CATCATCCCATGAGTCACAGAG

- **IL6**
  - Forward primer: CCAGAGTCCTTCAGAGAGATAC;
  - Reverse primer: CTCCTTCTGTGACTCCAGCTTATC

- **IFN-γ**
  - Forward primer: GAACGCTACACACTGCATCT:
  - Reverse primer: CCAGTTCCTCCAGATATCCAAG

- **β-actin**
  - Forward primer: CCTTCTTGGTATGGAGATCTCTG,
  - Reverse primer: GGCATAGAGGTCTTTACGGATG
Reverse Transcription Polymerase chain reaction (RT-PCR): RT-PCR for the presence of CXCL1 transcripts was done according to the manufacturer’s protocol (Promega, Madison, WI). The amplified products were resolved on 1% agarose gel. Primers for RT-PCR were as follows.

β-actin Forward primer: CCTTCTTGGGTATGGAATCCTG
β-actin Reverse primer: GGCATAGAGGTCTTTACGGATG
CXCL1 Forward primer: GGGATTCACCTCAAGAACATCC
CXCL1 Reverse primer: TCTGAACCAAGGGAGCTTCA

In vitro and in vivo virus (HSV-1) - drug interaction assay:

The effect of src kinase inhibitor TG100572 on viral replication (if any) was confirmed by incubating cells with inhibitor either before (pretreatment) or after (post treatment) inoculation with the virus and then quantifying infectious HSV kos yield by the viral plaque assay. Vero cells were infected with MOI=1 and were incubated with 100 nM (maximum below cytotoxicity levels) TG100572 or 0.1%DMSO either before (-1h) or after infection. The effect of the drug (TG100572) on production of HSV-1 was determined by titrating the virus in cell culture supernatant at different times following infection. For the in vivo assay, the virus (HSV-RE) was quantified in eye swabs following HSV-1 infection in TG100801 treated and control mice and titrated by viral plaque assay.
**Statistical analysis**

Most of the analyses for determining the level of significance were performed using either one way ANOVA (Dunnetts post hoc test) or two way ANOVA (Bonferroni test). Values of $p \leq 0.001$ (***) , $p \leq 0.01$ (**), and $p \leq 0.05$ (*) were considered significant. Results are expressed as means ± SD.
**Results:**

**Topical application of prodrug TG100801 inhibits angiogenesis and SK**

The prodrug was shown by others to convert to an active inhibitor of the src kinase involved in VEGF signaling following topical application to the eye (5, 23). Using the same conditions shown by others to inhibit retinal angiogenesis and vessel permeability, the effects on responses to ocular infection with HSV were evaluated. C57BL/6 animals infected with $10^4$ PFU of HSV-1 and divided into 3 groups. One group served as infected and untreated control and the other groups were treated with TG100801 either 0.3% or 0.6%, topically. Drug treatment was administered twice daily with therapy begun 1 day after infection until experiments were terminated on day 14 p.i. Three separate experiments were performed and cumulated results are shown in Figs 1a and b. As is evident, there was a significant inhibition of both angiogenesis and SK lesion severity with the higher dose of drug investigated compared to controls. The incidence of SK as recorded in three independent experiments was lower in TG100801 treated group (Fig 1c). Histological sections taken from sample animals from test and control groups revealed evident differences in ocular inflammatory responses (Figs.1d, e &f).

In another experiment mice were infected with HSV-1 and divided into two groups, One group was treated with TG100801 (0.6%), a concentration that resulted in significant inhibition of both angiogenesis and SK lesion severity, and the non treated groups receiving liposomal vehicle served as an infected control. The corneas from these mice were collected at the indicated time points post
infection and subjected to western blotting for the detection of FAK phosphorylation, an event known to be the biomarker of src kinase activity (6, 7, 27). Whereas phosphorylated FAK was undetectable in naïve corneal lysates, it could be demonstrated in infected corneas as early as day 1 post infection (Fig 2a). However there was a significant inhibition in FAK phosphorylation in TG100801 treated corneal lysates at the time points tested within the periods of elevated FAK-p861 (Fig 2b) suggesting that an important step in VEGF signaling, i.e src kinase activation and FAK phosphorylation was efficiently blocked by the inhibitor (TG100801).

**TG100572 controls the lesion severity independent of viral replication**

Ocular swabs from control and TG100801 treated animals, from the above mentioned experiments were taken in the early time periods to determine if the drug was inhibitory to virus replication. The corneas were homogenized and centrifuged and the viral titers were determined in the supernatants of the infected control and TG100801 treated animals. No significant differences were observed in the viral titers recovered from the corneas in different groups (Fig3a). To measure if the src kinase inhibitors had any antiviral activity, we examined the effect of TG100572 on HSV infection in vitro. Vero cells were infected with 1 MOI of HSV kos and treated with maximum below cytotoxic levels (100nM) of TG100572 or 0.1% DMSO as control. Quantitation of viral titers in culture supernatants by plaque assay revealed no significant effect on viral titers as
compared to DMSO treated controls (Fig.3b). Accordingly, we conclude that TG100572 does not have anti viral activity.

**Effect of active src kinase inhibitor given systemically on ocular HSV infection**

Repeatedly administering drugs topically to the infected mouse eye can be problematic and requires a general anesthetic to facilitate the procedure. In consequence, experiments to measure the effects of drug treatment on cellular and molecular events at different times after treatment were also done in infected animals given the active drug intra-peritoneally. Animals were **ocularly infected with HSV and** divided into different groups and treated with TG100572 (0.5, 1.5 or 5mg/kg body wt) once daily starting at 24 hours after infection. The pattern of angiogenic response, compared to sham treated controls, was recorded at day 15 p.i. Potent effects were obtained with a dose of 5mg/kg body weight (Fig 4a) with minimal effects on their general health. As shown in (Fig 4b&c), treatment started at day 1 p.i. (preventive) resulted in a major reduction in the extent of angiogenesis (which was >5 fold at day 12 post infection) and SK (3 fold at day 12 p.i.) along with a 6 fold reduction in CD11b*Gr1* cells (at day 15 p.i.) (Fig 4g) compared to infected and untreated control (Fig 4f). Additionally, commencing treatment at day 6p.i (Fig 4d&e) also resulted in significant inhibition of angiogenesis, lesion scores and CD11b*Gr1* infiltration (Fig 4h) measured at the termination of experiments at day 15 p.i. The visible angiogenesis and SK lesion severity was also significantly less in the treated mice (Fig 5a &b).
To compare our novel approach (TG100572) with a potential positive control bevacizumab (a monoclonal antibody that binds to VEGF with high specificity; thereby blocking VEGF mediated signaling pathways and thus angiogenesis) (21), experiments were done where mice were divided into three groups (n=6/group). One group received bevacizumab (Avastin) i/p (5 mg/kg body weight) at day 3, 6, 9 and 12 p.i and other was treated with TG100572 i/p (5mg/kg b wt) starting at day 3p.i until day 13p.i. The third group served as infected and untreated control. As shown (Fig 4i) at day 9 p.i., bevacizumab (Avastin) resulted in significant inhibition of angiogenesis and SK severity (p<0.0201) but this was less than observed in a group of animals that received TG100572 systemically. TG100572 treatment showed a highly significant inhibition of angiogenesis. At day 14p.i.(Fig 4j) there was around 1.6 fold and 2.9 fold reduction in angiogenesis following Bevacizumab (avastin) and TG100572 treatment respectively as compared to the control groups.

To evaluate whether the administration of the src kinase inhibitor could influence the expression of SK, animals were treated either with TG100572 starting from day 1 p.i till day 14 p.i. or day 6 to day 14 p.i and the extent of inflammatory ocular reactions were compared in the treated and control animals by sacrificing animals and recovering ocular cells from corneas following collagenase digestion. As is evident, there was a reduced infiltration of Gr1+CD11b+ cells (neutrophils) in treated mice at all indicated time points post infection (Fig 6a). The neutrophil infiltration peaked at day 2 and day 11 post infection in both the control and treated mice, however the control mice had 3 fold and 2.5 fold higher frequencies (Fig 6a)
and absolute numbers (Fig 6e) of neutrophil infiltrates at day 2 and day 11p.i. respectively, as compared to the treated group.

A significant reduction in the infiltration of CD11b⁺ F4/80⁺ cells (macrophages) was also observed following src kinase inhibition when evaluated at the later time points post infection (in the clinical phase). There were both reduced frequencies (Fig 6b) and total numbers (Fig 6f) of F4/80 cells in treated compared to the infected and untreated controls. Strikingly, around 4 fold decrease in the CD4⁺ T cells infiltration was evident in the src kinase inhibitor treated mice both in terms of percentages (Fig 6c) and absolute numbers (Fig 6g) at the clinical phase of the disease. Additionally, the frequencies and numbers of IFNγ⁺ CD4⁺ T cells were reduced 7-8 fold as a consequence of TG100572 treatment along with a highly significant reduction in IFNγ and IL-2 producing CD4⁺ T cells upon stimulation with anti-CD3 anti-CD28 (Figs 7a, b, e & f) or uv inactivated HSV kos (Fig 7c, d, g & h). Corneal single cell suspension stained for CD45 (a pan leukocyte marker) revealed significantly reduced frequencies (Fig 6d) and absolute numbers (Fig 6h) of CD45⁺ T cells (almost 2 fold) in the corneas of the treated mice at all indicated time points analyzed post infection. Taken together our data indicates that both topical and systemic administration of small molecule inhibitors of src kinases results in significant reduction in both the extent of neovascularization and severity of SK.
Inhibition of src activity blocks CXCL1 and proinflammatory cytokines

Our observation that src kinase inhibitors caused a reduction in neutrophil infiltration could mean that the drug inhibited the expression of molecules involved in neutrophil recruitment such as the chemokine CXCL1. In support of this, CXCL1 mRNA was present in the infected eye at higher levels than the scarified controls. Fig 8a depicts the expression of CXCL1 in HSV-1 infected corneas as early as day 1 p.i. For the quantification of CXCL1 gene expression, mice were ocularly infected with HSV and 6 corneas from each group (control and treated) were collected, pooled and were analyzed by QPCR at indicated time points. Corneas from src kinase treated mice subjected to QPCR revealed a decrease in CXCL1 levels at all time points tested with maximum reduction observed during the clinical phase of the disease (Fig 8b). In addition, the same situation was evident with the protein levels measured by ELISA. The corneal supernatants were assayed for CXCL1/IL-8 by ELISA as indicated in materials and methods. Fig 8c depicts the kinetics of CXCL1 protein expression levels in the control and src kinase inhibitor treated mice throughout the course of ocular infection. There was a significant reduction in the CXCL1 at day 2 and also during the clinical phase (around 8 fold reduction at day 11 p.i.) of the disease in the treated mice. Thus, our data could mean that Src kinases may regulate critical “downstream” signaling pathways that might contribute to expression of CXCL1, a pro-angiogenic and pro-inflammatory chemokine in murine cornea. Additionally treated mice represented diminished levels of proinflammatory
cytokines notably IL-6, IFN-γ and IL-1β as compared to infected and untreated controls (Fig 8d).

**TG100572 may down regulate CD49d on CD4^+ T cells in lymphoid organs resulting in fewer cells to migrate to the ocular site**

SK is well known to be orchestrated by CD4^+ T cells (25). The frequencies and absolute numbers of CD4^+ T cells recovered by collagenase digestion of corneas were diminished in TG100572 treated animals at all time points tested after ocular HSV infection. To address the cause of reduced CD4^+ T cells in the treated mice, animals that were ocularly infected and begun TG100572 treatment i/p at day 1 p.i and continued daily were sacrificed and the phenotype of CD4^+ T cells isolated from the DLN as well as from corneal lesions after collagenase digestion was evaluated at indicated time points (day 5, 7, 9, 11 post HSV-1 ocular infection). In these experiments, lesion severity was greater in the control animals as compared to the drug treated animals. FACS analysis revealed that whereas there was no difference in the expression of CD62L (data not shown) and a modest difference in the expression of CD44 (Fig 9b), interestingly CD49d on CD4^+ T cells in CLN (Fig 9a) and corneas (Fig 9c) was significantly down regulated at all time points in the src kinase inhibitor recipients. This observed down regulation of CD49d on CD4^+ T cells could be relevant since previous studies indicate that blocking CD49d reduces SK (25). Taken together, we interpret these observations to mean that src kinases are involved in controlling the expression of integrin molecules such as CD49d that are involved in
migration of inflammatory cells to the ocular lesion site (10) which could explain the overall impaired infiltration of CD4⁺T cells in the cornea.

**TG100572 inhibits CD4⁺T cell proliferation in vitro**

Src knockout mice have shown marked reduction in inflammatory responses to a variety of physiological insults (10). Measurement of the absolute numbers of draining cervical lymph node (CLN) cells revealed diminished numbers of total lymph node cells (Fig 9d) and also CD4⁺T (Fig 9e) cells in the kinase inhibitor (TG100572 i.p) recipient mice at all the time points tested. This likely means that in addition to effects on VEGF, TG100572 may also interfere directly with CD4⁺T cell activation and proliferation. To explore this possibility, draining CLN cells from HSV infected mice at day 15 p.i were enriched for CD4⁺T cells and stimulated with anti-CD3 anti-CD28 (as described in materials and methods). In some wells, different concentrations of TG100572 (non cytotoxic concentrations) were added and the effects on proliferation responses recorded. Whereas a minimal proliferation was observed with anti-CD3 alone, anti-CD3 anti-CD28 resulted in a significant CD4⁺T cell proliferation. TG100572 inhibited CD4⁺T cell proliferation in a dose dependent manner (Fig 9f) suggesting that by diminishing immune activation and CD4⁺T cell proliferation, TG100572 may additionally serve to limit the size of immunopathogenic CD4⁺T cells involved in lesion expression.
Discussion

Neovascularization of the otherwise avascular cornea represents a pathological hallmark of ocular HSV-1 infection. Present approaches for clinical management of corneal neovascularization rely on anti-virals, corticosteroids or anti VEGF antibody treatment. In this study we show that a small molecule inhibitor of src kinases, results in suppression of angiogenesis and lesion severity in a murine model of SK. Administered topically as a prodrug (TG100801) or the active form (TG100572) systemically, resulted in inhibition of several key events in the pathogenesis of SK. These included diminished cellular infiltration in the corneas, of CD4+ T cells and neutrophils, the cells primarily involved in SK. There was also a reduction in levels of the chemokine CXCL1 and proinflammatory cytokines such as IFNγ, IL1β and IL-6. Importantly treatment resulted in inhibited FAK phosphorylation in the corneal tissues; an essential step in VEGF mediated angiogenesis (1). Additionally TG100572 administered systemically resulted in down regulation of CD49d on CD4+ T cells in the DLN and cornea. However, the src kinase inhibitor had no demonstrable proinflammatory effect and failed to express antiviral activity.

The current anti-angiogenic approach shows effective control of newly proliferating vascular endothelial cells and since the phosphorylation of FAK and src activation appears to be very early events post infection, src blockade by the inhibitors early during the course of infection could be advantageous for significant and complete anti angiogenic effects. Additionally achieving efficacious drug concentration in the corneal tissues following the topical delivery
route (TG100801) is generally considered a technical challenge. However recent clinical trials with these drugs have shown that TG100801, while lacking anti kinase activity of its own, quickly generated active TG100572 within the eye upon topical delivery, however neither compound was detectable in plasma, indicating that delivery to the eye occurs by local penetration and not systemic absorption (5).

Systemic treatment with the src kinase inhibitors also significantly reduced angiogenesis and cellular infiltration, particularly of neutrophils, one plausible explanation being that by inhibition of FAK phosphorylation, src kinase inhibitors preserves the junctional integrity of the endothelial cells (13) and thus inhibited the paracellular transport of neutrophils. However, src kinases were also shown to modulate the expression of the pro angiogenic neutrophil attracting chemokine CXCL1/mouse KC/homologue of IL8 (26) and that src kinase activation correlates with the amount of IL-8 produced (13, 16, 26). Consistent with this, src kinase inhibitors resulted in inhibition of CXCL1 expression. Thus it is possible that significant reduction in the neutrophils in the treated group may be a consequence of reduced chemokine levels, which is the prominent regulator of neutrophil infiltration into the inflamed cornea (22).

In humans, SK may lead to permanent loss of vision and the current treatment modalities that are used for the clinical management of SK includes anti VEGF antibody treatment such as bevacizumab (4, 22), antivirals and corticosteroids but none of them are considered ideal. Antiviral compounds that block virus entry or reduce viral replication can be prophylactic and may not be
efficacious against SK. As a result, small-molecule inhibitors targeting cellular responses that contribute to disease may have a substantial advantage over antiviral approaches. Corticosteroid therapy, on the other hand when continued for a longer duration might exhibit several side effects (14), and anti VEGF antibody (bevacizumab) although shown to reduce VEGF induced neovascularization (21, 22), however abnormal vascular permeability is frequently associated with neovascularization (23). Thus, an antivasular permeability compound that is also antiangiogenic such as src kinase inhibitor should have added therapeutic benefit. Endothelial cell barrier functions are disrupted by a number of viruses and a very recent study suggests that VEGFR2 and SFK inhibitors may be of therapeutic utility in stabilizing vasculature during viral infections (30). Additionally both VEGF and FGF growth factors have been shown to be potent proangiogenic factors in HSV-1 induced corneal neovascularization. The binding of angiogenic growth factors (VEGF, bFGF) to their receptors is known to result in activation of non receptor tyrosine kinases (src kinases) which in turn regulates endothelial cell proliferation, migration and survival (by inhibiting apoptosis of endothelial cells) (6). A compound that inhibits redundant pathways of angiogenesis has the potential of being therapeutically advantageous (23). In support of this our results clearly indicate that bevacizumab (avastin), a VEGF antagonist, inhibit HSV-1 induced corneal neovascularization, but the levels of inhibition achieved were less than that caused by TG100572. Although we do not preclude the possibility that the observed lesser reduction in angiogenesis in the avastin treated mice could be
due to a weaker binding affinity of avastin to mouse VEGF-A (4). Our findings therefore rationalize testing of these kinase inhibitors for additional indications and clinical application in reducing HSV induced immunopathology. The fact that VEGFR2 and SFK inhibitors are already FDA approved for use in humans (30), they could be immediately rationalized for use in clinical cases of HSK.

A significant anti-angiogenic and anti-inflammatory effect was also observed following systemic treatment with TG100572. This mode of treatment also produced additional effects such as CD49d expression (on CD4^+^T cells) and attenuation of T cell function. It may be argued that these effects might be due to the limited specificity of the src kinase inhibitors (19). However the drug was well tolerated systemically with no adverse effects on the general health except for some reduction in the body weight of the animals. Our observations that systemically administered TG100572 influences the severity of SK lesions is supported by the observation of significantly less CD4^+^T cell infiltration in the cornea in the clinical phase suggesting that TG100572 (src kinase inhibitor, i/p) could control the lesion development by limiting the migration of pathogenic T cells to the extra lymphoid inflammatory site. The SK model represents a situation where normally immunoprotective CD4^+^T cells exert an immunopathological function in the cornea of the eye (17). The Integrin CD49d (VLA-4) is known to be involved in the migration of pathogenic CD4^+^T cells to the ocular site and that in SK 70–80% of corneal T cells in disease expresses CD49d (VLA-4) early in lesion development (25). Thus, it was intriguing to observe that the CD49d integrin was expressed on a significantly lower percentage of
lymphoid CD4+ T cells in the src kinase inhibitor treated mice compared to controls. The down regulation of CD49d in src kinase inhibitor treated mice was not unexpected since there is mounting evidence that α4 integrins use the src family kinases to transduce intracellular signals (20). Our observations could shed light on the relative merits of various cellular targets as candidates for therapeutic intervention during an ongoing immune-inflammatory reaction such as is SK. Src kinase inhibition also resulted in a less activated phenotype of CD4+T cells as shown by decreased CD44 expression on cells in the draining cervical lymph nodes. Additionally src kinase inhibitors resulted in diminished levels of inflammatory cytokines which is in accordance with findings that the reduction in proinflammatory cytokines resulted in impairment of Th1 differentiation (11).

In conclusion, we demonstrated that the blockade of Src kinase activation (which is an essential step in VEGF signaling) with VEGFR2/src kinase inhibitor resulted in reduced SK lesion severity and diminished cellular infiltration, probably by inhibition of vascular leak and removal of a corneal chemokine gradient. In the present approach, which is novel for infectious ocular angiogenesis, we did achieve highly significant but not complete anti angiogenesis. We advocate that combining src kinase inhibitor with additional therapeutic approaches could be valuable for use in the clinic to manage herpetic ocular lesions.
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References:


Figure legends

Fig.1: Effect of topical administration of src kinase inhibitor (TG100801) on the severity of SK

C57BL/6 animals infected with 10⁴ PFU of HSV-1 were treated with TG100801 (0.3%, 0.6%, topically). TG100801 was applied topically starting at day 1 till day 14 post infection (p.i.). (a,b) Cumulative data of angiogenesis (a) and HSK scores (b) at day 12 p.i as measured by slit lamp biomicroscopy, of three independent experiments. The levels of significance was determined by one way ANOVA using Dunnett’s post hoc setting. (c) The bar diagram demonstrates the percentage severity of each group of mice infected with 10⁴ PFU of HSV-1 RE at day 15 p.i. in three independent experiments. The SK scores of three or greater than three were counted as SK incidence (black bars= infected control; white bars represents TG100801 treated). (d-f) H&E staining of the corneal sections of naïve (d) control (e) and TG100801-treated animals (f).

Fig.2: TG100801 inhibits FAK-y861 phosphorylation in the murine cornea

(a) Western blot analysis for phosphorylated FAK⁸⁶¹ in corneal cell lysate at different time points following HSV-1 infection. (b) Inhibition of FAK phosphorylation: Mice were infected with 10⁴ pfu of HSV-1 RE. Twenty four hrs following infection, one group was treated topically (cornea) with a 5μl drop of 0.6% TG100801, twice daily till 14 dpi. Second group received the vehicle control in the same way. Corneal cell lysate was used to detect level of phosphorylated FAK⁸⁶¹ by anti-rabbit FAK antibody in Western blot. Drug treated mice showed
inhibition of FAK phosphorhylation at all time points tested within the periods of elevated phosphorylated FAK. (N=naïve; C=control; T=treated).

**Fig.3: TG100572 controls SK lesion severity independent of viral replication**

(a) Viral titers in corneal swabs in control and TG100801 treated mice at day 2 and 4 p.i. is shown. (b) Vero cells were infected with MOI=1 and incubated with 100 nM of TG100572 (maximum below cytotoxicity level) after infection or 0.1% DMSO. The effect of the drug (TG100572) on production of HSV-1 was determined by titrating the virus in cell culture supernatant at different times following infection.

**Fig.4: Effect of systemic administration of src kinase inhibitor (TG100572) on angiogenesis and SK lesion severity**

(a) C57BL/6 animals infected with $10^4$ PFU of HSV were treated with different concentrations of TG100572 (0.5, 1.5 and 5 mg/kg body weight), intraperitoneally daily starting from either 24 hrs post infection (preventive) or day 6 p.i. (therapeutic manipulation) until day 14. The comparative angiogenesis scores of HSV infected animals at day 14, treated with indicated concentrations of TG100572. The statistical significance was determined using one way ANOVA. (b-e) Kinetics of angiogenesis and lesion expression in control and TG100572 treated animals at 9-15 dpi is shown. Disease progression in control and TG100572 treated mice following infection with $10^4$ pfu of HSV-1 to C57Bl/6 animals under preventive (b & c) and therapeutic (d & e) mode of treatment. The
level of significance was calculated by two way ANOVA. (i & j) Comparative angiogenesis scores in bevacizumab and TG100572 treated mice compared to infected but untreated controls at day 9p.i (i) and day 14 p.i (j). The statistical significance was determined by student’s t test. (f-h) Infiltration of CD11b*Gr1* polymorph nuclear neutrophils in the corneas of control (f), following preventive (g) and therapeutic (h) treatment at day 15 p.i. All experiments were repeated at least three times.

**Fig.5:** Representative eye photograph (at day 15 p.i.) of control (a) and TG100572 treated (b) mice.

**Fig.6:** Kinetics of Cellular infiltration in the corneas of control and TG100572 treated mice. Effects of TG100572 treatment on kinetics of cellular infiltration in corneas of HSV infected animals analyzed by flow cytometry. C57BL/6 infected with 10^4 PFU of HSV were either treated with TG100572 5 mg/kg body weight i/p daily starting from 24 hr p.i. until day 14 p.i. or untreated controls. Single cell suspension of the infected corneas were prepared from pooled 6 corneas (n=3) at indicated time points from each group (TG100572 treated or control) of mice. The cells were labeled for: (a, e) Gr1*CD11b* (polymorph nuclear cells), (b, f) CD11b*F4/80* (macrophage), (c, g) CD4* and (d, h) CD45* (leucocyte common antigen; pan leucocyte marker). The numbers on the dot plots indicates the percentage of the cells expressing the particular markers in control and kinase inhibitor treated mice at indicated time points p.i.
The experiment was repeated three times and data are representative of a single experiment.

**Fig.7:** TG100572 treatment diminishes the infiltration of pathogenic Th1 cells in the cornea. C57BL/6 infected with 10^4 PFU of HSV-1 was treated with TG100572 (5 mg/kg body weight) i/p daily starting from 24 hr p.i. until the termination of the experiments. Single cell suspension of the infected corneas were prepared from pooled 6 corneas at day 15 p.i. from each group (control and treated) of mice. The cells were stimulated with either anti-CD3 anti-CD28 or UV inactivated HSV Kos and stained for CD4^+T cells producing IFN\_γ and IL-2. Frequencies (a & b) and absolute numbers (per cornea) (e & f) of IFN\_γ^+ and IL2^+ T cells respectively in control and treated group was observed following stimulation by anti-CD3 anti-CD28. The frequencies (c & d) and absolute numbers (g & h) of IFN\_γ^+ and IL2^+ T cells respectively in control and treated group following stimulation by UV inactivated HSV Kos is shown. The experiment was repeated three times and data are representative of a single experiment.

**Fig.8:** TG100572 treatment results in the blockade of CXCL1 in the cornea. (a) Agarose gel analysis for CXCL1 (132bp) (Lane 4) transcripts from infected corneas is shown. Lane M is Marker; lane 2 is beta actin (92bp). Lane1 and lane 3 are RT negative control for beta actin and CXCL1 respectively. Lane 5 is negative control (water) (b) Kinetic analysis for the expression of CXCL1 mRNA by QPCR at different time p.i after src kinase inhibitor or mock treatment is
shown. WT mice were infected with HSV and treated with mock or TG100572. Corneas were harvested from respective groups at an indicated time points, pooled and subjected to quantification by QPCR for CXCL1 mRNA. (c) Quantification of CXCL1 protein in HSV-1 infected corneas by ELISA after mock or TG100572 treatment is shown. At each time point, 6 corneas were harvested from HSV infected mice treated with mock or TG100572 and levels of CXCL1 protein were determined by ELISA. The level of significance was determined using two way ANOVA with Bonferroni post test. (d) Reduction in IL6, IFN-γ and IL-1β after TG100572 treatment is shown. The mice infected with HSV were treated with mock or TG100572 and corneas collected from respective groups at an indicated time points were subjected to QPCR for IL-6, IFN-γ and IL-1β mRNAs. The values are represented as fold change in mRNA compared to infected control. The above experiments were repeated three times.

**Fig.9: Src kinase inhibition may result in the attenuation of T cell function**

(a) Kinetic analysis of CD49d expression on the CD4+T cells in the control and treated animals after ocular HSV-1 infection. C57BL/6 mice were infected with 10^4 PFU of HSV. Mice (n=3) were sacrificed at each indicated time points and their draining cervical lymph nodes and corneas were analyzed for surface expression of CD4 and CD49d by flow cytometry. (a) Histograms representing the percentage of CD49d expression on CD4+T cells in draining cervical lymph node at the indicated time points post infection. Data are shown from one representative experiment. (b) Reduced expression of CD44 on CD4+T cells in
draining CLN of TG100572 treated mice. (c) FACS plots showing the percentage of CD4+ CD49d+ T cells in the corneas in control and TG100572 treated animals at day 11 post infection. All kinetic experiments were repeated at least twice. (d, e) Absolute numbers (x10^6) of the total lymph node cells and (d) and CD4+ T cells (e) in control and treated animals is shown at indicated time points p.i. The level of significance was determined using two way ANOVA with Bonferroni post test. (f) DLN cells were enriched for CD4+ T cells and stimulated with anti-CD3 anti-CD28 in the presence or absence of drug at the indicated concentrations. Cell proliferation results are expressed as mean CPM from triplicate cultures.
Figure 1

a

![Graph a: Angiogenesis scores comparison](image)

b

![Graph b: HSK Scores comparison](image)

c

![Graph c: Protein expression](image)

d  Naive

e  Control D15 p.i.

f  Treated D15 p.i.
Figure 2

**a**

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**b**

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Figure 3

a

b
Figure 5

(a) Control  (b) Treated
Figure 7

a) CD3/CD28 STIMULATED
   Control  Treated
   IFN-γ → CD4

b) CD3/CD28 STIMULATED
   Control  Treated
   IL-2 → CD4

c) UV-HSV-KOS STIMULATED
   Control  Treated
   IFN-γ → CD4

d) UV-HSV-KOS STIMULATED
   Control  Treated
   IL-2 → CD4

e) Absolute numbers of IFN+ CD4 T cells in the cornea
   control  treated

f) Absolute numbers of IL-2+ CD4 T cells in the cornea
   control  treated

g) Absolute numbers of IFN+ CD4 T cells in the cornea
   control  treated

h) Absolute numbers of IL-2+ CD4 T cells in the cornea
   control  treated