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Therapeutic dosing of FTY720 (Fingolimod) prevents cell infiltrate, rapidly suppresses ocular inflammation and maintains blood-ocular barrier

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Abstract

Fingolimod (FTY720) is a FDA-approved therapeutic, with efficacy demonstrated in experimental models of MS, and phase III human MS trials. FTY720 prevents T-cell migration to inflammatory sites, by down-regulating expression of the sphingosine-1 phosphate receptor normally required for egress from secondary lymphoid tissue. Experimental autoimmune uveoretinitis (EAU) serves as a preclinical model of human uveitis, permitting assessment of immunotherapeutic efficacy. Murine EAU is initiated by activation of retinal-antigen specific CD4+ T cells that infiltrate the eye, and previous studies demonstrate that high dose FTY720 treatment administered before disease onset reduces ocular infiltrate within hours of administration and suppresses clinico-pathological expression of EAU. The present study investigates the efficacy of FTY720 treatment for established disease. Single dose treatment is effective and its immunosuppressive ability is maintained through a dose range of FTY720, demonstrating significant and rapid reduction in the CD4+ cell infiltrate at clinically relevant therapeutic doses. Furthermore, a repeated treatment regimen using a comparable dose to current MS patient protocols significantly reduces infiltrate within 24 hours of administration, and importantly repeated doses do not compromise the vascular integrity of the blood-ocular barrier. Upon withdrawal of FTY720, drug induced remission is lost and recrudescence of clinical disease is observed. These results support the great therapeutic potential for FTY720 as an acute rescue therapy for the treatment of ocular immune-mediated inflammation.

Keywords

FTY720; Experimental autoimmune uveoretinitis; immuno-modulation; autoimmunity

Introduction

Intraocular inflammatory disease (Uveitis) and immune mediated retinal degenerative disorders, such as age-related macular degeneration account for the majority of visual handicap in the adult population. Non-infectious uveitis is considered an autoimmune disease initiated by loss of immune tolerance to retinal proteins, mediated and characterised by infiltration of leukocytes including T cells and tissue damaging macrophages ³⁻⁴.

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Currently, uveitis is estimated to affect up to 115 people per 100,000 in Western populations ¹, 25% of whom require systemic immuno-suppression with corticosteroid treatment for sight-threatening disease ², and overall around 35% remain visual disabled, often after a debilitating chronic relapsing disease course.

Experimental Autoimmune Uveoretinitis (EAU) provides a platform to dissect the mechanisms responsible for immune-mediated tissue damage, in addition to acting as a preclinical clinico-pathological model of non-infectious uveitis ³⁻⁵. Murine EAU develops following immunisation with specific ocular antigens and subsequent local activation of ocular-specific CD4⁺ T cells within the retina ⁶⁻¹⁰. Disease occurs when activated CD4⁺ Th1 and Th17 cells infiltrate the eye, neutrophils and macrophages are recruited and consequent cell activation generates structural damage ¹¹. Correlation between the clinical appearance, cellular analysis and underlying histopathology enables reliable and rapid assessment of disease features and severity. This liberates information detailing the infiltrate dynamics, phenotype and quantity, as assessed by flow cytometric analysis of the retina ¹²⁻¹³, alongside clinical appearance and histological changes including documenting the integrity of blood-ocular vascular barriers ¹⁴. Therefore EAU, as a preclinical model of uveitis, permits assessment of immunotherapeutic efficacy, and has also proven successful in the translation of current, clinically used therapeutics including cyclosporine A, Tacrolimus, Cellcept and biologics such as tumour necrosis factor-alpha (TNF- α) blockade ¹⁵.

Direct manipulation of immune cell traffic to the target organ represents a paradigm shift in the treatment of autoimmune diseases. Fingolimod (FTY720) is a potent immuno-modulator, with proven efficacy in both Experimental Autoimmune Encephalomyelitis (EAE), the MS inflammatory disease model and phase II & III human MS trials, resulting in FDA approval in 2010 for clinical use in patients with relapsing and remitting disease ¹⁶⁻¹⁹. FTY720 treatment generates lymphopenia due to selective lymphocyte retention in secondary lymphoid tissue, thus preventing migration to the periphery and sites of inflammation ²⁰⁻²². This effect is mediated by FTY720 as it acts as an agonist analogue by binding and down regulating expression of the sphingosine 1-phosphate receptor 1, which is required for T cell egress from lymph nodes ²³⁻²⁷.

Repeated treatment regimes initiated before onset of clinical disease have demonstrated suppression of disease severity in EAU and thus the relevance of FTY720 for the treatment of ocular inflammation ²⁸⁻²⁹. However, the impact and potential for treatment of human disease is increased by the observation that administration of single high dose FTY720 rapidly (within hours) reduces ocular infiltrate and prevents retinal damage. These results infer a significant clinical potential to serve as a rescue therapy for active acute onset sight-threatening intraocular inflammation ³⁰. The potency to prevent cellular influx into the eye and suppress acute disease may enable restoration of tissue immune homeostasis thereby offering clinical advantages over current high dose steroid therapy. Therefore, the purpose of this study was firstly to determine the efficacy of clinically safe and therapeutic doses of FTY720 as a steroid sparing approach to suppress active intraocular inflammation, secondly to assess whether following such dosing regimens long-term disease remission is induced and finally, whether tissue homeostasis, particularly with respect to restoration of ocular vascular barriers can be achieved. Together, the results provide an experimental proof of concept which supports the initiation of phase II clinical studies in man.

Materials & Methods

Mice

B10.RIII mice were originally obtained from Harlan UK Limited (Oxford, UK) and breeding colonies were established within the Animal Services Unit at University of Bristol,

UK. Mice were housed in specific pathogen-free conditions with water and food available continuously. Female mice immunised for disease were aged between 6 and 8 weeks. All mice were kept in the animal house facilities of the University of Bristol, according to Home Office regulations. Treatment of animals conformed to the ARVO statement for the use of animals in ophthalmic and vision research.

Reagents

Human RBP-3₁₆₁₋₁₈₀ peptide (SGIPYIISYLHPGNTILHVD) was obtained from Sigma (Poole, UK). Peptide purity was determined by HPLC. Peptide preparations were aliquoted and stored at -80°C. FTY720 and control analogue AAL149 were provided by Novartis, Switzerland.

EAU Induction and treatment

B10.RIII mice were immunized subcutaneously in one flank with 50 µg RBP-3₁₆₁₋₁₈₀ in phosphate buffered saline (PBS) emulsified with Complete Freund's Adjuvant (CFA) supplemented with 1.5mg/ml *Mycobacterium tuberculosis* complete H37 Ra (BD Biosciences, Oxford, UK) (1:1 vol/vol). The mice also received 1 µg *Bordetella pertussis* toxin (Tocris, Bristol, UK) intraperitoneally (i.p.). Doses of FTY720 or control analogue AAL149, in a maximum volume of 150 µl water, were administered by oral gavage on the days indicated.

EAU Clinical Assessment

Using a method adapted from Paques et al.,³¹ an endoscope with a 5cm long teleotoscope of 3mm outer diameter (1218AA; Karl Storz, Tuttlingen, Germany) was connected to a Nikon D80 digital camera with a 10-million pixel charge-coupled device image sensor and Nikkor AF 85 /F1.8 D objective (Nikon, Tokyo, Japan), with an additional +4.00 dioptre magnifying lens. Through pupils dilated with topical tropicamide 1% and phenylephrine 2.5% (Minims, from Chauvin Pharmaceuticals, UK), and topical oxybuprocaine 0.4% (Minims) and Viscotears (Novartis Pharmaceuticals, UK) for corneal anaesthesia, images were obtained by direct corneal contact with the endoscope. Images were processed using Adobe Photoshop (Adobe Corporation, Mountain View, CA). Using an adapted clinical grading system, fundal images were scored according to inflammatory changes to the optic disc and retinal vessels in addition to retinal lesions and structural damage³². All scores were added together to calculate a final disease score (Table 1).

Collagenase digest

Retinal-infiltrating cells were isolated by dissecting retinas and digesting them in complete RPMI supplemented with 5% vol/vol fetal calf serum, 1mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Invitrogen, Paisley, Scotland), 0.5 mg/ml collagenase D, and 750 U/ml DNase I (Sigma-Aldrich, Poole, UK). After 20 minutes at 37°C, an additional 0.5 mg/ml collagenase D and 750 U/ml DNase I were added. The mixture was then incubated for an additional 10 minutes at 37°C. Cell suspensions were then forced through a 40-µm cell strainer using a syringe plunger, washed and resuspended in staining buffer (balanced salt solution with 0.1% BSA and 0.08% sodium azide).

Flow cytometry

Cells were incubated with 24G2 cell supernatant for 10 minutes at 4°C before incubation with fluorochrome-conjugated monoclonal antibodies (mAbs) against cell surface markers including, CD4, CD11b, Ly6G and CD45 at 4°C for 20 minutes. Cell suspensions were acquired using a 3-laser BD™ LSR-II flow cytometer (BD Cytometry Systems, Oxford, UK). Analysis was performed using FlowJo software (Treestar, San Carlos, California). Cell

numbers were calculated by reference to a known cell–standard, as previously reported¹³. Briefly, splenocytes at a range of known cell concentrations were acquired using a fixed and stable flow rate for 1 minute. Based on total cell number acquired during this time, a standard curve was generated and used to interpolate cell concentrations of ocular infiltrating cells acquired at the same flow rate and time.

Assessment of vasculature and immuno-fluorescence

To evaluate micro-vascular permeability in the retina following FTY720 or AAL149 treatment in EAU-immunized or normal mice, 100µl of 2% (wt/vol) Evans Blue dye (Sigma-Aldrich) was injected through the tail vein. Evans Blue is an acid dye that binds to albumin in the blood, allowing visualisation of sites of blood retinal barrier breakdown. Animals were sacrificed 10 minutes later by an injection of lethal anaesthetic. The eyes were removed and immediately immersed in fresh 2% (wt/vol) para-formaldehyde (PFA) for 2 hours. Retinal whole mounts were prepared, by removing the anterior segment, and peeling the retina away from the choroid. Retinas were washed twice in cold PBS for 15 minutes then spread on clean glass slides and mounted, vitreous side up, under coverslips with mounting media (Vectashield, Vector Laboratories, Burlingame, CA).

To evaluate the expression of tight junction proteins following treatments, retinal and choroidal whole mounts were prepared. Eyes were enucleated, the anterior segment removed and the retina and choroid carefully dissected and separated in ice-cold PBS. Both retina and choroid staining protocols were identical, with the exception that all fixation, wash and antibody incubation buffers for the retinas contained 0.3% Triton. For ZO-1, Occludin & Claudin-1 (Invitrogen, UK) immuno-fluorescence staining, tissues were fixed in 2% PFA for 30 minutes at 4°C, washed and incubated in blocking buffer (5% BSA/Goat serum) for 2 hours. Primary antibodies were incubated in 1% BSA at 4°C overnight, washed, and incubated with Alexa-488-conjugated goat anti-rabbit immunoglobulin (Ig) (Invitrogen) for 1 hour at room temperature. For mounting, tissues were washed and mounted on slides as before.

To evaluate whether treatment induced apoptosis of cells in the retina, eyes from treatment mice were enucleated at various time-points and cryosections prepared. TUNEL staining was performed according to manufacturer's instructions and counterstained with DAPI (Roche, UK).

Confocal Microscopy

All retinal and choroidal whole-mounts were examined for Evans Blue or Alexa-488 by a confocal scanning laser imaging system fitted with krypton-argon lasers (Leica TCS-SP2-AOBS). Using dual blue and green fluorescence, the Evans Blue appeared red and the Alexa-488 stains green.

Results

Titration of FTY720 dose maintained the rapid reduction in retinal cell infiltrate

Previously, we have demonstrated that a short-term, high dose treatment with FTY720 could rapidly reduce the retinal immune cell infiltrate in EAU, and prevent subsequent retinal damage³⁰. Therefore, we proposed that Fingolimod has therapeutic potential as an acute rescue intervention for human non-infectious posterior-segment intraocular inflammatory disease. As our previous report had utilised a clinically unsuitable dose of FTY720, for translational application it was necessary to perform a dose titration of the single dose regimen to assess efficacy within a known therapeutic range.

To assess the efficacy of FTY720 in EAU, and as in our previous study, we utilized the highly susceptible B10.RIII mouse strain in which the immunizing regimen produces consistent moderate disease severity¹². Using topical endoscopic fundal imaging (TEFI)^{12, 32}, clinical changes which correlate with significant inflammatory cell tissue infiltrate are evident from around day 12. During the acute period these are progressive and include raised optic nerve, peri-vascular infiltrate and vasculitis, vitritis and retinal detachments, which resolve by day 28, leaving a persistent chorioretinal inflammatory infiltrate for months¹². For these experiments, TEFI enabled us to screen the mice and select experimental groups that displayed early signs of disease onset, namely raised optic nerve or early vasculitis.

Groups of selected mice were treated on day 13 with a single oral dose of FTY720 at different concentrations (10, 1 and 0.1 mg/kg), which were calculated using the FDA Guidance notes as human equivalent doses (HED) of 56, 5.6 and 0.56mg respectively, or control vehicle. The clinical appearance was assessed, and the retinal infiltrate examined and enumerated by flow cytometric methods, 24 hours following drug administration. Clinically at this time point, control treated mice demonstrate typical disease features with raised optic disc, vasculitis and choroidal lesions, which contrasts mice that received FTY720 which only exhibit low grade disease, with signs of raised optic disc (Figure 1A). Flow cytometric analyses of the retinas of the FTY720 mice demonstrate reduced CD4, CD11b and Ly6G positive infiltrate (Figure 1B; Supp. Fig 1A). There is an 80% reduction of CD4⁺ cells in mice receiving the highest dose of FTY720 compared to control animals, and this is dose dependent with significant reductions of 72% and 68% at 1 and 0.1 mg/kg, respectively (Supp. Fig 1B).

Repeated FTY720 dose regimen reduces retinal inflammatory infiltrate

Considering the acute and significant effect of reducing ocular cell infiltrate and rescue from retinal inflammation observed at a range of single FTY720 dose concentrations, it was important to determine the efficacy of a clinically relevant dose within a repeated treatment regimen. For the MS clinical trials (Phase II & III), daily oral doses ranging from 0.5 to 5mg have been evaluated, with treatment regimens spanning 6-24months¹⁷⁻¹⁸. Therefore, in subsequent experiments designed to assess a repeated dosing regimen, a low dose regimen of 0.3mg/kg was selected, which equates to a HED of 1.68mg.

In these experiments, groups of immunized mice were treated from disease onset for 5 consecutive days (12-16) with FTY720 or a control analogue, AAL149, at the stated dose. The clinical appearance of the retinas on day 17, from mice that received FTY720 were relatively normal with only signs of low grade disease, including raised optic disc and low level vasculitis; compared to AAL149 control mice that exhibited significantly higher disease severity, with pan-vasculitis and choroidal lesions (Fig 2A). The clinical observations in FTY720 treated animals were reciprocated in the retinal cell infiltrate analysis with an overall reduction in the total retinal infiltrate (Fig 2B) and a 75% decrease in CD4⁺ infiltrate (Supp Fig 2B). Furthermore, the degree of macrophage and neutrophil infiltrate was also reduced by 60% and 50% respectively, as compared to control treated animals (Supp Fig 2C).

FTY720 treatment restores vascular integrity

Our data thus far shows that repeated treatment is highly effective in rapidly reducing the retinal cell infiltrate. Such protective effects are likely a result of effective T-cell sequestration within secondary lymphoid tissues, as a result of down modulation of S1P1 on T cells^{25, 33}. In recent MS clinical trials, macular oedema has been reported in patients treated with FTY720, although the mechanisms responsible for this (via breakdown of the

blood-retinal barrier) remain unknown^{19, 34}. As such, it was important to determine whether FTY720 treatment promoted vascular dysregulation and tight junction breakdown in the context of both normal retinal vasculature and during inflammatory responses.

To elucidate whether repeated FTY720 treatment could mediate changes to the vasculature and blood ocular barrier (including both retinal and choroidal vasculature and retinal pigment epithelium (RPE)), eyes were assessed on day 17 from both normal and immunized mice. Changes to the micro-vascular permeability and barrier integrity were evaluated by Evans Blue staining and expression of tight-junction proteins including ZO-1, Occludin-1, Claudin-1 and E-cadherin, on retinal or choroidal flat-mounts, respectively. In normal un-immunized mice, repeated treatment with either FTY720 or AAL149 does not alter the clinical appearance of the retina, the vascular integrity or expression of tight-junction proteins, ZO-1 in retinal venules or RPE (Fig 3), or Occludin, Claudin and E-cadherin expression in RPE (Supp Fig 3 A-C).

EAU mice receiving AAL149 treatment demonstrated high clinical disease severity compared to untreated animals and showed diffuse Evans Blue leakage from retinal vasculature indicative of inflammatory-mediated increased vascular permeability and blood ocular barrier breakdown. This is corroborated by loss of ZO-1 expression (Fig 3). In contrast, retinas from FTY720 treated mice not only appear clinically normal and healthy, but maintain intact vasculature and expression of ZO-1 in retinal venules and RPE. Further indication that FTY720 treatment affords protection to the barrier, and prevents breakdown is the maintained expression of Occludin and Claudin in the RPE layer as compared to AAL149 treated mice (Supp Fig 3 A & B). Expression of E-cadherin across the RPE was similar between normal and diseased animals (Supp Fig 3C).

Rapid reduction of retinal infiltrate is not a result of *in situ* death following FTY720 treatment

It was important to determine that treatment induced rapid resolution of retinal cell infiltrate as a result of the inhibition of influx of cells and was not due to extensive cell apoptosis in the tissue. We therefore utilised a high dose FTY720 treatment regimen³⁰, and using the TUNEL assay, examined retinal sections for the presence of apoptotic cells.

Confocal images demonstrate the presence of cells that have undergone apoptosis on days 15, 18 & 21 post-immunization. Sections from control mice show the presence of apoptotic cells at all time-points examined, with an increased number of positively stained cells at day 18, located primarily in retinal folds. In contrast, the retina of FTY720 treated mice demonstrates no signs of apoptosis alongside maintenance of retinal morphology and architecture (Figure 4A & B).

Continued suppression of retinal infiltration requires maintained treatment

The efficacy of FTY720 demonstrated in reducing ocular infiltrate and maintenance of the blood retinal barrier following repeated treatment, indicates potential for clinical translation as a rescue therapy in active ocular inflammatory conditions. Previous EAU studies had utilized different high dose regimens of FTY720, administered before disease onset to show the effectiveness of treatment in maintaining the eye with reduced disease severity to late time-points²⁹⁻³⁰. Therefore, the longer term effect of FTY720 on disease suppression was important to determine in the context of the 0.3mg/kg therapeutic dosing.

To investigate this, clinical assessment of the retina from disease onset, during the active treatment phase (day 12-16), and then for an extended period until day 27, following FTY720 withdrawal was performed. A total clinical score reflecting combined assessment of optic disc, retinal vessels, retinal lesions and structural damage changes to the retina, from

TEFI images obtained on days 12, 15 and 27, was calculated. In groups of mice receiving AAL149 control treatment, disease presents and progresses as per published EAU course with low scores at onset, that increase to a peak of clinical inflammation on day 15, followed by gradual reduction during the clinically low grade persistent phase of disease¹²⁻¹³. FTY720 administration leads to significantly reduced disease scores on days 12 and 15, however, following drug withdrawal at day 16, levels of disease are comparable to control treated animals by day 27 (Figure 5). The only clinical signs of disease evident during FTY720 treatment are optic nerve swelling, which rapidly manifests to full expression of disease following drug withdrawal (Supp Fig 5A). Furthermore, assessment of the vasculature in both groups at day 27 reveal a similar appearance; equivalent breakdown of the blood ocular barrier with loss of barrier protection during treatment are evident with the diffuse Evans Blue staining on retinal flat-mounts, (Supp Fig 5B).

Discussion

The present study investigates the efficacy and potential of Fingolimod for future translatory studies to treat human intraocular inflammatory disease, in which the control mechanisms that maintain normal immune homeostasis and vascular integrity of the eye are dysregulated. Therefore, the ability to limit cellular infiltration has a potential knock-on to suppress subsequent bystander activation of non-specific infiltrating mononuclear cells and result in suppression of overt inflammatory responses, ultimately protecting the tissue. Although previous studies have demonstrated the benefit of this type of immuno-modulatory approach to suppress target organ infiltrate, the therapeutic doses utilized were either clinically unsuitable for human translation, did not define ability to acutely suppress active disease or did not identify the ability to protect or restore tissue tone in terms of maintenance of blood ocular barrier integrity²⁹⁻³⁰. In this study, we clearly demonstrate that clinically relevant doses of Fingolimod can acutely suppress active intraocular inflammation, maintain disease remission and support the vascular barrier integrity of the eye. Overall, the work provides evidence to facilitate translation of this drug to the clinic, as a highly effective rescue therapy for patients with acute onset or acute-relapsing intraocular inflammatory disease (uveitis).

Utilizing the experimental platform of EAU, we demonstrate that therapeutic dosing acutely suppresses ocular infiltrate comprised of T cells, macrophages and neutrophils. Confirmation of previous EAU studies reveal that within hours of a single high-dose treatment of FTY720, there is rapid decrease in the overall retinal infiltrate, with 80% reduction in the CD4⁺ T cell component as compared to controls. Furthermore, efficacy in reducing T cell infiltrate is maintained through a dose range of FTY720, where significant reduction in the CD4⁺ infiltrate occurs even at low therapeutic doses. A repeated regimen using comparable dosing to current MS patient treatment protocols reduces the overall CD45⁺ retinal infiltrate following FTY720 administration, as illustrated by a 75% reduction in CD4⁺ T cell infiltrate, compared to control animals. The dramatic and acute reduction in infiltrate is not a result of cells undergoing apoptosis in the tissue, as retinas from mice receiving high dose FTY720 treatment displayed no signs of death (increased TUNEL signal), and retinal morphology and architecture was maintained.

In EAU, inflammation is initiated by the activation of ocular antigen specific CD4⁺ T cells that infiltrate the eye and recruit macrophages, which in turn become classically activated, expressing Nitric Oxide Synthase 2 (NOS2) that effects structural damage^{8, 12-13, 35-37}. The activation of macrophages is an important determinant of disease outcome, and is controlled by inflammatory signals from the microenvironment, including IFN- from T cells³⁸⁻³⁹. Therefore, promoting S1P1 activation, via FTY720, blocks both T cell entry and requisite

signals for recruitment to the tissue, and thus subsequent mononuclear cell activation within the tissue.

This data supports previous observations that recruitment of macrophage mononuclear cell infiltrate to inflammatory sites, like the eye, is a dynamic process with rapid migration into and out of the retina regulated by control mechanisms, such as tumour necrosis factor receptor-1 (TNFRI) signalling⁴⁰. It is also probable that FTY720 effects are exerted directly on the myeloid component of ocular infiltrate as infiltrating macrophages and microglia express S1P1⁴¹⁻⁴³. S1P1 specific receptor agonists or S1P can reduce expression of pro-inflammatory cytokines by macrophages and regulate Arginase-1 and NOS2 expression, promoting a switch to an anti-inflammatory macrophage phenotype⁴². This is an effective method of reducing tissue damage in the retina, as previously shown by inhibiting TNF-induced macrophage activation³⁶⁻³⁷ or activation of inhibitory CD200R⁴⁴. Hence in EAU, treatment may also elicit additional effects through suppression of classical IFN- mediated tissue damage and suppression of monocyte infiltration and microglial migration.

The S1P1 receptor is expressed predominantly on immune, neural, and endothelial cells, and genetic deletion studies suggest a key role in angiogenesis, neurogenesis, as well as the regulation of immune cell trafficking and endothelial barrier function²⁶. *In vivo*, FTY720 phosphorylation (FTY720-P) allows interaction and activation of the S1P1 surface receptor²³⁻²⁴, which in turn induces an irreversible internalisation via endocytosis and subsequent proteosomal degradation⁴⁵. The natural S1P1 receptor ligand, S1P, does not stimulate these mechanisms, and thus the effects of FTY720-P are considered as a result of resistance to degradation or changes in the receptor conformation, when bound⁴⁶⁻⁴⁷. The effect of FTY720 modification leaves immune cells refractory to the normal action of S1P, and prevents their egress from secondary lymphoid tissues, and ultimately migration to sites of inflammation.

However, down regulation of S1P1 receptor by FTY720 could also influence the normal function of endothelial cells⁴⁸, as S1P is essential for maintaining the vascular endothelium, and thus provides a mechanism to resist vascular leak associated with inflammation⁴⁹. Recent evidence that endothelial cell expressed S1P1 is critical for the control of barrier permeability was provided by a study that demonstrated a single dose FTY720 treatment in mice could mediate S1P1 degradation and contribute to pulmonary vascular leak *in vivo*⁴⁷. Furthermore, this work also suggests that heterogeneity in receptor expression or degradation machinery and modulation of the receptor, could explain both the efficacious as well as adverse effects observed in MS patients receiving long-term FTY720 treatment regimens. Clinical trial reports detail a range of adverse effects associated with treatment, including the occurrence of macular oedema in 0.3% and 1.1% of recipients receiving 0.5mg and 1.25mg FTY720 doses respectively^{18, 34}. Although no direct evidence, this may be a result of increased permeability of the vascular network of the eye.

Considering these observations, and combined with the enormous potential that FTY720 offers for treatment of ocular inflammatory disease, it was necessary to determine whether FTY720 had any adverse effects via S1P1 receptors within the vasculature of the eye. Our results indicate that short-term repeated administration of therapeutically relevant doses of FTY720 does not adversely influence vascular integrity, as demonstrated by an intact retinal vasculature and maintained expression of tight junction proteins in the retina and RPE of normal and treated EAU mice. This is a key observation as cell passage during EAU disease progression is associated with barrier breakdown, vascular leak and the extravasation of a small number of leukocytes¹⁴, all of which is mediated by transient expression and redistribution of tight-junction proteins in the presence of inflammatory cytokines⁵⁰.

Furthermore, clinical monitoring indicated no signs of oedema or exudative retinal detachments in normal and treated EAU mice.

The immunization regimen in both EAE and EAU experimental disease models initiates a dominant and robust CD4⁺ T cell response, and subsequent non-specific immune cell activation, both of which are abrogated by the protective effect of FTY720 treatment. However, following on from the initial acute inflammatory stage, the late persistent disease phase is driven by a lowered threshold of T cell activation and a quantitatively lower and largely non-specific immune response^{13, 51}. As such, the direct effects of FTY720 on an already compromised vasculature become increasingly evident and may explain the adverse effects observed in MS patients with prolonged Fingolimod use¹⁹. As we propose FTY720 as a short-term acute rescue therapy, the risk of such adverse effects associated with long-term use are unlikely, and in the future could be circumvented by development of pharmacological receptor analogues/agents that specifically target S1P1 expressed on lymphocytes and not endothelial cells. Notwithstanding, as mentioned previously, preclinical studies have utilized high dose regimens of FTY720, administered before disease onset to show the effectiveness of treatment in maintaining disease remission, with reduced histological disease severity and retinal infiltrate to late time-points²⁹⁻³⁰. Pertinent to the clinical translatability of this study is that long-term suppression was not maintained in mice receiving low therapeutic doses of FTY720 and recrudescence of clinical disease with clear signs of compromised vascular integrity following drug withdrawal was evident. However, this in turn supports short term use in acute inflammation without compromise to vascular barriers.

In summary, the data presented supports the potential of Fingolimod as an effective agent for an acute rescue therapy for sight-threatening intraocular inflammation and offers proof for immediate translation into clinical trials. Further, although the effectiveness of persistent low dose treatment regimens still requires validation in terms of ensuring long term vascular integrity, the potential to personalise healthcare by tailoring efficacy when used in combination with other immunosuppressive therapies, may ultimately provide long-term disease remission.

Acknowledgments

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Abbreviations

EAU	experimental autoimmune uveoretinitis
RBP-3	retinol binding protein-3
FTY720	Fingolimod

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Figure 1. Single Dose regimen of FTY720 reduces CD4⁺ infiltrate

Mice were immunized for EAU, and eyes monitored by TEFI from day 10 onwards to select animals with clinically evident disease. Groups of mice (n=3) were treated with FTY720 (0.1, 1, or 10 mg/kg) or PBS control on day 13. Eyes were enucleated 24 hours after treatment and retinal infiltrate characterised and quantified. (A) – Representative TEFI image and disease score on day 14. In control mice, disease features indicated by arrows include, raised optic disc (1) and vasculitis (2). FTY720 treated mice only display low grade disease with raised optic disc (3). (B) – Graph showing combined cell numbers for CD4, CD11b and Ly6G positive infiltrate populations on day 14.

Figure 2. FTY720 repeated dose regimen reduces CD4⁺ infiltrate by 75%

Groups of immunized mice (n=6) were treated with a repeated low dose regimen (0.3mg/kg) of FTY720 or AAL149 analogue control on days 12-16. Eyes were enucleated 24 hours following final treatment and retinal infiltrate characterised and quantified. (A) – representative TEFI image and disease score on day 17. AAL149 treated mice display severe disease features indicated by arrows and include, raised optic disc (1) and vasculitis (2), and choroidal lesions (3). FTY720 treated mice have low grade disease with a raised optic disc (4). (B) – Graph showing combined cell numbers for CD4, CD11b and Ly6G positive infiltrate populations on day 17.

Figure 3. Maintenance of retinal vasculature and blood ocular barrier following repeated FTY720 treatment

Groups of normal and immunized mice (n=3) were treated with a repeated low dose regimen (0.3mg/kg) of FTY720 or AAL149 analogue control on days 12-16. Representative images following treatment on day 17, demonstrating the clinical appearance (TEFI), vasculature (Evans Blue), and ZO-1 expression in the retina and RPE.

Figure 4. Rapid reduction of retinal infiltrate is not as a result of in situ death following FTY720 treatment

Mice were treated with a high dose of FTY720 (10mg/kg) on days 13 & 14. Eyes were harvested on days indicated and sections prepared. The presence of apoptotic cells was visualised using the TUNEL (red) assay and counterstained with DAPI (blue). (A)

Representative images showing apoptotic cells in the retina of EAU mice on days 15, 18 and 21 post-immunisation. (B) Graph showing the mean number of TUNEL positive cells, counted in three different fields (* P < 0.05; ** P < 0.01).

Figure 5. Recrudescence of disease following drug withdrawal

Groups of immunized mice (n=3) were treated on days 12-16, and clinically monitored until day 27. TEFI images from days 12, 15 and 27 were assessed and a total clinical score reflecting changes to the optic disc, retinal vessels, retinal lesions and structural retinal damage calculated.

Table 1

Score	Optic Disc	Retinal vessels	Retinal Tissue Infiltrate	Structural damage
1	Minimal inflammation	1-4 mild cuffings	1-4 small lesions or 1 linear lesion	Retinal lesions or retinal atrophy involving 1/4 to 3/4 retina area
2	Mild inflammation	>4 mild cuffings or 1-3 moderate cuffings	5-10 small lesions or 2-3 linear lesions	Pan retinal atrophy with multiple small lesions (scars) or <3 linear lesions (scars)
3	Moderate inflammation	>3 moderate cuffings	>10 small lesions or >3 linear lesions	Pan-retinal atrophy with >3 linear lesions or confluent lesions (scars)
4	Severe inflammation	>1 severe cuffing	Linear lesion confluent	Retinal detachment with folding
5.	Not visible (white out or extreme detachment)	Not visible (white out or extreme detachment)	Not visible (white out or extreme detachment)	Not visible