SYK inhibition attenuates autoantibody production and reverses Experimental Autoimmune Glomerulonephritis

Stephen. P. McAdoo,1 John Reynolds,2 Gurjeet Bhangal,1 Jennifer Smith,1 John McDaid,1 Anisha Tanna,1 William D. Jackson,1 Esteban S. Masuda,3 H. Terence Cook,4 Charles D. Pusey,1* Frederick W.K. Tam1*

1Renal and Vascular Inflammation, Imperial College London, UK
2Dept of Biomedical/Forensic Science, University of Bedfordshire, UK
3Rigel Pharmaceuticals, South San Francisco, CA
4Centre for Complement and Inflammation Research, Imperial College London, UK

*These authors contributed equally to this work

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Corresponding Author: Dr Frederick WK Tam, Renal and Vascular Inflammation, Department of Medicine, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK
Email: f.tam@imperial.ac.uk; Tel: 44-20-8382354; Fax: 44-20-83832062
ABSTRACT

Spleen tyrosine kinase (SYK) has an important role in immunoreceptor signaling and SYK inhibition has accordingly been shown to attenuate immune-mediated injury in several in vivo models. However, the effect of SYK inhibition on autoantibody production remains unclear, and SYK inhibition has not been studied in an autoimmune model of renal disease. We have therefore studied the effect of SYK inhibition in experimental autoimmune glomerulonephritis, a rodent model of anti-glomerular basement membrane disease. We demonstrate glomerular SYK expression and activation by immunohistochemistry in both experimental and clinical disease, and show that treatment with fostamatinib, a small molecule kinase inhibitor selective for SYK, completely prevented the induction of experimental autoimmune glomerulonephritis. In established experimental disease, introduction of fostamatinib treatment lead to cessation of autoantibody production, reversal of renal injury, preservation of biochemical renal function, and complete protection from lung haemorrhage. B cell ELISpot and flow cytometric analysis suggest short-term fostamatinib treatment inhibits the generation and activity of antigen-specific B cells, without affecting overall B cell survival. In addition, fostamatinib inhibited the production of pro-inflammatory cytokine production by nephritic glomeruli ex vivo, and cultured bone marrow-derived macrophages in vitro, suggesting additional therapeutic effects independent of effects on autoantibody production, likely related to inhibited Fc receptor signaling within macrophages in diseased glomeruli. Given these encouraging results in an in vivo model that is highly applicable to human disease, we believe clinical studies targeting SYK in glomerulonephritis are now warranted.
INTRODUCTION

Spleen tyrosine kinase (SYK) is a non-receptor tyrosine kinase that has a well characterised role in the intracellular signaling cascade for classical immunoreceptors, such as activatory Fc receptors (FcR) and the B cell receptor (BCR).\(^1\) SYK is critical for mediating FcR-induced responses in a variety of cell types, including myeloid cells,\(^2,3\) dendritic cells,\(^4\) and mast cells.\(^5\) In B cells, SYK-mediated BCR signaling is necessary for cell maturation and survival, and SYK deficient cells developmentally arrest at the pro-B cell stage.\(^6,7\)

SYK has therefore emerged as a potential therapeutic target in autoimmune and allergic disease. Genetic disruption of SYK expression using siRNA, antisense oligonucleotides or inducible deletion has been shown to attenuate responses in animal models of airway hyper-responsiveness and asthma.\(^8,9\) A number of small-molecule inhibitors directed against SYK are also in development. One such agent – fostamatinib - has progressed to late-phase clinical trials, where it has shown biological activity in patients with rheumatoid arthritis (RA).\(^10,11\)

We have previously reported that SYK inhibition with fostamatinib - the orally bioavailable prodrug of active moiety R406 - is remarkably effective in reducing injury in rat nephrotoxic nephritis (NTN), even when treatment was delayed until disease was well-established.\(^12\) It has similarly shown efficacy in models of autoimmune disease, including murine lupus,\(^13,14\) collagen-induced arthritis (CIA),\(^15,16\) and spontaneous diabetes in non-obese diabetic (NOD) mice.\(^17\)

Whilst inhibition of antibody-dependent FcR-mediated responses has been shown to contribute to the reduction in injury seen in these models, the specific impact of SYK inhibition on autoantibody production remains unclear. No effect on circulating autoantibody levels was observed in CIA or murine lupus. Conversely, in NTN, there was a significant reduction in autologous rat anti-rabbit
antibody titre in animals pre-treated with fostamatinib. In NOD mice, treatment resulted in a reduction in anti-glutamate decarboxylase (GAD) antibodies, but not anti-insulin antibodies. These conflicting results are of particular interest given that the role of SYK in antibody production in mature B cells and plasma cells is not defined, since constitutively SYK deficient B cells arrest at the pro-B cell stage. Several factors may account for these discrepancies, such as timing and duration of SYK inhibitor exposure, and potential differences in response to auto- or alloantigens. Notably, whilst modeling autoimmune diseases, all of the reported non-spontaneous models rely on immunisation with alloantigen, or passive transfer of antibody that acts as planted alloantigen in target tissue, and so their translation to clinical autoimmunity is limited.

To address these issues, we have studied the effects of SYK inhibition in experimental autoimmune glomerulonephritis (EAG). This rodent model closely recapitulates the immunobiology and pathology of Goodpasture’s (or anti-glomerular basement membrane, GBM) disease. In our laboratory, it is induced by immunising susceptible rat strains with a well-defined recombinant rat protein (non-collagenous domain of the alpha-3 chain of type IV collagen – α3(IV)NC1), the universal Goodpasture autoantigen that is germane to human disease. Both the model and clinical disease are critically dependent on the development of autoantibodies directed against this autoantigen, and both manifest features of crescentic glomerulonephritis and alveolar haemorrhage. EAG, therefore, can be regarded as a genuine model of autoimmunity, and since it is characterised by the ongoing production of a directly pathogenic, disease-relevant autoantibody, it more accurately reproduces clinical disease than our previous studies in NTN, and in particular allows for study of pathogenic humoral responses, in addition to renal and lung end-organ damage.

Here, we first demonstrate the presence of the target protein, SYK, in both experimental and clinical anti-GBM disease. We show that SYK inhibition prevents the induction of autoimmunity
in this model. In established EAG we report, for the first time, significant attenuation of pathogenic humoral autoimmune responses, along with reversal of end-organ damage. Taken together, these data provide a clear rationale for targeting SYK in autoimmune glomerular disease.
RESULTS

SYK is expressed and activated in experimental and clinical anti-GBM disease

In normal rat kidney (Figure 1A), staining for total SYK (T-SYK) was often positive in distal tubular epithelial cells, but consistently negative in glomeruli. Staining for phosphorylated SYK (P-SYK) was negative in both glomeruli and tubular epithelial cells, suggesting that SYK may be expressed but not activated in the latter. In nephritic glomeruli from animals 18 days after induction of EAG (Figure 1B), the same pattern of tubular staining was observed. Within diseased glomeruli, staining for both T-SYK and P-SYK was positive, and localised to areas of endocapillary and extracapillary proliferation. Double staining confirmed strong co-localisation of P-SYK to ED-1 positive macrophages (Figure 1G).

In renal tissue from patients with benign, non-proliferative glomerular pathology (thin basement membrane lesion), a pattern of distal tubular expression for T-SYK was observed comparable to that seen in rat tissue, and glomerular staining for both T-SYK and P-SYK was negative (Figure 1C). In biopsies from patients with anti-GBM disease, there was strong staining for both T-SYK and P-SYK that localised predominantly to areas of crescent formation within abnormal glomeruli (Figure 1D), and occasionally to proliferating cells within the glomerular tuft.

We also noted that immunostaining of both rat and human lymphoid tissue (Figures 1E & 1F, respectively) for T-SYK and P-SYK was strongly positive, and centered on follicles and marginal zone areas, consistent with the well-characterised role for SYK in the generation of adaptive immune responses.
SYK inhibition with fostamatinib prevents the induction of autoimmunity

In order to establish if SYK has a role in the pathogenesis of autoimmunity in EAG, we examined the effect of SYK inhibition using fostamatinib in a preventive in vivo study. Wistar Kyoto rats (n=8/group) were treated with either vehicle or fostamatinib by oral gavage from one hour prior to immunisation with α3(IV)NC1 to day 18 after disease induction. This time-point was chosen since previous studies have shown reproducible disease in all animals by day 18. From day 18 onwards, treatment was discontinued, and animals were monitored for a further 18 day treatment-free period, in order to observe any progression of disease after treatment withdrawal. A dose of 40mg/kg twice daily was chosen based on our previous dose-response study in NTN that demonstrated maximal biological effect without toxicity, and is consistent with the dose used in murine lupus studies¹³.

During the treatment period, fostamatinib treated rats were completely protected from haematuria (100% reduction compared to vehicle controls; p=0.0005; Figure 2A) and proteinuria (97% reduction; p=0.0003; Figure 2B). Circulating autoantibodies to the GBM were virtually undetectable in treated animals (84% reduction; p=0.0003; Figure 2C). Fostamatinib treated animals had entirely normal renal histology at day 18, whereas vehicle treated animals had severe pathology (i.e. >50% of the glomerular tuft affected by necrosis or crescent formation) affecting 26% of glomeruli (100% reduction; p=0.021; Figure 2E). Similarly, fostamatinib treatment completely prevented glomerular macrophage infiltration at day 18 (100% reduction; p=0.021; Figure 2F). These observations suggest that SYK activity is an absolute requirement for the induction of autoimmunity in this model.

When treatment was withdrawn at day 18, the treated group developed typical features of disease, though with sustained protection compared to controls at day 36. Fostamatinib treated animals had
preserved biochemical renal function as reflected by normal serum urea levels (73% increase in vehicle treated group; p=0.009; Figure 2G), and there remained significant differences in haematuria (66% reduction in fostamatinib group; p=0.031), proteinuria (40% reduction; p=0.014), and severe glomerular pathology (38% reduction; p=0.0014). These indices of disease severity approximated those in untreated animals at day 18, suggesting that ‘disinhibition’ of SYK by treatment withdrawal at day 18 allowed the natural history of disease to be restored. It is notable, however, that anti-GBM antibody levels in the fostamatinib treated animals reached a lower plateau level than vehicle controls (51% reduction; p=0.021; Figure 2C), suggesting possible immunomodulatory effects following exposure to SYK inhibition, as previously suggested in murine lupus. In keeping with lower levels of circulating autoantibodies, there was less deposited antibody detected in the glomeruli of fostamatinib treated animals at day 36 (60% reduction; p=0.002, Figure 2D).

**SYK inhibition is an effective treatment in established EAG**

In a second *in vivo* study, we examined the effects of SYK inhibition in established EAG, to more accurately reflect the potential effect of treatment in clinical practice. Rats were treated with either fostamatinib 40mg/kg or vehicle by twice daily oral gavage from day 18 to day 36, and then assessed for disease severity.

At day 18, all animals had comparable degrees of haematuria (Figure 3A) and proteinuria (Figure 3B). Histological assessment in vehicle treated control animals at this time-point confirmed the presence of severe segmental necrotizing injury and crescent formation in approximately 26% of glomeruli. Disruption of the glomerular basement membrane was confirmed by Jones methenamine silver stain, and crescents were acute in nature, being characterised by extravasation of fibrin and cellular proliferation.
Following the introduction of fostamatinib treatment at day 18, there was a rapid and complete resolution of urinary abnormalities that was sustained until day 36, whereas vehicle treated animals had marked progression of disease (100% reduction of both haematuria and proteinuria at day 36; p=0.0001 and p=0.0009, respectively). Examination of renal histology (Figures 3C & 3D) at day 36 confirmed severe pathology (>50% of the glomerular tuft affected by necrosis or crescent formation) affecting 76% of glomeruli in vehicle treated animals, whereas fostamatinib treated animals had essentially normal glomerular histology (100% reduction; p=0.0007). When compared to histology in untreated animals at day 18, this implied that SYK inhibitor treatment led to reversal of necrosis and crescent formation (100% reduction; p=0.005). In keeping with the normal histology, fostamatinib treated animals had preserved levels of serum urea (103% increase in vehicle group; p=0.0023; Figure 3E). Fostamatinib treated animals had minimal evidence of ED-1 or CD8 positive cell infiltration into glomeruli (95% reduction in both; p=0.0009 and p=0.0014, respectively; Figure 4). SYK inhibition also resulted in complete protection from pulmonary haemorrhage (Figure 5), a late feature of disease in this model that develops after day 18, as assessed by macroscopic inspection (100% reduction in fostamatinib group; p=0.0013) and histological quantification of haemosiderin-laden cells (95% reduction; p=0.031).

**SYK inhibition suppresses autoantibody production in established disease**

The introduction of SYK inhibitor treatment at day 18 caused circulating autoantibody levels to plateau, at a time when levels were rising rapidly in the vehicle treated group (Figure 6A). Since the half-life of these autoantibodies exceeds 2-3 weeks, this suggests that there was no ongoing antibody production after the introduction of treatment, implying significant effects of SYK inhibition on mature, antibody secreting B cells or plasma cells. At the end of the treatment period, there was a 75% reduction in circulating antibody levels (p=0.0006) with a concomitant decrease
in the amount of deposited antibody (37% reduction; \( p=0.039 \); Figure 6B). There was also a decrease in deposited complement component C3 (47% reduction; \( p=0.015 \); Figure 6C), in keeping with reduced activation of the classical complement pathway.

SYK inhibition reduces the generation and activity of antigen-specific autoantibody-producing splenocytes *in vivo*

Since we observed significant effects of SYK inhibition on humoral immune responses in both *in vivo* studies, we went on to enumerate antigen-specific splenic B cells using B-cell ELISpot assays. We found that fostamatinib treatment from day 0-18 after disease induction reduced the generation of antigen-specific cells by 63% (\( p=0.0015 \)) (Figure 6C). However, in animals treated from day 18-36, once disease was established, there was no significant difference in the number of antigen-specific splenic B cells at the end of the treatment period (\( p=1.00 \)). This supports our observation that the introduction of fostamatinib treatment at day 18 had a direct effect on antibody production by mature antigen-specific cells, since there was no ongoing antibody production during this period, despite equal numbers of antigen-specific cells being present in the spleen.

We found only modest changes in the overall proportion of CD8, CD4 and CD45RA positive splenocyte subsets after 18 days treatment with fostamatinib (Figures 6D & 6E). The maintenance of CD45RA positive population, in particular, suggests that B cell survival was not adversely affected by a short period of treatment, and implies a direct effect of SYK inhibition on autoantibody production.
SYK inhibition has additional effects in EAG, independent of effects on autoantibody production

Based on our previous studies in NTN and our immunohistochemical findings, we hypothesised that in addition to inhibiting the production of autoantibodies, fostamatinib may prevent their downstream function via inhibition of FcR-signaling. To investigate a role for SYK independent of autoantibody production in EAG we isolated nephritic glomeruli from untreated animals 28 days after disease induction, in order to examine the effects of SYK inhibition in diseased tissue dissociated from the systemic humoral response. When these nephritic glomeruli were incubated with R406, the active metabolite of fostamatinib, there was a dose-dependent reduction in the spontaneous production of a number of pro-inflammatory cytokines (Figure 7A), including monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor alpha (TNF-α) and interleukin 12 (IL-12), each of which has been implicated in the pathogenesis of experimental glomerulonephritis. In addition, we studied the effects of R406 on cytokine production by primary bone marrow-derived macrophages (BMDM) in vitro, following stimulation by cross-ligation of Fc receptors using heat-aggregated IgG. We observed a dose-dependent reduction in pro-inflammatory cytokine production (Figure 7B), similar to that as seen in glomerular preparations.
DISCUSSION

Here we report several important observations that suggest SYK warrants clinical investigation as a therapeutic target in the autoimmune glomerulonephritides. These include the first description of SYK expression in experimental and clinical anti-GBM disease; the novel finding that SYK inhibition has a significant impact on the production of pathogenic autoantibody, in addition to its known effects on antibody-dependent FcR-mediated responses; and that this is accompanied by reversal of crescentic glomerulonephritis and complete protection from alveolar haemorrhage, the life-threatening manifestations of anti-GBM disease, in a highly accurate pre-clinical autoimmune model.

SYK inhibition using fostamatinib or other small molecule inhibitors has been studied in a number of in vivo models of immune-mediated injury. However, confirmation of SYK expression by immunohistochemistry in these models is limited. T-SYK expression has been described in the skin lesions of MRL/lpr lupus-prone mice\textsuperscript{14} and in the synovium of CIA rats,\textsuperscript{16} although the identification of SYK in its activated, phosphorylated state is limited to the alloimmune heterologous phase of rat nephrotoxic nephritis.\textsuperscript{27} This is therefore the first report of P-SYK detection by immunohistochemistry in an autoimmune model of renal injury, and the observation that P-SYK localised to areas of pathology in diseased glomeruli robustly implicates SYK in the pathogenesis of EAG. P-SYK co-localised strongly (although not exclusively) to ED-1 positive macrophages, the predominant infiltrating leucocyte in the model. Macrophages are well-recognised mediators of glomerular injury in clinical glomerulonephritides,\textsuperscript{28} and this observation supports potential targeting of SYK in clinical disease. To this end, we have previously reported both T-SYK and P-SYK expression in IgA nephropathy,\textsuperscript{23} and P-SYK detection is also described in post-infectious glomerulonephritis.\textsuperscript{27} The identification of P-SYK in anti-GBM disease, the most severe form of glomerulonephritis, suggests that SYK may contribute to the pathogenesis of
a range of proliferative glomerulonephritides, and that these may be responsive to SYK inhibitor therapy.

In addition to SYK expression in diseased end-organ renal tissue, we observed strong staining for both T-SYK and P-SYK in the lymphoid tissue of both rats and humans, consistent with a well characterised role for SYK in the generation of adaptive immune responses, and in both in vivo studies we observed significant effects of SYK inhibition on the induction and progression of autoimmunity. In particular, we found considerable attenuation of humoral responses after the introduction of treatment in the second study. This finding is in contrast to previous reports in murine lupus, CIA, and spontaneous diabetes in NOD mice, where no clear-cut effects on anti-dsDNA, anti-collagen, or anti-insulin antibody levels, respectively, were observed. However, the exposure period in these studies was not optimal for studying humoral responses, since treatment was initiated after maximal autoantibody responses were established, and did not continue beyond the lifetime of these pre-existing antibodies. Previous studies in mice have suggested that prolonged exposure to fostamatinib (>1-3 months) is associated with a decline in total B cell number and altered proportions of certain B cell subpopulations.13, 17 Our data, however, suggest that overall B cell survival was not affected by short-term fostamatinib treatment in rats, and that the effect on autoantibody production was due to a direct inhibitory effect on mature antibody producing B cells and plasma cells. Since constitutively SYK deficient B cells arrest at the pro-B cell stage, it has only been possible to study the role of SYK in mature cells with the advent of specific small molecule inhibitors (and potentially conditional genetic techniques29) and our novel observation suggests that SYK inhibition may prevent the production of pathogenic autoantibody, even after aberrant clonal responses have been established. Further analysis of the effects of fostamatinib treatment on B lymphocyte subsets or on B cell function in vitro, however, is limited by the paucity of validated B cell markers in the rat.
In addition to preventing the production of pathogenic autoantibodies, SYK inhibition appeared to have the potentially therapeutic second effect of inhibiting their downstream effector functions. Spontaneous pro-inflammatory cytokine production by nephritic glomeruli was inhibited by incubation with R406, the active metabolite of fostamatinib, independent of its effects on systemic humoral immunity. We observed a similar pattern of attenuated cytokine production by primary BMDM following FcR ligation, suggesting that the effect in glomeruli was mediated, at least in part, by inhibition of antibody-dependent, FcR-mediated responses in macrophages. Notably, the effect on IL-12 production by BMDM was less dramatic than that seen in whole glomerular preparations, in keeping with previous reports that IL-12 production by intrinsic renal cells is important in the pathogenesis of glomerulonephritis\textsuperscript{30}, and that resident renal cells may also respond to SYK inhibition.\textsuperscript{23}

The combined effect of SYK inhibition on antibody production and antibody-mediated effector functions, was a striking reversal of severe glomerular pathology in EAG, confirming our previous observations in NTN, where we observed approximately 20% reduction in glomerular crescent formation when treatment was initiated in severe disease with greater than 90% established glomerular crescents. We also observed complete protection from alveolar haemorrhage, both by macroscopic assessment and as a reduction in haemosiderin laden-cells, a characteristic finding in the broncho-alveolar lavage fluid of patients with lung haemorrhage\textsuperscript{31}, a life-threatening phenomenon that may accompany certain forms of glomerulonephritis.

Whilst our data suggest an important effect of SYK inhibition on B cells and macrophages in this model, our study has not addressed the role of other cell types that express SYK and that have been implicated in the pathogenesis of glomerulonephritis, such as neutrophils,\textsuperscript{32} mast cells\textsuperscript{33} and dendritic cells.\textsuperscript{34} Systemic exposure to a small molecule inhibitor such as fostamatinib limits the extent to which the effect in individual cells can be delineated, and only future work using genetic
approaches will allow complete dissection of the potential cellular mechanisms involved in vivo. Strengths of our study, however, include the use of a genuine autoimmune model using a well-characterised autoantigen that is relevant to human disease, along with a selective SYK inhibitor that has already demonstrated biological activity and an acceptable toxicity profile in several clinical studies.10, 11, 35, 36 Although fostamatinib did not demonstrate sufficient efficacy in a Phase III study programme to support regulatory submissions in rheumatoid arthritis, we believe the striking findings in this experimental model suggest that clinical studies in glomerulonephritis are now desirable.
CONCISE METHODS

A detailed description of all methods is provided in the Supplementary Material.

Study Approval

Human renal tissue samples were obtained from patients under local ethics committee approval (04/Q0406/25 Hammersmith and Queen Charlotte’s & Chelsea Hospitals Research Ethics Committee). Lymph node samples were provided the Imperial College Healthcare NHS Trust Tissue Bank. Animal studies were carried out with local ethics committee approval and in accordance with the UK Animals (Scientific Procedures Act) 1986.

Immunohistochemistry for SYK

All immunohistochemistry was performed on formalin-fixed paraffin-embedded tissues using the following primary antibodies: T-SYK N-19 (Santa Cruz Biotechnology, Dallas TX); human P-SYK Tyr525/526 (Cell Signaling Technology, Danvers MA); rodent P-SYK Try323 (Abcam, Cambridge UK); and developed using the Envision + System (DakoCytomation, Cambridge UK). For double staining, sections were first stained for P-SYK as described, then incubated with primary antibody to ED-1 (Serotec, Oxford UK) and developed using the Vector Blue ALP Substrate Kit III (Vector Labs, Burlingame CA).

SYK inhibitors
Fostamatinib and its prodrug, R406, were provided by Rigel Pharmaceuticals (South San Francisco, CA) and AstraZeneca (London, UK). The details of these molecules have been reported previously.13, 15

Experimental Autoimmune Glomerulonephritis

Disease was induced by immunising 6 week old female Wistar Kyoto rats (n=8/group) with 100mcg α3(IV)NC1 emulsified in complete Freund’s adjuvant as previously described.18 To obtain comparative histology at day 18, an additional two groups of n=4 were used. Fostamatinib 40mg/kg or vehicle was administered by twice daily oral gavage.

Assessment of renal disease

Haematuria was quantified by dipstick analysis (Multistix 8 SG, Siemens Healthcare, Rarrytown NY) and proteinuria by sulphosalicylic acid method.37 Kidney sections stained with periodic acid-Schiff, haematoxylin and eosin, and Jones methenamine silver stain were assessed for glomerular injury - 50 consecutive glomeruli were graded as normal, abnormal (<50% tuft affected by necrosis or crescent formation) or severely abnormal (>50% tuft affected) by a blinded observer, and results expressed as the mean proportion for each animal. Macrophages and CD8+ cells were immunostained using mAb to ED-1 and CD8 (Serotec), respectively, and the number of positive cells per cross section counted in 20-50 consecutive glomeruli by a blinded observer, with results expressed as the mean for each animal.

Assessment of lung injury
Lung surfaces were inspected at time of cull, and severity of lung haemorrhage graded as: 0 (normal), 1 (<10 petechaie), 2 (>10 petechaie), 3 (>20 petechaie), or 4 (massive haemorrhage). Lung sections were stained with Perls’ Prussian blue, without counterstain, and quantified by a blinded observer using automated image analysis software, with results expressed in arbitrary units.

**Assessment of autoantibody response**

Circulating α3(IV)NC1 antibodies were assayed in serum by ELISA. Deposited antibodies and complement C3 were detected on frozen kidney sections using a FITC-labeled anti-rat IgG (SigmaAldrich, St Louis MO) and a FITC-labeled anti-rat C3 antibody (Nordic MUbio, Susteren, The Netherlands), respectively. Fluorescence intensity was graded (0 to 3+) in 20 consecutive glomeruli by a blinded observer, and the results expressed as mean per animal.

**B Cell ELISpots**

Splenocytes were obtained from rats after treatment from either day 0-18 or day 18-36 (n=3/group). Cells were suspended in cell culture medium and incubated (500,000 cells/well) in ELISpot plates (Multiscreen HTS 96 well filter plates; Millipore, Billerica MA) previously coated with α3(IV)NC1 50mcg/ml in sterile PBS. Cells were incubated for 48 hours without moving, then plates were washed and developed using a biotin-streptavidin based secondary system. Spots were counted using an ELISpot platereader and dedicated software (ELISpot 4.0, Autoimmun Diagnostika, Strassberg Germany). Replicate results from 1 of 3 pairs of animals are reported in the manuscript.

**Flow cytometry**
Analysis was performed on splenocytes obtained from animals after treatment from day 0-18 after disease induction (n=4). Cells were stained with the following antibodies: CD45RA-PE (OX-33; Becton Dickenson (BD) Biosciences, San Diego CA); CD8-PECy7 (OX8; eBiosciences); CD4-FITC (W3/25; eBiosciences, San Diego CA) and run on a BD Accuri C6 flow cytometer. Analysis was performed on FlowJo X software (Tree Star, Ashland OR).

*Ex vivo* glomeruli and bone marrow-derived macrophages (BMDM)

Glomeruli were extracted from untreated animals 28 days after induction of EAG, by differential sieving of whole kidney tissue, and cultured in full culture media with 0.1% DMSO (vehicle) and 0.2μM, 1μM, or 2μM R406 for 48 hours. MCP-1, TNF-α and IL-12 levels in the media were quantified by ELISA or cytometric bead array (OptEIA Rat MCP-1 ELISA Set, Cytometric Bead Array Rat TNF-α Flex Set; both BD Biosciences; Rat IL-12+p40 ELISA Kit, Invitrogen, Paisley UK) used according to the manufacturer’s instructions. Primary BMDM were grown in L929-conditioned culture media to day 7. Cell culture supernatants were collected 24 hours following stimulation with heat-aggregated rat IgG2b (250mcg/ml; Sigma) and/or incubation with R406/vehicle in serum-free conditions, and cytokine levels quantified as above.

Statistics

Statistical analysis was conducted using Graphpad Prism 5.0 (Graphpad Software, San Diego, CA). All data are reported as median per group ± interquartile range unless otherwise stated. Comparison between groups was by Mann-Whitney *U* test and Kruskal-Wallis test with Dunn’s multiple comparison.
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Components of this work have been presented in abstract form at the American Society of Nephrology Renal Week 2012 and at the 16th International Vasculitis and ANCA Workshop 2013. It has not been published in whole or part elsewhere.

CONFLICT OF INTEREST STATEMENT

ESM is an employee and owns stocks/stock options of Rigel Pharmaceuticals. FWKT has received research project grants from AstraZeneca Limited, and has a consultancy agreement with Rigel Pharmaceuticals. CDP has received a research project grant from GlaxoSmithKline and has a consultancy agreement with Genzyme.
**FIGURES & FIGURE LEGENDS**

Figure 1

**SYK is expressed and activated in experimental and clinical anti-GBM disease.** Immunostaining for total (T-SYK) and phosphorylated (P-SYK) SYK in (A) normal rat kidney shows tubular SYK expression without activation (phosphorylation) and no evidence of glomerular expression; (B) nephritic rat kidney, showing a similar pattern of tubular staining accompanied by both SYK expression and activation within inflamed glomeruli; (C) thin basement membrane (TBM) disease shows no glomerular SYK expression; (D) anti-GBM disease shows SYK expression and activation localised to areas of crescent formation and in occasional endocapillary cells (thin arrows). (E) & (F) T-SYK and P-SYK expression and activation in lymphoid follicles in rat spleen tissue and human lymph node. (G) Double immunostaining for ED-1 (blue) and P-SYK (brown) shows significant co-localisation of P-SYK to infiltrating macrophages (solid arrows), the predominant infiltrating leucocyte in this model, along with a small number of ED-1 negative cells (hollow arrow) that express P-SYK. Images A-F are T-SYK N-19 (Santa Cruz), rat P-SYK Tyr323 (Abcam) and human P-SYK Tyr535/536 (Cell Signaling) immunoperoxidase stains with haematoxylin counterstain, x200 magnification. Image G is rat ED-1 (Serotec) immunophosphatase stain and rat P-SYK Tyr 323 immunoperoxidase stain, without counterstain, x400 magnification.
**SYK inhibition prevents induction of autoimmunity in experimental anti-GBM disease.**

(A) Haematuria, (B) proteinuria and (C) circulating anti-GBM antibody levels in fostamatinib (Fosta; dashed plots) and vehicle (solid plots) treated animals during the 18 day treatment period (shaded) and 18 day withdrawal period (unshaded), showing complete protection from these key autoimmune phenotypes during fostamatinib exposure. Protection was sustained after treatment withdrawal until day 36. (D) In keeping with reduced circulating antibody levels, fostamatinib treatment resulted in less deposited anti-GBM antibody, as detected by direct immunofluorescence (IF), at day 36. (E) Quantification of glomerular pathology in fostamatinib and vehicle (Veh) treated animals at the end of the 18 day treatment period (D18), shows full protection from nephritis with fostamatinib treatment; at the end of the 18 day withdrawal period (D36), glomerular injury was still reduced in the treated group. (F) Fostamatinib treatment completely prevented macrophage infiltration until day 18. After treatment withdrawal, macrophage infiltration proceeded and, by day 36, was comparable to day untreated animals at day 18. (G) Fostamatinib treated animals had significantly lower levels of serum urea at day 36; dashed line represents upper reference limit for serum urea in healthy rats (5.4mmol/l). Animals n=8/group. Data reported as median per group ± interquartile range. *p<0.05, **p<0.01, ***p<0.001.
**SYK inhibition is an effective treatment for established experimental anti-GBM disease.**

(A) Haematuria and (B) proteinuria in fostamatinib (Fosta; red plots) and vehicle (blue plots) treated animals during the 18 day treatment-free period (unshaded) and 18 day treatment period (shaded), showing complete resolution of urinary abnormalities following treatment initiation. At day 36, there was 100% reduction in haematuria and proteinuria in fostamatinib treated animals. (C) Glomerular pathology in untreated animals at day 18 prior to initiation of treatment (repeated from figure 2E for comparison), and in fostamatinib and vehicle treated animals after 18 days treatment at day 36, showing significant reversal of severe glomerular abnormalities with fostamatinib. (D) Representative photomicrographs of haematoxylin and eosin stained sections (upper panel) and Jones methenamine silver stained sections (lower panel) demonstrating typical glomerular pathology in vehicle and fostamatinib treated animals at day 18 and day 36. Notably, silver stain confirmed disruption of the glomerular basement membrane and extracapillary crescent formation at day 18 in untreated animals. All images x400 magnification. (E) Fostamatinib treated animals had lower levels of serum urea at day 36; dashed line represents upper reference limit for serum urea in healthy rats (5.4mmol/l) Animals n=8/group. Data reported as median per group ± interquartile range. *p<0.05, **p<0.01, ***p<0.001, ns – not significant.
SYK inhibition abrogates glomerular inflammatory cell infiltration in experimental anti-GBM disease.

(A) Glomerular macrophages were identified using anti-ED-1 mAb, and quantified per glomerular cross section (GCS). Fostamatinib treated animals had 95% reduction in glomerular macrophage infiltration at day 36. (B) Representative photomicrographs showing ED-1 staining in vehicle and fostamatinib treated animals at day 36. (C) Fostamatinib treated animals also had 95% reduction in glomerular CD8+ cells at day 36. (D) Representative CD8 staining for vehicle and fostamatinib treated animals at day 36. All images with haematoxylin counterstain, x400 magnification. Animals n=8/group. Data reported as median per group ± interquartile range. *p<0.05, **p<0.01, ***p<0.001.
SYK inhibition protects from the development of lung haemorrhage in experimental anti-GBM disease.

(A) Lung injury was assessed by macroscopic inspection of the lung surfaces at the point of cull on day 36, and scored on the basis of number of visible petechiae. Fostamatinib treated animals were completely protected from developing macroscopic lung haemorrhage. (B) Lower panel shows representative lung appearances for vehicle and fostamatinib treated animals. (C) Lung sections were also stained for haemosiderin with Perls’ Prussian blue (without counterstain) and the number of haemosiderin positive cells quantified using automated image analysis. Fostamatinib treated animals had minimal evidence of haemosiderin deposition. (D) Lower panel photomicrographs shows representative Perls’ stain in fostamatinib and vehicle treated group, x400 magnification. Animals n=8/group. Data reported as median per group ± interquartile range. *p<0.05, **p<0.01, ***p<0.001.
SYK inhibition terminates pathogenic autoantibody production in experimental anti-GBM disease.

(A) Circulating anti-GBM antibody levels in fostamatinib (Fosta; red plots) and vehicle (blue) treated animals during the 18 day treatment-free period (unshaded) and 18 day treatment period (shaded) showing cessation of autoantibody production after introduction of fostamatinib treatment. (B) At day 36, fostamatinib treated animals had less deposited anti-GBM antibody, as detected by direct immunofluorescence (IF). Lower panel photomicrographs show representative linear IF for deposited anti-GBM antibodies in fostamatinib and vehicle treated animals at day 36, x400 magnification (C) Fostamatinib treated animals also had less complement C3 deposition within glomeruli, suggested reduced activation of the classical complement pathway secondary to decreased deposition of antibody. Lower panel photomicrographs show representative immunofluorescence for C3 in glomeruli of vehicle and fostamatinib treated rats. (D) α3-specific splenic B cells were enumerated by B cell ELISpot assay. Fostamatinib treatment from day 0-18 inhibited the generation of α3-specific splenic B cells (measured at day 18). Fostamatinib treatment from day 18-36 did not affect the overall number of α3-specific splenic B cells at day 36. Replicate results (x8) from one of three representative biological replicated experiments are shown. (D) Gating strategy for flow cytometric analysis of splenocyte subsets. (E) Fostamatinib treatment for 18 days did not significantly affect the survival of CD45RA or CD8 positive splenocytes, although there was a reduction in CD4 positive cells. Animals n=8/group, except flow cytometric analysis, n=4/group. Data are reported as median per group ± interquartile range. *p<0.05, **p<0.01, ***p<0.001, ns – not significant.
SYK inhibition has additional effects in EAG, independent of effects on autoantibody production. (A) Incubation with R406, the active metabolite of fostamatinib, reduced spontaneous pro-inflammatory cytokine production by nephritic glomeruli \textit{ex vivo} in a dose dependent manner. MCP-1, monocyte chemoattractant protein-1; TNF-\(\alpha\), tumour necrosis factor alpha; IL-12, interleukin12. Results from four biological replicate experiments are shown, with data from each experiment normalised to vehicle group measurements. (B) R406 inhibited the production of pro-inflammatory cytokines by primary bone-marrow derived macrophages \textit{in vitro}, following stimulation with heat-aggregated IgG (aIgG). Replicate results (x3) from one of at least two biological replicate experiments are shown. All Data reported as median ± interquartile range. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\).
REFERENCES


