In vitro target validation and *in vivo* efficacy of p38 MAP kinase inhibition in established chronic collagen-induced arthritis model: a pre-clinical study

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Abstract

Objective

The aim of the present study was to determine the in vivo *efficacy of p38 mitogen-activated protein kinase (MAPK) inhibitors, namely GW856553X and GSK678361, in murine models of arthritis.*

Methods

The effect of p38 MAPK inhibitors was tested in 2 variants of the collagen-induced arthritis model (CIA) in DBA/1 mice, acute arthritis induced by heterologous collagen and chronic relapsing arthritis induced by homologous collagen. Animals were treated after onset of arthritis. Furthermore, post-onset disease efficacy of GSK678361 was tested in the chronic model, so as to determine the effects on established arthritis. In vitro *studies were carried out with GW856553X, using human umbilical vein endothelial cells, to determine potential effects of GW856553X on the vasculature.*

Results

In both acute and chronic arthritis, GW856553X reduced signs and symptoms of disease, and protected joints from damage. The effect of GW856553X in chronic CIA was confirmed using an alternative compound, GSK678361. Importantly, treatment with GSK678361 from 14 days post-onset of chronic arthritis completely reversed signs of established disease and joint destruction. Mechanism of action studies demonstrated that GW856553X inhibited endothelial cell migration and angiogenesis in vitro*, with reduced pro-inflammatory cytokine production.*

Conclusions

Suppression of murine CIA by the p38 MAPK inhibitors GW856553X and GSK678361 suggests that they may have therapeutic potential for future use in RA if safe clinical dosing achieves adequate compound exposure.

Key words

Arthritis, mouse, p38 MAP kinase, cytokine, joint.

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Received on May 13, 2009; accepted in revised form on October 29, 2009.

Abbreviations:
A-SAA: acute phase serum amyloid A;
ATP: adenosine triphosphate;
CIA: collagen-induced arthritis;
CFA: complete Freund's adjuvant;
ELISA: enzyme-linked immunosorbent assay;
BLC1/CXCL13: B lymphocyte chemoattractant;
FCS: foetal calf serum
HUVEC: human umbilical vein endothelial cells;
IL: interleukin;
LPS: lipopolysaccharide;
MAPK: mitogen-activated protein kinase;
MAPKAP 2: mitogen-activated protein kinase-activated protein kinase 2;
MCP: monocyte chemoattractant protein;
MKP: MAPK phosphatise;
MSK: mitogen- and stress-activated kinase;
RA: rheumatoid arthritis;
STAT: signal transducers and activators of transcription;
TNF-α: tumour necrosis factor α;
VEGF: vascular endothelial growth factor

Conflict of interest: This work was sponsored by GlaxoSmithKline (GSK) through the Alternative Discovery Initiative. Drs Rioja, Buckton and Binks are employees of GSK; Dr Malhotra is a former employee of GSB; Dr Ray is a former employee of GSB and declares a competing financial interest; the other authors declare no competing interests.

Introduction

The host immune system is highly regulated in terms of space and time. Loss of this control can lead to a number of diseases, including rheumatoid arthritis (RA), chronic inflammatory bowel diseases, neurodegenerative disorders and septic shock. RA is a chronic and systemic disorder, characterised by progressive destruction of articular cartilage and bone, and thought to be triggered by a combination of genetic susceptibility and exposure to environmental factors.

The p38 mitogen-activated protein kinase (MAPK) pathway is involved in a number of cellular processes critical to the development of RA, for example, activation and infiltration of leukocytes, as well as production of inflammatory cytokines. Mitogen-activated protein kinase p38 is a serine/threonine kinase, and four p38 MAPK isoforms have been characterised, namely p38α p38β, p38γ, and p38δ, which share a 12-amino-acid activation loop containing a TGY motif located at amino acid positions 180-182, which are subsequently phosphorylated at the residues Thr180 and Tyr182 leading to p38 MAPK activation. The inactivation process of p38 MAPK occurs by tyrosine-specific MAPK phosphatases (MKP) such as tyrosine and threonine phosphatase 1 (MKP1) (1). The most extensively studied isoform is p38α MAPK, which was shown to be involved in the biosynthesis of tumour necrosis factor α (TNF-α) and interleukin (IL)-1β at the transcriptional and translational level. More importantly, p38 MAPK is involved in the regulation of pro-inflammatory mediators to initiate leukocyte recruitment and activation, and cytokine gene expression (2).

Many p38 MAPK inhibitors have demonstrated efficacy in animal models of arthritic disease and at least two p38 inhibitors have been in phase II clinical trials for RA; however many have failed due to unacceptable side-effects. In the present study, we investigated the MAPK inhibitory compounds GW856553X and its close structural analogue GSK678361 for *in vivo* efficacy in mouse models of arthritis, and in *in vitro* validation studies, in order to determine whether the compounds modulate inflammation and/or angiogenesis. The aims of this study were to investigate differences in these structurally similar compounds, by studying the efficacy and dose responses and most importantly adverse reactions or side-effects in the animal setting. GW856553X is currently being evaluated for the treatment of chronic obstructive pulmonary disease in human phase II clinical trials. We determined the effect of single daily dosing with GW856553X in acute and chronic collagen-induced arthritis (CIA). The chronic model of arthritis we have used closely mimics RA and hence is a potentially good predictor of clinical efficacy (3, 4). The inhibition by GW856553X of acute CIA was mirrored by disease amelioration in chronic CIA, which was confirmed using another inhibitor, namely GSK678361. Furthermore, when GSK678361 was administered to mice 2 weeks after onset of symptoms of chronic CIA, this compound was able to abolish symptoms and reverse joint inflammation. Moreover, mechanism of action studies demonstrated that GW856553X inhibited human endothelial cell angiogenesis *in vitro*, and reduced pro-inflammatory cytokine production (IL-6, IL-8 and MCP-1). These data suggest that p38 MAPK inhibitors may have therapeutic potential for future use in RA.

Materials and methods

Materials

GW856553X and GSK678361 are structurally related inhibitors of p38α MAPK, which also inhibit p38β but exhibit a high degree of selectivity with respect to other protein kinases, enzymes and receptors (5). In assays measuring the effects of GW856553 and GSK678361 on LPS-stimulated human peripheral blood mononuclear cells, the compounds were found to inhibit production of the pro-inflammatory cytokines TNF-α (IC50 values of 31nM and 12nM respectively, data not shown). Similar potencies were found against IL-1β, IL-6 and IL-8.

Human recombinant vascular endothelial growth factor (VEGF) and human recombinant TNF-α were from R&D Systems, Abingdon, UK, and human

recombinant interleukin IL-1β was from Invitrogen Ltd, Paisley, UK.

Induction and monitoring of CIA

Bovine type II collagen was extracted from bovine articular cartilage and murine type II collagen was purified from mouse sternums (6). Collagen was dissolved in 0.05M acetic acid and emulsified with an equal volume of Freund's complete adjuvant (CFA; Difco Becton Dickinson, Cowley, Oxford, UK).

Mice used in this study were male DBA/1 (H-2q) purchased from Harlan UK Limited (Bicester, Oxon, UK). Animals were housed in individual ventilated cages over a 12-hour light/dark cycle and were fed standard laboratory chow and water *ad libitum*. Animal work was conducted under the Home Office project licence PPL no: 70/5446 *Pathogenesis and therapy for RA* under the operatives of the Animals (Scientific Procedures) Act 1986.

For induction of acute CIA, 10-12 week old mice received a single intradermal injection at the base of the tail of 100μg bovine type II collagen emulsified in CFA, containing paraffin oil, and lyophilised *Mycobacterium tuberculosis H37 Ra* (Difco Becton Dickinson, Cowley, Oxon, UK). The first clinical signs of arthritis were assessed by oedema and/or erythema involving any of the four paws. Onset of disease was observed from 2 weeks after collagen administration, as previously described (3, 7-14).

For the induction of chronic relapsing arthritis, 10-12 week old mice were immunised with 100μg of homologous type II collagen emulsified in CFA by intradermal injection at the base of the tail. On day 14 after immunisation the mice were boosted with 100μg of homologous type II collagen. The first clinical signs of arthritis were assessed as for acute CIA, with onset of disease occurring from 2 weeks after murine type II collagen boost in incomplete Freund's adjuvant (4 weeks after primary immunisation), as previously described (3, 4, 15).

The mice were randomised into untreated, inhibitor-treated (GW856553X or GSK678361) and vehicle-treated (1% DMSO plus 99% methyl cellulose) groups, at the onset of the disease. Treatments were administered intra-peritoneally (i.p.), from the day of arthritis onset. Paw swelling was measured with 0-10 mm callipers (Kroeplin, Schluchern, Germany). In both models, mice were monitored daily and each limb was assigned a clinical score as follows: 0: normal paws and no clinical features of inflammation; 1: slight oedema or erythema; 2: pervading oedema/erythema involving the entire paw; 3: pronounced oedema and erythema leading to incapacitated limb mobility.

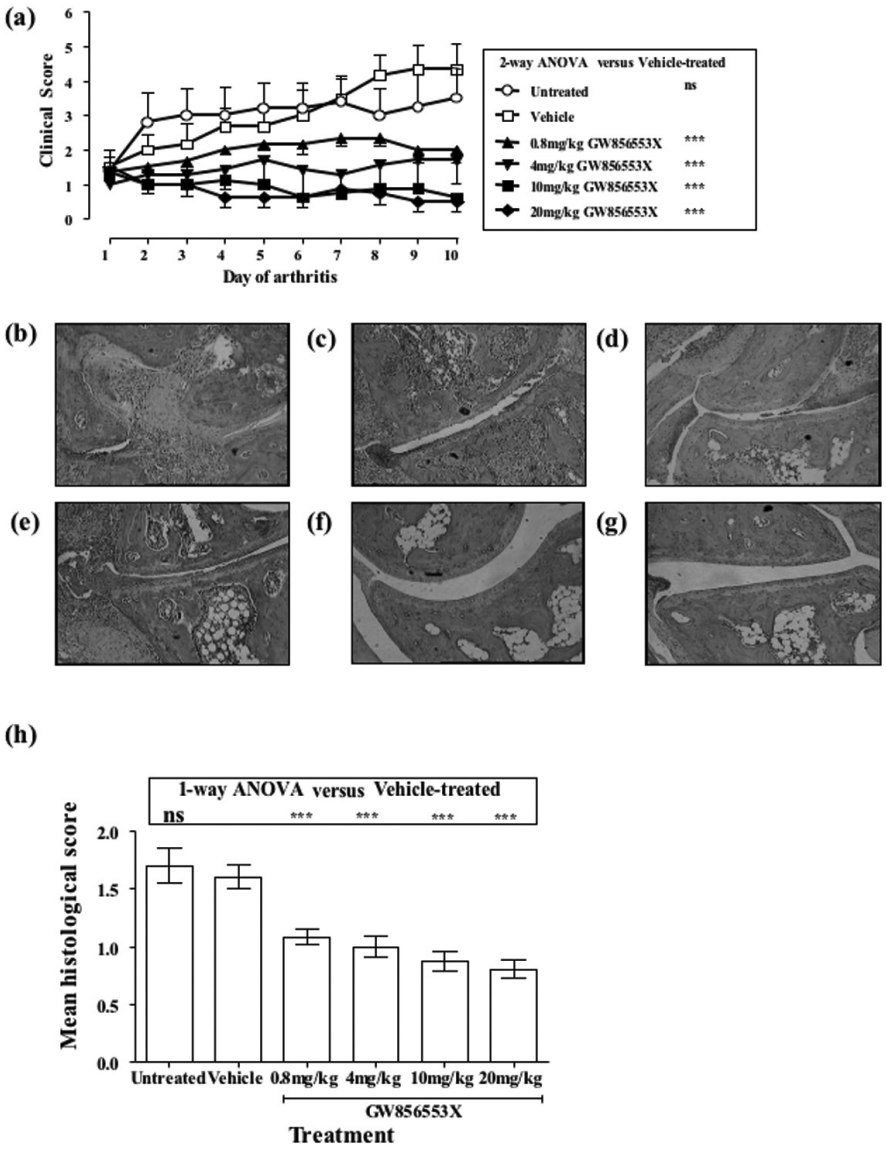

Fig. 1. GW856553X reduces acute CIA.
Following onset of arthritis, mice were treated with GW856553X (0.8mg/kg, 4mg/kg, 10mg/kg daily or 20mg/kg daily), vehicle alone or left untreated (n=5-8 per group). (**a**) Clinical score was assessed over a 10 day period, and data are expressed as mean ± SEM. Statistical analysis was carried out using a 2-way ANOVA *versus* vehicle-treated mice: $^{***}p<0.001$. At the end of the study (day 10), animals were sacrificed and paws were sectioned for histological evaluation. Representative sections are shown as follows: (**b**) untreated, (**c**) vehicle-treated, (**d**) 0.8mg/kg GW856553X, (**e**) 4mg/kg GW856553X, (**f**) 10mg/kg GW856553X, (**g**) 20mg/kg GW856553X. (**h**) Haematoxylin and eosin sections were scored in a blinded fashion for pannus formation, synovitis, and bone and cartilage erosion. Paws were assigned one of three grades: 0=normal; 1=moderate synovitis with some loss of cartilage; 2=extensive synovial hyperplasia, destruction of cartilage and some bone erosions. Results are mean (± SEM) histological score (metatarsal, tibia-tarsus, proximal interphalangeal and distal interphalangeal joints). Statistical analysis was carried out by 1-way ANOVA *versus* vehicle-treated mice: $^{***}p<0.001$.

Histological analysis

Hind paws were removed post-mortem and fixed in 4%. Fixed specimens

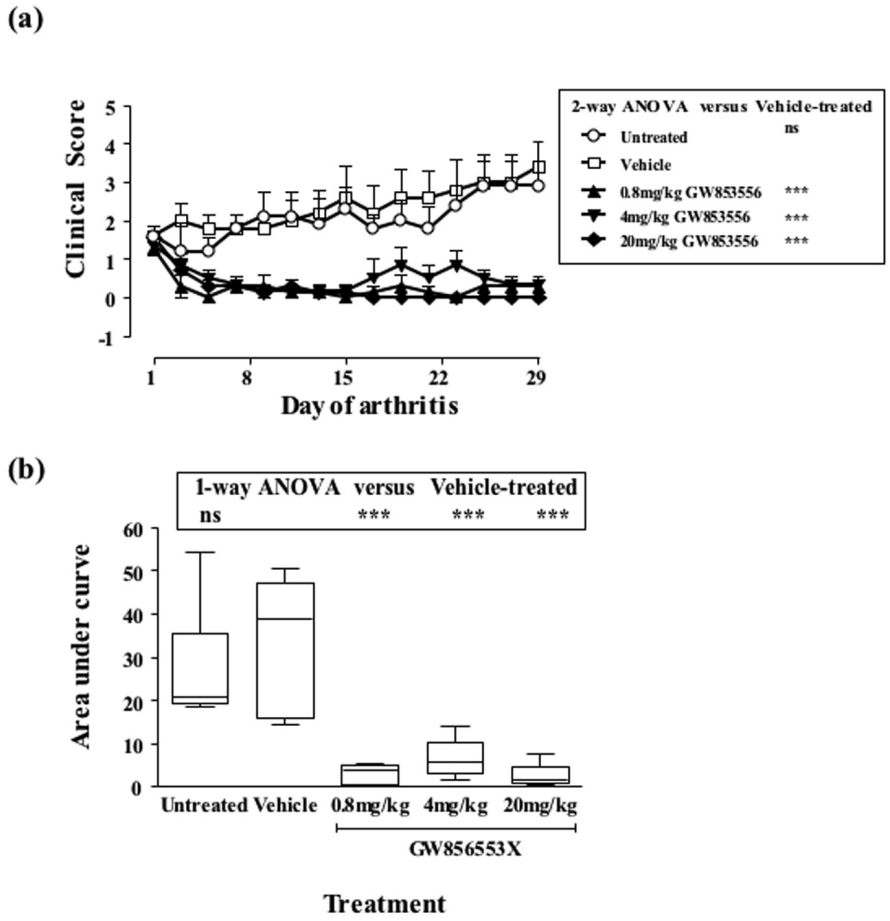

Fig. 2. GW856553X reduces chronic CIA.
From the day of onset of arthritis, mice were treated with GW856553X (0.8mg/kg, 4mg/kg or 20mg/kg daily), vehicle alone or left untreated (n=5-7 per group). (**a**) Clinical score was assessed over a 4 week period, and data are expressed as mean ± SEM. Statistical analysis was carried out using a 2-way ANOVA *versus* vehicle-treated mice: ***p<0.001. (**b**) Area-under-curve analysis of clinical score data, represented as box-and-whiskers plots. Statistical analysis was carried out using a 1-way ANOVA *versus* vehicle-treated mice: ***p<0.001.

were decalcified (Rapid-Cal™, BBC Biochemical, Dallas, TX, USA) and embedded in paraffin wax. Serial sections of 5μm thickness were dewaxed and stained with haematoxylin and eosin. The stained sections were scored microscopically for changes to joint architecture by an observer blinded to the study groups. Arthritic changes in the ankle, metatarsophalangeal, proximal interphalangeal, and distal interphalangeal joints were individually graded as follows: 0=normal, 1=moderate (pannus formation and erosions limited to the cartilage-pannus formation), and 2=severe (more extended bone and cartilage erosions, with loss of joint architecture).

Endothelial cell studies

Human umbilical cords were collected from Chelsea and Westminster Hospital (London, UK) in accordance with the guidelines of the Riverside Research Ethics Committee (RREC 2948). Human umbilical vein endothelial cells (HUVEC) were isolated by digestion of umbilical cord veins in 0.025mg/ml collagenase A (Roche Diagnostics, Mannheim, Germany) (3, 16). Cells were maintained in RPMI 1640 (Cambrex, Berkshire, UK) containing 10% foetal calf serum (FCS; Biowest, Nuaillé, France), 10% new born calf serum (Gibco, Paisley, UK), 5U/ml heparin (CP Pharmaceuticals, Wrexham, UK), and 15μg/ml endothelial cell growth supplement (Sigma Aldrich, Poole, UK). To measure HUVEC viability, a modified assay for mitochondrial activity was used. Wells were treated with GW856553X (1-100nM) for 24 hours. Following treatment as above, 0.5mg/ml MTT (3-[4,5-dimethyl-2-yl]-2,5-diphenyltetrazolium; Sigma Aldrich, Poole, UK) was added to the cells overnight. Cells were lysed using 10% SDS containing 0.001M HCl, and absorption was measured after 24 hours at 620nm. In order to analyse the effect of GW856553X on angiogenesis, a commercially available angiogenesis kit was used (AngioKit; TCS Cell Works, Buckingham, UK). Wells were treated on day 0 with VEGF, in the absence or presence of increasing concentrations of GW856553X or 0.01% DMSO as vehicle control. Culture medium was replenished after 4, 7 and 9 days according to manufacturer's instructions. On day 11, the medium was aspirated and the cells were fixed at room temperature in ice-cold 70% ethanol. Expression of CD31 was visualised by staining with mouse anti-human CD31 antibody (TCS Cell Works, UK, Buckingham, UK) for 60 minutes at 37°C, followed by goat anti-mouse IgG alkaline phosphatase conjugate for 10 minutes at room temperature. CD31 ELISA substrate was prepared by dissolving p-nitrophenol phosphate in Tris buffer and was added according to manufacturer's instructions. The plate was read at 405nm. Subsequently, an insoluble substrate prepared from BCIP (5-bromo-4-chloro-3-indolyl phosphate)/NBT (nitro blue tetrazolium; TCS Cell Works, UK) was added. Once the substrate was filtered, 0.5ml was added per well and incubated at 37°C until tubules developed a dark colour (5-15 minutes). Wells were then washed three times with distilled H_2O and air dried before microscopic capture using a BH2 microscope (Olympus Optical, Japan) linked to a KY-F55BE video camera (Victor Company, Japan).

To assess migration, $2x10^5$ HUVEC in RPMI 1% FCS were seeded onto gelatin-coated polycarbonate cell culture inserts 8μm (Becton Dickinson, Cowley, Oxon, UK) and allowed to migrate for 6 hours. GW856553X or 0.01% DMSO as vehicle control was added to both upper and lower wells. After the migration period, non-migrating cells were removed from the upper side of the membrane, and the insert was transferred to a clean well containing 300μl

Cell Stain Solution (Cambridge Bioscience Ltd, Cambridge, UK). Inserts were air-dried and migrated cells were placed in Extraction Solution (Cambridge Bioscience Ltd, Cambridge, UK). The plate was read at 560nm.

The effect of GW856553X on cytokine release was determined. HUVEC ($8x10^4$ per $200mm^2$) were stimulated with 10ng/ml TNF-α for 24 hours, in the absence or presence of GW856553X or 0.01% DMSO as vehicle control. Release of MCP-1, IL-6 and IL-8 were measured by ELISA as previously described (3, 16).

Statistical analyses

Two-way ANOVA test was used to evaluate the clinical scoring data, assessing the dose and the time of treatment. One-way ANOVA test was used to evaluate the histological scoring data as a function of the dose. *P*-values less than 0.05 were considered to be statistically significant.

Results

Therapeutic effect of p38 MAPK inhibitor in acute and chronic relapsing CIA

The acute CIA model was first used to establish the dosing regime for GW856553X. We observed that daily intra-peritoneal administration of GW856553X ameliorated acute arthritis (Fig. 1a). This effect was seen at the higher dosages of 10-20mg/kg GW856553X ($p<0.001$ *versus* vehicle-treated animals) while stabilisation was also achieved ($p<0.001$ *versus* vehicle-treated animals), at lower doses of 0.8-4mg/kg, significantly reducing the scores ($p<0.001$ in both cases). These observations were supported by reduced joint inflammation and destruction in GW856553X-treated mice, which was evident across all joints (proximal and distal interphalangeal, first metatarsal and tibia-tarsal joint) at all doses of GW856553X (Fig. 1b-h).

Having established the dosing regime for GW856553X in acute CIA, we subsequently assessed the effect of GW856553X in a chronic model of arthritis. Immunisation of mice with murine, rather than bovine, collagen leads to disease which more closely resembles RA. Animals were treated intra-peritoneally from day 1 of arthritis with either vehicle (1% DMSO plus 99% methyl cellulose) or GW856553X at doses of 0.8mg/kg, 4mg/kg or 20mg/kg once daily, and compared to an untreated arthritic group of animals. GW856553X was found to be effective at all doses, in terms of ameliorating chronic CIA (Fig. 2). For example, on day 29 of arthritis, the clinical scores were 0.29±0.18, 0.33±0.21 and 0.00±0.00 for mice treated with 0.8mg/kg, 4mg/kg and 20mg/kg GW856553X respectively, compared with 3.40±0.68 for animals treated with vehicle (clinical score for untreated animals 2.90±0.64; Fig. 2a).

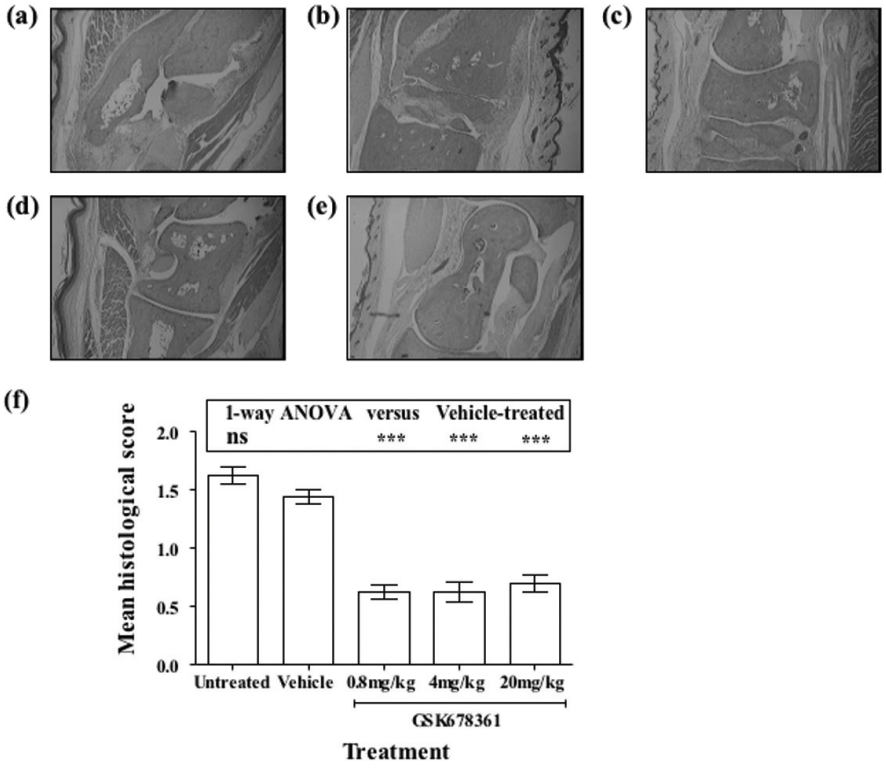

Fig. 3. GW856553X reduces joint destruction in chronic CIA.
From the day of onset of arthritis, mice were treated with GW856553X (0.8mg/kg, 4mg/kg or 20mg/kg daily), vehicle alone or left untreated (n=5-7 per group). At the end of the study (day 29), animals were sacrificed and paws were sectioned for histological evaluation. Representative sections are shown as follows: (**a**) untreated, (**b**) vehicle-treated, (**c**) 0.8mg/kg GW856553X, (**d**) 4mg/kg GW856553X, (**e**) 20mg/kg GW856553X. (**f**) Results are mean (± SEM) histological score (metatarsal, tibia-tarsus, proximal interphalangeal and distal interphalangeal joints). Statistical analysis was carried out by 1-way ANOVA *versus* vehicle-treated mice: $^{***}p<0.001$.

Table I. GW856553X reduces joint destruction in chronic CIA
From the day of onset of arthritis, mice were treated with GW856553X (0.8mg/kg, 4mg/kg or 20mg/kg daily), vehicle alone or left untreated (n=5-7 per group). Haematoxylin and eosin sections were scored in a blinded fashion for pannus formation, synovitis, bone and cartilage erosion. Paws were assigned one of three grades: 0=normal; 1=moderate synovitis with some loss of cartilage; 2=extensive synovial hyperplasia, destruction of cartilage and some bone erosions. Data for number of distal interphalangeal joints scored are shown.

Treatment	Histological score (number of affected joints)		
	Normal (0)	Moderate (1)	Severe (2)
Untreated	1	9	6
Vehicle	0	8	5
0.8mg/kg GW856553X	8	6	0
4mg/kg GW856553X	6	8	0
20mg/kg GW856553X	6	9	0

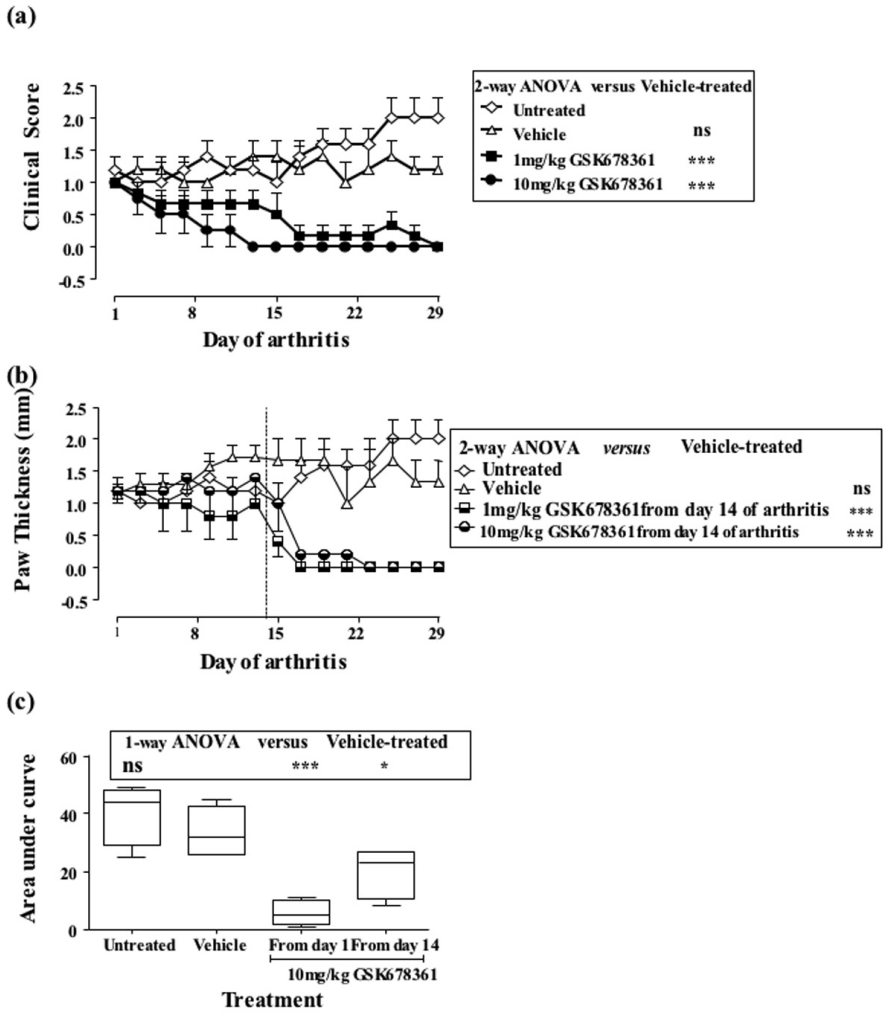

Fig. 4. p38 MAPK inhibition reduces established chronic CIA.
To determine the effect on established disease, mice (n=5 per group) were treated with either 1mg/kg or 10mg/kg GSK678361 daily from day 1 of arthritis (**a**), or with vehicle from day 1 followed by either 1mg/kg or 10mg/kg GSK678361 daily from day 14 of arthritis (**b**). Further groups were treated with either vehicle alone for the full period of the study or left untreated. Clinical score was assessed over a 4 week period, and data are expressed as either mean ± SEM (a, b) or as area-under-curve (**c**). Statistical analyses were carried out *versus* vehicle-treated mice either (a, b) by 2-way ANOVA or (c) by 1-way ANOVA: $^{*}p<0.05$, $^{***}p<0.001$.

Area-under-curve analysis confirmed a significant reduction in disease severity in GW856553X-treated animals, relative to vehicle-treated mice (Fig. 2b). Such analysis allowed for additional comparison of disease severity in this complex model, in which remission and relapse occurred on different days for individual animals. A similar effect was seen when paw thickness was measured. On day 29 of arthritis, paw thickness measurements were 1.91±0.02mm, 1.96±0.03mm, and 1.88±0.02mm for mice treated with 0.8mg/kg, 4mg/kg and 20mg/kg GW856553X respectively, compared with 2.18±0.04mm for animals treated with vehicle alone (untreated animals 2.13±0.04mm; data not shown).

Histological analysis provided supporting evidence, showing reduced joint inflammation and destruction in GW856553X-treated mice, which was evident at all doses of GW856553X (Fig. 3a-e). The data shown for distal interphalangeal joints were mirrored for the other joints (proximal interphalangeal, first metatarsal joint and tibia-tarsal joints) and are summarised in Table I and Fig. 3f.

The effectiveness of p38 MAPK inhibition in chronic CIA was confirmed using an alternative p38 MAPK inhibitor, namely GSK678361, which has comparable *in vivo* efficacy to GW856553X in acute CIA (data not shown). GSK678361 was administered at either 1mg/kg or 10mg/kg daily either from day 1 of arthritis, and dose-dependently reduced arthritis in this model (Fig. 4a).

Effect of p38 MAPK inhibitor on established chronic CIA

We further assessed the effect on established chronic CIA using GSK678361, which was administered at either 1mg/kg or 10mg/kg daily from day 14 of arthritis (with prior treatment using vehicle). Drug-treated animals were compared to untreated arthritic mice, or mice receiving vehicle for the full period of the study (4 weeks). Delaying the start of treatment with GSK678361 to day 14 of arthritis reduced established disease (Fig. 4b). Prior to commencing GSK678361 treatment (day 13), all groups of animals were indistinguishable. However, by day 23 of arthritis, there was no disease in the groups receiving GSK678361 from day 14, comparable with animals who had received GSK678361 for the entire duration of the study. In contrast, untreated and vehicle-treated mice continued to progress with clinical signs. Area-under-curve analysis are shown in Fig. 4c (data for 1mg/kg GSK678361 treatment groups not shown).

Importantly, it appears that GSK678361 was able to reverse joint destruction associated with chronic CIA. As illustrated in Fig. 5, 4 weeks of chronic CIA was associated with synovitis, inflammation and bone erosions, as evidenced in the typical sections illustrated for untreated animals (Fig. 5a) and vehicle-treated animals (Fig. 5b). Treatment with GSK678361 from day 1 of arthritis significantly ($p<0.001$ *versus* vehicle-treated mice) reduced joint inflammation and destruction (Fig. 5c and 5f). Changes in joint pathology were evident even after 14 days vehicle treatment (Fig. 5e), with 30% and 70% joints showing moderate or severe changes respectively (data not shown). Treatment from day 14 of arthritis until the end of the study (day 29) resulted in the joints appearing comparable to those from animals who received

GSK678361 from the very first day of disease (Fig. 5d). The percentage of joints with severe changes was 8% for animals treated from day 1 of arthritis, compared with 13% for animals treated from day 14. In contrast, untreated or vehicle-treated mice showed 75% and 67% severely affected joints. These data are illustrated in Fig. 5f.

Differential effects of GW856553X on HUVEC responses

To study the potential mechanism of action of GW856553X *in vivo*, we determined the effect of this inhibitor in a panel of *in vitro* endothelial cell-based assays. In order to determine the effects of GW856553X on endothelial inflammatory responses, HUVEC were stimulated with TNF-α, in the absence or presence of 100nM GW856553X. The release after 24 hours of cytokines and chemokines (namely IL-8, IL-6, and MCP-1) was measured. We observed modest but consistent (and statistically significant) inhibition of cytokine release (Figs. 6a-c). However, inhibition was less than 50% (24% for MCP-1, 37% for IL-6, 41% for IL-8 in Figs. 6a-c). Addition of higher concentrations of GW856553X did not result in further inhibition (data not shown). A comparable modest inhibitory effect was seen when IL-1β was used as a stimulus (data not shown). Under these conditions, HUVEC viability, assessed using MTT, was unaffected (data not shown). VEGF, a key angiogenic stimulus *in vivo*, was used to promote HUVEC migration across an 8μm polycarbonate membrane *in vitro*. However, 100nM GW856553X did not affect the chemotactic response (Fig. 6d). In contrast, tubule formation by AngioKit (see *Materials and Methods*), as assessed by CD31 expression, was inhibited in a concentration-dependent fashion (Fig. 7).

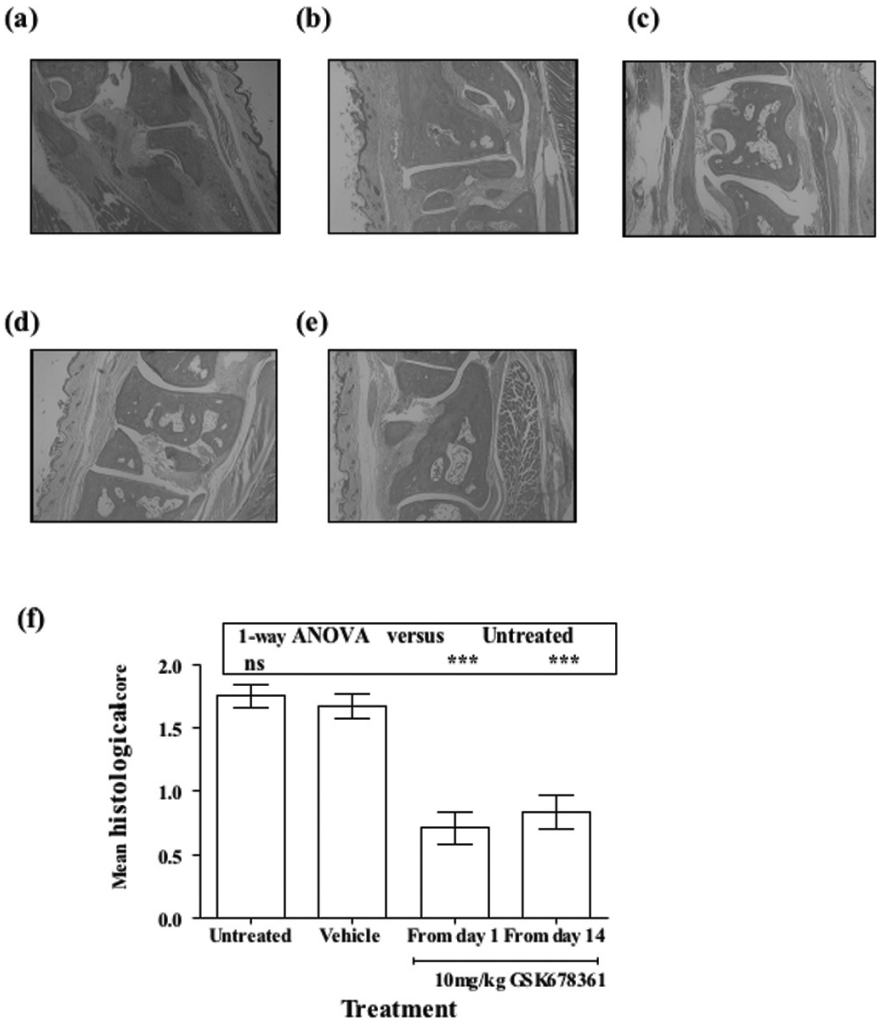

Fig. 5. p38 MAPK inhibition reverses joint destruction in chronic CIA.
To determine the effect on established disease, mice were treated with either GSK678361 10mg/kg daily from day 1 of arthritis, or with vehicle from day 1 followed by 10mg/kg daily GSK678361 from day 14 of arthritis (n=5-7 per group). At the end of the study (day 29), animals were sacrificed and paws were sectioned for histological evaluation. Haematoxylin and eosin sections were scored in a blinded fashion for pannus formation, synovitis, bone and cartilage erosion. Representative sections are shown as follows: (**a**) untreated, (**b**) vehicle-treated, (**c**) 10mg/kg GSK678361 from day 1 of arthritis (**d**) 10mg/kg GSK678361 from day 14 of arthritis. (**e**) Panel shows a paw section from a vehicle-treated animal sacrificed on day 14 of arthritis. (**f**) Paws were assigned one of three grades: 0=normal; 1=moderate synovitis with some loss of cartilage; 2=extensive synovial hyperplasia, destruction of cartilage and some bone erosions. Results are mean (± SEM) histological score (metatarsal, tibia-tarsus, proximal interphalangeal and distal interphalangeal joints). Statistical analysis was carried out by 1-way ANOVA *versus* vehicle-treated mice: $^{***}p<0.001$.

Discussion

The characterisation of p38 MAPK as a key player in inflammation more than 10 years ago has led to the development of several p38 MAPK inhibitors for the treatment of inflammatory autoimmune diseases. Several synthetic p38 MAPK inhibitors have demonstrated protective anti-inflammatory effects in animal models of arthritis, such as CIA in mice or adjuvant-induced arthritis in Lewis rats (17-22). However, none of these molecules has yet successfully passed early clinical trials for the treatment of human autoimmune diseases such as RA, because of safety concerns related to possible cross-reactivities with other kinases. These compounds can bind to both the active and inactive forms of p38, providing an advantage over ATP and resulting in a very potent inhibitory capacity, regardless of high intracellular ATP concentrations (23). Two different p38 MAPK inhibitors, BIRB-796 and RWJ-67657, have demonstrated clinical efficacy in a human endotoxin challenge model in which inhibition of p38 MAPK was shown to decrease LPS-induced cytokine and C-reactive protein production *in vivo* and to reduce LPS-induced clinical symptoms, for example those of sepsis (such as increased heart rate, decreased blood pressure, fever, and headache) (24, 25).

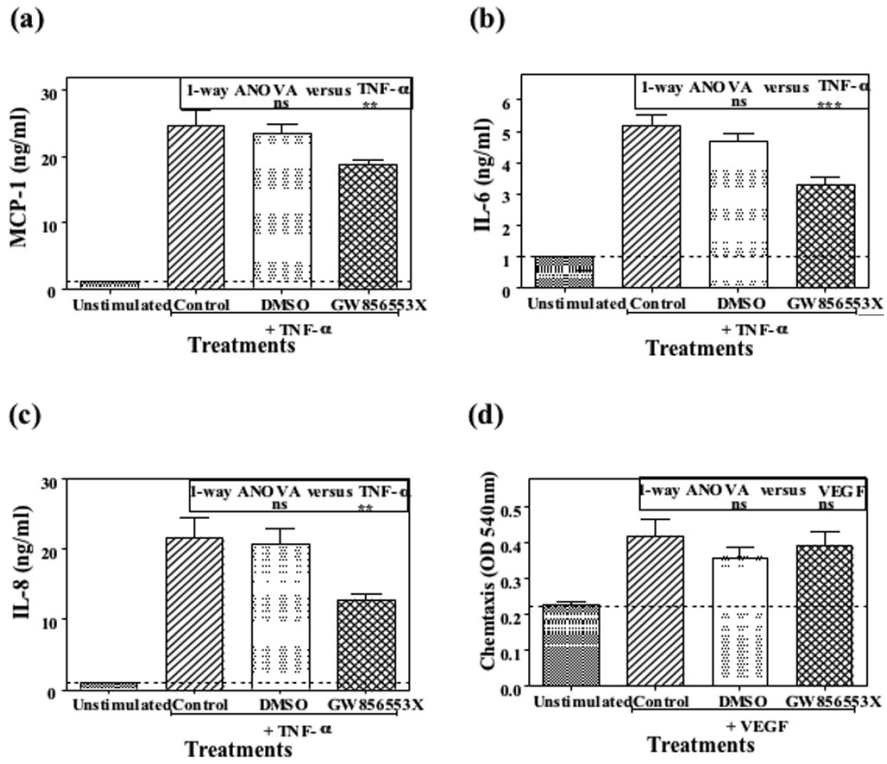

Fig. 6. Differential effects of GW856553X on endothelial inflammatory responses.
To assess release of cytokines and chemokines, HUVEC were stimulated for 24 hours with 10ng/ml TNF-α in the absence or presence of 100nM GW856553X (or DMSO as vehicle). Release of (**a**) MCP-1, (**b**) IL-6 and (**c**) IL-8 was measured by ELISA. Dashed line shows release from unstimulated cells. (d) Endothelial migration was assessed using 8.0μm gelatinised polycarbonate membranes. HUVEC were allowed to migrate for 6 hours in response to 10ng/ml VEGF (or medium alone as control). GW856553X (100nM) was added to the upper and lower chambers 30 minutes prior to addition of VEGF (using DMSO as vehicle). All data are means ± SD, from a single experiment, representative of 3 similar experiments, and were analysed by 1-way ANOVA *versus* HUVEC in the presence of stimulus alone: $^{**}p<0.01$, $p<0.001$.

To date, it remains to be defined which stimulus induces TNF expression in autoimmune inflammation and whether p38 MAPK controls TNF production induced by this stimulus.

New p38 MAPK inhibitors that are unable to cross the blood-brain barrier are now in clinical trials in RA and may delineate more precisely the role of p38 MAPK in Th1-driven chronic inflammatory diseases. These new molecules have helped to clarify the role of p38 MAPK *in vitro* and to define the mechanisms by which p38 MAPK controls, for example, LPS-induced cytokine expression in macrophages (26). The current study has an advantage over these previous reports in that these inhibitors can easily cross the blood-brain barrier towards the CNS (5). However, in view of recent advances underlining the essential role of p38 MAPK in IL-10 expression and in Th2 cell function, and of the regulatory capacities of IL-10 and Th2 cells in Th1-driven inflammation, p38 MAPK inhibitors might be associated with some unwanted effects on the immune system, enhancing rather than ameliorating the underlying inflammatory response in Th1-driven diseases. In contrast to Th1-mediated autoimmune disorders, allergic disorders are mediated by Th2 cells through the production of IL-4, IL-5, and IL-13. Indeed, it has been shown that in the ovalbumin-induced airway inflammation model, eosinophilia was decreased by inhibition of p38 MAPK in mice (27) and guinea-pigs (28, 29). However, its precise function in chronic inflammatory processes, such as those mediating autoimmune diseases, including RA, remains unclear. The specificity of the 4 different isoforms of p38 MAPK to the substrate, namely transcription factors and protein kinases (30), is provided by a docking motif which is usually composed of 3 domains: the basic region, the LXL motif, and the hydrophobic region with the latter of particular importance for the determination of the p38 MAPK substrate specificity (31). The development of models to predict p38 MAPK docking-domain specificities may permit the design of inhibitory peptides to block the phosphorylation of specific subsets of substrates so as to block specific pathways mediated by p38 MAPK.

In our study in order to assess the effects of p38 MAPK inhibition, we examined the effect of once daily dosing of GW856553X and demonstrated that this schedule is sufficient to provide efficacy in the acute CIA model. Furthermore, both GW856553X and the related compound GSK678361 showed good efficacy in the chronic model of CIA which is considered to more closely reflect RA. GW856553X is a novel compound first tested in chronic joint inflammation in arthritis models, while GSK678361 is a newly profiled benchmark compound, both exhibit p38 MAPK inhibition with higher selectivity to other kinases, proteins or receptors. Importantly, in the chronic CIA model, therapeutic dosing with GSK678361 14 days after the onset of arthritis was adequate to reduce clinical signs of arthritis until the end of the study. The observation that reductions in disease achieved in response to therapeutic dosing were comparable to the levels seen in the group which received the treatment at the onset was supported by the histopathological findings. Mechanism of action studies demonstrated that GW856553X inhibited human endothelial cell angiogenesis *in vitro*, and reduced pro-inflammatory cytokine production (IL-6, IL-8 and MCP-1). More importantly, in our animal studies in both models, acute and chronic CIA, mice had well tolerated the administration of both compounds, and their efficacy in the selected dosing regime was not compromised by any observed clinical side-effects as the mouse weight was unaffected (data not shown). Compounds of similar chemical class to GW856553X and GSK678361 have been used in human clinical trials with a low incidence of side effects or toxicity

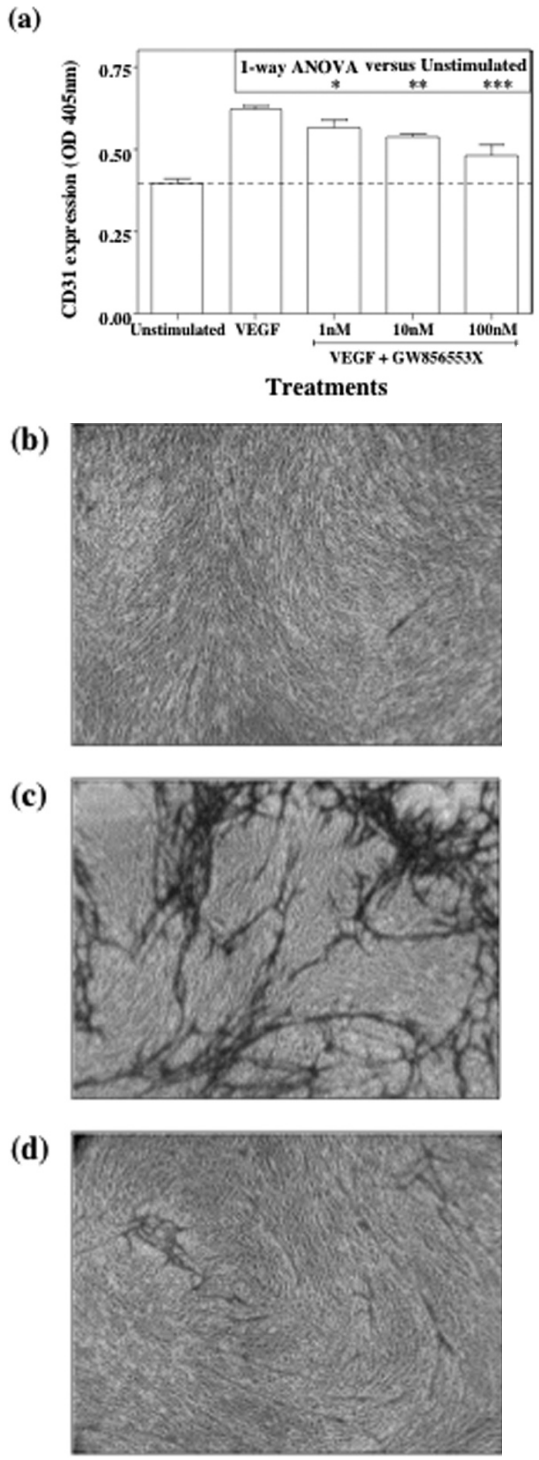

Fig. 7. GW856553X inhibits angiogenesis induced by VEGF. Angiogenesis in response to VEGF (1ng/ml) was assessed after 11 days. (**a**) CD31 expression was measured using alkaline phosphatase-mediated conversion of p-NPP followed by colorimetric assay. Data are means ± SD, representative of 3 similar experiments, and were analysed by 1-way ANOVA *versus* response in the absence of inhibitor: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Dashed line shows release from unstimulated cells. **(b-d)** Representative images showing morphology of the formed tubes stained for CD31 at day 11, following stimulation with (**b**) medium alone, (**c**) VEGF or (**d**) VEGF plus 100nM GW856553X (objective magnification ×40).

(32). The invention of these p38 MAP kinase compounds provides methods for treating or reducing symptoms in human or animals suffering from cytokine-driven inflammatory diseases such as rheumatoid arthritis, and many other inflammatory or putative p38 dependent diseases (5). Combination therapies are also considered, in order to adapt treatment modalities to the humans and enhance patient's benefit but also to reduce adverse effects. Lastly and not least the amounts of the inhibitors, the timing(s) of administration, the form of the compound, the age and the medical condition under treatment should be important factors to assess their benefit in the clinic. Potential limitations for the use of these non-steroid anti-inflammatory drugs is to prevent or treat the aforementioned clinical conditions are their inhibition activity of Cox enzymes, which can lead to unwanted side effects, and their limitation in CNS penetration (33). Third generation of p38 MAPK inhibitors that are currently in clinical trials will eventually permit a better characterisation of the role of p38 MAPK in humans by virtue of improved kinase selectivity profiles.

The inhibition of murine chronic relapsing arthritis by p38 MAPK targeted compounds, GW856553X and GSK678361, as demonstrated in the present study indicates that p38 MAPK inhibition as a therapeutic target for RA may have therapeutic potential for future use in RA if safe clinical dosing achieves adequate compound exposure.

Acknowledgements

We are grateful to the staff of the Biological Services Unit (Kennedy Institute of Rheumatology, Imperial College London, UK) for their help in the care and maintenance of laboratory mice used in our studies; and to the Histopathology Department, Charing Cross Hospital, London, for the sectioning and staining of paw specimens. The Kennedy Institute of Rheumatology is supported by the Arthritis Research Campaign of Great Britain (arc). The authors also thank Richard Williams, Mino Medghalchi, Ian Buysschaert and Nasser Malik for their assistance in the *in vivo* studies.

References

1. FAROOQ A, ZHOU MM: Structure and regulation of MAPK phosphatases. *Cell Signal* 2004, 16: 769-79.
2. KAMINSKA B: MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits. *Biochim Biophys Acta* 2005, 1754: 253-62.
3. BAINBRIDGE J, MADDEN L, ESSEX D, BINKS M, MALHOTRA R, PALEOLOG EM: Methionine aminopeptidase-2 blockade reduces chronic collagen-induced arthritis: potential role for angiogenesis inhibition. *Arthritis Res Ther* 2007, 9: R127.
4. MALFAIT AM, WILLIAMS RO, MALIK AS, MAINI RN, FELDMANN M: Chronic relapsing homologous collagen-induced arthritis in DBA/1 mice as a model for testing disease-modifying and remission-inducing therapies. *Arthritis Rheum* 2001, 44: 1215-24.
5. ASTON NM, BAMBOROUGH P, BUCKTON JB *et al.*: p38alpha mitogen-activated protein

kinase inhibitors: optimization of a series of biphenylamides to give a molecule suitable for clinical progression. *J Med Chem* 2009; 52: 6257-69.
6. MILLER EJ: Structural studies on cartilage collagen employing limited cleavage and solubilization with pepsin. *Biochemistry* 1972, 11: 4903-9.
7. WILLIAMS RO, FELDMANN M, MAINI RN: Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc Natl Acad Sci USA* 1992, 89: 9784-8.
8. WILLIAMS RO, MASON LJ, FELDMANN M, MAINI RN: Synergy between anti-CD4 and anti-tumor necrosis factor in the amelioration of established collagen-induced arthritis. *Proc Natl Acad Sci USA* 1994, 91: 2762-6.
9. WILLIAMS RO, GHRAYEB J, FELDMANN M, MAINI RN: Successful therapy of collagen-induced arthritis with TNF receptor-IgG fusion protein and combination with anti-CD4. *Immunology* 1995, 84: 433-9.
10. MIOTLA J, MACIEWICZ R, KENDREW J, FELDMANN M, PALEOLOG E: Treatment with soluble VEGF receptor reduces disease severity in murine collagen-induced arthritis. *Lab Invest* 2000, 80: 1195-205.
11. WILLIAMS RO, MARINOVA-MUTAFCHIEVA L, FELDMANN M, MAINI RN: Evaluation of TNF-alpha and IL-1 blockade in collagen-induced arthritis and comparison with combined anti-TNF-alpha/anti-CD4 therapy. *J Immunol* 2000, 165: 7240-5.
12. AFUWAPE A, FELDMANN M, PALEOLOG E: Adenoviral delivery of soluble VEGF receptor 1 (sFlt-1) abrogates disease activity in murine collagen-induced arthritis. *Gene Therapy* 2003, 10: 1950-60.
13. SUMARIWALLA P, CAO Y, WU H, FELDMANN M, PALEOLOG E: The angiogenesis inhibitor protease-activated kringles 1-5 reduces the severity of murine collagen-induced arthritis. *Arthritis Res Ther* 2003, 5:R32-R39.
14. SUMARIWALLA PF, JIN P, ZHANG J *et al*.: Antagonism of the human epidermal growth factor receptor family controls disease severity in murine collagen-induced arthritis. *Arthritis Rheum* 2008, 58: 3071-80.
15. HOLMDAHL R, JANSSON L, LARSSON E, RUBIN K, KLARESKOG L: Homologous type II collagen induces chronic and progressive arthritis in mice. *Arthritis Rheum* 1986, 29:106-13.
16. GROSJEAN J, KIRIAKIDIS S, REILLY K, FELDMANN M, PALEOLOG E: Vascular endothelial growth factor signalling in endothelial cell survival: A role for NFkappaB. *Biochem Biophys Res Commun* 2006, 340: 984-94.
17. BADGER AM, BRADBEER JN, VOTTA B, LEE JC, ADAMS JL, GRISWOLD DE: Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. *J Pharmacol Exp Ther* 1996, 279: 1453-61.
18. BARONE FC, IRVING EA, RAY AM *et al*.: SB 239063, a second-generation p38 mitogen-activated protein kinase inhibitor, reduces brain injury and neurological deficits in cerebral focal ischemia. *J Pharmacol Exp Ther* 2001, 296: 312-21.
19. DE DIOS A, SHIH C, LOPEZ DE URALDE B *et al*.: Design of potent and selective 2-aminobenzimidazole-based p38alpha MAP kinase inhibitors with excellent *in vivo* efficacy. *J Med Chem* 2005, 48: 2270-3.
20. DUMAS J, HATOUM-MOKDAD H, SIBLEY RN *et al*.: Synthesis and pharmacological characterization of a potent, orally active p38 kinase inhibitor. *Bioorg Med Chem Lett* 2002, 12: 1559-62.
21. REVESZ L, BLUM E, DI PADOVA FE *et al*.: Novel p38 inhibitors with potent oral efficacy in several models of rheumatoid arthritis. *Bioorg Med Chem Lett* 2004, 14: 3595-9.
22. WADA Y, NAKAJIMA-YAMADA T, YAMADA K *et al*.: R-130823, a novel inhibitor of p38 MAPK, ameliorates hyperalgesia and swelling in arthritis models. *Eur J Pharmacol* 2005, 506: 285-95.
23. FRANTZ B, KLATT T, PANG M *et al*.: The activation state of p38 mitogen-activated protein kinase determines the efficiency of ATP competition for pyridinylimidazole inhibitor binding. *Biochemistry* 1998; 37: 13846-53.
24. BRANGER J, VAN DEN BLINK B, WEIJER S *et al*.: Anti-inflammatory effects of a p38 mitogen-activated protein kinase inhibitor during human endotoxemia. *J Immunol* 2002, 168: 4070-7.
25. FIJEN JW, ZIJLSTRA JG, DE BOER P *et al*.: Suppression of the clinical and cytokine hresponse to endotoxin by RWJ-67657, a p38 mitogen-activated protein-kinase inhibitor, in healthy human volunteers. *Clin Exp Immunol* 2001, 124: 16-20.
26. BIRRELL MA, WONG S, MCCLUSKIE K *et al*.: Second-generation inhibitors demonstrate the involvement of p38 mitogen-activated protein kinase in post-transcriptional modulation of inflammatory mediator production in human and rodent airways. *J Pharmacol Exp Ther* 2006, 316: 1318-27.
27. DUAN W, CHAN JH, MCKAY K *et al*.: Inhaled p38alpha mitogen-activated protein kinase antisense oligonucleotide attenuates asthma in mice. *Am J Respir Crit Care Med* 2005, 171: 571-8.
28. TRIFILIEFF A, KELLER TH, PRESS NJ *et al*.: CGH2466, a combined adenosine receptor antagonist, p38 mitogen-activated protein kinase and phosphodiesterase type 4 inhibitor with potent *in vitro* and *in vivo* anti-inflammatory activities. *Br J Pharmacol* 2005, 144: 1002-10.
29. UNDERWOOD DC, OSBORN RR, KOTZER CJ *et al*.: SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. *J Pharmacol Exp Ther* 2000, 293: 281-8.
30. DODELLER F, SCHULZE-KOOPS H: The p38 mitogen-activated protein kinase signaling cascade in CD4 T cells. *Arthritis Res Ther* 2006, 8: 205.
31. BARSYTE-LOVEJOY D, GALANIS A, SHARROCKS AD: Specificity determinants in MAPK signaling to transcription factors. *J Biol Chem* 2002, 277: 9896-903.
32. GALE EA, BINGLEY PJ, EMMETT CL, COLLIER T: European Nicotinamide Diabetes Intervention Trial (ENDIT): a randomised controlled trial of intervention before the onset of type 1 diabetes. *Lancet* 2004, 363: 925-31.
33. PERETTO I, RADAELLI S, PARINI C *et al*.: Synthesis and biological activity of flurbiprofen analogues as selective inhibitors of beta-amyloid(1)(-)(42) secretion. *J Med Chem* 2005, 48: 5705-20.
34. LEE MR, DOMINGUEZ C: MAP kinase p38 inhibitors: clinical results and an intimate look at their interactions with p38alpha protein. *Curr Med Chem* 2005, 12: 2979-94.