REPORT

Mitogen activated protein kinase inhibitors: where are we now and where are we going?

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Ann Rheum Dis 2006;**65**(Suppl III):iii83–iii88. doi: 10.1136/ard.2006.058388

Orally bioavailable compounds that target key intracellular signalling molecules are receiving increasing attention for the treatment of rheumatic diseases. The mitogen activated protein (MAP) kinases are especially attractive because they regulate both cytokine production and cytokine action. The MAP kinases are expressed and activated in rheumatoid arthritis (RA) synovium. Preclinical studies using MAP kinase inhibitors are very effective in animal models of arthritis, supporting their potential utility in human disease. Although the available data suggest a rationale for MAP kinase blockade, development of drugs has been hampered by toxicity and limited efficacy. Alternative strategies, such as targeting other kinases in the cascade or development of allosteric inhibitors have been proposed. These approaches might permit effective use of MAP kinase inhibitors for the treatment of rheumatic and immune-mediated diseases.

The introduction of biological therapy for rheumatoid arthritis (RA) and other inflammatory diseases represents a major therapeutic advance. These proteins typically bind to cytokines (for example, etanercept), lineage specific cell surface receptors (for example, rituximab), or proteins that modulate costimulatory signals (for example, abatacept). Although quite effective, biological agents have some disadvantages:

- the drugs can only target a single cytokine or cellular receptor, which in a complex disease such as RA might limit the number of patients who respond
- the agents must be administered by injection
- the drugs are very expensive compared with traditional pharmaceutical agents.

As a result, orally bioavailable small molecules that target key intracellular signalling molecules have garnered increasing attention because they can regulate both cytokine production and cytokine action. The myriad of potential targets is daunting, but kinase inhibitors represent an especially attractive approach. Of these, the mitogen activated protein (MAP) kinases have been the focus of many drug development programmes due to their prominent role in the regulation of cytokines, chemokines, degradative enzymes, programmed cell death, and cell proliferation.

MAP KINASE PATHWAYS

Kinases are enzymes that phosphorylate serine, threonine, or tyrosine residues on regulatory and structural proteins in order to modulate their structure, function, or metabolism. The MAP kinases are a family of enzymes that participate in many cellular activities and are divided into three subfamilies (fig 1). The extracellular signal related kinases (ERKs) 1 and 2 are widely expressed and typically regulate cellular

proliferation and differentiation. p38 MAP kinase has four isoforms (α , β , γ , δ) and plays an especially important role in the production of cytokines such as interleukin (IL)-1, tumour necrosis factor α (TNF α), and IL-6. Three genes encode for the third MAP kinase, c-Jun N-terminal to produce c-Jun-N-terminal kinase (JNK)1, JNK2, or JNK3. Each of the JNK isoforms can be alternatively spliced to produce numerous distinct JNK proteins that play a major role in extracellular matrix regulation through the production of metalloproteinases.¹

The MAP kinases are tightly associated with other proteins in signalling complexes which include scaffold proteins and upstream kinases that carefully regulate their function. Like other cascades, this structure serves as an amplifying mechanism to enhance signal intensity. MAP kinase families include at least three tiers, beginning with the MAP kinase kinase kinases (MAP3Ks), which can be activated by small GTPases such as Rho and Rac. The MAP3Ks subsequently activate the MAP kinase kinases (MKKs), which in turn, phosphorylate individual MAP kinases. In addition to amplifying the signal, the upstream kinases also serve as a mechanism to integrate extracellular stimuli and orchestrate the correct balance of ERK, JNK, and p38 phosphorylation.

Figure 1 The mitogen activated protein (MAP) kinase signalling pathways. Complex parallel and crossover signalling cascades link the three main MAP kinase families, extracellular regulating kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38. The top level shows the MAP kinase kinase kinases (MAP3Ks), the second tier shows the MAP kinase kinases (MKKs), and the third tier consists of the MAP kinases that regulate various genes through transcriptional and post-transcriptional mechanisms. ASK, apoptosis signal regulating kinase; ATF, activating transcription factor; IL, interleukin; MAPKAF, MAPK activated protein; MEKK, MKK kinase; TAK, TGF β associated kinase; TNF, tumour necrosis factor.

Abbreviations: ACR, American College of Rheumatology; ERK, extracellular signal related kinase; IL, interleukin; JBD, JNK binding domain; JIP, JNK interacting protein; JNK, c-Jun-N-terminal kinase; LPS, lipopolysaccharide; MAP, mitogen activated protein; MAP3K, MAP kinase kinase kinase; MKK, MAP kinase kinase; MMP, matrix metalloproteinase; RA, rheumatoid arthritis; TLR, toll-like receptor; TNF, tumour necrosis factor

Once the MAP kinases are engaged, they can phosphorylate a variety of transcription factors and other proteins that regulate gene transcription, mRNA stability, and gene translation.

The MAP kinase cascade is activated when cells detect environmental stress, such as cytokines, toll-like receptor (TLR) ligands, ultraviolet irradiation, growth factors, adhesion molecule ligation, and reactive oxygen. Ligation of certain cytokine receptors and growth factor receptors leads to ERK phosphorylation, whereas lipopolysaccharide (LPS), proinflammatory cytokines, and osmotic shock activate p38. Ultraviolet light, protein synthesis inhibitors, and cytokines such as IL-1 and TNF α stimulate the JNK pathway. The individual MAP3Ks are then matched with specific MKK-MAPK complexes, which integrate the responses to extracellular stimuli by activating the correct proportion of ERK, JNK, and p38. There is considerable flexibility and overlap, however, and many extracellular signals activate more than one MAP kinase.

MAP KINASE EXPRESSION AND REGULATION IN RA SYNOVIUM

MAP kinases contribute to inflammatory and destructive mechanisms in rheumatoid synovium. All three families are expressed in synovial tissue and are present in their active phosphorylated forms.^{2,3} In each case, the level of MAP kinase phosphorylation is significantly higher in joint samples from RA compared with osteoarthritis. p38 is widely distributed in the synovium, and the phosphorylated form is mainly found in the intimal lining where most of the cytokines and proteases are produced. Additional phospho-p38 can be found in sub-lining blood vessels. Phospho-p38 α is the main isoform expressed in macrophages and fibroblast-like synoviocytes, but p38 δ is expressed at sites of invasion into the extracellular matrix.⁴ Phosphorylated MKK3 and MKK6, the two major upstream regulators of p38, are also localised to the intimal lining and, to a lesser extent, in perivascular lymphoid aggregates.⁵ Phospho-ERK has been detected in synovial blood vessels.

JNK activation in rheumatoid synovium was first demonstrated by western blot analysis, which confirmed that both JNK1 and JNK2 are phosphorylated in RA but not osteoarthritis synovium.³ JNK3 is primarily expressed in neurological tissue and is not present in the rheumatoid joint. The major substrate of JNK is c-Jun, which, along with activator protein (AP)-1 and matrix metalloproteinases (MMPs), is mainly localised to the synovial intimal lining. Immunostaining has also localised a significant amount of the phospho-JNK to the sub-lining. MKK4 and MKK7, which are the upstream activators of JNK, are also expressed in rheumatoid synovium.⁵ Both are highly activated in RA as determined by western blot analysis and immunohistochemistry.

ERK and p38 regulate many of the genes that participate in synovial inflammation, including TNF α , IL-1, and MMPs. The mechanism by which p38 induces expression of these genes involves a combination of increased transcription, mRNA stability, and translation, depending on the specific cell type and method of stimulation. JNK also modulates many of the same genes, although it is primarily through transcriptional events mediated by phosphorylation of c-Jun in the transcription factor AP-1.

MAP KINASES IN ANIMAL MODELS OF ARTHRITIS

Preclinical studies using MAP kinase inhibitors provide considerable support for their use in diseases such as RA. p38 inhibitors have been tested in a variety of arthritis models, including adjuvant arthritis and collagen induced arthritis.^{6,7} MAP kinase blockade decreases synovial

inflammation, bone destruction, and cartilage destruction compared with controls in most models of inflammatory arthritis. Inhibition of bone erosions appears to result from suppressed osteoclast differentiation and activation and decreased expression receptor activator of nuclear factor κ B ligand (RANKL).⁸ Blocking upstream regulators of p38 is also effective in preclinical models. MKK3 deficiency markedly suppresses joint swelling, synovial p38 activation, and articular cytokine production in the passive K/BxN model⁹ (fig 2). The defect is reversed by administration of exogenous IL-1 early in the model. Of interest, IL-6 production in MKK3^{-/-} mice is normal in animals that have been injected with endotoxin, indicating that some aspects of innate immunity remain intact despite the defect in synovial cytokine gene expression.

The ability of JNK to regulate matrix degradation and cytokine production makes it an attractive target for diseases with significant damage to the extracellular matrix. However, the functions of JNK extend beyond MMP and cytokine expression. For example, studies in JNK1^{-/-} and JNK2^{-/-} mice indicate that this MAP kinase also regulates T cell differentiation into the T helper (Th) 1 subset.¹⁰ Because Th 1 cells are thought to play an important role in RA, targeting JNK could modify adaptive immune responses and suppress synovial lymphokine production in addition to blocking metalloproteinase production by synoviocytes.

To evaluate the role of JNK in arthritis, the selective JNK inhibitor SP600125 was tested in the rat adjuvant model.¹¹⁻¹³ The compound is a reversible ATP-competitive inhibitor that blocks all three JNK isoforms. The adjuvant arthritis model is induced by immunisation with complete Freund's adjuvant and results in T cell dependent, severe polyarticular, destructive arthritis. Administration of SP600125 decreased paw swelling, but the effect was relatively modest. In contrast, animals treated with SP600125 demonstrated a dramatic decrease in bone and cartilage damage as determined by radiographic analysis. The effect was more likely due to suppression of effector mechanisms, like synoviocyte MMP production, rather than the initial immune response because the drug was administered a week after the initial immunisation. Evaluation of joint extracts from animals treated with SP600125 supported this finding because the JNK inhibitor significantly decreased AP-1 binding and MMP

Figure 2 MAP kinase kinase (MKK)3 deficiency protects from passive K/BxN arthritis. Wild-type (WT) and MKK3^{-/-} mice were injected with K/BxN serum and evaluated for arthritis. Deletion of the MKK3 gene markedly suppressed synovial p38 activation and inflammatory arthritis. These data suggest that upstream kinases can potentially be targeted rather than the MAP kinases. Used with permission from Inoue T. *Proc Natl Acad Sci USA* 2006;103:5484-9.⁹

expression. In vitro kinase assays also showed that JNK activity was suppressed in the synovium.

Although SP600125 inhibits all three isoforms of JNK, it is possible that an isoform selective inhibitor could have the same benefit with decreased the risk of toxicity. This question has been addressed by evaluating animal models of arthritis in JNK1 and JNK2 knockout mice. Because JNK2 is the major isoform expressed by synoviocytes, initial studies were carried out in JNK2^{-/-} animals using passive collagen arthritis.¹⁴ The passive transfer model was used because it is independent of T cells and involves mainly the effector phase of arthritis. Although a modest degree of joint protection was observed in JNK2^{-/-} mice, the benefit was much less than observed in the adjuvant arthritis model using a JNK inhibitor. JNK2 deficiency had no effect on clinical arthritis or articular expression of AP-1 and MMP13.

The protective effect of JNK1 deficiency has also been examined in TNF α transgenic mice. JNK1^{-/-} mice were backcrossed with human TNF transgenic mice and the clinical course was evaluated. No differences in synovial inflammation, bone erosion, cartilage damage, or cellular infiltrate of the synovium were noted in the JNK1^{-/-} hTNFtg compared with controls.¹⁵ Evaluation of JNK signalling demonstrated decreased phosphorylation of JNK in the JNK1^{-/-} hTNFtg mice. However, phospho-c-Jun levels in the synovial membrane were similar in both groups. These data suggest that JNK2 can compensate for the deficiency of JNK1 in this model. Therefore, a JNK inhibitor probably needs to target both JNK1 and JNK2.

An orally bioavailable JNK inhibitor AS601245 has also been tested in preclinical models.¹⁶ This compound resulted in a dose dependent decrease in TNF α release in a model of murine endotoxin shock. AS601245 was also effective in collagen induced arthritis, decreasing paw swelling and clinical arthritis scores. Histological analysis revealed decreased cartilage erosion and synovial inflammation. Unlike SP600125, this optimised compound demonstrated potent anti-inflammatory and matrix protecting effects. Selectivity tests against a large panel of kinases suggested that the compound has little or no effect on closely related kinases, indicating the in vivo effects are likely due to inhibition of the JNK.

Peptide based approaches that can target or disrupt JNK signalling complexes have also been reported. The JNK pathway is distinct from other MAP kinases because it uses the JNK interacting protein (JIP) family scaffold proteins.^{17,18} Overexpression of full length JIP1 or specific fragments of JIP, such as the JNK binding domain (JBD), inhibit JNK activity in a variety of cell types. Purified JBD protein (JIP1 127–202) inhibits JNK. Purified JBD protein activity in an in vitro kinase assay, and residues 144–163 of JIP1 JBD are essential for interaction with JNK.¹⁹ The sequence was resolved to an 11 amino acid peptide in the JBD region of JIP1 that binds JNK and inhibits kinase activity. The short JIP1 JBD derived peptides are quite selective and appear to inhibit only JNK and its upstream activators, MKK4 and MKK7.²⁰ Biochemical analysis showed that JIP1 derived peptide acts as a competitive inhibitor of the kinase interaction motif of c-Jun substrate.²¹ In vivo studies are limited to gene transfer of the JIP1 JBD derived peptides protecting neurones in a mouse model of Parkinson's disease.²²

MAP KINASES IN OTHER AUTOIMMUNE DISEASES

MAP kinases have been implicated in many other inflammatory and immune mediated diseases. For instance, JNK and ERK expression is increased in the nuclei of psoriatic epidermal cells, suggesting that these MAP kinases might participate in the inflammatory skin lesions. Western blot analysis has also shown phosphorylated JNK and ERK in the

involved psoriatic regions.²³ These studies suggest that JNK and ERK activation contributes to dermal hyperproliferation and abnormal differentiation. However, other studies suggest that combined ERK and p38 activation is more important in psoriasis than the JNK/ERK combination.²⁴ However, the role of MAP kinases in skin inflammation might be less likely in light of the novel animal studies demonstrating that inducible deletion of JunB/AP-1 activation in epidermal keratinocytes causes psoriatic-like skin lesions.²⁵ p38 has also been implicated in animal models of other diseases, including inflammatory bowel disease and allergic airway disease.^{26,27}

JNK activation has been demonstrated in systemic lupus erythematosus (SLE), psoriasis, asthma, and inflammatory bowel disease. For instance, B cells from the peripheral blood of SLE patients contained higher levels of phosphorylated JNK, ERK, and p38 compared with normal individuals as determined by flow cytometry.²⁸ JNK blockade with SP600125 decreases inflammatory cell migration into the airway in murine allergen induced asthma.²⁹ SP600125 was also used to evaluate the role of JNK in a second murine model of airway remodelling.³⁰ The inhibitor decreased cell infiltration into the airways, suppressed eosinophilic inflammation in bronchial submucosa, and decreased bronchial responsiveness.

MAP KINASES AS THERAPEUTIC TARGETS

Although p38 inhibitors have been extensively evaluated in preclinical models of arthritis, information in humans is far less comprehensive. The results of clinical trials typically have been released in the form of abstracts or press releases, so the data have rarely been vetted by peer review and are not comprehensive. Some publications describing clinical trials are available and permit a more careful appraisal of the risks and benefits. For instance, the p38 α/β selective inhibitor RWJ-67657 has been evaluated in a human model involving injection with small quantities of LPS. The inhibitor significantly suppressed the fever response and partially blocked the increase in serum cytokines such as TNF α .³¹ (fig 3). A similar study of a second p38 inhibitor, BIRB 796, showed that phosphorylation of the p38 substrate activation transcription factor (ATF)-2 in peripheral blood cells was inhibited after LPS injection.³² Although the LPS data clearly demonstrate that p38 can regulate cytokine responses in humans, its relevance to RA or other rheumatic disease is uncertain. BIRB 796 was also tested in a phase 2 study in RA, although limited information is available.

The first clinical efficacy data in an RA trial involved VX-745, which was tested in a placebo controlled study involving 44 patients. Of the patients in the treatment arm, 43% achieved an American College of Rheumatology (ACR) 20 response compared with 17% for placebo, and the results were stated to be statistically significant. Although the differences were not great, the exposure to the drug was limited by hepatotoxicity. Other undefined gastrointestinal toxicity was also observed in a significant percentage of patients.

VX-702, another selective p38 inhibitor, was examined in a short-term study of acute coronary syndrome (unpublished data, press release). Although not reported in a peer review setting, p38 inhibition in this single dose study appeared to suppress C-reactive protein levels compared with placebo treated patients. The same compound was also evaluated in a phase 2 study in RA, known as the VeRA study. This was a prospective, placebo controlled study of 315 Eastern European patients, of whom 278 completed 12 weeks of treatment. Because hepatotoxicity has been observed with other p38 inhibitors, concomitant treatment with methotrexate was not allowed. Although the data are limited, VX-702 appeared to provide to a dose dependent, statistically

Figure 3 A p38 inhibitor (RWJ-67657) blocks fever after lipopolysaccharide (LPS) challenge in humans. Normal individuals received an injection of LPS and were observed for 24 hours. (A) The compound significantly decreased endotoxin induced fever in a dose dependent fashion. (B) Peak tumour necrosis factor α (TNF α) levels were measured in the serum by enzyme-linked immunosorbent assay (ELISA). The p38 inhibitors significantly suppressed cytokine production after LPS challenge. Adapted with permission, from Fijen *et al. Clin Exp Immunol* 2001;**124**:16–20.³¹

significant increase in ACR 20 responders. Overall the response was not robust, with a 44% ACR 20 response rate compared with 31% for placebo. The most common adverse events were rash, infection, and gastrointestinal intolerance. Information on hepatotoxicity is limited, but apparently there was not a significant increase of patients with threefold or greater increases in liver enzymes in the treatment group. The compound still needs to be evaluated in combination with methotrexate to determine the effect on liver function and whether this approach is more effective than use of the compound as a single agent.

A phase 1 safety study and a phase 2 efficacy study in RA have been completed using the p38 inhibitor SCIO-469. Whereas information on the efficacy in RA is limited, the company has reported a randomised controlled study involving 263 patients who had dental pain (unpublished data, press release). The rationale for this study relates to extensive preclinical data demonstrating that p38 in the spinal cord modulates nociception, especially reports that intrathecal administration of p38 inhibitors markedly suppresses acute and chronic pain.³³ SCIO-469 was compared with either placebo or ibuprofen in a prospective, placebo controlled, double blind study. The compound significantly extended the time needed for ibuprofen rescue (from 4.1 hour in the placebo group to 8.1 hours), suggesting that it possesses analgesic properties.

The major issues that have interfered with the development of p38 inhibitors generally relate to preclinical and clinical toxicity. Several compounds have demonstrated an unusual inflammatory condition of the central nervous system (CNS) in dogs, although this does not appear to occur in other species. As a result, new compounds with more limited CNS penetration have been proposed. One concern with this approach is that a decrease in CNS access for p38 inhibitors might eliminate the analgesic effects as well. In humans, hepatotoxicity has been a frequent dose limiting concern. It is not certain whether this is compound specific or mechanism based. For instance, 16% of patients with RA treated with the lowest dose of VX-745 had elevated liver enzymes (unpublished data, press release). Structurally distinct compounds have exhibited evidence of hepatotoxicity suggesting that this side effect might be target based. However, this is not certain, and it is still possible that the liver issues are unrelated to p38 inhibition.

JNK inhibitors also have significant therapeutic potential in a wide variety of diseases, including cancer, diabetes, and inflammatory disorders. The first JNK inhibitor, SP600125, had a number of issues related to specificity and pharmacokinetics. However, the elucidation of the crystal structure of JNK3 helped guide the synthesis of more suitable compounds.³⁴ For instance, Celgene (Summit NJ) has recently disclosed a second series of JNK inhibitors, such as CC-401 for which a phase 1, double blinded, placebo controlled, ascending single intravenous dose study in healthy human volunteers has been completed. Cephalon (Frazer PA) has also announced that the JNK inhibitor CEP-1347 will be tested in early Parkinson's disease.

A series of JNK compounds developed by Serono (Rockland MA) have been synthesised as inhibitors of JNK2 and JNK3 for the treatment of autoimmune and neurodegenerative disorders. These benzazoles are more potent inhibitors of JNK3 than JNK2. An interesting series of sulphonamide, sulphonyl amino acid, and sulphonyl hydrazide, which inhibits both JNK2 and JNK3, has also been disclosed by the same company. One of the initial sulphonamides was further screened for its structure–activity relation and allowed identification of the areas that impart potency to the kinase inhibiting motif. Based on these experiments, one JNK inhibitor of this class, AS600292, has been synthesised and profiled.³⁵ AS600292 protected against neuronal death by serum and growth factor starvation in vitro. Another compound, AS601245, has demonstrated efficacy in murine collagen induced arthritis.

MAP kinase inhibitors have also been evaluated in Crohn's disease. In a small open label study, the combined JNK and p38 inhibitor CNI-1493 demonstrated evidence of clinical benefit with more rapid healing of ulcers.³⁶ After initial promising results, larger studies were terminated because of lack of efficacy at doses that could be tolerated and infusion site reactions. BIRB 796 was also tested in a larger placebo controlled study in Crohn's disease involving 284 patients.³⁷ There was no significant improvement in the primary endpoints, which included the Crohn's disease activity index (CDAI) and endoscopy. C-reactive protein levels only transiently decreased in the drug treated group, which suggests that there could be mechanisms to escape p38 regulation or that there might be pharmacokinetic/drug penetration issues. One confounding aspect of the study

was the inclusion of a large percentage of patients from Russia, in whom placebo responses and other clinical characteristics appeared to differ from the Western European cohort.

ERK inhibitors for rheumatic disease have not progressed as far as the other two MAP kinase families. Most of the attention has focused on cancer due to the prominent role that ERK plays in the regulation of cell growth. Rather than inhibit ERK directly, most current efforts target the upstream kinases that regulate ERKs (MEK1 and MEK2). PD0325901 has been evaluated in a phase I study involving 41 patients with melanoma and several other types of cancer (press release). The compound decreased ERK phosphorylation in the tumours, and several partial remissions were observed. Toxicity related to skin rash and visual changes were observed with some frequency. These side effects were thought to be mechanism based, but may be managed by adjusting the dose or the dosing schedule for MEK inhibitors.

STRATEGIES FOR THE FUTURE DEVELOPMENT OF MAP KINASE INHIBITORS

Definitive proof-of-concept evidence for the utility of MAP kinase inhibitors in human inflammatory, neurodegenerative, or malignant diseases is still lacking. The studies to date are generally confounded by toxicity or lack of efficacy; in some cases, these two problems are related because side effects prevent investigators from achieving optimal drug exposure for a sufficient period of time. Nevertheless, data on p38 inhibitors to date are not inspiring. Greater selectivity for p38 over other kinases, increased specificity for the alpha isoform of p38, or the development of allosteric inhibitors rather than ATP competitors might improve the toxicity profile. Specificity issues can be particularly difficult when drugs are targeted at the ATP site of a kinase because there is considerable homology between different enzymes in the kinome. Hence, allosteric inhibitors that bind to other sites offer an attractive alternative.

Side effects may also be minimised by targeting a downstream kinase, such as MAPKAPK-2, instead of p38 itself, because this protein is probably responsible for the cytokine regulating properties of p38 in macrophages.³⁸ One could also target upstream kinases in order to suppress signalling responses that contribute to inflammatory arthritis but not host defence and/or homeostasis. The studies of MKKs suggest that inhibiting MKK3 might provide the benefit of p38 inhibition for cytokine mediated inflammation while sparing host defence and TLR responses. MKK7 rather than MKK4 regulates cytokine mediated JNK activation and represents another potential target.³⁹ Therefore, targeting an individual MKK might permit separation of pathogenic MAP kinase activation from some normal responses.

CONCLUSIONS

MAP kinases remain attractive targets for rheumatic diseases despite the setbacks. They regulate the cytokines and other mediators that are known to participate the pathogenic processes implicated in inflammation. The kinases are expressed at the sites of disease, and functional studies demonstrate that they are highly activated. Preclinical studies confirm remarkable efficacy when MAP kinases are inhibited. Hence, the rationale for MAP kinase blockade in humans is quite clear. Issues related to toxicity and limited efficacy have hampered drug development. A number of alternative strategies have been proposed to overcome some of these problems, including development of allosteric inhibitors or drugs that target other kinases in the cascade. Although the optimism of a few years ago must be tempered, there is still considerable hope that targeting this signal

transduction pathway will offer novel treatment to patients with inflammatory and immune mediated diseases.

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Competing interests: none declared

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