British Journal of Pharmacology (2008) 154, 153–164

0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

A potent and selective p38 inhibitor protects against bone damage in murine collagen-induced arthritis: a comparison with neutralization of mouse TNFα

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Background and purpose: The p38 kinase regulates the release of proinflammatory cytokines including tumour-necrosis factor-α (TNFα) and is regarded as a potential therapeutic target in rheumatoid arthritis (RA). Using the novel p38 inhibitor Org 48762-0, we investigated the therapeutic potential of p38 inhibition and compared this to anti-mouse (m)TNFα antibody treatment in murine collagen-induced arthritis (CIA).
Experimental approach: Pharmacological profiles of Org 48762-0 were characterized in kinase assays, cellular assays and in lipopolysaccharide (LPS)-induced inflammation in mice. The effects of Org 48762-0 and of mTNFα-neutralization on established arthritis were examined in murine CIA.
Key results: Org 48762-0 potently inhibited p38α kinase with a high degree of kinase selectivity. In cellular assays, Org 48762-0 reduced LPS-induced TNFα release. Oral administration of Org 48762-0 in mice showed drug-like pharmacokinetic properties and inhibited LPS-induced cytokine production. These pharmacological characteristics of Org 48762-0 prompted a comparison of therapeutic efficacy with mTNFα-neutralization in CIA. Org 48762-0 and anti-mTNFα antibody treatment equally inhibited development of arthritis when evaluated macroscopically. Radiological analyses revealed protection against bone damage for both treatments, although statistical difference was reached with Org 48762-0 treatment only. Further, micro-computed tomographical and histopathological analyses confirmed the protective effects of Org 48762-0 on joint damage.
Conclusions and implications: Pharmacological targeting of p38 kinase provided good protection against joint tissue damage in CIA. In our experiments, neutralization of mTNFα produced less prominent suppression of bone damage. Our data suggest a therapeutic potential for selective and potent p38 inhibitors in RA.
British Journal of Pharmacology (2008) **154,** 153–164; doi:10.1038/bjp.2008.53; published online 25 February 2008

Keywords: p38 MAPK; Org 48762-0; anti-TNFα therapy; collagen-induced arthritis; inflammation; rheumatoid arthritis; proinflammatory cytokines; bone damage

Abbreviations: CIA, collagen-induced arthritis; IL, interleukin; IMAP, immobilized metal ion affinity-based fluorescence polarization; LPS, lipopolysaccharide; MK2, MAP kinase-activated protein kinase-2; RA, rheumatoid arthritis; TNF, tumour-necrosis factor

Introduction

At least three different mitogen-activated protein kinases, including p38, c-Jun N-terminal kinase and extracellular signal-regulated protein kinase, are expressed in eukaryotic cells. The p38 kinase is known to play an important role in inflammatory processes. Among its four isoforms (α, β, γ and δ), p38α kinase is believed to be an important regulator of inflammation (Kumar *et al.*, 2003; Schieven, 2005). Diverse environmental stressors and cytokines can activate p38 kinase, which results in phosphorylation and activation of downstream targets, such as MAP kinase-activated protein kinase-2 (MK2) (Almholt *et al.*, 2004), heat shock protein 27 (Guay *et al.*, 1997) and transcription factors, subsequently

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Received 12 November 2007; revised 17 January 2008; accepted 31 January 2008; published online 25 February 2008

leading to the production of proinflammatory cytokines. Activation of p38 kinase was also reported to enhance production of tumour-necrosis factor-α (TNFα) by stabilizing the TNFα mRNA (Mahtani *et al*., 2001; Deleault *et al*., 2008).

Rheumatoid arthritis (RA) is characterized by a chronic and progressive inflammatory process that leads to immunological abnormalities of the joints, ultimately resulting in functional loss. The disease can be therapeutically approached by neutralizing cytokines, such as TNFα, or by blocking the receptors for interleukin (IL)-1 or IL-6 (Jorgensen *et al*., 1999; Choy *et al*., 2002). In particular, the TNFα neutralization therapies are known to be efficacious in RA patients. In various inflammatory conditions, the p38 kinase is known to play important roles in regulating cellular signalling initiated via a variety of proinflammatory cytokines including TNFα, IL-1β and IL-6 as well as in modulating the release of such cytokines (Saklatvala *et al*., 1999; Branger *et al*., 2002; Guo *et al*., 2003). Furthermore, a recent report showed that, among p38 kinase isoforms in inflamed RA tissue, the α- and γ-isoforms are predominantly expressed (Korb *et al*., 2006). Taken together, these findings suggest a prominent role of p38 kinase in RA pathology.

Although cytokine-targeting biopharmaceuticals, including anti-TNFα treatments, are effective in many patients suffering from inflammatory diseases such as RA (Gaffo *et al*., 2006; Gartlehner *et al*., 2006; Smolen *et al*., 2007), they are costly and require parenteral administration. Therefore, there is a clear need for a lower cost, orally active and low molecular weight drug that would reduce production of, or cellular signalling by, TNFα and other proinflammatory cytokines. In the past few years, some p38 inhibitors have entered clinical trials and shown a strong interference with production of TNFα and other cytokines after lipopolysaccharide (LPS) stimulation during the phase I clinical studies in healthy subjects (Fijen *et al*., 2001; Branger *et al*., 2002). These results were supportive of the potential utility of p38 inhibitors for clinical application. However, conclusive clinical evidence for the benefit of such inhibitors for the treatment of RA patients is yet to be obtained.

The therapeutic efficacy of p38 inhibition in preclinical arthritis models has been demonstrated before (Mbalaviele *et al*., 2006; Medicherla *et al*., 2006). However, the question remains whether pharmacological targeting of p38 kinase shows any therapeutic advantages over TNFα neutralization therapy in RA, in such preclinical settings. To address this question in an unbiased manner, the pharmacological profile of a p38 inhibitor including kinase selectivity profiles and pharmacokinetic properties ought to be well characterized first. Here, we report a pharmacological profile of a structurally novel and highly selective p38 inhibitor Org 48762-0 that we discovered recently (Soliva *et al*., 2007) and its therapeutic potential in comparison with anti-mTNFα antibody treatment in murine collagen-induced arthritis (CIA).

Methods

Animals

All animal procedures and experiments received ethics approval and were according to the recognized guidelines. Male or female Balb/c mice (Charles River, Sulzfeld, Germany) and male DBA/1J/BOM mice (Bomholtgard, Ry, Denmark) were group-housed under controlled conditions with a constant temperature (19–21 °C), a 12-h light/dark cycle and *ad libitum* access to water and standard pelleted food.

Immobilized metal ion affinity-based fluorescence polarization assay

The immobilized metal ion affinity-based fluorescence polarization (IMAP) (Molecular Devices, Sunnyvale, CA, USA) technology (Loomans *et al*., 2003) was employed to determine p38α kinase activity. As previously described (Zaman *et al*., 2006), a 384-well assay was initiated by adding 38 nM (0.3 U ml^{-1}) activated p38α kinase (Upstate, Dundee, Scotland), 240 nM fluorescein-labeled peptide substrate (LVEPLTPSGEAPNQK-fluorescein) and 20 μM ATP in reaction buffer (pH 7.4). The reaction was stopped after 2 h incubation at room temperature by the addition of IMAP binding buffer (Molecular Devices). After incubation for 30 min at room temperature, fluorescence polarization was measured to determine kinase activity on an Analyst multimode fluorescence plate reader (Molecular Devices).

Kinase selectivity assays

The selectivity profiles of p38 inhibitors were determined against a panel of 50 human kinases (Figure 2). Experimentation was performed at Upstate according to Upstate's standard protocols (KinaseProfiler Assay Protocols, detailed protocols not described here) that employ purified human kinases, generic peptide substrates and a radioactive filter-binding assay format. Compounds were tested in duplicate at 10 μM in the presence of 100 μM ATP for each kinase assay.

LPS-induced TNFα production in cellular assay

To profile activity of p38 inhibitors in a functional cellular assay, healthy donors provided peripheral blood mononuclear cells (PBMC, Sanquin Blood Bank, the Netherlands). PBMC (7×10^5 cells per well (200 μl), 1 day old buffy coats) were plated in 96-well plates in DMEM F12 medium (Gibco BRL, Grand Island, NY, USA). Cells were pre-incubated with compounds (0.32 nM–10 μM in 0.1% dimethyl sulphoxide) for 30 min and subsequently stimulated with 1 μg ml^{-1} LPS (Sigma, St Louis, MO, USA, *Escherichia coli* serotype O111:B4). After 4 h incubation at 37 °C and 5% CO_2, supernatants were collected and assayed for TNFα by enzyme-linked immunosorbent assay (Biosource, Camarillo, CA, USA).

MK2 redistribution assay

Upon stress-induction (for example anisomycin), p38 kinase directly phosphorylates regulatory sites of MK2 resulting in activation and translocation of MK2 from nucleus to cytoplasm. Therefore, the inhibition of p38 kinase activity interferes with such MK2 translocation. Experimentation was performed at BioImage (Soeborg, Denmark) as

previously described (Almholt *et al*., 2004) using the cell line BHK-ps1362cl.15B-FS that overexpresses a green fluorescent protein–MK2 fusion protein. Briefly, the assay was initiated by adding 450 nM anisomycin (agonist of MK2 translocation) and p38 inhibitors in the concentration range of 3.16 nM ~ 31.6 μM. After 1 h, cells were fixed and stained with a nuclear counterstain to monitor the translocation of the green fluorescent protein–MK2 fusion protein from the nucleus to the cytoplasm. Assay response was quantified in an Amersham IN Cell 3000 Analyzer. The effect of the tested compounds was assessed as the percent activity relative to the SB203580 control.

Western blotting

Human monocytic THP-1 cells were obtained from American Type Culture Collection. The THP-1 cells (1×10^6 cells per ml) were pre-incubated with compounds (0.1 ~ 10 μM in 0.1% dimethyl sulphoxide) for 30 min before stimulation with 10 ng ml^{-1} IL-1β (R&D Systems, Minneapolis, MN, USA). After 30 min of stimulation, cells were washed twice in ice-cold phosphate-buffered saline (PBS) and then lysed for 30 min on ice using a cell lysis buffer (Biosource) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany), 1 mM PMSF (Fluka, Buchs, Switzerland) and phosphatase inhibitor cocktail I/II (Sigma). Proteins were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and phosphorylation status of p38 kinase (pTpY180/182, Biosource) and heat shock protein 27 (pS82; Biosource) was evaluated via western blotting using β-actin (Cell Signaling Technology, Danvers, MA, USA) as a loading control.

Pharmacokinetic study in mice

The pharmacokinetic characteristics of Org 48762-0 were profiled in male Balb/c mice. Org 48762-0 dissolved in a vehicle (0.5% dimethyl sulphoxide and 0.5% cremophore in 5% mannitol aqueous solution) was administered intravenously and orally at a dose of 1.6 and 4.0 mg kg^{-1}, respectively. At different time points, blood samples were isolated. The serum level of Org 48762-0 was determined via LC-MS/MS and pharmacokinetic parameters were calculated by WinNonlin (Pharsight, Cary, NC, USA).

Mouse model of LPS-induced endotoxaemia

Female Balb/c mice (7- to 8-week old) were intraperitoneally challenged with LPS (20 μg per mouse, Sigma, *E. coli* serotype 055:B5) dissolved in PBS to induce inflammatory responses. Animals were treated orally with different doses (Figure 5a) of compounds or intraperitoneally with anti-TNFα antibodies or rabbit IgG prepared in saline at a dose of 30 mg kg^{-1} 1 h before LPS challenge. Ninety minutes after LPS injection, blood samples were obtained from each animal. The serum TNFα level was quantified by enzyme-linked immunosorbent assay (BD Biosciences, San Diego, CA, USA) to assess the efficacy of p38 inhibitors as well as neutralization of TNFα by the antibodies. Other parameters (IL-1β, IL-6, macrophage inflammatory protein-1α/β, regulated upon activation, normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein (MCP)-1) were measured using Luminex technology and are expressed as percentage of the levels found in the placebo-treated control group.

Murine CIA model

The experiment was essentially performed as described previously (Smeets *et al*., 2003; Dulos *et al*., 2004). Male DBA1/J mice were immunized at the base of the tail with 100 μg bovine type II collagen in Freund's complete adjuvant enriched with 2 mg ml^{-1} *Mycobacterium tuberculosis* H37Ra. Three weeks after the immunization, the animals were given a booster i.p. injection of 100 μg bovine type II collagen dissolved in saline. After disease onset, animals with arthritis scores ranging from 0.25 to 1.25 were divided into separate groups of 11–12 mice so that the mean arthritis score of all treatment groups was comparable at the start of treatment (day 0). In the first experiment, Org 48762-0 and prednisolone (to provide a positive control) were dissolved in vehicle (dimethyl sulphoxide/cremophore/mannitol aqueous solution; see above) and dosed daily for 3 weeks (day 0–21) at doses of 5 and 1.5 mg kg^{-1}, respectively. Further, efficacy of Org 48762-0 was confirmed in a dose–response experiment using a different vehicle (0.5% gelatin/5% mannitol in water) that provides a stable and homogeneous suspension and is commonly used for clinical studies. To neutralize TNFα in mice, anti-TNFα polyclonal antibodies obtained from rabbits immunized with murine TNFα were injected i.p. three times a week for 3 weeks at a dose of 25 mg kg^{-1}. As a control, rabbit IgG was injected. These experiments were performed without knowledge of the treatments.

Assessment of CIA

All assessments were carried out without knowledge of the treatments applied. The clinical severity of arthritis (arthritis score) was graded as described previously (Joosten *et al*., 2000) on a scale of 0–2 for each paw, according to changes in redness and swelling, and measured three times per week to monitor time course profiles of arthritis development. To assess the effects of compounds, the area under the curve of arthritis score with baseline correction (subtracting baseline area under the curve of arthritis score on day 0) was used. The data were further normalized to an average value of the placebo-treated group and indicated as normalized area under the curve$_{(\text{arthritis score})}$. At the end of the experiments, knee and ankle joints were used for radiological or histological analyses. Joints were X-rayed using the radiography system equipped with direct digital imaging system (Faxitron X-ray, Model MX-20 Digital). X-ray pictures were displayed on a monitor using the software program Specimen (Version 2.0.1) and carefully examined. The bone destruction of a joint was scored on a scale of 0–5 ranging from no damage to complete destruction of the joint as described previously (Joosten *et al*., 1999). The cumulative scores of 2 joints (right ankle and knee) were used as radiological scores, which were further normalized to the average value of the placebo-treated group.

For histology, the knee joints of naive and placebo- and Org 48762-0 (5 mg kg^{-1})-treated animals were fixed for 4 days in phosphate-buffered 4% paraformaldehyde. After decalcification in 5% formic acid, the specimens were processed for paraffin embedding. Tissue sections (7 μm) were stained with Safranin-O, which visualizes the articular cartilage in bright red colour, and representative histological pictures were shown. Further, cartilage destruction was evaluated and scored microscopically on a scale of 0–3, ranging from no damage to complete destruction.

Microcomputed tomography

The microcomputed tomographical analyses of the right ankle joints were performed using Skyscan 1076 microCT-40 system (Skyscan, Aartselaar, Belgium) equipped with a high-resolution, low-dose X-ray scanner. The X-ray tube was operated with photon energy of 70 kV, current of 142 μA, exposure of 790 ms and a 0.5 mm-thick filter. The scanning conditions were set at 35 mm width, 35 μm pixel sizes and scanning time of approximately 10 min. After standardized reconstruction of the scanned image, the data sets for each ankle sample were resampled using software CTAn (Skyscan) so that each sample is oriented in the same manner. The same measurement conditions such as thresholds were used for all samples. Hereafter, a three-dimensional bone characteristic parameter (bone surface to bone volume ratio) was analysed by taking 60 consecutive slices from the base of the toe towards the ankle (bone of the tarsus excluding calcaneus and talus). The results were calculated as a percentage of naive control values. Three-dimensional images of the samples were obtained using the software program CTVol (Skyscan).

Data analysis

Data are expressed as the mean ± s.e.mean. Nonlinear regression analysis of the data and calculation of ED_{50} values were performed using Prism 4.03 (GraphPad Software, San Diego, CA, USA). An unpaired Student's *t*-test was used to determine the differences in EC_{50} values obtained for Org 48762-0 and SB203580 in biochemical and cellular assays. The cytokine levels after LPS challenge *in vivo* were analysed using two-factor ANOVA followed by Fisher's least significant difference test. The same statistical test was used to analyse CIA data. The bone characteristic parameter was analysed using ANOVA followed by Tukey's multiple comparison test. Statistical significance was defined as $P<0.05$.

Materials

Org 48762-0 (4,6-Bis(4-fluorophenyl)-2-methyl-5-(4-pyridyl)-2H-pyrazolo[3,4-b]pyridine (structure shown previously (Soliva *et al*., 2007)) is a low molecular weight inhibitor of p38α/β kinase and originated from Palau Pharma (Barcelona, Spain). Endotoxin-free polyclonal antibodies against mouse TNFα (IgG class) and its control (rabbit IgG) were generated at BioGenes (Berlin, Germany) according to the supplier's standard procedures. The neutralizing activity of the antibodies was confirmed in *in vitro* assays before *in vivo* testing (data not shown).

Results

Potent and selective inhibition of p38α and β kinases

The inhibitory potency of Org 48762-0 on p38α kinase activity was determined in the enzyme activity IMAP assay. Org 48762-0 showed an EC_{50} value of 0.10 ± 0.01 μM, which was comparable to that of a reference p38 inhibitor, SB203580 (0.10 ± 0.01 μM) (Figure 1). There was no statistical difference between these two EC_{50} values. The kinase selectivity of Org 48762-0 was evaluated in comparison to SB203580 at a panel of 50 human kinases. This assessment showed a high degree of kinase selectivity for p38α and p38β over other kinases for Org 48762-0 at a concentration of 10 μM, whereas SB203580 displayed a promiscuous selectivity profile (Figure 2). Among 50 human kinases, p38α and p38β were the only two kinases potently inhibited by Org 48762-0, whereas less than 25% inhibition was seen for the other 48 kinases. The data indicate that EC_{50} values of Org 48762-0 for these 48 kinases are most likely at least higher than the tested concentration (10 μM), supportive of a more than 100-fold selectivity of Org 48762-0 for p38α kinase (EC_{50}: 0.10 μM, (Figure 1)) over other kinases tested.

Demonstration of p38 kinase inhibition in cells

To further investigate the inhibitory potential of Org 48762-0, the effect on LPS-induced TNFα production was examined in a functional cellular assay. Org 48762-0 completely inhibited LPS-induced TNFα release from PBMC with an EC_{50} value of 0.06 ± 0.01 μM, which was approximately fourfold lower ($P<0.05$) than that for SB203580 (0.28 ± 0.07 μM) (Figure 3). Mechanistically, the cellular activity of Org 48762-0 was further examined by measuring redistribution of MK2, a kinase downstream of p38 kinase, in cells expressing a green fluorescent protein–MK2 fusion protein. Both Org 48762-0 and SB203580 dose-dependently inhibited stress-induced

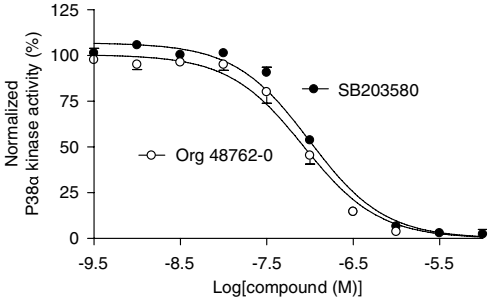

Figure 1 Profiles of Org 48762-0 and SB203580 in the p38α kinase activity assay. Compounds were tested in the p38α enzyme activity IMAP assay. Data were normalized to enzyme activity obtained in the absence of inhibitors. Org 48762-0 showed an EC_{50} value of 0.10 ± 0.01 μM ($n=3$), which was identical to that of SB203580 (0.10 ± 0.01 μM, $n=3$). IMAP, immobilized metal ion affinity-based fluorescence polarization.

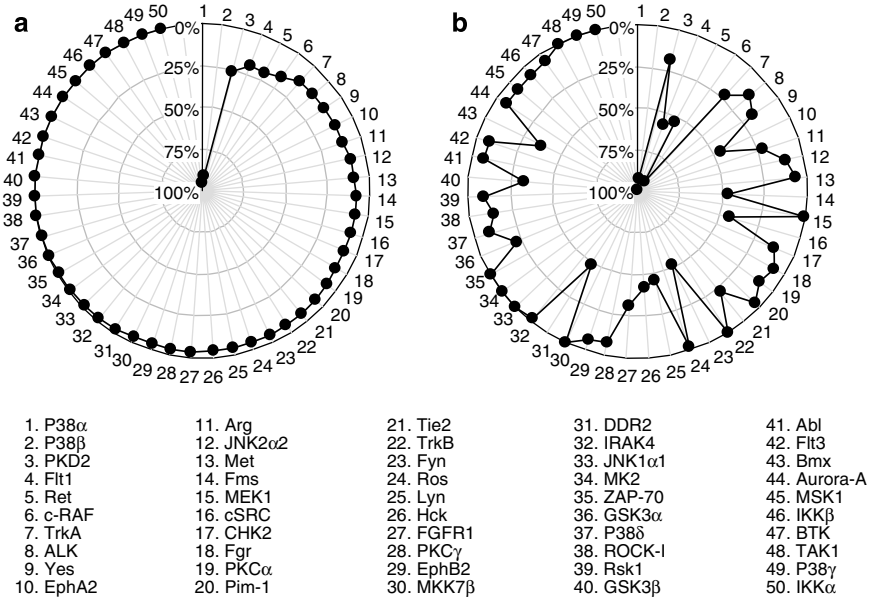

Figure 2 Profiles of Org 48762-0 (**a**) and SB203580 (**b**) in the human kinase selectivity assays. Selectivity profiles against a panel of 50 human kinases were determined in duplicate at a compound concentration of 10 μM. The plots indicate the percentages of inhibition per individual kinase.

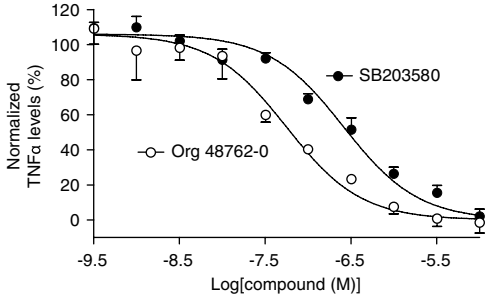

Figure 3 Pharmacological profiles of Org 48762-0 and SB203580 in a functional cellular assay. PBMC were pretreated with a range of concentrations of p38 inhibitor for 30 min before the LPS stimulation. After 4 h incubation, the amount of TNFα released in the culture medium was quantified by enzyme-linked immunosorbent assay. Data were normalized to TNFα levels obtained in the absence of inhibitors. Org 48762-0 showed an EC_{50} value of 0.06 ± 0.01 μM ($n = 3$), which was significantly different from that for SB203580 (0.28 ± 0.07 μM, $n = 3$) ($P < 0.05$, Student's *t*-test). PBMC, peripheral blood mononuclear cell; TNF, tumour-necrosis factor.

MK2 translocation, with significantly different ($P < 0.01$) EC_{50} values of 0.69 ± 0.12 and 4.67 ± 0.29 μM, respectively (Figure 4a). This observation in cells is in agreement with the mode of action of both compounds, being a direct interference with p38 kinase activity. As IL-1β is known to trigger p38 signalling cascades, we also examined whether Org 48762-0 could inhibit such signalling in THP-1 cells. IL-1β-induced phosphorylation of p38 kinase as well as of heat shock protein 27, a downstream marker of p38 kinase activity, was clearly inhibited by Org 48762-0 and SB203580 but not by specific inhibitors of other MAP kinases, that is by SP600125, a c-Jun N-terminal kinase inhibitor, or by PD98059, a MEK inhibitor (Figure 4b). Taken together, these data indicate that Org 48762-0 is capable of inhibiting both LPS- and IL-1β-induced signalling cascades, consequently interfering with production of proinflammatory cytokines.

Drug-like pharmacokinetic properties in mice

In mice, Org 48762-0 showed an oral bioavailability of 85% and exhibited sustained systemic exposure (>1 μM) for 24 h after a dose of 4.0 mg kg^{-1}. When Org 48762-0 was administered intravenously (1.6 mg kg^{-1}), the compound showed a low volume of distribution in steady state (V_{ss}: 50.0 ml kg^{-1}) and a low clearance (CL: 9.0 ml h^{-1} kg^{-1}), resulting in a mean residence time of 5.3 h. These drug-like pharmacokinetic properties of Org 48762-0 in mice allowed for a once-daily oral treatment regimen in the experiments with murine CIA.

Inhibition of LPS-induced endotoxaemia in mice

After establishing the potent cellular activity and given the good pharmacokinetic properties of Org 48762-0, we further evaluated its *in vivo* potency and efficacy in a mouse model of LPS-induced endotoxaemia. We also assessed the effects of a polyclonal antibody against mouse TNFα in the same model. Org 48762-0 dose-dependently and significantly inhibited LPS-induced TNFα production (Figure 5a). At a dose of 3 mg kg^{-1}, almost complete inhibition (~90%) was observed with Org 48762-0, whereas a 10-fold higher dose was required for SB203580 to obtain a similar effect. Preliminary observation of the effects of anti-mTNFα antibodies at 10 mg kg^{-1} (i.p.) in the model showed that already most of the TNFα was neutralized (data not shown). Supported by the initial data, we further tested the effects of 30 mg kg^{-1} anti-mTNFα antibodies in the model and

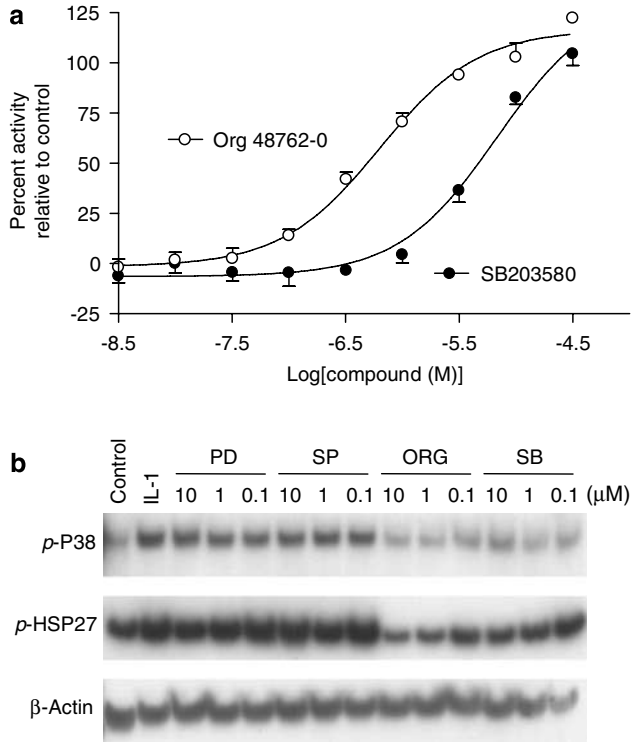

Figure 4 Selective interference of Org 48762-0 with p38 kinase activity in cellular assays. (**a**) An interference with anisomycin-induced translocation of MK2 was tested using BHK-ps1362cl. 15B-FS cells that overexpress a green fluorescent protein–MK2 fusion protein. In the assay, Org 48762-0 and SB203580 were tested at concentrations ranging from 3.2 nM to 32 μM. The EC_{50} value for Org 48762-0 (0.69 ± 0.12, $n=3$) was statistically different from that for SB203580 (4.67 ± 0.29 μM, $n=3$) ($P<0.01$, Student's *t*-test). (**b**) Selective inhibition of p38 kinase versus the other two mitogen-activated protein kinases was assayed in THP-1 cells stimulated with IL-1β. Cells were pre-incubated with kinase inhibitors (c-Jun N-terminal kinase inhibitor: SP600125 (SP), MEK inhibitor: PD98059 (PD) and p38 inhibitors: Org 48762-0 (ORG)/SB203580(SB)) at concentrations of 10, 1 and 0.1 μM for 30 min and subsequently stimulated with 10 ng ml^{-1} IL-1β. Proteins were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis and phosphorylation status of p38 kinase and its downstream substrate, heat shock protein 27, was evaluated via western blotting using specific antibodies. Detection of β-actin is shown as a control of comparable loading per lane. IL, interleukin.

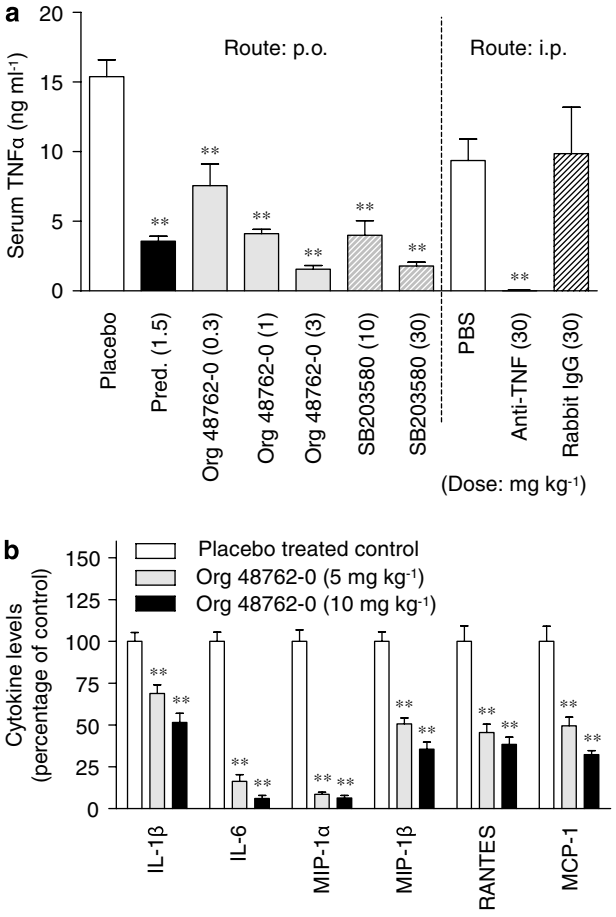

Figure 5 *In vivo* pharmacological characterization of Org 48762-0. (**a**) *In vivo* potency and efficacy of Org 48762-0 and SB203580 to interfere with TNFα production, as well as neutralizing capacity of polyclonal antibodies against mouse TNFα, were tested in the mouse model of LPS-induced endotoxaemia. The data represent the mean ± s.e.mean of five animals. $^{**}P<0.01$, significantly different from control group (placebo or rabbit-IgG), ANOVA plus Fisher's least significant difference test. (**b**) In a separate study, the inhibitory effects of Org 48762-0 on the production of a range of cytokines and chemokines, as shown, were further investigated in the same model. The data represent the mean ± s.e.mean of five animals and are depicted as a percentage of the placebo-treated control group. $^{**}P<0.01$, significantly different from control group. TNF, tumour-necrosis factor.

found complete neutralization of TNFα *in vivo* at this dose, whereas rabbit IgG as a negative control showed no inhibitory effects compared to the PBS-treated group (Figure 5a). As the p38 inhibitor BIRB-796 was reported to inhibit several cytokines and chemokines in human endotoxaemia (Branger *et al*., 2002), we further examined the effects of Org 48762-0 on the production of a range of six cytokines and chemokines in a separate experiment in the mouse endotoxaemia model. Org 48762-0 significantly reduced serum levels of IL-1β, IL-6, macrophage inflammatory protein-1α/β, RANTES and MCP-1 induced by LPS challenge (Figure 5b). Guided by pharmacokinetic properties and the *in vivo* efficacy profiles of both Org 48762-0 and anti-mTNFα polyclonal antibodies, the treatment regimen for the murine CIA experiments was then chosen.

Therapeutic potential of Org 48762-0 in comparison to TNFα neutralization in murine CIA

The therapeutic effects of Org 48762-0 on established arthritis were investigated in two separate experiments. In the first experiment, Org 48762-0 at a daily dose of 5 mg kg^{-1} inhibited clinical signs of arthritis by ~70%, which was somewhat less than the effect of prednisolone (~90%) treatment at 1.5 mg kg^{-1} (Figures 6a and b). For both compounds, the level of inhibition of clinical signs of arthritis corresponded with their protective effects on bone as reflected by the radiological scores of knee and ankle joints (Figure 6c). Subsequently, a dose–response study was performed with Org 48762-0 at three levels of daily doses (1, 5 and 25 mg kg^{-1}). A clear dose–response relationship was observed with improvement of the clinical arthritis scores by

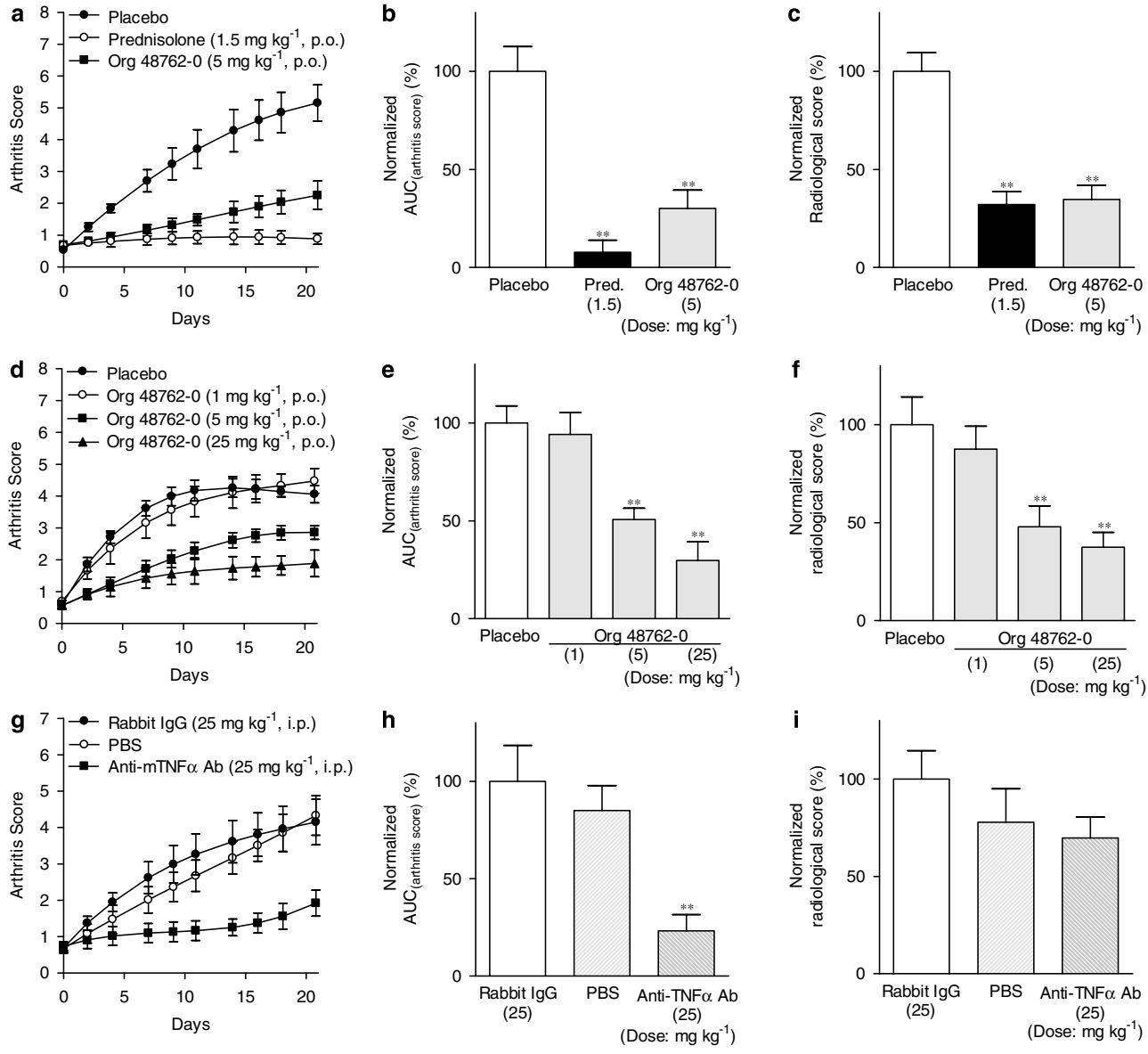

Figure 6 Therapeutic effect of Org 48762-0 (**a**–**f**) or anti-TNFα antibody (**g**–**i**) on collagen-induced arthritis in mice. Compounds were given daily p.o. for 3 weeks, whereas anti-TNFα antibody was injected i.p. three times a week for 3 weeks. Efficacy of Org 48762-0 was confirmed in two independent studies (**a**–**c** and **d**–**f**). For each study, a time course profile of mean arthritis score (**a**, **d**, **g**) and normalized area under the curve$_{(\text{arthritis score})}$ (**b**, **e**, **h**) and radiological score (**c**, **f**, **i**) of respective groups are shown. The results are means ± s.e.mean of 10–12 animals. $^{**}P<0.01$, significantly different from control group (placebo or rabbit IgG), ANOVA plus Fisher's least significant difference test. TNF, tumour-necrosis factor.

49 and 70% at doses of 5 and 25 mg kg^{-1}, respectively (Figures 6d and e). Org 48762-0 also dose-dependently improved the radiological scores by 52 and 63% at doses of 5 and 25 mg kg^{-1}, respectively (Figure 6f). When the effects of Org 48762-0 at a dose of 5 mg kg^{-1} were compared in two separate studies, the second study showed less efficacy. This may be explained by the use of a suspension vehicle in the second study (versus solution vehicle in the first), possibly yielding somewhat lower plasma levels. Some biological variation, which is common in animal experimentation, may have added to that.

The therapeutic efficacy of anti-mTNFα polyclonal antibodies (25 mg kg^{-1}, three times per week, i.p.) was tested and compared to treatment with purified rabbit IgG at the same dose or PBS as negative controls in the same model. The rabbit IgG-treated control group showed a clinical arthritis score and a radiological score comparable to the scores in the PBS-treated group (Figures 6g–i). Neutralization of mTNFα during the established CIA resulted in marked suppression of clinical disease activity as compared to the rabbit IgG-treated control group (Figures 6g and h). The effect of anti-mTNFα antibody treatment on clinical signs and symptoms was at least as pronounced as that observed with 5–25 mg kg^{-1} Org 48762-0 (Figures 6b, e and h). Although data analysis by ANOVA showed no statistical differences, neutralization of mTNFα tended to decrease the bone erosion in knee and

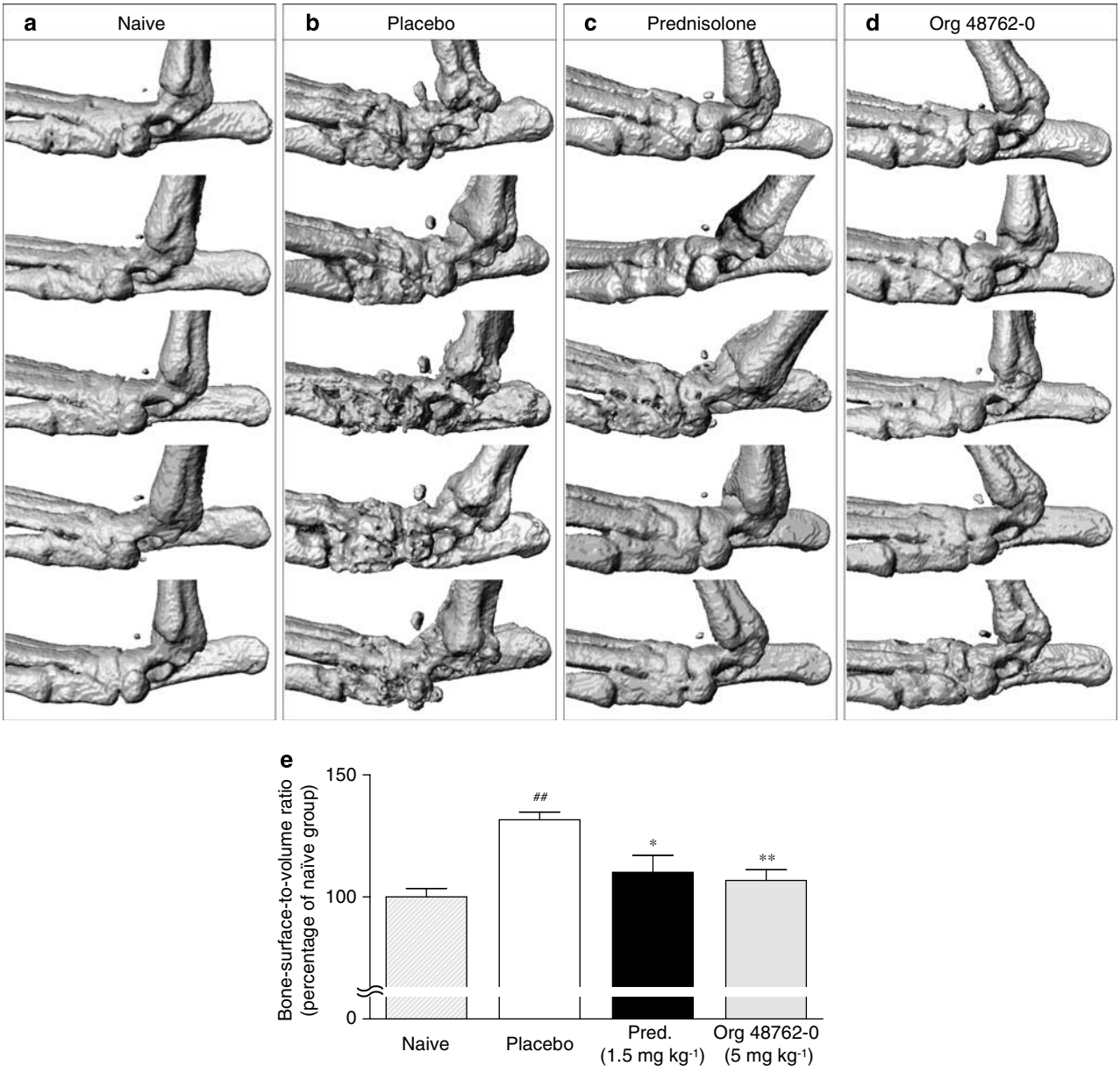

Figure 7 Microcomputed tomographical analysis of the ankle joints. The right ankle joints of naive mice (**a**) or mice treated with placebo, (**b**) prednisolone (1.5 mg kg^{-1}, **c**) or Org 48762-0 (5 mg kg^{-1}, **d**) for 21 days were used. For each group, representative microcomputed tomographic pictures of five animals are shown. (**e**) As a quantitative bone characteristic parameter, the bone surface to bone volume ratio was measured and results were expressed as percentages of naive control values. The results are means ± s.e.mean of six to eight animals ($n = 6$ for naive mice). $^{\#\#}P < 0.01$, significantly different from naive control group and $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from placebo-treated group, ANOVA plus Tukey's multiple comparison test.

ankle joints assessed by radiological analyses (Figure 6i). A direct comparison using Student's *t*-test confirmed a trend towards reduced bone damage by treatment with anti-mTNFα antibody, compared to the control group, treated with rabbit-IgG ($P = 0.058$).

Protective effects of Org 48762-0 on bone and cartilage destruction in murine CIA

The protective effects of Org 48762-0 on bone degradation, statistically significant in established CIA, were further substantiated by microcomputed tomography data of the ankle joints as shown in Figure 7. The ankle joints of placebo-treated animals showed marked bone destruction as compared to intact joints obtained from naive animals (Figures 7a and b). Daily oral administration of prednisolone (1.5 mg kg^{-1}) or Org 48762-0 (5 mg kg^{-1}) displayed a clear protection against bone destruction as compared to the placebo-treated control group (Figures 7c and d). As a quantitative parameter of the condition of bone, the bone surface to bone volume ratio was measured and results were expressed as percentages of naive control values (Figure 7e).

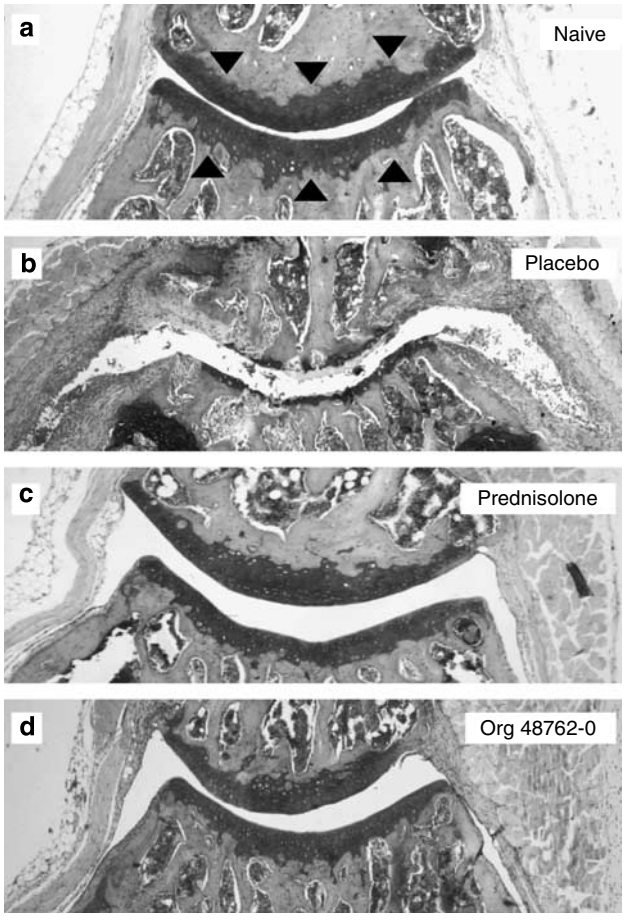

Figure 8 Histology of knee joints of naive, placebo- or Org 48762-0-treated mice. Knee joints of mice treated with placebo, prednisolone (1.5 mg kg^{-1}) or Org 48762-0 (5 mg kg^{-1}) were taken at the end of the experiment. After fixation and decalcification, sections were stained and representative sections of joints from animals with no CIA (naive; **a**), with CIA and treated with placebo (**b**) or prednisolone-treated (**c**) or Org 48762-0-treated (**d**) are shown. Tissue sections were stained with Saffranin-O, which visualizes articular cartilage in bright red colour. The intact cartilage is indicated by black arrows in the picture of the naive animal. When cartilage damage was assessed by histological score on a scale of 0–3, ranging from no damage to complete destruction, the placebo-treated animals showed almost complete destruction of the cartilage (score: 2.8 ± 0.2). Daily treatment with prednisolone (1.5 mg kg^{-1}) or Org 48762-0 (5 mg kg^{-1}) clearly and significantly reduced the score for the cartilage destruction (1.2 ± 0.5; $P<0.01$ for prednisolone and 1.3 ± 0.4; $P<0.05$ for Org 48762-0) as compared to placebo-treated animals (ANOVA plus Fisher's least significant difference test). CIA, collagen-induced arthritis.

In the placebo-treated animals, the value was significantly increased by approximately 30% in mice with CIA, compared to naive animals, owing to fragmentation of bone as shown in Figure 7b. Treatments with Org 48762-0 in murine CIA potently and significantly inhibited such bone fragmentation. Further, an apparent protective effect on proteoglycan depletion and subsequent cartilage destruction, as measured by Saffranin-O-staining, was observed with both prednisolone and Org 48762-0 treatments as compared to the placebo-treated group (Figures 8a–d). When cartilage damage was assessed by histology scores on a scale of 0–3, ranging from no damage to complete destruction, the placebo-treated animals showed almost complete destruction of the cartilage (score: 2.8 ± 0.2). Daily treatment of prednisolone or Org 48762-0 clearly and significantly reduced the score for the cartilage destruction (1.2 ± 0.5; $P<0.01$ for prednisolone and 1.3 ± 0.4; $P<0.05$ for Org 48762-0) as compared to placebo-treated animals.

Discussion and conclusions

Current biopharmaceuticals for the treatment of RA, in particular the TNFα blockers, have been proven to be of high therapeutic value (Maini and Taylor, 2005). Still, in a substantial proportion of patients treated with anti-TNFα biologicals, resistance to TNFα blockade or residual disease activity is seen, suggesting some degree of TNFα-independent RA pathophysiology in these patients. In addition to the high costs and parenteral administration, this lack of response to TNFα-based therapy reflects another unmet need and an opportunity for new drugs in RA that mechanistically add to the blockade of TNFα and enhance therapeutic options to bring patients into clinical remission. In this context, we investigated the therapeutic potential of p38 inhibition using a structurally novel inhibitor and compared this to anti-mTNFα antibody treatment in a preclinical model of arthritis.

Although clinical efficacy of p38 inhibitors remains to be shown, one potential liability of kinase inhibitors is their propensity for off-target activity. Most kinase inhibitors are designed to target the ATP binding site, which is an intrinsic part of all protein kinases, implying possible cross-reactivity. For a fair profiling of the concept of p38 inhibition as well as an unbiased efficacy profiling in preclinical and clinical studies, the selectivity of an inhibitor is regarded as a key element. In this study, we demonstrated that Org 48762-0 potently inhibits p38α and β kinases with a high degree of selectivity (>100-fold) over a broad range of human kinases including potential targets for inflammation such as c-Jun N-terminal kinases, c-RAF, transforming growth factor-β-activated kinase-1 and IκB kinase (IKK)α/β. In addition, Org 48762-0 exhibited a sustained exposure (>1 μM over 24 h) in pharmacokinetic studies in mice at dose levels that are relevant to those applied in our *in vivo* studies. As Org 48762-0 was shown to inhibit p38α kinase as a target with EC_{50} values well below 1 μM in all biochemical and cellular assays described in this study, such pharmacokinetic properties seem to support long-lasting and selective pharmacological inhibition of the target *in vivo*. Its *in vivo* potency was confirmed by the almost complete inhibition (>90%) of LPS-induced TNFα production in mice after a dose of 3 mg kg^{-1}. These pharmacological properties of Org 48762-0 enabled us to evaluate the biological concept and the therapeutic potential of p38 kinase inhibition in a preclinical model of arthritis, with comparison to anti-mTNFα antibody treatment.

Reduction of phosphorylation of p38 itself by Org 48762-0 and SB203580 (Figure 4b) may have been caused by interference with p38 autophosphorylation in the alternative activation path as suggested by ten Hove *et al*. (2007) or

by compound-induced conformational change affecting the availability of its phosphorylation sites. SB203580 was reported to be capable of inducing p38 conformational change, although functional consequences were not studied (Vogtherr *et al*., 2006).

In the murine CIA model, Org 48762-0 substantially ameliorated the clinical arthritis and radiological scores when dosed daily at 5 or 25 mg kg^{-1} upon onset of disease. Microcomputed tomographic and histological analyses of joints further support that Org 48762-0 has strong anti-inflammatory effects as well as protective effects on joint tissue damage during established CIA. These results are in line with previous findings obtained using other p38 inhibitors (Nishikawa *et al*., 2003; Medicherla *et al*., 2006). In a mouse model of LPS-induced endotoxaemia, we showed that the treatment with i.p. anti-mTNFα antibody completely neutralized the abundantly produced serum levels of TNFα (>10 ng ml^{-1}). Considering that immunoglobulins generally have a half-life of 7–12 days (Bazin and Malet, 1969), three times per week treatment with the antibody was expected to cause complete neutralization of the target molecules in the course of a murine CIA study. Indeed, TNFα neutralization with anti-mTNFα antibody treatment showed clear suppression of clinical signs and symptoms of the CIA. Radiological analyses of knee and ankle joints showed a trend towards reduced bone destruction in animals treated with anti-mTNFα antibody ($P=0.058$, when Student's *t*-test was used for a direct comparison with the control group). A similar observation was reported previously by other investigators (Joosten *et al*., 1999). Taken together, these data suggest that p38 kinase inhibition in the current experimental setting compares favourably to anti-mTNFα antibody treatment in murine CIA.

The p38 kinase is known to exhibit exceedingly broad functions, making it difficult to define the detailed mechanism behind the tissue-protective effects as observed in CIA. Still, one of the most important mechanisms is arguably the modulation of the release of proinflammatory cytokines. In this study, we showed that our new p38 inhibitor, Org 48762-0, potently reduced production of TNFα as well as other proinflammatory cytokines such as IL-1β and IL-6 and chemokines in an acute inflammation model in mice, whereas no significant inhibition of the latter cytokines was observed with anti-mTNFα antibody treatment (unpublished observation). These results are fully compatible with previous findings that another p38 kinase inhibitor suppressed the production of cytokines such as IL-1β and IL-6 in the joint tissues of arthritic animals (Medicherla *et al*., 2006). A pivotal involvement of these proinflammatory cytokines in driving tissue damage has been extensively described. For example, the critical role of IL-1α/β but not TNFα in tissue damage including cartilage and bone destruction in murine CIA has been clearly demonstrated (Joosten *et al*., 1999). Further, it was reported that IL-6 deficient mice are protected against articular cartilage damage in an experimental arthritis model (Ohshima *et al*., 1998). Thus, downregulation of a set of proinflammatory cytokines and chemokines by Org 48762-0 in the course of murine CIA may well have contributed to the observed protective effects on tissue damage, which were less prominent with anti-mTNFα antibody treatment.

Not only modulation of the release of proinflammatory cytokines but also cellular signalling triggered by such proinflammatory cytokines is regulated by p38 kinases. It was recently reported that p38 kinase mediates IL-1-induced downregulation of aggrecan gene expression in human chondrocytes, suggesting that inhibition of this pathway could be of importance for the protection of cartilage tissue in arthritis (Radons *et al*., 2006). In this study, we showed that Org 48762-0 interfered with IL-1β-mediated signalling in activated monocytic cells. Interestingly, Mbalaviele *et al*. (2006) also demonstrated an important role of p38 kinase in osteoclast differentiation when induced by the ligand of receptor activator of nuclear factor NF-κB (RANKL) or by TNFα. As osteoclasts with their primary role of bone resorption are involved in bone degrading processes, the protective effects of Org 48762-0 on bone may at least partly be attributed to the inhibition of osteoclast differentiation. Taken together, these underlying mechanisms could explain the protective effects on tissue damage seen with Org 48762-0 in murine CIA.

The beneficial systemic and local bone effects of anti-TNFα therapies have been demonstrated clinically in patients with RA (Maini *et al*., 2004) and active Crohn's disease (Ryan *et al*., 2004). Recently, Saito *et al*. (2007) demonstrated that clinically used anti-TNFα monoclonal antibody (infliximab) clearly and potently prevented CIA-induced bone destruction at the knee joints in mice. In our study, anti-mTNFα antibody treatment showed a trend towards improved radiological scores by some 30% as compared to rabbit-IgG-treated control group, although this was not statistically significant. The reason for a difference in degree of bone protection observed in these two studies warrants further exploration. It cannot be ruled out that the less favourable effect on radiological score observed with mTNFα neutralization, as compared to Org 48762-0-treatment in the current study, may be determined by the characteristics of the antibody used. Still, although preclinical evaluation of drugs in the arthritis models have contributed to our understanding of mechanisms relevant to RA, it is generally accepted that the predictive value of mouse models remains limited. Therefore, further dedicated clinical research and reporting will be required to assess the therapeutic potential of the most promising p38 kinase inhibitors. Currently, several phase II clinical trials are being conducted to evaluate the efficacy of various p38 inhibitors in RA patients (Dominguez *et al*., 2005; Ding, 2006). The clinical output from those studies will at least provide us with a first perspective of the therapeutic potential of those p38 inhibitors in RA.

In conclusion, we showed the structurally new Org 48762-0 to be a potent and selective inhibitor of p38 kinase exhibiting favourable pharmacokinetic properties in mice. In murine CIA, interference with p38 kinase activity with this selective inhibitor yielded pronounced reduction of inflammatory symptoms as well as joint tissue damage. In terms of the suppression of bone damage assessed by radiological analyses, anti-mTNFα antibody treatment produced weaker effects in the current experimental setting. We consider our

observations in mice to be a strong motivation for further preclinical and clinical efforts to elucidate the therapeutic potential of highly selective p38 kinase inhibitors in RA.

Acknowledgements

The authors are grateful to Monika Gorecka, Laura Sapulete, Trudy Pullens, Miranda van der Lee, Lonneke Voorneveld-Dijkmans, Frank Wijnands and Jesse Wong for developing and performing *in vitro* assays; Gerard Vogel for coordinating PK studies; Cindy van Doorn for her technical support on CIA study; Dr Roel Arends for his support and scientific discussion on micro-CT analyses; Desiree Veening for her work on Luminex assays; Dr Pedro-Manuel Grima-Poveda and Dr Nicole van Straten for substantial scientific contributions in the project team as well as synthesis of the compounds; Dr Guido Zaman and Dr Javier Bartrolí (Palau Pharrma) for scientific advice. The study was supported by NV Organon and Palau Pharma.

Conflict of interest

The authors state no conflict of interest.

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