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Effects of a mitogen-activated protein kinase inhibitor on allergic airways inflammation in the rat studied by magnetic resonance imaging

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Abstract

We recently described a new model to study non-invasively with magnetic resonance imaging (MRI) the effects of compounds to prevent and/or resolve airway inflammation induced by ovalbumin in the lungs of actively sensitised rats. We report here the effects of 4-(4-fluorophenyl)-2-(1-methylpiperidin-4-yl)-5-(2-(1-(*S*)-phenylethyl)amino-4-pyridinyl)thiazole fumarate (Compound 1), which exhibits inhibitory activity against p38α and p38β2 and residual activity on c-*Jun* amino-terminal kinase (JNK)2 mitogen-activated protein (MAP) kinases, on the oedematous signals detected by MRI and generated by antigen challenge in the lungs of sensitized rats. Compound 1 (10 mg kg^{-1}) given orally 1 h prior to allergen challenge significantly reduced the oedematous signal measured at 24 h. Similar effects were seen with a synthetic corticosteroid, mometasone furoate (0.3 mg kg^{-1}), given intratracheally 3 h prior to challenge. For both compounds, inhibition of the oedematous signal was accompanied by reductions in the inflammatory parameters in the bronchoalveolar lavage fluid measured 24 h after challenge with ovalbumin. Compound 1 (10 mg kg^{-1}) administered 24 h after challenge with ovalbumin did not change the rate of resolution of the signal detected by MRI in the lungs. In contrast, mometasone furoate (0.3 mg kg^{-1}) significantly increased resolution of these signals, which was evident 3 h after drug administration and maintained to 48 h post challenge. Collectively, our data suggest that the p38 MAP kinase inhibitor Compound 1 shows a different profile than glucocorticosteroids since its ability to resolve existing inflammation is limited.

Keywords: Allergen; Allergic pulmonary inflammation; Asthma; Glucocorticosteroid; Lung; Magnetic resonance imaging (MRI); Mitogen-activated protein kinase inhibitor; Oedema; Oedema resolution

1. Introduction

Mitogen-activated protein (MAP) kinase pathways are involved in inflammatory processes in diseases such as rheumatoid arthritis, asthma and chronic obstructive pulmonary disease (Herlaar and Brown, 1999). They are components of intracellular signalling pathways which play a role in several cellular functions including gene expression, cell proliferation and death (Herlaar and Brown, 1999). Five distinct mammalian MAP kinase pathways have been identified and implicated in inflammatory responses among which extracellular signal regulated protein kinase (ERK), p38 and c-*Jun* amino-terminal kinase (JNK)/stress-activated protein kinase are the best characterized (Pearson et al., 2001).

Among the MAP kinase isoforms, p38 has received particular attention since it plays a critical role in regulating multiple inflammatory responses in many cell types, such as inhibition of the synthesis and activation of inflammatory cytokines and chemokines (New and Han, 1998; Lee et al., 1999). Selective inhibitors of p38 kinase, e.g. SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole] and SB220025 [5-(2-amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinyl)imidazole)], have been

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shown to inhibit the production of tumor necrosis factor-α and interleukin-1β in cultured cells (Lee et al., 1994). Recent reports demonstrated that p38 MAP kinase inhibitors are also effective anti-inflammatory agents in in vivo models of airway inflammation (Escott et al., 2000; Underwood et al., 2000).

Recently, magnetic resonance imaging (MRI) has been introduced as a means of assessing non-invasively the inflammatory response in the lungs of actively sensitised rats following allergen challenge (Beckmann et al., 2001). Changes in oedematous signal volume detected by MRI followed closely the changes in the parameters of inflammation determined in the bronchoalveolar lavage fluid (Tigani et al., 2002). In the current study we applied MRI to investigate the capacity of 4-(4-fluorophenyl)-2-(1-methylpiperidin-4-yl)-5-(2-(1-(*S*)-phenylethyl)amino-4-pyridinyl)thiazole fumarate (Compound 1), an inhibitor of p38 kinase, to prevent and/or resolve the pulmonary oedematous signal induced by ovalbumin challenge in the actively sensitised Brown Norway rats. Mometasone furoate, a new glucocorticosteroid effective in reducing airway inflammation and improving lung function in asthma (Inman et al., 2001), was used as a comparison compound.

2. Materials and methods

2.1. Kinase assays

2.1.1. Kinase activity of p38α

The substrate GST-ATF-2 (Novartis Pharma, Basel, 50 μl/well) was coated onto the wells of microtiter plates overnight at 4 °C. The following day, the microtiter plates were washed four times with phosphate buffer saline (PBS)/0.5% Tween20/0.02% Na azide and blocked with PBS/2% bovine serum albumin/0.02% Na azide for 1 h at 37 °C. Plates were washed again four times with PBS/0.5% Tween20/0.02% Na azide. The kinase cascade reaction was then started by adding a phosphorylated form of human His-p38α MAP kinase (10 ng/well) to the kinase buffer containing 125 mM HEPES, pH 7.4; 125 mM β-glycerophosphate; 125 mM $MgCl_2$; 0.5 mM sodium orthovanadate, 10 mM 1,1,1,-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) in the presence of and 120 μM cold ATP. Compound 1 was tested in the concentration range of 0.001–10 μM. Incubation was for 1 h at 37 °C in 96-well plates and the reactions were stopped by washing the plates four times with PBS/0.5 Tween20/0.02% Na azide. The phosphorylated GST-ATF-2 was detected by adding 50 μl of 1mM rabbit polyclonal antibody (PhosphoPlus ATF-2 (Thr^{71})). The plates were washed four times with PBS/0.5% Tween20/0.02% Na azide, and 50 μl of 0.3 mM biotin labeled goat anti-rabbit immunoglobulin G (IgG) added to all wells. After washing the plates four times with PBS/0.5% Tween20/0.02% Na azide, 50 μl of 0.2 mM streptavidin-alkaline phosphatase and 100 μl substrate (phosphatase substrate tablets, 1 mg/ml in substrate buffer of a mixture of 97 mg/l of diethanolamine, 100 mg/l of $MgCl_2 \cdot 6\ H_2O$, 0.2 g/l of Na azide and 1 M HCl) were added. The plates were read in a Bio-Rad microplate reader in a dual wavelength mode (measurement filter 405 nm and reference filter 490 nm).

2.1.2. Kinase activity of p38β2, p38δ, JNK1, JNK2 and MKK6

Methods for measuring kinase activities of p38β2, p38δ, JNK1, JNK2 and mitogen-activated protein kinase kinase (MKK)6 were broadly similar to that described above for p38α. Phosphorylated forms of human His-p38β2, His-p38δ, His-JNK1 and His-JNK2 MAP kinases (30, 3, 200 and 30 ng/well, respectively) were used to phosphorylate the immobilized substrate GST-ATF-2 in the presence of cold ATP (120 μM). The phosphorylated GST-ATF-2 was detected by a mouse monoclonal antibody produced in house (anti-phospho ATF-2 (Thr^{71})MAb 289-16), followed by biotin labeled goat anti-mouse IgG, streptavidin-alkaline phosphatase and substrate. After washing with PBS/0.5 Tween20/0.02% Na azide, the reaction was stopped with 50 μl of 1.5 M NaOH, and the plates read in a Bio-Rad microplate reader in a dual wavelength mode (measurement filter 405 nm and reference filter 490 nm).

For the MKK6 kinase assays, an active form of GST-MKK6b (30 ng/well) of human origin was used to phosphorylate the immobilized substrate GST-P38α (KM) in the presence of cold ATP (12 μM). A rabbit polyclonal antibody (anti-phospho-specific p38 MAP kinase (Thr^{180}/Tyr^{182})) was used for this assay.

For all kinase assays, means of duplicate optical density values were calculated. After background substraction percentages of inhibition versus the dimethylsulphoxide (DMSO) control were determined. These values were then used to calculate the IC_{50} for each experiment.

2.2. Sensitisation and challenge procedures

Male Brown Norway rats weighing approximately 300 g were used in this study (Iffa-Credo, L'Arbresle, France). They were kept at an ambient temperature of 22 ± 2 °C under a 12-h normal phase light–dark cycle. Food and water were freely available. All experiments were carried out with the approval of the Veterinary Authority of the City of Basel (Kantonales Veterinaeramt, Basel-Stadt). The animals were sensitised to ovalbumin, and further challenged with ovalbumin (or saline) by intratracheal (i.t.) instillation (0.3 mg kg^{-1}) as described previously (Hannon et al., 2001). For challenge, rats were anaesthetized (4% isofluran) in an anaesthetic chamber until surgical level was achieved. Ovalbumin was administered intratracheally via a cannula (which needed to be bent to an angle of approximately 120°), and the animals were allowed to recover.

2.3. Monitoring the inflammatory response by MRI

The inflammatory response in the lungs induced by ovalbumin challenge was assessed non-invasively by MRI. Protocols of MRI acquisition and determination of oedematous signal volume were described in detail in Beckmann et al. (2001). Briefly, measurements were carried out with a Biospec 47/40 spectrometer (Bruker, Karlsruhe, Germany) operating at 4.7 T. Rats were anesthetised with 2% Forene (Abbott, Cham, Switzerland) in a mixture of O_2/N_2O (1:2), administered via a face mask. The rats respired spontaneously during image acquisition, and neither respiratory nor cardiac triggering was applied. A gradient-echo sequence with repetition time 5.6 ms, echo time 2.7 ms, band width 100 kHz, flip angle of the excitation pulse approximately 15°, field of view 6×6 cm^2, matrix size 256×128 and slice thickness 1.5 mm was used throughout the study. A single slice image was obtained by computing the two-dimensional Fourier transform of the averaged signal from 60 individual image acquisitions and interpolating the data set to 256×256 pixels. There was an interval of 530 ms between individual image acquisitions, resulting in a total acquisition time of 75 s for a single slice. The entire lung was covered by 25 consecutive slices. For each animal a set of baseline images was acquired before the ovalbumin challenge.

2.4. Bronchoalveolar lavage fluid collection and analysis

A detailed description of the bronchoalveolar lavage fluid procedure and the analysis of the parameters of inflammation has been provided recently (Beckmann et al., 2001; Tigani et al., 2002). Briefly, animals were killed with sodium pentobarbital (250 mg kg^{-1} i.p.), and the lungs lavaged. For leukocyte numbers and cell differentiation the automatic cell analysing system was utilised (Cobas Helios 5Diff, Hoffmann-La Roche, Axon Laboratory, Switzerland). Determination of eosinophil peroxidase was based on the oxidation of *o*-phenylenediamine by eosinophil peroxidase in the presence of hydrogen peroxide. Myeloperoxidase activity was measured in a photometric assay based on the oxidation of *O*-dianiside dihydrochloride by myeloperoxidase in the presence of hydrogen peroxide. The level of protein in the bronchoalveolar lavage fluid supernatants was measured by a photometric assay, based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent.

2.5. Drug administration

Compound 1 (10 mg kg^{-1}) or vehicle was administered p.o. 1 h prior to ovalbumin challenge for the pretreatment experiments and 24 h after challenge for the post-treatment studies. Mometasone furoate (0.3 mg kg^{-1}) or vehicle was administered i.t. to anaesthetised rats 3 h prior to ovalbumin challenge for the pretreatment experiments, and 24 h after challenge for the post-treatment studies. Application of mometasone (or its vehicle) followed the procedure described in Hannon et al. (2001) and outlined above for ovalbumin administration.

2.6. Materials

Ovalbumin was obtained from Fluka (Buchs, Switzerland). Pentothal (thiopentalum natricum) and Forene (Isofluran 100%) were obtained from Abbott. 4-(4-Fluorophenyl)-2-(1-methylpiperidin-4-yl)-5-(2-(1-(*S*)-phenylethyl)amino-4-pyridinyl)thiazole fumarate (Compound 1) and mometasone furoate were synthesised in the research department of Novartis Pharma.

Ovalbumin solution was prepared in 0.9% w/v NaCl. Compound 1 was prepared in 4% DMSO and diluted in Neoral™ placebo (Novartis Pharma) for p.o. administration (2 ml kg^{-1}). Mometasone furoate (blended in lactose) was administered i.t. as a dry powder formulation.

2.7. Data analysis

Mean values with standard errors (S.E.M.) from *n* individual experiments are presented. Statistical analysis comprised ANOVA multiple comparison tests with the Bonferroni correction. Significance was assumed at $P < 0.05$.

3. Results

Compound 1 exhibited potent inhibitory activity against p38α and p38β2 MAP kinases and no activity against the δ isoform (Table 1). Inhibitory activity of Compound 1 was also observed against another member of the MAP kinase family member, JNK2 (IC_{50} 261 nM). By contrast, JNK1 and the MKK6 were at best only weakly inhibited (Table 1).

Pronounced oedematous signals were detected by MRI in the lungs of vehicle-treated animals 24 h following ovalbumin challenge (Fig. 1A; Beckmann et al., 2001; Tigani et al., 2002, 2003). Compound 1 (10 mg kg^{-1}, p.o.) or mometasone furoate (0.3 mg kg^{-1}, i.t.) markedly reduced allergen-induced oedematous signals measured at 24 h.

Table 1
Enzymatic selectivity of Compound 1

MAP kinases	IC_{50} (nM)
p38α kinase	18 ± 0.2 (3)
p38β2 kinase	30 ± 0.9 (3)
p38δ kinase	10,137 ± 1321 (3)
JNK1	1880 ± 579 (3)
JNK2	261 ± 84 (3)
MKK6(EE)	>10,000 (3)

Data are expressed as means ± S.E.M. from the number of individual experiments indicated in brackets.

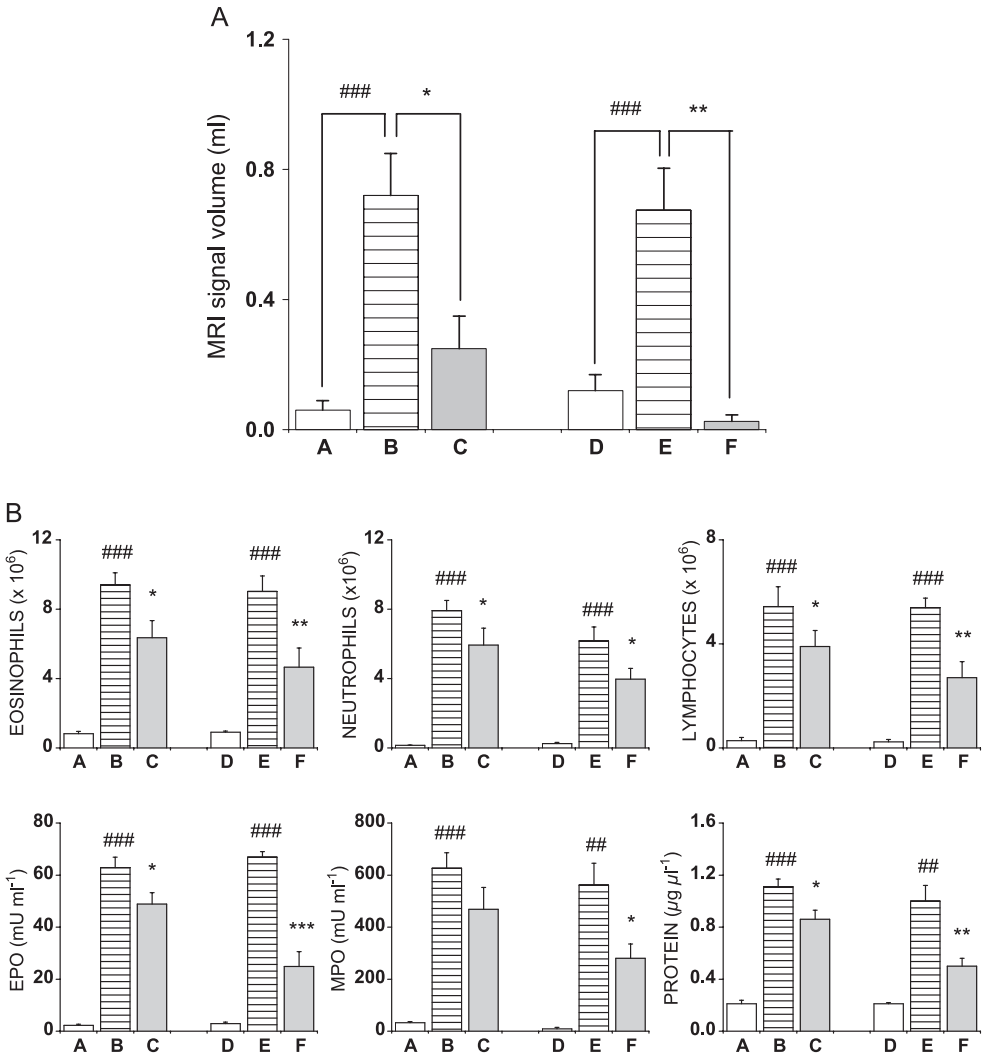

Fig. 1. Comparison between oedematous signals by MRI and BAL fluid parameters of inflammation in the lungs of Brown Norway rats: effect of pretreatment with Compound 1 (CP 1) or mometasone furoate (MF). (A) MRI signals and (B) Inflammatory cell infiltration and activation determined in the BAL fluid of the same animals. The data (means ± S.E.M.; $n = 5–6$/group) show the effects of CP1 or MF on the changes in MRI signals and in eosinophil, neutrophil and lymphocyte numbers, eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activities and protein levels in the lungs of actively sensitised Brown Norway rats, 24 h following challenge with ovalbumin (OA; 0.3 mg kg^{-1}, i.t.) or saline. CP 1 (10 mg kg^{-1}, p.o.), MF (0.3 mg kg^{-1}, i.t.) or their respective vehicles were given 1 or 3 h before the ovalbumin challenge, respectively. (A) Saline challenge, treatment with the vehicle for CP 1 (VEH-1, 4% DMSO in Neoral™ Placebo); (B) ovalbumin challenge, treatment with VEH-1; (C) ovalbumin challenge, treatment with CP 1; (D) saline challenge, treatment with the vehicle for MF (VEH-2, lactose in dry powder formulation); (E) ovalbumin challenge, treatment with VEH-2; (F) ovalbumin challenge, treatment with MF. $^{###}P < 0.001$ indicates significant difference from the animals challenged with saline. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences with respect to ovalbumin-challenged animals receiving the respective vehicle treatment.

Intratracheal instillation of ovalbumin led to an inflammatory response in the airways of sensitised BN rats when assessed by changes in the bronchoalveolar lavage fluid leukocyte numbers, myeloperoxidase and eosinophil peroxidase activities and protein concentration measured 24 h after challenge (Fig. 1B). Following pretreatment with mometasone furoate at a dose of 0.3 mg kg^{-1}, given i.t. 3 h prior to allergen challenge, all parameters of inflammation from the BAL fluid of challenged animals were significantly reduced (Fig. 1). Qualitatively similar results were obtained with Compound 1 (10 mg kg^{-1}) given p.o., 1 h prior to allergen (Fig. 1B).

The effects of Compound 1 on established pulmonary inflammation was assessed by giving 10 mg kg^{-1} orally 24 h after challenge with ovalbumin and compared to that of mometasone furoate (0.3 mg kg^{-1}) administered i.t. 24 h following ovalbumin. In confirmation of our previous data (Beckmann et al., 2001), intratracheal instillation of ovalbumin induced a marked pulmonary oedematous signal for up to 72 h after challenge (Fig. 2A). Oral administration of Compound 1 had no effect on the resolution of the oedematous signal at any time point. By contrast, mometasone furoate accelerated the oedematous signal resolution. A trend towards a reduction in the

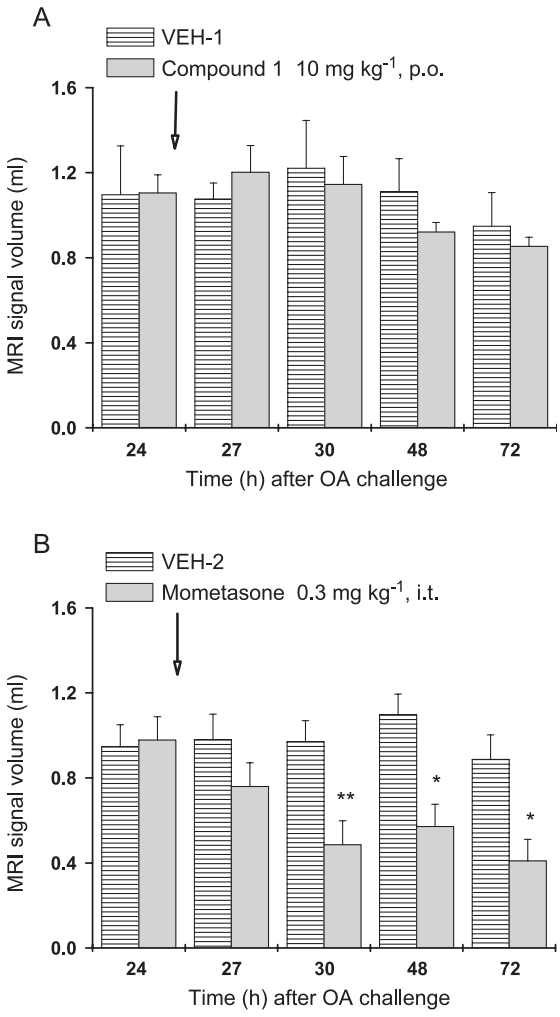

Fig. 2. Time-dependent changes in the volume of oedematous signals detected by MRI in the lungs of actively sensitised Brown Norway rats after intratracheal challenge with ovalbumin (OA; 0.3 mg kg^{-1} at time 0): effect of post-treatment with Compound 1 or mometasone furoate. The compounds (or their respective vehicles) were administered 24 h after allergen (indicated by the black arrows), immediately following an MRI acquisition. The data represent mean ± S.E.M. for $n=5-6$ rats/group. *$P<0.05$, **$P<0.01$ indicate a significant difference between the effect of the compound and that of the respective vehicle, at a given time point. VEH-1 (4% DMSO in Neoral™ Placebo) is the vehicle for Compound 1, and VEH-2 (lactose in dry powder formulation), the vehicle for mometasone.

signal was observed 3 h after drug administration, and the effect was statistically significant from 6 up to 48 h following the compound (Fig. 2B).

4. Discussion

MAP kinase pathways play an important role in inflammatory responses (Herlaar and Brown, 1999). Interest in the p38 family has been particularly intense following the findings that p38 inhibitors reduce mortality in a murine model of endotoxin-induced shock and display anti-arthritic activities in mice and rats (Badger et al., 1996). Because p38 MAP kinase plays a central role in regulating expression of pro-inflammatory mediators, leukocyte recruitment and inflammatory cell activation (New and Han, 1998; Herlaar and Brown, 1999), the concept that p38 MAP kinase might represent a potential therapeutic target for the treatment of lung inflammation has been extensively explored (Jackson et al., 1998; Underwood et al., 2000). However, there are no published reports which investigate the effects of a p38 MAP kinase inhibitor on both prevention and resolution of allergen-induced lung inflammation.

Compound 1 selectively inhibited the p38α and p38β2 isoforms of MAP kinase. Selectivity for p38α inhibition over JNK2 was 15-fold whereas other closely related MAP kinases, including JNK1 and MKK6, were only weakly inhibited.

To investigate the in vivo efficacy of Compound 1, we used the Brown Norway rat model of allergic-induced lung inflammation (Elwood et al., 1991; Underwood et al., 1997). In this model, Compound 1 potently inhibited the pulmonary inflammatory response, as shown by reduction in parameters of inflammation determined in bronchoalveolar lavage fluid and the oedematous signal detected by MRI. The inhibitory effects on pulmonary inflammation observed following pretreatment with Compound 1 seem likely to be due to blockade of the enzymatic activity of p38, and more probably of p38α rather than p38β2 activity (Herlaar and Brown, 1999; Schafer et al., 1999). However, Compound 1 also inhibits JNK activity (especially JNK2). We therefore can not rule out that blockade of JNK2 pathways, alone or in combination, may represent a mechanistic basis of action of Compound 1.

Similar inhibitory effects on the upregulation of markers of inflammation in bronchoalveolar lavage fluid and the generation of a lung oedematous signal detected by MRI were observed in animals treated with mometasone furoate 3 h prior to challenge with ovalbumin. However, although MRI signals significantly correlate to the inflammatory parameters determined in the bronchoalveolar lavage fluid following challenge with ovalbumin (Tigani et al., 2002), they probably better reflect the inflammatory status of the lung tissue. In particular, MRI signals highly significantly correlate to the perivascular oedema determined histologically (Tigani et al., 2003). Thus, the fully blockade of MRI signals following pretreatment with mometasone furoate reflects the reduction in perivascular oedema, but not necessarily means an inhibition of cell influx and activation by the same factor as oedema was reduced (Beckmann et al., 2001; Tigani et al., 2003). The data for mometasone furoate are in accord with previous results obtained with budesonide (Beckmann et al., 2001).

To further investigate whether p38 MAP kinase inhibitors may have potential as orally active anti-inflammatory agents in asthma, the effects of Compound 1 on the oedema associated with established pulmonary inflammation were determined. To evaluate this, we used an approach described

recently which showed that budesonide, a clinically used glucocorticosteroid, given 24 h after the challenge with ovalbumin, increased the rate of resolution of the established oedematous signal detected by MRI (Beckmann et al., 2001; Tigani et al., 2003). In the present study, mometasone furoate also accelerated the oedematous signal resolution, with a trend observed already 3 h after drug administration and a statistically significant effect from 6 to 48 h. In contrast, Compound 1 given p.o. had no effect on the established oedematous signal. Although the mechanism of the increased resolution of the existing oedematous signal induced by glucocorticosteroids is not understood (for a discussion on possible mechanisms, see Tigani et al., 2003), it is clear from the present observations that a fundamental difference exists between glucocorticosteroids and MAP kinase inhibitors in this respect.

In conclusion, despite having ''steroid-like'' anti-inflammatory effects when administered before challenge with ovalbumin, the concept of a ''steroid-like profile'' for p38 MAP kinase inhibitors remains under question because, unlike glucocorticosteroids, resolution of the oedematous signal associated with established pulmonary inflammation is not seen in this model.

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