Pharmacological profile of PKF242-484 and PKF241-466, novel dual inhibitors of TNF-α converting enzyme and matrix metalloproteinases, in models of airway inflammation

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Introduction

Chronic obstructive pulmonary disease and asthma are two inflammatory disorders of the airways. Chronic obstructive pulmonary disease is characterized by airway obstruction that is both, slowly progressive and irreversible. This disease encompasses two related conditions; chronic bronchitis and emphysema. Chronic bronchitis is characterized by mucus hypersecretion and an increased risk of chest infections, while the hallmark of emphysema is progressive airflow limitation, largely due to enzymatic destruction of elastin fibres in the lung parenchyma. Both conditions have an inflammatory component which is characterized by the infiltration of neutrophils and macrophages into the lungs (Jeffery, 1998).

Bronchial asthma is a chronic inflammatory disease of the airways, a prominent feature of which is an intense infiltration of inflammatory cells. Of these, the eosinophils are believed to be the main protagonist in inflicting injury to the bronchial mucosa. This damage contributes to bronchial obstruction, hyperreactivity and to the symptoms of asthma (Djukanovic et al., 1990).

Tumour necrosis factor-alpha (TNF-α) is produced as a 26 kDa integral membrane protein which is translocated to the cell surface (Kriegler et al., 1988). Soluble, 17 kDa TNF-α is released by a membrane enzyme called TNF-α converting enzyme (TACE). Its sequence (Moss et al., 1997) indicates that TACE is a member of the ADAM subfamily of Zn-dependent neutral proteinases (Black et al., 1997). There is considerable homology in primary and tertiary structure between enzymes from the ADAM and the matrix metalloproteinases (MMP) sub-families (Botos et al., 1997). Certain low molecular weight inhibitors of MMP enzymes have been shown to be effective towards TACE and in cellular assays of TNF-α release (Gearing et al., 1994). TNF-α is known to cause airway neutrophilia and eosinophilia in various animal models and humans and inhibition of TNF-α is in general associated with an inhibition of the infiltration of proinflammatory cells into the lung tissue (Kips et al., 1992; Lukacs et al., 1995;...
Zuany-Amorim et al., 1995; Yasui et al., 1995; Conroy et al., 1995). Significantly, increased levels of TNF-α have been found in sputum of patients with either chronic obstructive pulmonary disease (Keatings et al., 1996) or asthma (Shah et al., 1995) suggesting that this cytokine could play an important role in regulating both neutrophil and eosinophil trafficking. Thus, blocking the production of TNF-α would be expected to reduce the infiltration of inflammatory cells into the airways of patients with chronic obstructive pulmonary diseases and asthma and thereby, limit pathological changes induced by these cells in the airways.

MMPs are enzymes that have the ability to degrade most of the extracellular matrix proteins. They are expressed at low levels in normal subjects and their activities are tightly regulated by their natural inhibitors, tissue inhibitors of MMPs (Shapiro, 1994). Increasing evidence indicates a key role for at least some of the MMPs in the development of emphysema in chronic obstructive pulmonary diseases patients (Finlay et al., 1997; Hautamaki et al., 1997; Ohnishi et al., 1998) and lung remodelling in asthmatic subjects (Vignola et al., 2000). Beside their involvement in tissue degradation and/or remodelling, MMPs also play a crucial role in the trafficking of inflammatory cells in vitro (Leppert et al., 1995; Delclaux et al., 1996; Shipley et al., 1996; Okada et al., 1997) and may be involved in inflammatory cell migration in vivo (Kumagai et al., 1999; Shipley et al., 1996).

Based on the above, PKF242-484 and PKF241-466, two new dual inhibitors of both the TNF-α convertase enzyme and MMPs, may be predicted to have the potential to target both the inflammatory component and the tissue destruction and/or remodelling characteristically found in both chronic obstructive pulmonary diseases and asthma patients. In this report we have described the anti-inflammatory activity of PKF242-484 and PKF241-466 in animal models of airway inflammation.

**Methods**

**Enzymes assays**

In vitro inhibition of human recombinant MMP-1, -9, -13 (obtained from V. Ganu, Novartis Pharmaceuticals Corporation, Summit, NJ, U.S.A.) and -2 (Boehringer Mannheim, Germany) as well as rat collagenase (from J. Jeffrey, Albany, NY, U.S.A.) was measured using the fluorescence-quenched peptide substrate Mca-Lys-Pro-Leu-Dpa-Ala-Arg-NH₂ (5 μM) and MMP-concentrations of 0.1 nM in 0.1 M Tris-HCl pH 7.5 containing 0.1 M NaCl, 10 mM CaCl₂, 0.05% Brij-35 and 0.1% PEG6000, at 25°C. Compounds (11 concentrations), enzyme, and buffer were incubated in a black 96-well plate for 1 h. The reaction was started upon the addition of substrate and fluorescence (325/400 nm) was measured for 20 min in a Spectramax Gemini plate reader (Molecular Devices). The rates were fitted to the equation \( v = v_o(1 + [I]/K_i) \), where \( v \) and \( v_o \) are the rates in presence and absence of inhibitor, respectively, and \( K_i \) is the inhibitor constant. The substrate concentration of 5 μM is \(<K_M\) for all MMPs, and no correction for substrate competition is needed. MMP-3 was assayed using Ac-Pro-Leu-Ala(S)-Nvl-Trp-NH₂ and DTNB in 50 mM MES, 10 mM CaCl₂ at pH 6.5 (Stein & Izquierdo-Martin, 1994).

Inhibitors and enzyme were pre-incubated for 3 h, and reaction was followed at 410 nm in a BioTec Elx808 plate reader for 40 min.

**Oxidative burst from human neutrophils and eosinophils**

Blood was obtained from normal individual donors. Granulocytes were separated from mononuclear cells by Ficoll-hypaque gradient centrifugation. Erythrocytes were lysed by two cycles of hypotonic lysis and the remaining granulocytes were either used as enriched neutrophil preparation (>95% neutrophils) or incubated with anti-CD16 coated immunomagnetic particles to purify eosinophils. Magentically labelled neutrophils were then depleted by passing the granulocytes through a MACS (magnetic cell separation) column which resulted in a more than 98% pure eosinophil preparation. Both, neutrophils and eosinophils (5×10⁶ cells well⁻¹ in RPMI 1640 containing 0.1% HSA) were then stimulated with plate-bound human IgG (Sandoglobin coated 96 well microtitre plates, 50 μg well⁻¹) or fMLP (Sigma, Poole, U.K., 10 μM) and the ability of the cells to generate superoxide anions during an oxidative burst was measured using a cytochrome C reduction assay (Sigma, Poole, U.K.). The IgG and fMLP induced changes in substrate consumption were monitored over 60 min and results were calculated as percentage of IgG or fMLP induced cytochrome C reduction in the absence of compounds.

**Proliferation and cytokine production of human peripheral blood lymphocytes**

Mononuclear cells were isolated from the blood of normal individuals by Ficoll-hypaque gradient centrifugation (20 min at 800×g). The interface within the gradient was collected, washed twice in PBS and resuspended in RPMI 1640 supplemented with 10% FCS. Cell density was adjusted to 1×10⁶ cell ml⁻¹. For the proliferation assay, 100 μl of the mononuclear cells suspension was placed in 96 well culture plates and 50 μl of either medium or compounds at the indicated concentrations were added. After a 10 min preincubation, cells were stimulated with 50 μl of anti-CD3 monoclonal antibodies (OKT-3, 100 ng ml⁻¹) and then incubated for 42 h at 37°C in a humidified incubator with 5% CO₂. [3H]-Thymidine was added to the culture plates and after 6 h incorporated radioactivity was measured in an automated liquid scintillation counter. For the cytokine measurements, cultures were prepared in the same way as for the proliferation assays. Supernatants were harvested after 20 h of incubation. The levels of IL-5 and IFN-γ were measured by sandwich ELISA. Antibodies used for measuring IL-5 and IFN-γ were purchased from Pharmingen (San Diego, CA, U.S.A.). In all cases, binding of the second antibody was analysed by stepwise incubation with streptavidin-alkaline phosphatase conjugate (Sigma, Poole, U.K.) and 4-nitrophenylphosphate disodium salts (Sigma, Poole, U.K.). Optical density was measured at 405 nm and cytokine concentration was calculated relative to a standard curve plotted from serial dilutions of standard recombinant mouse IL-5 and IFN-γ, respectively. The sensitivity of the cytokine ELISAs was around 10 pg ml⁻¹.
TNF-α production by human peripheral blood mononuclear cells (PBMC)

Mononuclear cells were isolated as above and resuspended in RPMI 1640 supplemented with 10% FCS to a cell density of 10^6 cell ml^-1. Cells were stimulated with LPS (10 μg/ml, LPS, Salmonella Typhosa) and IFN-γ (50 ng/ml) and supernatants were harvested after 20 h of incubation at 37°C in a humidified incubator with 5% CO2. Concentration of TNF-α in the supernatants was measured by sandwich ELISA using two monoclonal antibodies recognizing different epitopes of the specific cytokine (mAb357/101-4 and biotinylated 2-179/E11, Novartis Pharma, Switzerland). Binding of the second antibody was analysed by stepwise incubation with streptavidin-

Animals

Female BALB/c mice or C57BL/6 (4–5 weeks old) were purchased from Harlan (Oxon, U.K.). The animals were housed in plastic cages in an air-conditioned room at 24°C in a 12 h light–dark cycle. Food and water were available ad libitum. The studies reported here conformed to the U.K. Animals (scientific procedures) Act of 1986.

LPS-induced accumulation of neutrophils into the lung

BALB/c mice were treated intranasally, under halothane/oxygen/nitrous oxide anaesthesia, with 0.3 mg kg^-1 of LPS (Salmonella Typhosa, Sigma, Poole, U.K.) in 50 μl of PBS or with PBS alone. At either 3 or 24 h following provocation, anaesthesia was induced with pentobarbitone sodium (60 mg kg^-1, i.p.).

Ovalbumin-induced lung inflammation

C57BL/6 mice were immunized intraperitoneally with 20 μg of ovalbumin, (grade V, Sigma, Poole, U.K.) in 0.2 ml of Alum (Serva, Heidelberg, Germany) on day 0, and again on day 7. On day 14, animals were exposed, for 20 min, to an aerosol of ovalbumin in PBS (50 mg ml^-1) or PBS alone. Twenty-four hours after the challenge, terminal anaesthesia was induced with pentobarbitone sodium (60 mg kg^-1, i.p.).

Assessment of lung inflammatory cells and bronchoalveolar lavage soluble mediators measurement

After anaesthesia, the abdominal cavity was opened and the animal exsanguinated by withdrawal of blood from a major blood vessel. The trachea was cannulated and bronchoalveolar lavage was performed by washing four times with 0.3 ml of PBS into the lung via the trachea. The fluid was immediately withdrawn and the cell suspension stored on ice. The total cell count was measured and cytospin preparation (Shandon Scientific Ltd, Cheshire, U.K.) prepared. Cells were stained with Diff-Quik (Baxter Dade AG, Dudingen, Switzerland) and a differential count of 200 cells performed using standard morphological criteria. The remaining lavage fluid was centrifuged at 200 × g for 10 min, the supernatant was either used fresh, or aliquoted and stored at –80°C.

Myeloperoxidase activity was measured on fresh bronchoalveolar lavage supernatant using a 96 well plate format colorometric assay. To duplicate 50 μl samples of bronchoalveolar supernatant was added 100 μl of the substrate buffer for 5 min at room temperature (sodium phosphate 50 mM, pH 6.0 containing, 0.5% hexadecyltrimethylammonium bromide, 0.167 mM O-dianiside dihydrochloride and 0.4 mM H2O2). The reaction was stopped with 100 μl of 5% sodium azide in distilled water and the OD read at 450 nm. Results were expressed as Unit ml^-1 using a standard curve established with human leukocyte myeloperoxidase (Sigma, Poole, U.K.).

Elastase activity was measured using a fluorogenic substrate specific for neutrophil elastase, N-methoxyxycyclo-

PKF242-484 and PKF241-466 in lung inflammation models

PKF242-484 and PKF241-466 were synthesized by the department of chemistry (Novartis Pharmaceutical Corporation, Summit, NJ, U.S.A.). For the LPS model, mice were treated intranasally, under halothane/oxygen/nitrous oxide anaesthesia, 30 min before and 6 h after the challenge, with compounds dissolved in PBS. Control mice received 50 μl of sterile PBS. In another experiment mice were treated orally, 1 h before the challenge, using 0.1 ml of polyethyleneglycol 300 as vehicle. For the ovalbumin model, mice were treated via the intranasal route, as described above, 15 min before and 3 h after the challenge.

Data analysis

Data are expressed as mean ± s.e.mean. Statistical compar-
isons were performed using a Kruskal Wallis test with Bonferroni correction for multiple comparison and a P value of less than 0.05 was considered significant.

Results

Activities in vitro

The structures of PKF242-484 and PKF241-466 are shown in Figure 1. Both compounds inhibited various human MMP enzymes and rat collagenase activities at low nanomolar concentrations...
range (Table 1). Both PKF242-484 and PKF241-466 were potent inhibitors of TNF-α release from human PBMC when stimulated in vitro with LPS and IFN-γ. PKF242-484 was about three times more potent than PKF241-466 (Table 2). None of the compounds were able to significantly inhibit the in vitro activation of neutrophils, eosinophils and T-cells by various stimuli (Table 2).

**Effect on LPS-induced lung inflammation**

The in vivo activity of PKF242-484 and PKF241-466 was first assessed in a model of LPS-induced neutrophil infiltration. At 3 h post-challenge, LPS induced a marked increase in neutrophil and lymphocyte numbers concomitant with a decrease in macrophage numbers in the bronchoalveolar lavage fluid. Intranasal administration of both PKF242-484 and PKF241-466 (30 min before the challenge) induced a dose-dependent inhibition of neutrophil and lymphocyte accumulation (Figure 2). Neither compound had an effect on the LPS-induced decrease in macrophage numbers. The LPS-induced TNF-α release in the bronchoalveolar lavage, was fully inhibited by PKF242-484 at the lowest dose used (3 mg kg⁻¹) and thus no dose response could be observed (Figure 3). In agreement with the in vitro results, PKF241-466 was less potent than PKF242-484 in inhibiting the LPS-induced TNF-α production (Figure 3).

Because, neutrophil activation parameters (i.e. myeloperoxidase and elastase activities) could not be measured at the 3 h time point (data not shown), a latter time point, 24 h post challenge was assessed. At this time point, the LPS-induced

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**Table 1**  
Kᵢ values (nM) for the inhibition of matrix metalloproteinases and TACE by PKF241-466 and PKF242-484

<table>
<thead>
<tr>
<th>Assay</th>
<th>PKF241-466 Kᵢ (nM)</th>
<th>PKF242-484 Kᵢ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1 (human collagenase-1)</td>
<td>2.3 ± 0.1 (3)</td>
<td>3.6 ± 0.1 (3)</td>
</tr>
<tr>
<td>MMP-2 (human gelatinase A)</td>
<td>0.7 ± 0.1 (3)</td>
<td>0.1 ± 0.01 (3)</td>
</tr>
<tr>
<td>MMP-9 (human gelatinase B)</td>
<td>25.3 ± 2.4 (3)</td>
<td>0.9 ± 0.1 (3)</td>
</tr>
<tr>
<td>MMP-3 (human stromelysin)</td>
<td>4.0 ± 0.6 (3)</td>
<td>4.5 ± 0.7 (3)</td>
</tr>
<tr>
<td>Rat collagenase</td>
<td>1.7 ± 0.2 (3)</td>
<td>0.04 ± 0.002 (3)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± s.e.mean of the number of experiments indicated in brackets.

**Table 2**  
Effects of PKF241-466 and PKF242-484 on human peripheral blood leukocyte activation

<table>
<thead>
<tr>
<th>Assay</th>
<th>PKF241-466 IC₅₀ (nM)</th>
<th>PKF242-484 IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC TNF-α production</td>
<td>141 ± 100 (4)</td>
<td>56.3 ± 28.4 (3)</td>
</tr>
<tr>
<td>Neutrophil oxidative burst (IgG)</td>
<td>&gt; 5000 (3)</td>
<td>&gt; 5000 (3)</td>
</tr>
<tr>
<td>Neutrophil oxidative burst (fMLP)</td>
<td>&gt; 5000 (3)</td>
<td>1625 ± 487 (3)</td>
</tr>
<tr>
<td>Eosinophil oxidative burst (IgG)</td>
<td>&gt; 5000 (3)</td>
<td>&gt; 5000 (3)</td>
</tr>
<tr>
<td>Eosinophil oxidative burst (fMLP)</td>
<td>&gt; 5000 (3)</td>
<td>&gt; 5000 (3)</td>
</tr>
<tr>
<td>T cell IL-5 production</td>
<td>&gt; 5000 (2)</td>
<td>&gt; 5000 (2)</td>
</tr>
<tr>
<td>T cell IFN-γ production</td>
<td>&gt; 5000 (2)</td>
<td>&gt; 5000 (2)</td>
</tr>
<tr>
<td>T cell proliferation</td>
<td>&gt; 5000 (2)</td>
<td>&gt; 5000 (2)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± s.e.mean of the number of experiments indicated in brackets.
increase in neutrophil and lymphocyte numbers was markedly increased when compared with the 3 h time point. Macrophage numbers were not affected by the LPS challenge. A small, non-significant, eosinophilia was also observed (PBS, 0.01±0.01; LPS, 0.03±0.02; P=0.14). Intranasal administration of both PKF242-484 and PKF241-466 (30 min before and 6 h after the challenge) induced a dose-dependent inhibition of neutrophil and lymphocyte accumulation (Figure 4). In parallel with the number of neutrophils, the levels of myeloperoxidase and elastase activities (Figure 5) were also partially inhibited by both compounds. However, this apparent inhibition of in vivo cellular activation is probably a reflection of the reduced inflammatory cell number. At the 24 h time point, no detectable levels of TNF-α was observed in the LPS-challenged animals (not shown). When applied without LPS challenge, at a dose of 30 mg kg⁻¹, PKF242-484 and PKF241-466 had no intrinsic effect on any of the parameters measured when examined at both the 3 h (Figures 2 and 3) and 24 h time point (Figures 4 and 5).

To assess whether these compounds were also active when given via the oral route, a comparison between oral and intranasal administration of PKF242-484, at 10 mg kg⁻¹, was made in a separate experiment. Groups of six mice were treated orally, 1 h before, and intranasally 30 min before the LPS challenge. Animals were sacrificed at 3 h post LPS challenge. For both routes of administration, PKF242-484 showed a similar profile of inhibition on the cellular infiltration and TNF-α levels (Figure 6).

**Effect on ovalbumin-induced lung inflammation**

Because PKF242-484 had a slightly better profile than PKF241-466 in vivo and in vitro, and in order to further characterize our class of compounds the effect of PKF242-484 in an ovalbumin-driven model of lung allergic inflamma-
Figure 4 Effect of PKF242-484 and PKF241-466 on bronchoalveolar lavage cellular infiltration 24 h after the LPS challenge. Animals were treated intranasally, 30 min before and 6 h after LPS or PBS challenge, with vehicle (Veh) or increasing doses of compounds (3 to 30 mg kg\(^{-1}\)). Data, from two different experiments, each included 6–10 mice per group, are expressed as mean ± s.e.mean. Significance (*) was determined versus vehicle-treated/LPS-challenged group (Veh/LPS).

Figure 5 Effect of PKF242-484 and PKF241-466 on bronchoalveolar lavage myeloperoxidase and elastase activities 24 h after the LPS challenge. Animals were treated intranasally, 30 min before and 6 h after LPS challenge or PBS challenge, with vehicle (Veh) or increasing doses of compounds (3 to 30 mg kg\(^{-1}\)). Data, from two different experiments, each included 6–10 mice per group, are expressed as mean ± s.e.mean. Significance (*) was determined versus vehicle-treated/LPS-challenged group (Veh/LPS).
tion was studied. In both sensitized and PBS-challenged animals, the majority of the cells recovered in the bronchoalveolar lavage were macrophages and lymphocytes (Figure 7). Twenty-four hours after the ovalbumin challenge, an increase in the number of neutrophils, eosinophils and lymphocytes was observed. Ovalbumin challenge did not affect macrophage number but did induce an increase in IL-5 levels in the bronchoalveolar lavage (Figure 7). PKF242-484, given intranasally 15 min before and 3 h after the challenge, significantly suppressed the number of neutrophils, eosinophils and lymphocytes present in the bronchoalveolar lavage of these animals and did not affect the number of macrophages (Figure 7). PKF242-484 had no significant effect on bronchoalveolar lavage IL-5 levels (Figure 7).
Discussion

In this study, murine models of lung inflammation were used to demonstrate the ability of PKF242-484 and PKF241-466, two novel dual TACE and MMP inhibitors, to inhibit TNF-α production whilst concomitantly reducing inflammatory cells trafficking into the lung. Although the in vivo efficacy for both compounds was studied mainly using intranasal dosing, orally active compounds are highly desirable as therapeutics. To assess whether these compounds were also active when given via the oral route, the efficacy of PKF242-484, given both orally or intranasally, in the LPS model was assessed. For both routes of administration, PKF242-484 showed a similar profile of inhibition of lung inflammation, suggesting that this compound has the potential to be an orally active drug.

The in vivo activity of PKF242-484 and PKF241-466 was first assessed in a model of LPS-induced neutrophil infiltration. Several lines of evidence indicate that TNF-α should play a significant role in this model. This study and the work of others (Goncalves de Moraes et al., 1996; Moreland et al., 2001) have shown that, following LPS provocation, a substantial release of TNF-α in the bronchoalveolar lavage is observed. Although these data demonstrated the presence of this cytokine during the early phase of neutrophil recruitment, it does not establish a causal link. Data from studies in mice, demonstrated that lung specific overexpression of TNF-α (Fujita et al., 2001) or direct application of TNF-α into the lung (Goncalves de Moraes et al., 1996) result in a massive influx of neutrophils. Moreover, it was shown that the lung neutrophil influx observed following LPS application in mice could be partially blocked using neutralizing anti-TNF-α antibodies (Goncalves de Moraes et al., 1996) or using TNF receptor p55-deficient mice (Smith et al., 1998). In the present study, when PKF242-484 was administered via the intranasal route, at a dose which was able to fully inhibit the release of soluble TNF-α in the bronchoalveolar lavage (3 mg kg⁻¹), no effect on the neutrophilic influx was observed. This apparent discrepancy can be explained by the fact that PKF242-484 does not inhibit the synthesis of TNF-α but only its release into its soluble form. It is well documented that membrane-bound TNF-α is functionally active. As such, it can mediate inflammation in experimental models of arthritis (Alexopoulos et al., 1997) and hepatitis (Kusters et al., 1997) and has also been shown to be increased in number and functionally more active on the surface of macrophages derived from subjects with acute respiratory distress syndrome (Armstrong et al., 2000). In summary, published data strongly support the idea that TNF-α is an important mediator that accounts for the large neutrophil influx into the airway following inhalation of bacterial products. Our data, together with that of previously published work (Alexopoulos et al., 1997; Kusters et al., 1997; Armstrong et al., 2000) suggests that the elimination of soluble TNF-α alone is not sufficient to abrogate the inflammatory response to LPS.

It was believed that the inhibition of the LPS-induced neutrophil and lymphocyte increases observed with higher doses (10 and 30 mg kg⁻¹) of both PKF242-484 and PKF241-466 is due to their inhibitory activity on the MMPs. Although we do not present any direct evidence of the involvement of these proteases in our model, LPS has been shown both in vitro (Suzuki et al., 2000) and in vivo (Pagenstecher et al., 2000) to induce a dramatic increase the expression of some of the MMPs. In response to LPS, human neutrophils are able to produce gelatinase-B (MMP-9) and this protease has been shown to play a crucial role in neutrophil migration across the basement membrane in vitro (Delclaux et al., 1996). Similarly, migration of human lymphocytes in vitro has also been shown to be highly dependent on the presence of active gelatinases (MMP-2 and -9) (Leppert et al., 1995; Xia et al., 1996). It should be noted that both PKF242-484 and PKF241-466 did not fully inhibit the LPS-induced neutrophilia (a maximum inhibitory effect of 50% observed at 10 mg kg⁻¹ and no further inhibition could be demonstrated at 30 mg kg⁻¹). Whereas, full inhibition was observed for the LPS-induced lymphocyte influx. This suggests that mediators other than MMPs are necessary for neutrophil transmigration following LPS stimulation. Indeed, the neutrophil influx observed following LPS instillation into Golden Syrian hamsters has been shown to be partially dependent on elastase activity (Yasui et al., 1995).

Because PKF242-484 had a slightly better profile in vivo and in vitro and in order to further characterize this class of compounds, it was decided to study the effect of PKF242-484 in an ovalbumin-driven model of lung allergic inflammation. Results obtained showed this compound to be a potent inhibitor of allergen-induced lung inflammatory cell infiltration (e.g. eosinophils, neutrophils and lymphocytes). In a very similar model to the one described in the present study, an increase of the release of MMP-2 and -9 in the bronchoalveolar lavage has been reported following allergen challenge (Kumagai et al., 1999). In the same study it was also shown that following treatment with a synthetic inhibitor of MMP (R-14138) or with natural MMP inhibitors such as tissue inhibitor of metalloproteinase-1 and -2, a reduction in the antigen-induced infiltration of lymphocytes and eosinophils into the lungs was observed (Kumagai et al., 1999). The role of TNF-α in this model is more controversial. Although it is clear that allergen challenge-induced an early increase in this cytokine (Lukacs et al., 1995; Zuzany-Amorim et al., 1995; Escott et al., 2000), the importance of TNF-α in the development of the subsequent inflammatory cell infiltration appears to differ depending on the specific phase of the allergic inflammation. The inhibition of TNF-α activity, using a TNF-α receptor fusion protein (Lukacs et al., 1995; Renzetti et al., 1996) or a neutralizing anti-TNF-α antibody (Zuzany-Amorim et al., 1995), was reported to reduce eosinophil recruitment into the lung following a single allergen challenge. However, if a multiple challenge protocol is used, TNF-α neutralization does not have a significant effect on the extent of lung inflammation (Hessel et al., 1997; Rudmann et al., 2000). Similar conclusions were drawn using mice deficient in TNF-α receptors (Rudmann et al., 2000). These published data, suggest that neutralization of TNF-α activity only transiently abrogates the inflammatory response following allergen challenge. In our model, which used a single allergen challenge, it is therefore difficult to distinguish the effects of PKF242-484 due to its inhibitory activity on TACE or the MMPs. The most likely explanation is that PKF242-484 inhibits the inflammatory reaction by acting on both families of enzymes. Cytokines of the Thelper 2 type, such as IL-5, are believed to play a central role in the induction of allergic airway inflammation (Anderson & Coyle, 1994). Interestingly, in our model, the allergen-induced
increase in bronchoalveolar lavage IL-5 levels was not affected by treatment with PKF242-484, whereas the inflammatory cell infiltration was significantly reduced. These results are in accordance with the in vitro data obtained following T-cells activation, that the inhibitory action of PKF242-484 on inflammatory cell infiltration was not due to a down regulation of IL-5.

One concern in using dual TACE/MMP inhibitors in the clinic is their possible pro-inflammatory properties. Indeed, in vitro, this class of compounds have been shown to decrease the ability of stimulated cells to shed various pro-inflammatory membrane bound proteins such as IL-6 receptor (Mullberg et al., 1995), IL-1 decoy receptor (Orlando et al., 1997) and L-selectin (Feehan et al., 1996). In addition, upon activation and when compared with wild type cells, TACE-deficient mice cells showed an increase in surface TNF-α expression (Black et al., 1997) and failed to shed the type II TNF-α receptor (Peschon et al., 1998). These observations, have been confirmed using synthetic inhibitors in vitro (Mohler et al., 1994; McGeehan et al., 1994; Williams et al., 1996). However, in contrast to these experimental situations, in humans, undesired proinflammatory effects such as enhanced expression of surface TNF-α or surface TNF-α receptor were not observed following a single dose treatment with a TACE/MMP inhibitor (Dekkers et al., 1999). Nevertheless, since TACE/MMP inhibitors are likely to be used in the treatment of various inflammatory diseases in the future, it is important to fully address their possible proinflammatory properties before this class of compound is tested in clinical setting.

In summary, we have demonstrated preclinical in vitro and in vivo evidence to suggest that PKF242-484 and PKF241-466, the novel combined TACE and MMP inhibitors, may have potential for the treatment of inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease.

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