PMX464, a thiol-reactive quinol and putative thioredoxin inhibitor, inhibits NF-κB-dependent proinflammatory activation of alveolar epithelial cells

ME Callister1, L Pinhu1, MC Catley2, AD Westwell3, R Newton4, SK Leaver1, GJ Quinlan1, TW Evans1, MJ Griffiths1 and A Burke-Gaffney1

1Critical Care, Critical Care, Pulmonary Vascular and Sleep Science, Respiratory Science, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, London, UK; 2Respiratory Pharmacology, Airways Disease, Respiratory Science, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, London, UK; 3Welsh School of Pharmacy, Cardiff University, Cardiff, UK and 4Department of Cell Biology and Anatomy, University of Calgary, Alberta, Canada

Background and purpose: Subtle changes in the intracellular reduction–oxidation (redox) state can modulate nuclear factor-κB (NF-κB) activity. Thioredoxin-1 (Trx) is a small, ubiquitous, redox-active thiol (-SH) protein that, with thioredoxin reductase-1 (TrxR), modifies the redox status of NF-κB pathway components. PMX464 is a novel thiol-reactive quinol thought to inhibit the Trx/TrxR system. The aim of this work was to investigate whether PMX464 inhibited NF-κB-mediated proinflammatory activation of human type II alveolar epithelial cells (A549).

Experimental approach: Intercellular adhesion molecule-1 (ICAM-1), granulocyte-macrophage colony-stimulating factor (GM-CSF) and CXCL8, NF-κB DNA binding, nuclear translocation of NF-κB p65 subunit, IκBα degradation, IκB phosphorylation and IκB kinase (IKK) activity were assessed in A549 cells stimulated with IL-1β with or without PMX464 pretreatment. Effects of PMX464 on ICAM-1 expression in human lung microvascular endothelial cells (HLMVEC) were also investigated. For comparison, selected measurements (ICAM-1 and IκB-α phospho-IκB-α) were made on A549 cells after RNA interference-mediated silencing (siRNA) of Trx.

Key results: PMX464 reduced ICAM-1, GM-CSF and CXCL8 expression in IL-1β-stimulated A549 cells and ICAM-1 in HLMVEC. PMX464 inhibited IL-1β-induced NF-κB DNA binding, nuclear translocation of NF-κB p65 subunit, IκBα degradation, IκB phosphorylation and IκB kinase (IKK) activity were assessed in A549 cells stimulated with IL-1β with or without PMX464 pretreatment. Effects of PMX464 on ICAM-1 expression in human lung microvascular endothelial cells (HLMVEC) were also investigated. For comparison, selected measurements (ICAM-1 and IκB-α phospho-IκB-α) were made on A549 cells after RNA interference-mediated silencing (siRNA) of Trx.

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Conclusion and implications: PMX464 inhibits a proinflammatory response in A549 cells targeting the NFκB pathway above IKK. The lack of effect with Trx siRNA suggests that PMX464 acts on thiol proteins, in addition to Trx, to elicit anti-inflammatory responses in lung epithelial cells.

British Journal of Pharmacology (2008) 155, 661–672; doi:10.1038/bjp.2008.258; published online 30 June 2008

Keywords: PMX464; quinol; A549 epithelial cells; ICAM-1; CXCL8; GM-CSF; thioredoxin; thioredoxin reductase; NF-κB pathway; Trx siRNA

Abbreviations: GM-CSF, granulocyte macrophage-colony stimulating factor; HLMVEC, human lung microvascular endothelial cells; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IKK, IκB kinase ; IL-1β, interleukin-1β; IκB, inhibitor of NF-κB; NF-κB, nuclear factor κB; siRNA, short interference RNA; TNF, tumour necrosis factor; Trx, thioredoxin, ; TrxR, thioredoxin reductase

Introduction

The transcription factor NF-κB plays a pivotal role in the expression of a wide range of genes involved in lung inflammation, including the chemokine CXCL8 (interleukin-8; IL-8), granulocyte macrophage-colony stimulating factor (GM-CSF) and intercellular adhesion molecule-1 (ICAM-1) (Lentsch and Ward, 2000). In unstimulated cells, NF-κB proteins are sequestered in the cytosol through interactions with inhibitory proteins (IκBα, β and ε). Tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1β), bacterial products and oxidants cause phosphorylation and degrada-
tion of IκB proteins through the ubiquitination-proteosome pathway. Free NF-κB enters the nucleus and induces gene expression. Phosphorylation of IκB by IκB kinase (IKK) complex is a point at which diverse signals converge to activate NF-κB. Key components of the IKK complex are IKK-α and IKK-β, also termed IKK-1 and -2 (Ghosh and Karin, 2002). Of the drug development strategies currently being pursued for inhibition of the NF-κB pathway, targeting IKK-β activity shows considerable potential as an anti-inflammatory therapeutic strategy (Birrell et al., 2005; Catley et al., 2005).

Subtle changes in intracellular reduction–oxidation (redox) state also modulate NF-κB activity and thus impact on the pulmonary inflammatory response (Rahman et al., 2006). Expression of thioredoxin (Trx), a small, ubiquitous thiol [-SH] protein involved in intracellular redox control, is raised during conditions associated with lung inflammation (Koura et al., 2000; Titto et al., 2003; Callister et al., 2006) and Trx is also known to regulate components of the NF-κB pathway (Lillig and Holmgren, 2007). The key to Trx redox activity lies in the two cysteine residues (Cys32 and Cys35) separated by two amino acids (Gly-Pro) in the active site. These cysteines exist as a dithiol (-[SH]2) in the reduced form and a disulphide (-S-S) in the oxidized form. Trx is oxidized when it transfers ‘reducing equivalents’ to disulphide groups in target proteins and is reduced back to the dithiol form by an nicotinamide adenine dinucleotide phosphate (reduced form); (NADPH)-dependent flavoprotein, thioredoxin reductase. Altogether, these enzymes and cofactors form the ‘trx system’ (Burke-Gaffney et al., 2005).

In the past decade, biological screening has identified several small organic compounds that inhibit the Trx system. PMX464 (previously AW464) is one of a group of (hetero) aromatic 4-hydroxycyclohexa-2,5-dienones (‘quinols’), which inhibit Trx redox cycling by forming an irreversible complex with the active-site thiol groups in the reduced form of Trx (Wells et al., 2003). Previous studies with PMX464 have shown antiproliferative effects on tumour cell lines and endothelial cells, in part, through the effects on hypoxia inducible factor-1a transcription factor and vascular endothelial growth factor release (Mukherjee et al., 2005, 2007). In addition to studies with pharmacological inhibitors, recent advances in post-transcriptional gene silencing using short interference RNAs (siRNAs), have provided another approach to downregulating expression of intracellular proteins and thus for evaluating the role of the Trx/TrxR system in cellular processes (Gorreta et al., 2005; Ravi et al., 2005; Freeman and Neuzil, 2006; Trigona et al., 2006).

The aim of the current study was to investigate the effect of PMX464 on the expression and function of markers of inflammation in the human alveolar epithelial cell line, A549. PMX464 reduced function and/or expression of ICAM-1, CXCL8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) and also activation of the NF-κB pathway subsequent to the inhibition of IKK activity. Likewise, PMX464 reduced ICAM-1 expression/function in normal human lung microvascular endothelial cells (HLMVEC). However, as Trx siRNA did not inhibit ICAM-1 expression or IκB-α degradation/phosphorylation in IL-1β-stimulated A549 cells, these results suggest that PMX464 has anti-inflammatory effects in lung epithelial cells that could be, at least in part, independent of direct effects on Trx.

Materials and methods

Cells

Human type II alveolar cell lines (A549, ECACC 86012804, European Collection of Cell Cultures, Porton Down, Salisbury, Wiltshire, UK) were maintained as described previously (Burke-Gaffney and Hellewell, 1996). 6xB cells, A549 cells harbouring the 6xB.k luciferase reporter, were prepared as described previously (Bergmann et al., 1998). HLMVEC were purchased from Cambrex (Wokingham, UK) and were maintained as previously described (Blese et al., 1999).

Cell viability

Viability was assessed using a method based on the mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to formazan as described previously (Burke-Gaffney and Hellewell, 1996). Preliminary screening experiments were carried out to select effective inhibitor concentrations and conditions that were not toxic to cells. Thus, for A549, cells were treated with PMX464 (10 and 30 μM) for 30 min and then the inhibitor was removed before cytokine treatment for 24 h. By contrast, HLMVEC were preincubated with PMX464 (0.6, 1 μM) for 30 min followed by the addition of cytokines to the PMX464 for 24 h. The difference in protocols could reflect a previously published observation that A549 cells are more resistant than endothelial cells to intracellular redox disturbance (Dandrea et al., 2002) and are therefore likely to require higher concentrations of PMX464 to disrupt inflammatory signalling.

ELISA measurement

A549 were seeded at 10⁴ cells cm⁻² into 96-well plates and after 2 days confluent monolayers were treated for 30 min with PMX464, which was then removed and IL-1β, TNF-α and interferon-γ (IFN-γ) added for 24 h. HLMVEC were seeded at 4.7 × 10⁴ cells cm⁻² and were confluent 4–5 days later. Supernatants were collected and assayed by ELISA for secretion of CXCL8 or granulocyte-macrophage colony-stimulating factor (GM-CSF) (R & D Systems, Abingdon, Oxford, UK). After removal of supernatants, ICAM-1 was detected by ELISA on the cell surface as described previously (Blese et al., 1999). A549 cells were also plated at 3.1 × 10⁴ cells cm⁻² in 6-well plates and at confluence were treated with PMX464 or AS602868 for 30 min and then IL-1β for 1 h. Whole cell extracts were prepared and ELISA based Trans-AM kits (Active Motif, Rixensart, Belgium) used to assess NF-κB p50 and p65 binding activity according to the manufacturer’s protocol (Gupta et al., 2002). Results are expressed as optical density (OD)₄₀₅ per milligram protein.

RNA isolation and reverse transcriptase-PCR

Total cellular mRNA was isolated from unstimulated, IL-1β, PMX464 and IL-1β plus PMX464 treated cells using
Tri-reagent (Chomczynski and Sacchi, 1987). The mRNA was converted into cDNA and regions of interest amplified using PCR as described previously (Blease et al., 1999). PCR fragments were resolved using agarose gel (1%) electrophoresis. Values for ICAM-1 expression were determined by densitometry and normalized to β-actin mRNA levels for each experimental condition.

**Adhesion and migration assays**

Neutrophils isolated from human peripheral blood by discontinuous plasma/percoll density gradient centrifugation were labelled with a fluorescent dye, Calcein-AM, as described previously (Blease et al., 1999). For adhesion assays, labelled neutrophils were resuspended at 1.25 × 10⁶ cells mL⁻¹ and 100 μL added to A549 monolayers that had been pretreated (and stimuli removed) with IL-1β plus or minus PMX464. Adhesion was measured and expressed as percent adherent neutrophils, as described previously (Blease et al., 1999). For migration assays, supernatants from A549 monolayers treated for adhesion experiments were diluted 1:10 and placed in wells of ChemoTx chemotaxis plates (Neutroprobe, Receptor Technologies, Gaithersburg, MD). Labelled neutrophils (25 μL of 3 × 10⁶ cells mL⁻¹) were placed on porous membranes (0.8 μm pores) positioned above wells containing stimuli or conditioned medium. Total fluorescence of 25 μL of labelled neutrophils and also fluorescence in wells after 60 min of migration was measured using a Biolite F1 plate reader (Labtech International, Ringmer, East Sussex, UK). Migration was expressed as percentage of neutrophils migrating to the lower chamber.

**Luciferase assay**

6xB cells were plated at 5 × 10⁴ cells cm⁻² in 24-well plates and treated with IL-1β or TNF-α (8 h) plus or minus PMX464 or the IKK-2 inhibitor, AS602868. Luciferase activity was measured in cell lysates using luciferase substrate (Promega, Offenburg, Germany) and normalized to the total protein concentration for each well. Results were expressed as luciferase activity units (mg protein)⁻¹.

**Immunoblotting**

Cells were plated at 3.1 × 10⁴ cells cm⁻² into 6-well plates, treated with medium or PMX464 for 30 min followed by medium or IL-1β for times between 5 min and 3 h. Cell lysate was separated on 4–12% SDS-polyacrylamide gel electrophoresis and probed with mouse polyclonal antihuman IkB-α (1:1000 dilution), mouse polyclonal antihuman phospho-IkB-α (1:2000 dilution) and mouse polyclonal antihuman β-actin (1:4000 dilution). The intensity of signal was quantified using densitometry and the values expressed as a ratio of β-actin band density.

**Kinase assay**

The in vitro kinase assay was performed as described previously (Catley et al., 2004). A549 cells in 6-well plates were incubated for 30 min with medium, PMX464 or PS-1145 (a specific and reversible IKK-2 inhibitor) followed by IL-1β for 3 min. Cells were then washed and cytoplasmic extracts prepared and precleared for 1 h using normal goat IgG agarose conjugated antibody. IKK-γ was precipitated from 200 μg of total protein using an agarose conjugated goat antihuman IKK-γ antibody for 2 h at 4°C. The immunoprecipitate was incubated at 37°C for 1 h in kinase reaction buffer containing [γ⁻³²P] ATP (80 μCi mL⁻¹; Amersham Bioscience, Little Chalfont, Buckinghamshire, UK) and a recombinant IKK substrate peptide (Upstate Biotechnology, Charlottesville, VA, USA). In selected experiments, PMX464 and PS-1145 were added directly to the kinase assay. Reactions were stopped and samples run on 12% NUPAGE SDS-PAGE gels (Invitrogen, Carlsbad, CA, USA). The gels were then cut at the 28 KDa marker and the upper portion analysed by Western blotting to determine equal loading of IKK-2, whereas the lower portion of the gel containing the substrate peptide was analysed by autoradiography.

**Thioredoxin activity assay**

The redox activity of pure Trx or that in lysates of A549 cells was assessed using a modification of the insulin reduction assay described by Holmgren and Bjornstedt (1995). Briefly, Trx or cell lysate together with PMX464 were added to a reaction mixture containing 6.4 mg mL⁻¹ insulin and incubated at 37°C for 20 min. The reaction was stopped and OD₄₁₂ and corrected for protein content of cell lysate samples.

**Transfection with siRNA**

Two target-specific siRNAs against Trx were designed and synthesized by Dharmacon (Lafayette, CO, USA), sense, GUGAUAAACUGUGAUAGUUA; and Santa Cruz Biotechnology (a pool of three strands against Trx mRNA; sense, CAUCUCUGUGCAAAAGAT; GAAGAGUGGCAUCCAU GAATT, and GGGAUCAACUCUGACGCAAATT). As a control, two non-targeting ‘scrambled’ siRNA were used (Dharmacon and Santa Cruz). Cells were seeded at 2 × 10⁴ cells per well onto 24-well plates and were transfected at 30–40% confluence approximately 16–24 h later, using a mixture of
siRNAs from Dharmacon and Santa Cruz (200 nM) with the Genesilencer siRNA transfection reagent (Genlantis, San Diego, CA, USA), according to the manufacturer’s instructions. Cells were either harvested 72 h after transfection and 20 µg of protein analysed by western blotting to confirm knockdown or stimulated for 5 min or 24 h with IL-1β (0.3 ng mL⁻¹) before measuring IkB-α degradation and phospho-IκB-α (5 min) or ICAM-1 (24 h).

Statistics
Results are expressed as mean ± s.e.m. of n experiments. Statistical analysis was carried out using a one-way ANOVA followed by the Bonferroni post-test. Data were log transformed before analysis, if variances were significantly different by Bartlett’s test. Graphpad Prism version 3.03 (GraphPad, San Diego, CA, USA) was used to perform statistical analysis; results were deemed significant if P<0.05.

Recombinant proteins and reagents
Human IL-1β, TNF-α and IFN-γ were purchased from Roche, (Lewes, East Sussex, UK). PMX464 (4-(benzothiazol-2-yl)-4-hydroxycyclohexa-2,5-dien-1-one hydrate) was synthesized according to a previously described method (Wells et al., 2003). 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide was from Sigma Aldrich (Poole, Dorset, UK). Rabbit polyclonal antihuman IkBα (sc-371), rabbit polyclonal antihuman p65 antibody (sc109) and agarose conjugated goat antihuman IKK-γ antibody were from Autogen Bioclear (Calne, Wiltshire, UK); mouse polyclonal antihuman phosphorylated (Ser 32 and 36) IκB-α (New England Biolabs, Hitchin, Hertfordshire, UK); and mouse polyclonal antihuman β-actin (AC-15, Sigma Aldrich); anti-Trx antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); donkey polyclonal antirabbit IgG antibody conjugated to Alexa 488 (Calne, Wiltshire, UK); mouse polyclonal anti-rabbit IκB-α (Ac-15, Sigma Aldrich); anti-Trx antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); donkey polyclonal antirabbit IgG antibody conjugated to Alexa 488 and Calcein-AM (Cambridge Bioscience, Cambridge, UK); PS-1145 (N-(6-chloro-9H-carbonyl-8-ly) nicotinamide) was a gift from Millennium Pharmaceuticals (Cambridge, MA, USA) and AS602868 from Serono (Geneva, Switzerland). AS602868 is an anilinopyrimidine derivative and ATP competitor, which inhibits IKK-2 (patent application PCTWO 02/46171). Human Trx was from IMCO (Stockholm, Sweden).

All drug and molecular target nomenclature conforms to the British Journal of Pharmacology’s Guide to Receptors and Channels (Alexander et al., 2008).

Results

PMX464 inhibits proinflammatory gene expression and function in A549 alveolar epithelial cells
Constitutive ICAM-1 was detected on non-stimulated A549 cells. Submaximal concentrations of cytokines were chosen to activate A549 cells, based on preliminary experiments (data not shown) and our previously published study (Burke-Gaffney and Hellewell, 1996), to facilitate the detection of inhibitory effects with PMX464. Incubation with TNF-α 2 ng mL⁻¹), IL-1β (0.3 ng mL⁻¹) and IFN-γ (5 ng mL⁻¹) significantly (P<0.001) increased ICAM-1 expression (Figure 1a). Pretreatment with 10 and 30 µM PMX464 reduced IL-1β- and TNF-α-, but not IFN-γ-induced, ICAM-1 expression (Figure 1a). PMX464 abolished IL-1β-induced...

![Figure 1](https://example.com/image1.png)

**Figure 1** PMX464 reduces expression of ICAM-1 protein and mRNA in A549 cells. A549 monolayers were preincubated with medium or PMX464 (10, 30 µM) for 30 min, then exposed to TNF-α (2 ng mL⁻¹), IL-1β (0.3 ng mL⁻¹), IFN-γ (5 ng mL⁻¹) or medium for 24 h. ICAM-1 expression was determined by ELISA and data are shown as mean ± s.e.m. (n=5; a). A549 monolayers were also preincubated with medium or PMX464 (30 µM) for 30 min, then exposed to IL-1β (0.3 ng mL⁻¹) or medium for 4 h. ICAM-1 mRNA levels were quantified by RT-PCR and compared to levels of β-actin mRNA and data shown as a representative blot for ICAM-1 and β-actin mRNA (b) and the ICAM-1/β-actin ratio (c) (normalized to the ratio for medium alone, n=5, o). *P<0.05, **P<0.01, ***P<0.001, asterisks above the bars indicate comparison with basal expression and those on connecting lines indicate comparison between these conditions. ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IL, interleukin, RT-PCR, reverse transcriptase-PCR.
ICAM-1 mRNA levels measured at 4 h; mRNA levels of the control β-actin were not altered (Figures 1b and c). PMX464 also reduced IL-1β- and TNF-α-induced expression of CXCL8 and GM-CSF in A549 (Figures 2a and b).

A functional consequence of ICAM-1 expression is neutrophil adhesion. The percentage of neutrophils adherent to A549 cells pretreated with PMX464 and IL-1β (0.3 ng mL⁻¹) was significantly less than that to A549 cells exposed only to IL-1β (Figure 3a). A functional consequence of CXCL8 release is neutrophil migration. Supernatants collected from PMX464/IL-1-β-treated A549 cells caused significantly less neutrophil migration than the supernatants from A549 cells treated only with IL-1β (0.3 ng mL⁻¹; Figure 3b). Neither IL-1β nor PMX464 had any direct effect on neutrophil migration (data not shown).

Inhibitory effects with PMX464 were not limited to A549 alveolar epithelial cells as treatment of HLMVEC with PMX464 also reduced cytokine-induced ICAM-1 expression (Table 1). As seen with A549 cells, PMX464 (1 μM) also significantly (P<0.05) reduced IL-1-β (1 ng mL⁻¹) induced ICAM-1 mRNA in HLMVEC from an ICAM-1/β-actin ratio of...
2.55 ± 0.48 to 1.35 ± 0.27 (n = 6). Likewise, neutrophil adhesion to IL-1β/PMX464-treated HLMVEC monolayers was also significantly (P < 0.01) reduced (8.3 ± 0.3% adhesion, n = 4) compared with adhesion to HLMVEC treated with IL-1β alone (19.4 ± 2.9% adhesion, n = 4). PMX464 also inhibited CXC8 release from HLMVEC (data not shown).

PMX464 inhibits NF-κB activation in A549 cells

We have reported previously using a variety of techniques, including small molecule inhibitors of IKK, that IL-1β-induced ICAM-1 and CXC8 expression in A549 cells is NF-κB dependent (Catley et al., 2005; Newton et al., 2007). In this study we investigated the effect of PMX464 on NF-κB activation in A549 cells. First, we looked at the effect of PMX464 on NF-κB-dependent transcription in A549 cells containing an NF-κB-dependent reporter gene (6κB cells). PMX464 and for comparison AS602868, an IKK-2 inhibitor, reduced IL-1β- (0.3 ng mL⁻¹), and TNF-α-induced luciferase reporter activity back to basal levels (Figure 4). Next, we assessed the inhibitory effect of PMX464 on the DNA binding activity of NF-κB p65 and p50 subunits present in A549 cell lysates using Trans-AM transcription factor assays. Binding of both p65 and p50 increased in cells treated for 1 h with IL-1β (0.3 ng mL⁻¹), compared to control cells, and preincubation with PMX464 abolished p65 and p50 binding activity (Figure 5a). Basal binding activity was also reduced with PMX464 alone. Similar effects were seen with AS602868 (Figure 5b).

Immunostaining and confocal microscopy were used to determine the subcellular distribution of p65 in A549 cells and the effect of PMX464 on its distribution. In resting and PMX464-treated cells, the p65 subunit was located in the cytoplasm, whereas in IL-1β- (0.3 ng mL⁻¹) treated cells, p65 was detected in the nucleus. When cells were pretreated with PMX464 followed by IL-1β, p65 remained in the cytoplasm (Figure 6). Similarly, cells pretreated with AS602868 before IL-1β also showed that p65 remained, predominantly, in the cytoplasm (Figure 6). The reason for the brighter red staining of the cytoplasm in cells treated with AS602868 is not clear. However, in this and two further experiments, AS602868 and PMX464 clearly prevented translocation of p65 into the nucleus of A549 cells.

### Table 1

| Medium (OD₄₅₀) | TNF-α (OD₄₅₀) | IL-1β (OD₄₅₀) |
|---------------|---------------|---------------|
| Medium        | 0.16 ± 0.02   | 0.98 ± 0.05   | 0.86 ± 0.05   |
| PMX464 0.6 μM | 0.11 ± 0.02   | 0.77 ± 0.04** | 0.69 ± 0.03*  |
| PMX464 1 μM   | 0.12 ± 0.02   | 0.62 ± 0.04***| 0.57 ± 0.03***|

Abbreviations: HLMVEC, human lung microvascular endothelial cells; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; TNF, tumour necrosis factor.

PMX464 inhibits NF-κB activity in A549 cells containing an NF-κB-dependent reporter gene (6κB cells). A549 cells stably transfected with the NF-κB reporter, 6NF-κB.luc (6κB), were preincubated for 30 min with PMX464 (30 μM), IL-1β (0.3 ng mL⁻¹) or medium, and then for 8 h with TNF-α (2 ng mL⁻¹) or medium. Luc activity for each condition (corrected for protein) was expressed as a proportion of baseline luc activity and data are expressed as mean ± s.e.m. (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 when compared to ICAM-1 expression with cytokine but without PMX464.

PMX464 inhibits rapid degradation and phosphorylation of IκB-α in IL-1β-treated A549 cells

NF-κB activity is controlled by the steady state level of IκB. Thus, we analysed the effect of PMX464 on IL-1β-induced IκB degradation by western blotting. IL-1β (0.3 ng mL⁻¹) induced a rapid loss of IκB with reexpression after 60 min (Figure 7). PMX464 prevented the rapid, initial loss of IκB in IL-1β-treated cells and, instead, induced a more gradual decline in protein levels over a 6 h period.

A key step required before degradation of IκB-α is IκK-2-dependent phosphorylation of the N-terminal residues Ser32 and Ser36. Using a specific IκK-2 phosphoserine antibody, we showed that phosphorylated IκBα was detected in IL-1β (0.3 ng mL⁻¹) treated cells at 5 or 15 min and also at 2 h (Figure 8). PMX464 prevented IL-1β-induced phosphorylation of IκB-α at all times between 5 min and 3 h (Figure 8).

PMX464 inhibits activation of processes upstream of the IKK complex in IL-1β-treated A549 cells

A decrease in IL-1β-induced IκBα phosphorylation with PMX464 suggests a possible effect on IKK complex activation. To investigate this, the IKK complex was immunoprecipitated from A549 cells and ability of the complex to phosphorylate a substrate peptide was assessed using kinase assays. IL-1β- (0.3 ng mL⁻¹) treated cells showed high levels of IKK activity, whereas activity from cells pretreated with
PMX464 (before IL-1β) was substantially reduced (Figure 9). Experiments in which treatments are added onto cells rather than directly onto the kinase assay are marked as OC in Figure 9. Treatment of A549 cells with the specific IKK-2 inhibitor, PS-1145 (Hideshima et al., 2002; Castro et al., 2003), did not inhibit IKK complex activity (Figure 9, OC), because it is a reversible inhibitor that washes off the complex during purification. These results suggest that PMX464 works as either a non-reversible inhibitor of the IKK complex or inhibits activation of IKK complex at an upstream step.

To investigate this, the IKK complex was immunoprecipitated from IL-1β- (0.3 ng mL⁻¹) treated A549 cells and then added onto kinases assays with or without PMX464 or PS-1145. These treatments are indicated OA in Figure 9. Applied in this manner, PS1145 but not PMX464 inhibited IKK activity (Figure 9). This suggests that PMX464 does not inhibit the IKK complex, directly, but most likely inhibits IKK activity at some upstream step. Similar data were obtained when TNF-α (2 ng mL⁻¹) replaced IL-1β in these experiments (data not shown). The upstream process inhibited is thus likely to be common to the IL-1β and TNF-α pathways.

PMX464 is unlikely to be a general suppressor of cell signalling as phosphorylation of p38 and ERK in PMX464/IL-1β-treated A549 was not decreased compared to treatment with IL-1 (0.3 ng mL⁻¹) alone (data not shown). Moreover, PMX464 is not an indiscriminate thiol inhibitor, because it fails to inhibit ficin a proteolytic enzyme, which contains an essential thiol (unpublished data, personal communication Dr T Bradshaw, University of Nottingham). In summary, these data suggest that PMX464 inhibited IL-1β-induced NF-κB activation by preventing processes leading to the activation of the IKK complex.

Trx siRNAs did not inhibit IL-1β-induced CXCL8 release, nor ICAM-1 expression or IKK activity in A549

One likely candidate for the upstream target of PMX464 is Trx. As previously reported (Pallis et al., 2003), we confirmed that the preincubation of Trx with PMX464 (30 μM) inhibited redox activity of Trx by 68% (n = 5). The IC₅₀ for this reaction was calculated to be 20 μM. Moreover, we also showed that when A549 cells were treated for 30 min with PMX464 (30 μM), the cell lysates showed significantly diminished (26%, P < 0.01) insulin reducing activity compared with the activity in untreated A549 cells (n = 6). These results suggest that PMX464 inhibits, at least in part, redox activity of the Trx system in A549 cells.

By western blotting, we showed that a combination of two different siRNA sequences that specifically target Trx lowered Trx in A549 by 70% (Figures 10a and b). However, neither intracellular levels of IκB-α or phosphorylated IκBα nor expression of ICAM-1 changed in siRNA-treated cells despite positive effects with PMX464 and AS602868 (Figures 10b and 10c).
IL-1 (interleukin; NF-κB) when conditions with and without PMX464 were compared using repeated measures ANOVA with mixed model to confirm results. IL, interleukin; NF-κB, nuclear factor-κB.

However, A549 cells did not display overt reductions in cell viability when Trx content was reduced with siRNA nor did a control, scrambled siRNA, have any effect on Trx protein expression (data not shown).

**Discussion**

Accumulation of neutrophils in the lung is a key event in the onset and development of lung inflammation and injury. Thus, blocking the processes contributing to neutrophil accumulation, namely adhesion and migration, remains an attractive target for anti-inflammatory therapies in lung disorders. In this study, we showed that the novel quinol compound and putative Trx inhibitor, PMX464, inhibited IL-1β-induced ICAM-1 expression and, correspondingly, reduced neutrophil adhesion to A549 epithelial cells. PMX464 also abolished GM-CSF and reduced CXCL8 release and the supernatants collected from IL-1β/PMX464-treated cells had reduced chemotactic ability. PMX464 showed similar inhibitory effects on ICAM-1 function and expression in HLMVEC. This finding suggests that the inhibitory effect of PMX464 on inflammatory markers is not limited to an alveolar epithelial cell line and moreover, it suggests that PMX464 could have therapeutic potential for the treatment of lung inflammatory disorders targeting endothelial cells in addition to epithelial cells. To our knowledge, this is the first study to report anti-inflammatory properties of PMX464.

Our findings with the PMX464, showing the inhibition of key processes involved in leukocyte recruitment, support two previous studies that showed a reduction of neutrophil and eosinophil accumulation in mouse models of intestinal ischaemia-reperfusion injury (Souza et al., 2005) and asthma (Henderson et al., 2002), respectively, with a structurally unrelated Trx inhibitor, MOL294. We also have unpublished data showing MOL294, like PMX464, reduced cytokine-induced ICAM-1 expression in A549 cells. Prompted by a further study showing that MOL294 inhibited NF-κB (Misra-Press et al., 2002) and the large body of evidence linking activation of the NF-κB pathway to lung inflammation,
we proposed to investigate whether PMX464 would also inhibit the NF-κB pathway in A549 lung epithelial cells. First, PMX464 was compared with AS602868, a known NF-κB inhibitor. Both inhibitors prevented cytokine-induced NF-κB controlled luciferase reporter activity suggesting that PMX464 has NF-κB inhibitory activity. AS602868, but not PMX464, reduced basal activity. AS602868 competes with ATP for binding to IKK-2 (Frelin et al., 2003). PMX464 is unlikely to compete with ATP and this probably accounts for

\[ \text{PMX464 inhibits IL-1β-induced activity of IκB-kinase complex immunoprecipitated from A549 cells. A549 monolayers were incubated for 3 min with either medium or IL-1β (0.3 ng mL}^{-1}) \text{. IKK complex was immunoprecipitated from A549 cell lysates using an anti-IKK-γ antibody. Immunoprecipitates were divided into two and one-half was subjected to IKK kinase assay using GST-IκB-α as a substrate (upper panel) and the other half to western blot analysis for IKK-β (lower panel). In some experiments, A549 cells were preincubated for 30 min with PMX464 (30 μM) or PS-1145 (10 μM) then for 3 min with IL-1β or medium. These experiments were designated OC (on cells) meaning that the inhibitor is added onto the cells and not directly onto the kinase assay. For other experiments, IKK was immunoprecipitated from IL-1β-stimulated cells and PMX464 (30 μM) or PS-1145 (10 μM) added directly to the kinase assay. These experiments were designated OA (on assay), meaning that the inhibitor is added to IKK complex and not incubated with the cells. Control experiments were also performed in which the anti-IKK-γ antibody was omitted from the immunoprecipitation protocol (PI) and also in which an equivalent concentration of vehicle (DMSO) was used instead of inhibitor. Results show a blot representative of three similar experiments. IKK, IκB kinase; IL, interleukin.} \]

\[ \text{Knockdown of Trx with siRNA did not alter IL-1β-induced levels of IκB-α or ICAM-1 expression. A549 monolayers were incubated with Trx siRNA (200 nM) and used in experiments 72 h later. Trx levels in A549 cells were determined by western blot on cell lysates and results are shown as a representative blot (a) and as a ratio to β-actin (n = 5, b). A549 monolayers were transfected with Trx siRNA or preincubated for 30 min with PMX464 (10 μM) or AS602868 (10 μM) before exposure to IL-1β (0.3 ng mL}^{-1}, 30 min). Western blots were performed on cell lysates to measure phosphorylated Ser32 and Ser36 IκB-α, total IκB-α, and β-actin as a loading control and a representative blot is shown (c). A549 cells were also exposed to Trx siRNA or inhibitor then treated with IL-1β or medium (24 h) and ICAM-1 measured (d). Results show expression (OD_{405}) ± s.e.m. (n = 5). ***p < 0.001; asterisks above the bars refer to comparison with basal expression and those above connecting lines refer to comparison between conditions indicated. Trx, thioredoxin-1; siRNA, short interference RNA; ICAM-1, intercellular adhesion molecule-1; IL, interleukin.} \]
the differences in effect on basal activity between the compounds. PMX464 inhibited IL-1β-induced NF-κB-controlled reporter luciferase activity by 90%. A similar level of inhibition was also seen with PMX464 on IL-1β-induced DNA-binding activity of the p50 and p65 subunits of NF-κB. Immunostaining of A549 cells, in conjunction with confocal microscopy, showed that PMX464 prevents movement of p65 from the cytoplasm to the nucleus in IL-1β treated cells.

The nuclear localization domain of p65 binds to IκB. Most pharmacological inhibitors that prevent NF-κB translocation do so by preventing IκB degradation. Thus, it was reasonable to assume that PMX464 similarly prevented translocation of p65. Our observation that PMX464 prevented rapid degradation of IκB and also prevented phosphorylation of IκBα, normally a prerequisite for degradation, would support this assumption. It also seemed likely that PMX464 would in some way inhibit, either directly or indirectly, IKK activity as this enzyme is solely responsible for phosphorylating IκB. A previous study with a gold compound, auranozin, which like PMX464 inhibits thiol groups, directly prevented IKK activity by modifying the Cys-179 of the IKK-β subunit (Jeon et al., 2003). It seems less likely that PMX464 has a similar direct effect on IKK, because our results suggested that PMX464 did not prevent IKK activity when added directly to the immunoprecipitated IKK. Our findings are also similar to those of a previous study showing that curcumin blocks intestinal epithelial cell gene expression by inhibiting the upstream signals leading to IKK activation (Jobin et al., 1999). As PMX464 prevented TNF-α- and IL-1β-induced IKK activity and, in general, IL-1β and TNF pathways vary considerably upstream of IKK, this would suggest that PMX464 acts in close proximity to IKK. Thus, it seemed plausible that PMX464 would block IKK activity, indirectly, possibly as a consequence of inhibiting Trx activity.

We confirmed that PMX464 inhibited Trx activity with an IC_{50} of 20 μM, which was similar to the previously published results of 23 μM (Pallis et al., 2003). We also showed that the insulin reducing activity of A549 cell lysates was reduced by pretreatment of the cells with PMX464. However, by contrast to the effect of PMX464, genetic silencing of Trx had no effect on the expression of ICAM-1 or, indeed, markers of activation in the NF-κB pathway. How could this difference be explained? We know that PMX464 is not an indiscriminate thiol inhibitor (Dr T Bradshaw, personal communication). However, other putative cellular protein targets have been identified as potential molecular targets for quinols including β-tubulin, heat-shock protein 60 and peroxiredoxin 1 (Chew et al., 2006). The extent to which inhibition of these proteins might impact on the proinflammatory response in lung epithelial cells is unclear. A recent paper showed that a number of antitumour quinols related to PMX464, inhibit thioredoxin reductase activity rather than Trx activity (Chew et al., 2008), a process that occurs, rapidly, in both cell-based assays and cell-free systems (Dr T Bradshaw, personal communication). Although a previous study showed no inhibition of TrxR with PMX464 in a cell-free system (Mukherjee et al., 2005, 2007) it is likely that the studies were not carried out sufficiently early to detect the inhibition of TrxR.

Certainly, we have unpublished data that would suggest that PMX464 can reduce, by approximately 25%, TrxR activity in cell lysates from A549 cells. Thus, one explanation for the discrepancy between inhibition seen with PMX464 and Trx siRNA is that in order to achieve an anti-inflammatory effect directed at the Trx system in lung epithelial cells, Trx and TrxR should both be targeted. In particular, this might be necessary with A549 cells as expression of TrxR is high in lung cancer cell lines (Soini et al., 2001). It is possible that an excess of TrxR could render the remaining Trx (30%) in Trx siRNA-treated A549 cells sufficiently active to cause ICAM-1 expression and increase levels and phosphorylation of IκBα.

Thus, it is of note that other studies investigating the intracellular role of the Trx system have used siRNA against either Trx or TrxR to demonstrate the inhibition. For example, siRNA against TrxR prevented TrxR and haem oxygenase (HO)-1 expression in endothelial cells, an effect that confirmed results with a pharmacological inhibitor, DNCB (Trigona et al., 2006). In this study, siRNA directed against a region located 30bp downstream of the ATG start codon of TrxR1, significantly decreased TrxR1 mRNA (87%), protein (63%) and activity levels (41%) strongly suggesting that complete elimination of protein expression is unusual in studies using siRNA. Another study used two different duplexes to inhibit Trx-1 and revealed a role of Trx in apoptosis in malignant mesothelioma cells (Freeman and Neužil, 2006). A further study showed that the down-regulation of Trx in MCF-7 cells decreased expression of p53 protein, caspase 7 and, in response to daunomycin, expression of cleaved PARP (Ravi et al., 2005). Finally, a study by Gorreta et al. (2005) used siRNA against TrxR and carried out RNA and cDNA microarrays; they concluded that the TrxR/Trx system affects a number of cellular processes by regulating the activity of transcription factors, leading to changes in gene expression. Thus, in future studies, we propose to employ siRNA against TrxR alone and in combination with siRNA against Trx to investigate the role of the Trx system in proinflammatory activation of A549 epithelial cells. Moreover, we also propose to compare the effects of Trx and/or TrxR siRNA in A549 cells to their effects in primary epithelial cells, as primary cells express lower TrxR levels (Soini et al., 2001) and thus, it might be possible to detect an inhibition that could be masked in A549 cells.

In summary, our data show that PMX464 downregulates IL-1β-induced proinflammatory activation of A549 lung epithelial cells through suppression of IKK activity and NF-κB activation. The effects with PMX464 were not mirrored by silencing Trx expression suggesting that PMX464 has therapeutic anti-inflammatory properties, which might result from the inhibition of other proteins with the Trx motif in addition to Trx. One possibility is TrxR, and this will be an important focus for future research.

Acknowledgements

MEC was supported by a Wellcome Trust Clinical Training Fellowship, AB-G by a Wellcome Trust University Award, LP
by the British Lung Foundation and SKL by the British Heart Foundation. We thank Dr Tracey Bradshaw for her helpful comments on the manuscript.

Conflicts of interest

ADW is a consultant to Pharminox, a company who hold a license for therapeutic applications of PMX464.

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