

RESEARCH PAPER

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

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Background and purpose: Subtle changes in the intracellular reduction–oxidation (redox) state can modulate nuclear factor- κ B (NF- κ B) 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- κ B 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- κ B-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- κ B DNA binding, nuclear translocation of NF- κ B p65 subunit, I κ B α degradation, I κ B phosphorylation and I κ B kinase (IKK) activity were assessed in A549 cells stimulated with IL-1 β 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 I κ B- α phospho-I κ B- 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-1 β -stimulated A549 cells and ICAM-1 in HLMVEC. PMX464 inhibited IL-1 β -induced NF- κ B DNA binding, nuclear translocation of NF- κ B p65 subunit and factors involved in NF- κ B activation; specifically, I κ B α degradation, I κ B phosphorylation and I κ B kinase (IKK) activity in A549. By contrast, Trx siRNA did not alter ICAM-1 expression or I κ B α degradation/phosphorylation in IL-1 β -stimulated A549 cells.

Conclusion and implications: PMX464 inhibits a proinflammatory response in A549 cells targeting the NF- κ B 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.

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

Introduction

The transcription factor NF- κ B 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- κ B proteins are sequestered in the cytosol through interactions with inhibitory proteins (I κ B α , β and ϵ). Tumour necrosis factor- α (TNF- α), interleukin-1 (IL-1 β), bacterial products and oxidants cause phosphorylation and degrada-

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tion of I κ B proteins through the ubiquitination-proteasome pathway. Free NF- κ B enters the nucleus and induces gene expression. Phosphorylation of I κ B by I κ B kinase (IKK) complex is a point at which diverse signals converge to activate NF- κ B. 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- κ B 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- κ B activity and thus impact on the pulmonary inflammatory response (Rahman *et al.*, 2006). Expression of thioredoxin (Trx), a small, ubiquitous thiol [sulphydryl (-SH)] protein involved in intracellular redox control, is raised during conditions associated with lung inflammation (Koura *et al.*, 2000; Tiitto *et al.*, 2003; Callister *et al.*, 2006) and Trx is also known to regulate components of the NF- κ B 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 (-S₂) 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-1 α 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- κ B 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 I κ B- α degradation/phosphorylation in IL-1 β -

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). 6 κ B cells, A549 cells harbouring the 6 κ B.tk luciferase reporter, were prepared as described previously (Bergmann *et al.*, 1998). HLMVEC were purchased from Cambrex (Wokingham, UK) and were maintained as previously described (Blease *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 μ M) 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 μ M) 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 (Dandreaa *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-1 β , TNF- α and interferon- γ (IFN- γ) added for 24 h. HLMVEC were seeded at 4.7 \times 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 (Blease *et al.*, 1999). A549 cells were also plated at 3.1 \times 10⁴ cells cm⁻² in 6-well plates and at confluence were treated with PMX464 or AS602868 for 30 min and then IL-1 β for 1 h. Whole cell extracts were prepared and ELISA based Trans-AM kits (Active Motif, Rixensart, Belgium) used to assess NF- κ B 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-1 β , PMX464 and IL-1 β 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 1.25×10^6 cells mL⁻¹ and 100 μ L added to A549 monolayers that had been pretreated (and stimuli removed) with IL-1 β 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 μ L of 3×10^6 cells mL⁻¹) were placed on porous membranes (0.8 μ m pores) positioned above wells containing stimuli or conditioned medium. Total fluorescence of 25 μ L 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

6 κ B cells were plated at 5×10^4 cells cm⁻² in 24-well plates and treated with IL-1 β 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, Southampton, UK) according to the manufacturer's protocol. Luciferase activity was measured using an automated luminometer (Fluostar Optima Microplate reader, BMG, Offenburg, Germany) and normalized to the total protein concentration for each well. Results were expressed as luciferase activity units (mg protein)⁻¹.

Immunofluorescence staining and confocal microscopy

Cells were plated onto sterilized glass cover slips in 24-well plates (4×10^5 cells cm⁻²), left overnight and then treated with medium or PMX464 for 30 min followed by medium or IL-1 β for 1 h. Cells were fixed, permeabilized and incubated, first, with rabbit antihuman p65 antibody and, secondly, with donkey antirabbit IgG antibody conjugated to Alexa 488 (stains p65 green). Cells were counterstained with 1% Evans blue solution (stains cytoplasm red) and images acquired using a Leica TCS 4D microscope at 488 nm for p65 and 568 nm for cytoplasmic counterstaining.

Immunoblotting

Cells were plated at 3.1×10^4 cells cm⁻² into 6-well plates, treated with medium or PMX464 for 30 min followed by medium or IL-1 β 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 I κ B- α (1:1000 dilution), mouse polyclonal antihuman phosphorylated I κ B- 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-1 β 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 μ g 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 μ Ci 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, GUGAUAAACUUGUAGUAGUUU); and Santa Cruz Biotechnology (a pool of three strands against Trx mRNA; sense, CAUCCUGGUGACAAAGAATT, GAAGGGAUGCAUCCAU GAATT, and GCGAUCAACUCUAGCCAAATT). As a control, two non-targeting 'scrambled' siRNA were used (Dharmacon and Santa Cruz). Cells were seeded at 2×10^4 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 μ g of protein analysed by western blotting to confirm knockdown or stimulated for 5 min or 24 h with IL-1 β (0.3 ng mL $^{-1}$) before measuring I κ B- α degradation and phospho-I κ B- α (5 min) or ICAM-1 (24 h).

Statistics

Results are expressed as mean \pm 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-1 β , 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 I κ B α (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) I κ B- 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 and Calcein-AM (Cambridge Bioscience, Cambridge, UK); PS-1145 (*N*-(6-chloro-9*H*- β -carbolin-8-yl) nicotinamide) was a gift from Millennium Pharmaceuticals (Cambridge, MA, USA) and AS602868 from Serono (Geneva, Switzerland). AS602868 is an anilino pyrimidine 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-1 β (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 μ M PMX464 reduced IL-1 β - and TNF- α -induced, but not IFN- γ -induced, ICAM-1 expression (Figure 1a). PMX464 abolished IL-1 β -induced

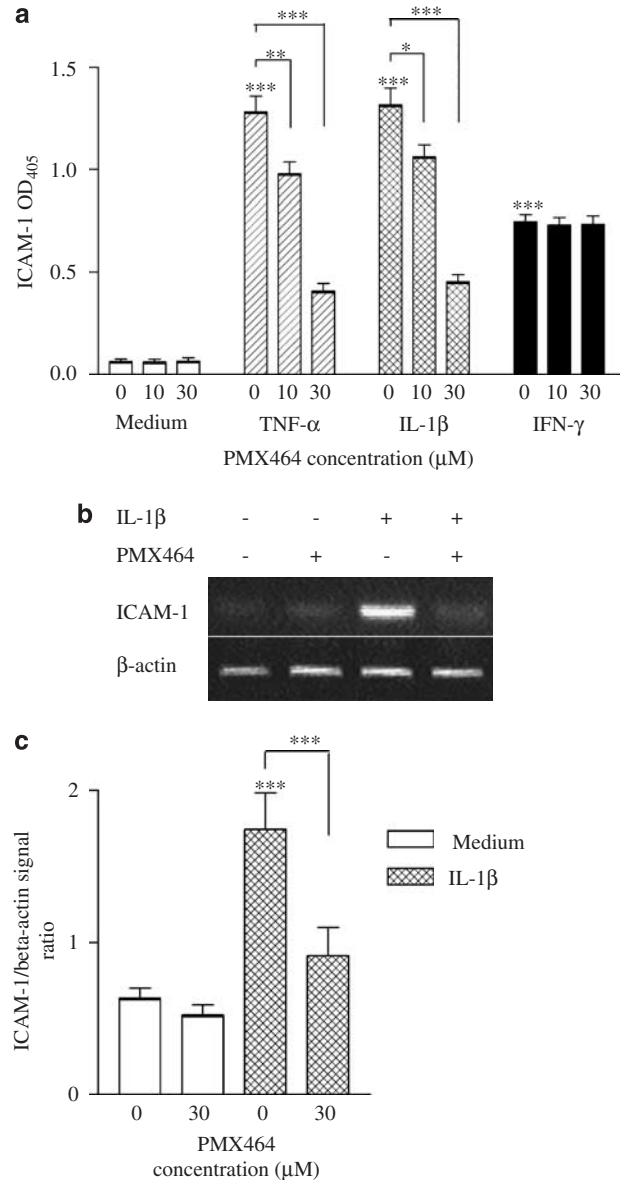


Figure 1 PMX464 reduces expression of ICAM-1 protein and mRNA in A549 cells. A549 monolayers were preincubated with medium or PMX464 (10, 30 μ M) for 30 min, then exposed to TNF- α (2 ng mL $^{-1}$), IL-1 β (0.3 ng mL $^{-1}$), IFN- γ (5 ng mL $^{-1}$) or medium for 24 h. ICAM-1 expression was determined by ELISA and data are shown as mean \pm s.e.m. ($n = 5$; a). A549 monolayers were also preincubated with medium or PMX464 (30 μ M) for 30 min, then exposed to IL-1 β (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 (normalized to the ratio for medium alone, $n = 5$, (c)). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, 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.

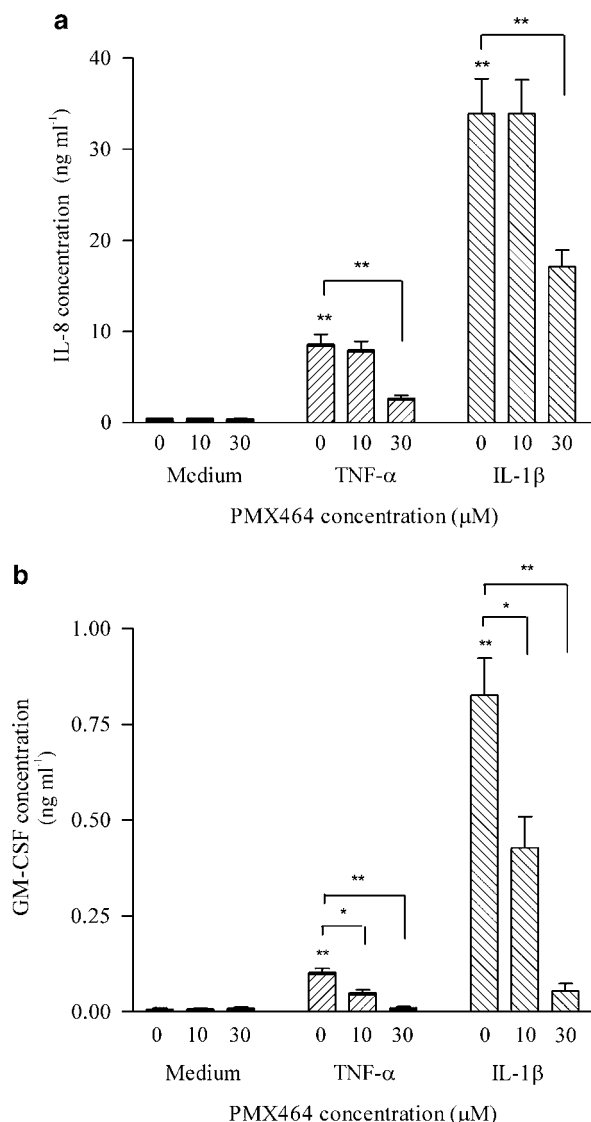


Figure 2 PMX464 reduces CXCL8 (IL-8) and GM-CSF release from A549 cells. A549 monolayers were preincubated with medium or PMX464 (10, 30 μ M) for 30 min, then exposed to TNF- α (2 ng mL⁻¹) or IL-1 β (0.3 ng mL⁻¹) for 24 h. CXCL8 (a) and GM-CSF (b) expression were determined by ELISA and data are shown as mean \pm s.e.m. ($n=8$ for CXCL8; $n=5$ for GM-CSF). * $P<0.01$, ** $P<0.001$, asterisks above the bars indicate comparison with basal expression and those on connecting lines indicate comparison between these conditions. GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; TNF, tumour necrosis factor; IL, interleukin.

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-1 β - 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-1 β (0.3 ng mL⁻¹) was significantly less than that to A549 cells exposed only to IL-1 β (Figure 3a). A functional consequence of CXCL8 release is neutrophil migration. Supernatants collected from PMX464/IL-1 β -treated A549 cells caused significantly less

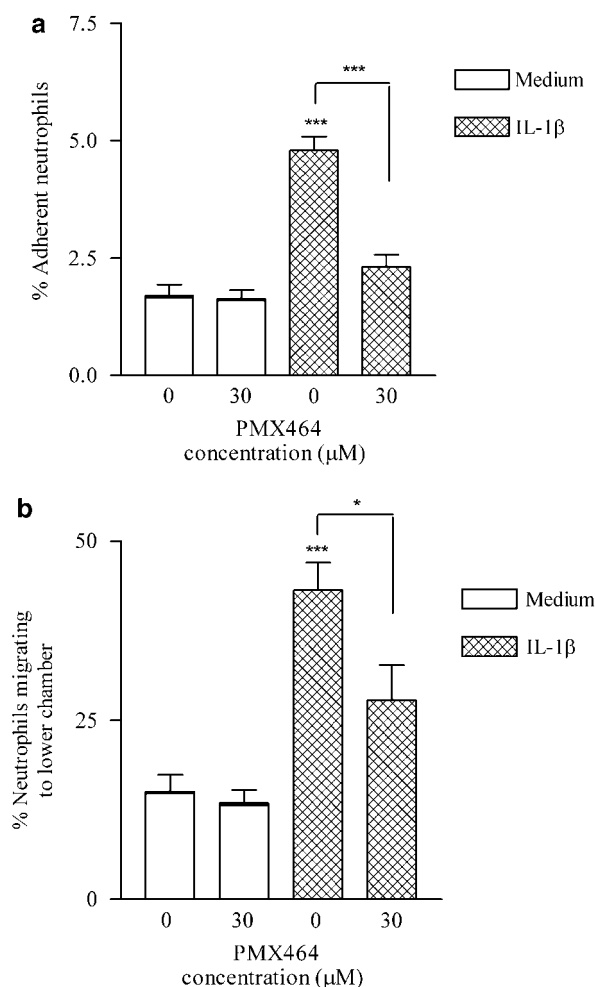


Figure 3 PMX464 inhibits neutrophil adhesion and migration, *in vitro*. Fluorescently-labelled neutrophils were placed onto A549 monolayers that had been preincubated with PMX464 (30 μ M) for 30 min, exposed to IL-1 β (0.3 ng mL⁻¹) for 24 h and stimuli removed before adding neutrophils. After 30 min, non-adherent cells were removed by washing, fluorescence measured and the number of neutrophils adhering expressed as a percentage \pm s.e.m. of total neutrophils added ($n=6$, a). For migration assays, A549 monolayers were treated as above with PMX464 and IL-1 β then the supernatants were collected and diluted 1:10, put into the lower chambers of the chemotaxis plate and then fluorescently-labelled neutrophils were placed on the porous membranes above. After 60 min, fluorescence in the lower chamber was measured and the number of migrating neutrophils expressed as a percentage \pm s.e.m. of total neutrophils placed on the top chamber ($n=6$, b). * $P<0.05$, *** $P<0.001$, asterisks above the bars refer to comparison with basal adhesion or migration and those above lines refer to comparison between indicated conditions. IL, interleukin.

neutrophil migration than the supernatants from A549 cells treated only with IL-1 β (0.3 ng mL⁻¹; Figure 3b). Neither IL-1 β 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 μ M) 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

Table 1 PMX464 inhibits ICAM-1 expression in HLMVEC

	Medium (OD_{405})	TNF- α (OD_{405})	IL-1 β (OD_{405})
Medium	0.16 \pm 0.02	0.98 \pm 0.05	0.86 \pm 0.05
PMX464 0.6 μ M	0.11 \pm 0.02	0.77 \pm 0.04**	0.69 \pm 0.03*
PMX464 1 μ M	0.12 \pm 0.02	0.62 \pm 0.04***	0.57 \pm 0.03***

Abbreviations: HLMVEC, human lung microvascular endothelial cells; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; TNF, tumour necrosis factor. HLMVEC monolayers were preincubated with medium or PMX464 (0.6, 1 μ M) for 30 min. TNF- α (0.3 ng mL⁻¹), IL-1 β (1 ng mL⁻¹) or medium was added to PMX464 for a further 24 h. ICAM-1 expression was determined by ELISA and data are shown as mean \pm s.e.m. ($n=5$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ when compared to ICAM-1 expression with cytokine but without PMX 464.

2.55 \pm 0.48 to 1.35 \pm 0.27 ($n=6$). Likewise, neutrophil adhesion to IL-1 β /PMX464-treated HLMVEC monolayers was also significantly ($P<0.01$) reduced (8.3 \pm 0.3% adhesion, $n=4$) compared with adhesion to HLMVEC treated with IL-1 β alone (19.4 \pm 2.9% adhesion, $n=4$). PMX464 also inhibited CXCL8 release from HLMVEC (data not shown).

PMX464 inhibits NF- κ B activation in A549 cells

We have reported previously using a variety of techniques, including small molecule inhibitors of IKK, that IL-1 β -induced ICAM-1 and CXCL8 expression in A549 cells is NF- κ B dependent (Catley *et al.*, 2005; Newton *et al.*, 2007). In this study we investigated the effect of PMX464 on NF- κ B activation in A549 cells. First, we looked at the effect of PMX464 on NF- κ B-dependent transcription in A549 cells containing an NF- κ B-dependent reporter gene (6 κ B cells). PMX464 and for comparison AS602868, an IKK-2 inhibitor, reduced IL-1 β - (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- κ B 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-1 β (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-1 β - (0.3 ng mL⁻¹) treated cells, p65 was detected in the nucleus. When cells were pretreated with PMX464 followed by IL-1 β , p65 remained in the cytoplasm (Figure 6). Similarly, cells pretreated with AS602868 before IL-1 β 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.

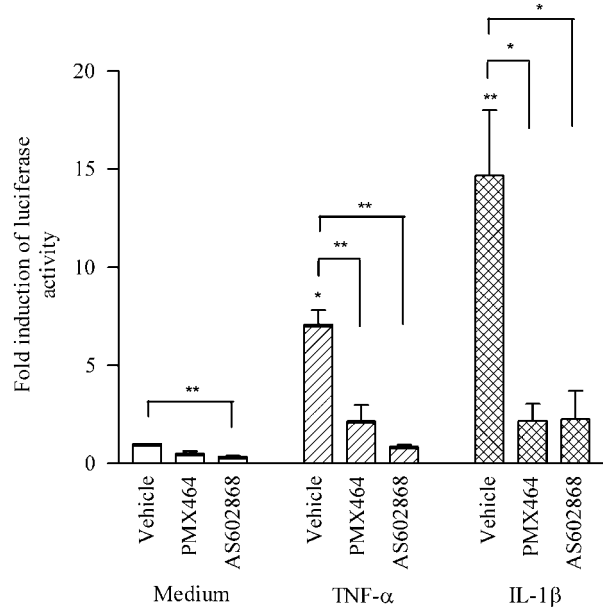


Figure 4 PMX464 and AS602868 inhibit luciferase activity in A549 cells containing an NF- κ B-dependent reporter gene (6 κ B cells). A549 cells stably transfected with the NF- κ B reporter, 6NF- κ B.tk luc (6 κ B), were preincubated for 30 min with PMX464 (30 μ M), AS602868 (10 μ M) or 0.01% DMSO vehicle (V) and then for 8 h with TNF- α (2 ng mL⁻¹), IL-1 β (0.3 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 \pm s.e.m. ($n=4$). * $P<0.01$, *** $P<0.001$, asterisks above the bars refer to comparison with basal activity and those above lines refer to comparison between conditions. IL, interleukin; Luc, luciferase; NF- κ B, nuclear factor- κ B; TNF, tumour necrosis factor.

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

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

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

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

A decrease in IL-1 β -induced I κ B α 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-1 β - (0.3 ng mL⁻¹) treated cells showed high levels of IKK activity, whereas activity from cells pretreated with

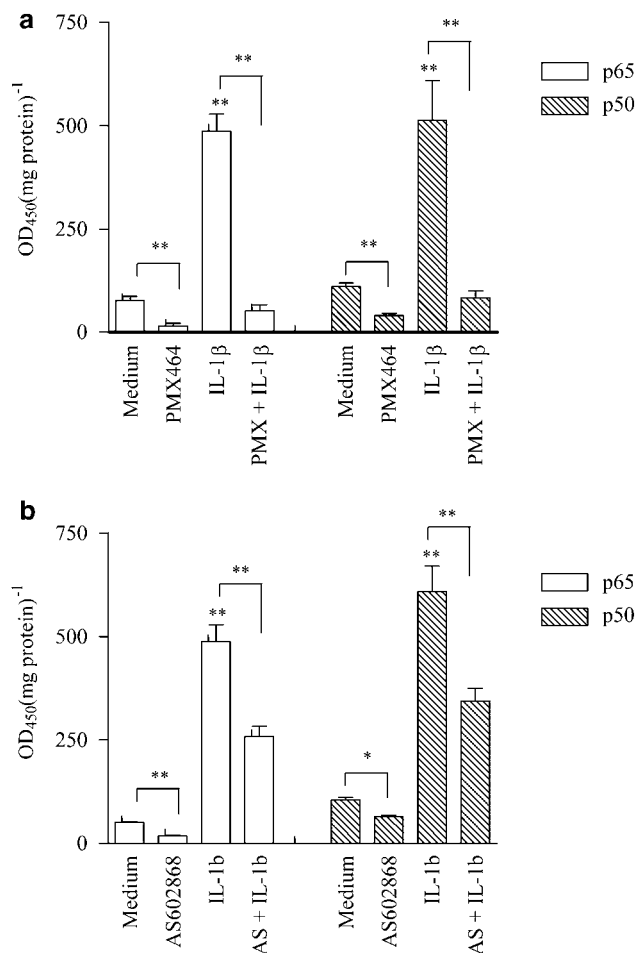


Figure 5 PMX464 and AS602868 prevent DNA-binding activity of p65 and p50 subunits of NF- κ B in A549 cells. A549 monolayers were preincubated for 30 min with PMX464 (30 μ M; **a**), AS602868 (10 μ M; **b**) or medium and then for 1 h with IL-1 β (0.3 ng mL⁻¹) or medium. DNA-binding activity of p65 and p50 in whole cell extracts were measured using TransAM transcription factors assays and results expressed as mean OD₄₅₀ per milligram protein \pm s.e.m. ($n=5$). * $P<0.01$, ** $P<0.001$, asterisks above the bars refer to comparison with basal activity and those above lines refer to comparison between conditions. IL, interleukin; NF- κ B, nuclear factor- κ B.

PMX464 (before IL-1 β) 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-1 β - (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

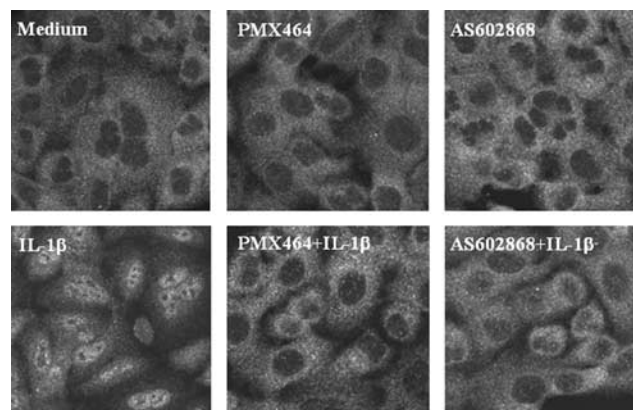


Figure 6 PMX464 and AS602868 alter cellular localization of p65 in A549 cells as assessed by confocal microscopy. Cells were preincubated for 30 min with PMX464 (30 μ M), AS602868 (10 μ M) or medium and then for 1 h with IL-1 β (0.3 ng mL⁻¹) or medium. The p65 subunit of NF- κ B was indirectly immunostained with Alexa 488-labelled antibody (green) and Evans blue (red) was used to counterstain cell cytoplasm. Images from an experiment representative of three separate experiments are shown. NF- κ B, nuclear factor- κ B.

IKK activity at some upstream step. Similar data were obtained when TNF- α (2 ng mL⁻¹) replaced IL-1 β in these experiments (data not shown). The upstream process inhibited is thus likely to be common to the IL-1 β and TNF- α pathways.

PMX464 is unlikely to be a general suppressor of cell signalling as phosphorylation of p38 and ERK in PMX464/IL-1 β -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-1 β -induced NF- κ B activation by preventing processes leading to the activation of the IKK complex.

Trx siRNAs did not inhibit IL-1 β -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 μ M) inhibited redox activity of Trx by 68% ($n=3$). The IC₅₀ for this reaction was calculated to be 20 μ M. Moreover, we also showed that when A549 cells were treated for 30 min with PMX464 (30 μ M), 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 I κ B- α or phosphorylated I κ B α nor expression of ICAM-1 changed in siRNA-treated cells despite positive effects with PMX464 and AS602868 (Figures 10b and

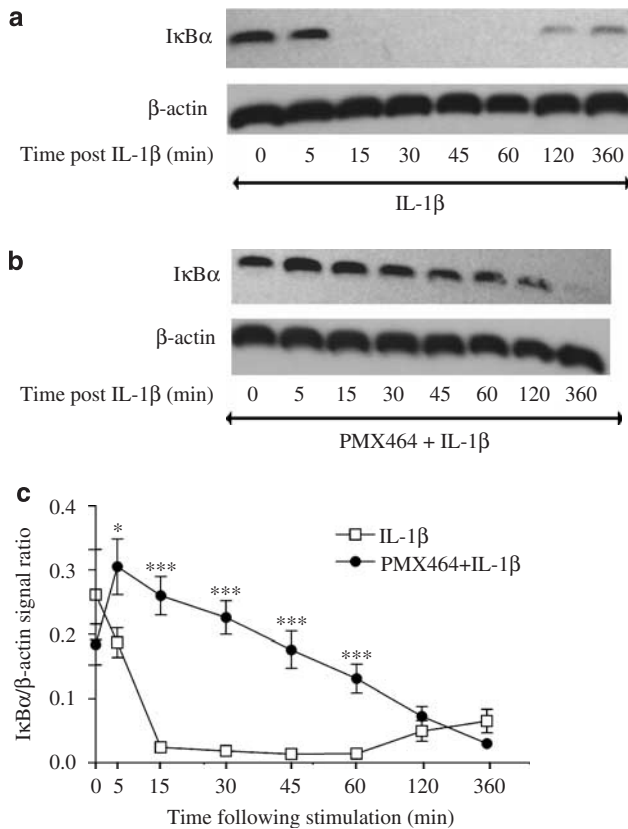


Figure 7 PMX464 delays IL-1 β -induced degradation of I κ B- α . A549 monolayers were preincubated for 30 min with PMX464 (30 μ M) or medium and then for times up to 360 min with IL-1 β (0.3 ng mL⁻¹). Western blots were performed on cell lysates and membranes probed for total I κ B- α protein and β -actin as a loading control. Representative blots are shown for cells stimulated with IL-1 β alone (a) and also cells preincubated with PMX464 and then exposed to IL-1 β (b). The mean ratio of I κ B- α / β -actin \pm s.e.m. (arbitrary units) for five separate experiments is also shown (c). * P <0.05, *** P <0.001 when conditions with and without PMX464 were compared using repeated measures ANOVA with mixed model to confirm results. IL, interleukin; NF- κ B, nuclear factor- κ B.

d). 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-1 β -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-1 β /PMX464-treated cells had reduced chemotactic ability. PMX464 showed

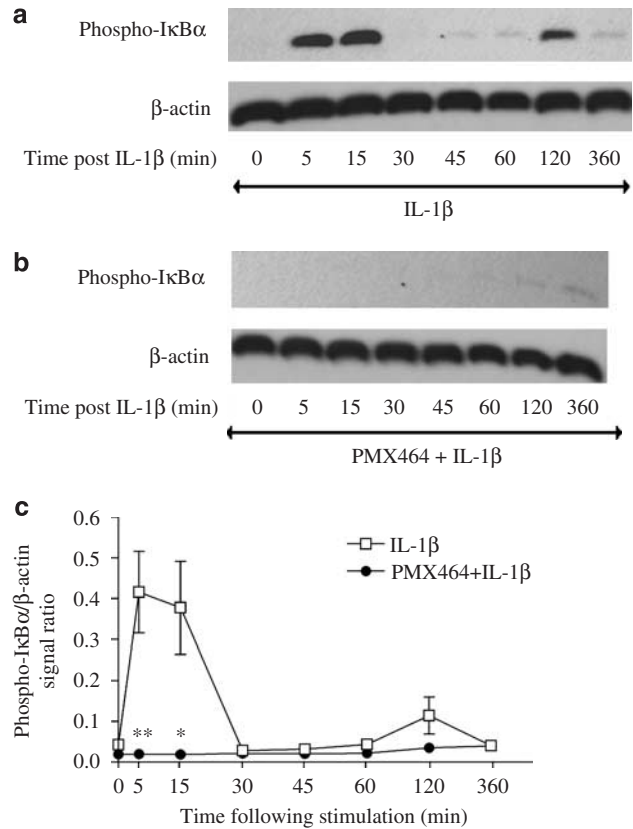


Figure 8 PMX464 prevented IL-1 β -induced phosphorylation of I κ B- α . A549 monolayers were incubated for 30 min with PMX464 or medium and then for times up to 360 min with IL-1 β (0.3 ng mL⁻¹). Western blots were performed on cell lysates and membranes probed for phosphorylated I κ B- α protein (Ser³² and Ser³⁶) and β -actin as a loading control. Representative blots are shown for cells stimulated with IL-1 β alone (a) and cells preincubated with PMX464 before IL-1 β stimulation (b). The mean ratio for phospho-I κ B- α / β -actin \pm s.e.m. (arbitrary units) for five separate experiments is also shown (c). * P <0.05, ** P <0.01 when conditions with and without PMX464 were compared using repeated measures ANOVA with mixed model to confirm results. IL, interleukin.

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- κ B (Misra-Press *et al.*, 2002) and the large body of evidence linking activation of the NF- κ B pathway to lung inflammation,

we proposed to investigate whether PMX464 would also inhibit the NF- κ B pathway in A549 lung epithelial cells.

First, PMX464 was compared with AS602868, a known NF- κ B inhibitor. Both inhibitors prevented cytokine-induced

NF- κ B controlled luciferase reporter activity suggesting that PMX464 has NF- κ B 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

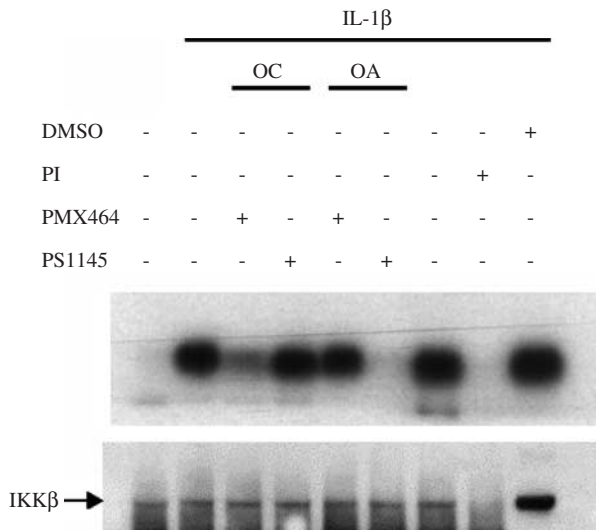


Figure 9 PMX464 inhibits IL-1 β -induced activity of I κ B-kinase complex immunoprecipitated from A549 cells. A549 monolayers were incubated for 3 min with either medium or IL-1 β (0.3 ng mL⁻¹). IKK complex was immunoprecipitated from A549 cell lysates using an anti-IKK- γ antibody. Immunoprecipitates were divided in two and one-half was subjected to IKK kinase assay using GST-I κ B- 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 μ M) or PS-1145 (10 μ M) then for 3 min with IL-1 β 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-1 β -stimulated cells and PMX464 (30 μ M) or PS-1145 (10 μ M) 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, I κ B kinase; IL, interleukin.

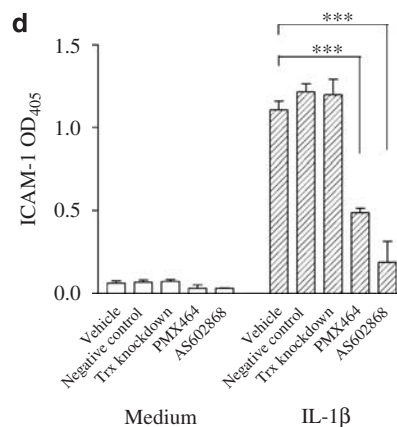
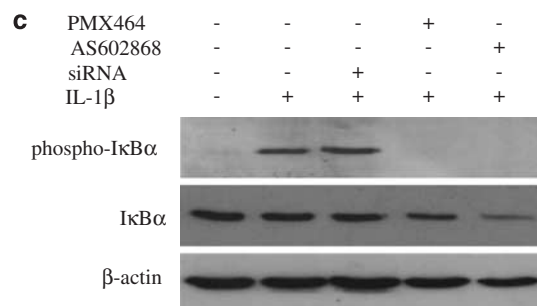
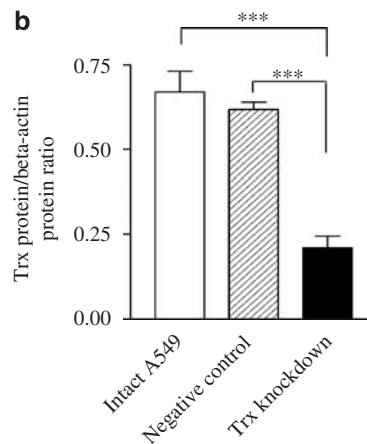
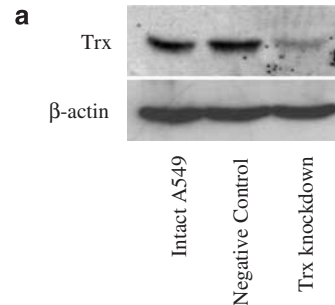


Figure 10 Knockdown of Trx with siRNA did not alter IL-1 β -induced levels of I κ B- α or ICAM-1 expression. A549 monolayers were incubated with Trx siRNA (200 nM) and used in experiments 72 h later. Trx levels in A549 cells were determined by western blot on cell lysates and results are shown as a representative 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 μ M) or AS602868 (10 μ M) before exposure to IL-1 β (0.3 ng mL⁻¹, 30 min). Western blots were performed on cell lysates to measure phosphorylated Ser³² and Ser³⁶ I κ B- α , total I κ B- 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-1 β or medium (24 h) and ICAM-1 measured (d). Results show expression (OD₄₀₅) \pm 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-1 β -induced NF- κ B-controlled reporter luciferase activity by 90%. A similar level of inhibition was also seen with PMX464 on IL-1 β -induced DNA-binding activity of the p50 and p65 subunits of NF- κ B. Immunostaining of A549 cells, in conjunction with confocal microscopy, showed that PMX464 prevents movement of p65 from the cytoplasm to the nucleus in IL-1 β treated cells.

The nuclear localization domain of p65 binds to I κ B. Most pharmacological inhibitors that prevent NF- κ B translocation do so by preventing I κ B degradation. Thus, it was reasonable to assume that PMX464 similarly prevented translocation of p65. Our observation that PMX464 prevented rapid degradation of I κ B and also prevented phosphorylation of I κ B α , 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 I κ B. A previous study with a gold compound, auranofin, 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-1 β -induced IKK activity and, in general, IL-1 β 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 IC₅₀ of 20 μ M, which was similar to the previously published results of 23 μ M (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- κ B 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 I κ B- α .

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 duplces to inhibit Trx-1 and revealed a role of Trx in apoptosis in malignant mesothelioma cells (Freeman and Neuzil, 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-1 β -induced proinflammatory activation of A549 lung epithelial cells through suppression of IKK activity and NF- κ B 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.

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Conflict of interest

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

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