# RESEARCH PAPER

# Montelukast inhibits neutrophil pro-inflammatory activity by a cyclic AMP-dependent mechanism

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**Background and purpose:** The objective of this study was to characterize the effects of the cysteinyl leukotriene receptor antagonist, montelukast (0.1–2 $\mu mol \cdot L^{-1}$), on $Ca^{2+}$-dependent pro-inflammatory activities, cytosolic $Ca^{2+}$ fluxes and intracellular cAMP in isolated human neutrophils activated with the chemoattractants, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (1 $\mu mol \cdot L^{-1}$) and platelet-activating factor (200 $nmol \cdot L^{-1}$).
**Experimental approach:** Generation of reactive oxygen species was measured by lucigenin- and luminol-enhanced chemiluminescence, elastase release by a colourimetric assay, leukotriene $B_4$ and cAMP by competitive binding ELISA procedures, and $Ca^{2+}$ fluxes by fura-2/AM-based spectrofluorimetric and radiometric ($^{45}Ca^{2+}$) procedures.
**Key results:** Pre-incubation of neutrophils with montelukast resulted in dose-related inhibition of the generation of reactive oxygen species and leukotriene $B_4$ by chemoattractant-activated neutrophils, as well as release of elastase, all of which were maximal at 2 $\mu mol \cdot L^{-1}$ (mean percentages of the control values of 30 ± 1, 12 ± 3 and 21 ± 3 respectively; $P < 0.05$). From a mechanistic perspective, treatment of chemoattractant-activated neutrophils with montelukast resulted in significant reductions in both post-peak cytosolic $Ca^{2+}$ concentrations and store-operated $Ca^{2+}$ influx. These montelukast-mediated alterations in $Ca^{2+}$ handling by the cells were associated with a significant elevation in basal cAMP levels, which resulted from inhibition of cyclic nucleotide phosphodiesterases.
**Conclusions and implications:** Montelukast, primarily a cysteinyl leukotriene (CysLT₁) receptor antagonist, exhibited previously undocumented, secondary, neutrophil-directed anti-inflammatory properties, which appeared to be cAMP-dependent.
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## Introduction

Montelukast, a highly selective antagonist of cysteinyl leukotriene (CysLT) receptors, is widely used in the treatment of bronchial asthma, primarily as an adjunct to corticosteroids (Anonymous, 2004; Currie *et al.*, 2005; Diamant and van der Molen, 2005; Riccioni *et al.*, 2007). In this setting, the therapeutic activity of montelukast is achieved through antagonism of CysLT-mediated bronchoconstriction, increased

vascular permeability and mucus secretion, following release of these mediators, mainly from monocytes/macrophages, eosinophils, mast cells and basophils, as well as by anti-inflammatory actions targeting type 2 helper CD4⁺ T-lymphocytes (Peters-Golden and Henderson, 2007). Unlike corticosteroids, montelukast has been reported to modulate airway remodelling in patients with chronic asthma, compatible with an extended spectrum of anti-inflammatory activity (Henderson *et al.* 2006; Muz *et al.*, 2006).

Montelukast has also been reported to possess therapeutic activity in other diseases such as chronic obstructive pulmonary disease, a disorder that is believed to be of neutrophilic aetiology (Rubinstein *et al.*, 2004; Celik *et al.*, 2005). Although

they do not produce CysLTs, neutrophils do possess receptors for $LTC_4$ and $LTD_4$, activation of which triggers relatively modest pro-inflammatory responses in these cells (Lärfars *et al*., 1999; Zhu *et al*., 2005). Interference with neutrophil activation by CysLTs released from other cell types, such as monocytes/macrophages, mast cells or eosinophils, may therefore underlie the neutrophil-directed therapeutic efficacy of montelukast. Alternatively, montelukast may possess secondary anti-inflammatory properties that are distinct from conventional antagonism of CysLT receptors. These include interference with activation of the transcription factor, nuclear factor kappa B in immune and inflammatory cells, promotion of sustained production of interleukin-10 in inflamed airways or by inhibition of signalling pathways triggered by P2Y receptors (Mamedova *et al*., 2005; Wu *et al*., 2006). However, the contribution of these mechanisms to the possible neutrophil-targeted anti-inflammatory activity of montelukast is unclear.

In the current study, we have investigated the effects of montelukast, at therapeutically relevant concentrations, on the mobilization of stored and extracellular $Ca^{2+}$ by chemoattractant-activated human neutrophils, as well as on several $Ca^{2+}$-dependent, pro-inflammatory activities of the cells. Our results demonstrate that montelukast antagonizes the pro-inflammatory activities of neutrophils by a mechanism involving inhibition of cyclic nucleotide phosphodiesterases (PDE) and cAMP-mediated attenuation of $Ca^{2+}$ influx.

## Methods

### Preparation of neutrophils

The study was approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria, and prior informed consent was obtained from all blood donors.

These cells were isolated from heparinized venous blood (5 units of preservative-free heparin per ml of blood) from healthy adult volunteers. Neutrophils were separated from mononuclear leucocytes by centrifugation on Histopaque-1077 (Sigma Diagnostics) cushions at $400 \times g$ for 25 min at room temperature. The resultant pellets were suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatine to remove most of the erythrocytes. Following centrifugation ($280 \times g$ at 10°C for 10 min), residual erythrocytes were removed by selective lysis with 0.83% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), determined by flowcytometric procedures, were re-suspended to $1 \times 10^7$ $ml^{-1}$ in PBS and held on ice until used.

### Measurement of reactive oxygen species

These were measured using lucigenin (bis-*N*-methylacridinium nitrate)- and luminol (5-amino-2,3-dihydro-1, 4-phthalazine dione)-enhanced chemiluminescence (CL) procedures that predominantly detect superoxide and reactive oxygen species (ROS) generated by the myeloperoxidase/$H_2O_2$/halide system respectively (Minkenberg and Ferber, 1984). Briefly, neutrophils ($10^6$ cells) were pre-incubated for 10 min at 37°C, without and with montelukast (0.1–2 $\mu mol \cdot L^{-1}$) in 900 $\mu l$

of Hanks' balanced salt solution (HBSS) containing either lucigenin (0.2 $mmol \cdot L^{-1}$) or luminol (0.1 $mmol \cdot L^{-1}$), followed by addition of either 100 $\mu l$ of HBSS (unstimulated control systems) or the chemoattractant, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 $\mu mol \cdot L^{-1}$) and CL responses recorded using a Lumac Biocounter (Model 2010, Lumac Systems Inc., Titusville, FL, USA). The final volume in each vial was 1 ml, and the results, which are expressed in relative light units (rlu), are the peak values for FMLP-activated systems that were reached 40–50 s after addition of the stimulant.

MK886, an inhibitor of 5-lipoxygenase-activating protein, was used to investigate the possible contribution of LTs generated by neutrophils, as well as by contaminating cells in the neutrophil suspensions, to superoxide generation by FMLP-activated cells, especially the involvement of $LTC_4$ and $LTD_4$. Neutrophils were pre-incubated with MK886 (0.5 $\mu mol \cdot L^{-1}$, final) for 5 min at 37°C followed by addition of montelukast (0.5 $\mu mol \cdot L^{-1}$) and a further pre-incubation of 5 min followed by addition of FMLP (1 $\mu mol \cdot L^{-1}$) and measurement of lucigenin-enhanced CL. Control systems included neutrophils only, as well as cells treated with either MK886 or montelukast only. The efficacy of MK886 as an inhibitor of 5-lipoxygenase in FMLP-activated neutrophils was measured according to the magnitude of inhibition of production of $LTB_4$ by the cells using the method described below.

The superoxide-scavenging potential of montelukast (2 $\mu mol \cdot L^{-1}$) was measured using a cell-free xanthine (1 $mmol \cdot L^{-1}$)/xanthine oxidase (130 mU $ml^{-1}$) lucigenin-dependent CL procedure.

### NADPH oxidase in isolated neutrophil membranes

Neutrophils ($1 \times 10^6$ $ml^{-1}$) were pre-incubated for 10 min at 37°C without or with montelukast at a fixed concentration of 2 $\mu mol \cdot L^{-1}$, followed by addition of FMLP (1 $\mu mol \cdot L^{-1}$). After 3 min of incubation at 37°C, the reactions were terminated by addition of a large volume of ice-cold HBSS and the tubes transferred to an ice bath. The cells were then pelleted by centrifugation at 4°C and the pellets pooled and re-suspended to $5 \times 10^6$ $ml^{-1}$ in 0.34 M sucrose supplemented with 0.5 $mmol \cdot L^{-1}$ phenylmethylsulphonyl fluoride (PMSF, Calbiochem Corp., La Jolla, CA, USA) and disrupted by sonication. Cellular debris was removed by centrifugation and the membrane fractions in the supernatants were harvested after centrifugation at $70\,000 \times g$ for 30 min. The membrane pellets were dispersed in 1 ml of sucrose and assayed for NADPH oxidase activity using lucigenin-enhanced CL. Reaction mixtures (1 ml) contained lucigenin, membrane fractions (200 $\mu l$) and NADPH (2 $mmol \cdot L^{-1}$), which was added last to initiate superoxide generation.

### Oxygen consumption

This was measured using a three-channel oxygen electrode (Model DW1, Hansatech Ltd, King's Lynn, Norfolk, UK). Neutrophils ($2 \times 10^6$ $ml^{-1}$) were pre-incubated for 10 min at 37°C in HBSS without or with montelukast at a fixed concentration of 1 $\mu mol \cdot L^{-1}$ followed by addition of FMLP (1 $\mu mol \cdot L^{-1}$) and measurement of $PO_2$ over a 5 min time course.

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## Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule enzyme, elastase. Neutrophils were incubated at a concentration of $2 \times 10^6$ ml$^{-1}$ in HBSS with and without montelukast (0.1–2 μmol·L$^{-1}$) for 10 min at 37°C. FMLP (1 μmol·L$^{-1}$) in combination with a submaximal concentration of cytochalasin B (0.5 μmol·L$^{-1}$, final) was then added to the cells that were incubated for 15 min at 37°C. The tubes were then transferred to an ice bath, followed by centrifugation at 400× *g* for 5 min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for elastase using a micromodification of a standard colourimetric procedure (Beatty *et al.*, 1982). Briefly, 125 μl of supernatant were added to the elastase substrate, *N*-succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide [3 mmol·L$^{-1}$ in dimethylsulphoxide (DMSO)] in 0.05 M Tris-HCl (pH 8.0), and elastase activity was monitored spectrophotometrically at a wavelength of 405 nmol·L$^{-1}$.

## Spectrofluorimetric measurement of cytosolic Ca$^{2+}$

Fura-2/AM was used as the fluorescent, Ca$^{2+}$-sensitive indicator for these experiments (Grynkiewicz *et al.*, 1985). Neutrophils ($1 \times 10^7$ ml$^{-1}$) were incubated with fura-2/AM (2 μmol·L$^{-1}$) for 30 min at 37°C in PBS, washed and re-suspended in indicator-free HBSS (pH 7.4), containing 1.25 mmol·L$^{-1}$ CaCl$_2$. The fura-2-loaded cells ($2 \times 10^6$ ml$^{-1}$) were then pre-incubated for 5 min at 37°C with montelukast (0.25–2 μmol·L$^{-1}$) or an equivalent volume of DMSO in control systems, after which they were transferred to disposable reaction cuvettes, which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500 nm respectively. After a stable baseline was obtained (±1 min), the neutrophils were activated by the addition of the chemoattractants FMLP (1 μmol·L$^{-1}$, final), or platelet-activating factor (PAF, 200 nmol·L$^{-1}$, final) and alterations in fluorescence intensity monitored over a 5–10 min time course. Cytosolic calcium concentrations were calculated as described previously (Grynkiewicz *et al.*, 1985).

## Radiometric assessment of Ca$^{2+}$ influx

A radiometric procedure was also used to measure the net influx of $^{45}$Ca$^{2+}$ into FMLP (1 μmol·L$^{-1}$)- or PAF (200 nmol·L$^{-1}$)-activated neutrophils uncomplicated by concomitant efflux of the radiolabelled cation. The cells were pre-incubated for 10 min at 37°C in Ca$^{2+}$-replete (1.25 mmol·L$^{-1}$) HBSS to ensure that intracellular Ca$^{2+}$ stores were full to minimize spontaneous uptake of $^{45}$Ca$^{2+}$ (unrelated to activation with FMLP or PAF) in the influx assay. The cells were then pelleted by centrifugation and re-suspended to a concentration of $1 \times 10^7$ ml$^{-1}$ in HBSS containing 25 μmol·L$^{-1}$ cold, carrier CaCl$_2$. The Ca$^{2+}$-loaded neutrophils ($2 \times 10^6$ ml$^{-1}$) were then incubated for 5 min at 37°C in HBSS containing 25 μmol·L$^{-1}$ CaCl$_2$ in the absence or presence of montelukast (2 μmol·L$^{-1}$) followed by simultaneous addition of FMLP or PAF and 2 μCi ml$^{-1}$ $^{45}$Ca$^{2+}$ (as $^{45}$[Ca]Cl$_2$, specific activity 24.3 mCi mg$^{-1}$, Perkin Elmer Life and Analytical Sciences, Boston, MA, USA), or $^{45}$Ca$^{2+}$ only to control, unstimulated systems. The cells, in a final volume of 5 ml, were then incubated for 5 min at 37°C, after which chemoattractant-activated, store-operated uptake of Ca$^{2+}$ is complete (Steel and Anderson, 2002), and the reactions stopped by the addition of 10 ml of ice-cold, Ca$^{2+}$-replete HBSS to the tubes, which were transferred immediately to an ice bath. The cells were then pelleted by centrifugation at 400× *g* for 5 min followed by washing with 15 ml of ice-cold, Ca$^{2+}$-replete HBSS and the cell pellets dissolved in 0.5 ml 0.1% Triton X-100/0.1 M NaOH and the radioactivity measured in a liquid scintillation spectrometer. The results are presented as the amount of cell associated radioactivity (pmol $^{45}$Ca$^{2+}$·10$^7$ cells$^{-1}$).

## Measurement of LTB$_4$ and cyclic AMP

Competitive binding enzyme immunoassay procedures (Correlate-EIA™, Assay Designs Inc., Ann Arbor, MI, USA) were used to measure LTB$_4$ in the supernatants of neutrophils activated with PAF (200 nmol·L$^{-1}$), while cAMP was measured in the extracts of unstimulated neutrophils, in the absence and presence of montelukast (0.25–2 μmol·L$^{-1}$). In the case of LTB$_4$, neutrophils ($2 \times 10^6$ ml$^{-1}$, final) in HBSS were pre-incubated for 10 min at 37°C with montelukast after which PAF was added to the cells and the reactions stopped after 3 min incubation at 37°C (predetermined in preliminary time course experiments) by the addition of an equal volume of ice-cold HBSS to the tubes, which were then held in an ice bath prior to pelleting the cells by centrifugation. The cell-free supernatants were then assayed for LTB$_4$ using the enzyme immunoassay procedure. Supernatants from cells activated with PAF were diluted 1:4 prior to assay. These results are expressed as pg 10$^7$ cells$^{-1}$.

In the case of cAMP, neutrophils ($2 \times 10^6$ ml$^{-1}$, final) were pre-incubated for 10 min at 37°C followed by the addition of montelukast (0.25–2 μmol·L$^{-1}$) after which the cells were incubated for a further period of 5 min at 37°C and the reactions were stopped by the addition of an equal volume of ice-cold HBSS to the tubes, which were then held on ice prior to pelleting the cells by centrifugation. Following centrifugation, the supernatants were discarded and cAMP extracted from the cell pellets by addition of 1 ml of 0.1 M HCl for 10–15 min followed by centrifugation to remove cell debris and the supernatants decanted and assayed for cAMP. These results are expressed as pmol cAMP 10$^7$ cells$^{-1}$.

In an additional series of experiments, the cells were exposed to montelukast (2 μmol·L$^{-1}$) or vehicle (0.05% DMSO) for 5 min at 37°C followed by the addition of salbutamol (β$_2$-adrenoreceptor agonist, 5 μmol·L$^{-1}$), CGS21680 (adenosine A$_{2A}$ receptor agonist, 1 μmol·L$^{-1}$) or rolipram (type 4 PDE inhibitor, 0.1 μmol·L$^{-1}$) for 3–5 min at 37°C after which cAMP was assayed in the cell extracts.

## PDE activity

To prepare neutrophil cytosol, the cells ($5 \times 10^6$ ml$^{-1}$) in PBS were pelleted by centrifugation, then re-suspended in 0.34 M sucrose and 0.5 mmol·L$^{-1}$ PMSF. The cells were then disrupted by sonication and cellular debris removed by centrifugation. The sonicates were then fractionated by ultracentrifugation at 70 000× *g* for 30 min and the supernatants harvested for

assessment of PDE activity using a scintillation proximity assay (SPA, Amersham Biosciences, UK). Briefly, assays were performed at 30°C for 10 min in buffer containing 50 mmol·L$^{-1}$ Tris-HCl (pH 7.5), 8.3 mmol·L$^{-1}$ MgCl$_2$, 17 mmol·L$^{-1}$ EGTA and 0.3 mg ml$^{-1}$ bovine serum albumin. Each assay was performed in a reaction volume of 200 μl containing neutrophil cytosol (20 μl) as a source of PDE and approximately 0.05 μCi [$^3$H]cAMP or [$^3$H]cGMP in the absence and presence of montelukast (0.25–20 μmol·L$^{-1}$), as well as rolipram (20 μmol·L$^{-1}$), or the non-specific PDE inhibitor, 3-isobutyl-1-methylxanthine (50 μmol·L$^{-1}$) in control systems. Reactions were terminated by the addition of 75 μl of PDE SPA beads suspended in 18 mmol·L$^{-1}$ zinc sulphate and PDE-mediated hydrolysis of [$^3$H]cAMP or [$^3$H]cGMP determined by liquid scintillation spectrometry.

The effects of montelukast on the activity of PDE in a preparation isolated from bovine heart (Sigma Chemical Co.) were also investigated, using the enzyme preparation at a fixed, final concentration of 1 mU ml$^{-1}$.

In an additional series of experiments, the effects of pretreatment of neutrophils with montelukast (2 μmol·L$^{-1}$) on the activities of cAMP PDE in matched, isolated membranes and cytosol fractions prepared from both unstimulated and FMLP (1 μmol·L$^{-1}$)-activated cells were investigated. Briefly, neutrophils were pre-incubated for 10 min at 37°C in the absence and presence of montelukast, followed by the addition of FMLP (or an equal volume of HBSS to control cells) and termination of reaction 1 min later by addition of ice-cold HBSS. The cells were then pelleted by centrifugation, re-suspended in 0.34 M sucrose/0.5 mmol·L$^{-1}$ PMSF, sonicated, and membrane and cytosol fractions prepared as described above and assayed for cAMP PDE activity by SPA. For purposes of comparison, the membrane and cytosol fractions were assayed for protein content and the results expressed as enzyme activity min$^{-1}$ mg protein$^{-1}$.

### Inositol triphosphate (inositol-1,4,5-triphosphate)

Neutrophils at a concentration of $4 \times 10^6$ ml$^{-1}$ were pre-incubated for 5 min at 37°C in HBSS without or with montelukast (2 μmol·L$^{-1}$) after which the cells were activated with PAF (200 nmol·L$^{-1}$) in a final volume of 1 ml. The reactions were terminated and the inositol-1,4,5-triphosphate (IP$_3$) extracted by the addition of 1 ml of 20% perchloric acid at 5 and 10 s after the addition of PAF. Following a 20 min incubation on ice, the tubes were centrifuged at 2000× *g* for 15 min and the supernatants decanted and titrated to pH 7.5 with 5 M KOH followed by centrifugation at 2000× *g* for 15 min to remove precipitated KClO$_4$. The supernatants were assayed for IP$_3$ using the inositol-1,4,5-triphosphate ($^3$H) radioreceptor assay kit (Perkin Elmer Life and Analytical Sciences), which is a competitive ligand binding assay, and the results expressed as pmol 10$^7$ cells$^{-1}$.

### Cellular ATP levels

To determine the effects of montelukast (2 μmol·L$^{-1}$) on neutrophil viability, intracellular ATP concentrations were measured in cell lysates (1 × 10$^6$ cells ml$^{-1}$) following exposure of the cells to the drug for 15 min at 37°C, using a luciferin/

**Table 1** Effects of MK886 and montelukast, separately and in combination, on the lucigenin-enhanced chemiluminescence (LECL) responses of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated neutrophils

| System | LECL (relative light units) | P-values |
|---|---|---|
| a. FMLP only | 6401 ± 496 | |
| b. FMLP + MK886 (0.5 μmol·L$^{-1}$) | 6448 ± 571 | NS[a] |
| c. FMLP + Montelukast (0.5 μmol·L$^{-1}$) | 4707 ± 354 | <0.01 to <0.001[a] |
| d. FMLP + MK886 + Montelukast | 4741 ± 349 | NS[b] |

The results of four experiments are expressed as the mean peak LECL values ± SEM.
[a]For comparison with the FMLP-activated, drug-free control system.
[b]For comparison between systems c and d.

luciferase CL procedure (Holmsen *et al.*, 1972). These results are expressed as nmol ATP 10$^7$ cells$^{-1}$.

### Statistical analysis

With the exception of the results of the fura-2 fluorescence experiments, some of which are presented as representative traces, the results of each series of experiments are presented as the mean values ± SEM, either as the absolute values or as mean percentages of the corresponding drug-free control systems where *n* = the number of different donors used in each series of experiments, with the number of replicates for each drug concentration and drug-free control system for each experiment shown in the figure legends and table footnotes. Levels of statistical significance were determined by comparing the absolute values for each drug-treated system with the corresponding values for the relevant drug-free control systems for each assay using the Friedman repeated measures ANOVA with Dunn's multiple comparisons post-test, or the Wilcoxon matched pairs signed-ranks test where appropriate, while a two-way repeated measures ANOVA with the Bonferroni post-test was used to analyse the data shown in Table 1.

### Chemicals and reagents

Montelukast sodium, 2-[1[[1-3-[2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-2[1-hydroxy-1-methyl-ethyl)phenyl]-propyl]sulphanyl-methyl]-propyl]cyclopropyl]ethanoic acid, was kindly provided by Merck Research Laboratories, Rahway, NJ, USA, and dissolved in DMSO to a stock concentration of 10 mmol·L$^{-1}$. Unless indicated, all other chemicals and reagents were purchased from Sigma-Aldrich, St Louis, MO, USA. In the various assay systems described below, montelukast was used at final concentrations of 0.1, 0.25, 0.5, 1 and 2 μmol·L$^{-1}$. Peak serum concentrations of 0.5–1 μmol·L$^{-1}$ are attainable during oral administration of montelukast (Cheng *et al.*, 1996; Knorr *et al.*, 2001). The final DMSO concentration was 0.05%, and DMSO control systems were included in each assay.

## Results

### Production of ROS

The effects of montelukast (0.1–2 μmol·L$^{-1}$) on the FMLP-activated generation of ROS using the lucigenin- and luminol-
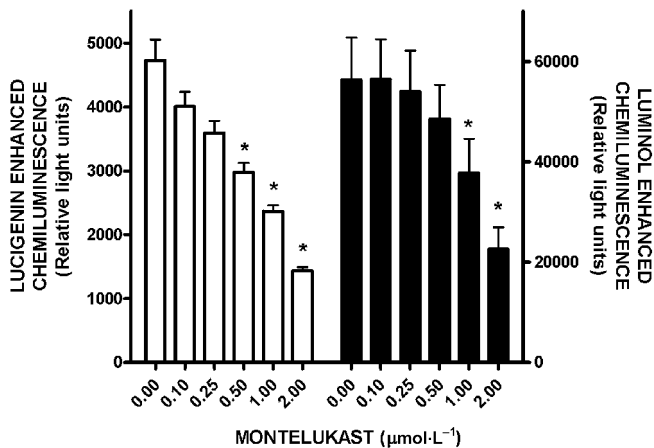
Figure 1 Effects of montelukast (0.1–2 µmol·L$^{-1}$) on the lucigenin- and luminol-enhanced chemiluminescence responses of neutrophils activated by N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (1 µmol·L$^{-1}$). The results are expressed as the mean peak chemiluminescence values in relative light units measured 30–50 s after the addition of FMLP and vertical lines show SEM. In the case of lucigenin-enhanced chemiluminescence (left graph, $n = 3$ with three to four replicates for each drug concentration and control system in each experiment), the absolute values for unstimulated neutrophils and for cells activated with FMLP in the absence of montelukast were 1086 ± 147 and 4729 ± 325 respectively, while the corresponding values for luminol-enhanced chemiluminescence (right graph, $n = 5$ with two to three replicates for each drug concentration and control system in each experiment) were 3047 ± 127 and 56 397 ± 8394 relative light units. *$P < 0.01$ to $P < 0.001$ for comparison with the FMLP-activated, montelukast-free control system.



Figure 2 Effects of montelukast (0.1–2 µmol·L$^{-1}$) on the release of elastase from neutrophils activated with N-formyl-L-methionyl-L-leucyl-L-phenylalanine (1 µmol·L$^{-1}$)/cytochalasin B (0.5 µmol·L$^{-1}$). The results ($n = 4$ with duplicate data sets for each experiment with 10 replicates for each drug concentration and control system in each experiment) are expressed as the mean values for total extracellular elastase (milliunits 10$^7$ cells$^{-1}$) and vertical lines show SEM. The absolute values for the unstimulated control system and for cells activated with N-formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B in the absence of montelukast were 221 ± 44 and 892 ± 9.6 milliunits elastase 10$^7$ cells$^{-1}$ respectively. *$P < 0.001$ for comparison with the drug-free control systems.

enhanced CL procedures are shown in Figure 1. Treatment of the cells with montelukast resulted in dose-related inhibition of the generation of ROS, which was evident using both procedures and which achieved statistical significance at concentrations of 0.5 µmol·L$^{-1}$ (lucigenin, $P < 0.001$) or 1µmol·L$^{-1}$ (luminol, $P < 0.01$). Maximal inhibition was observed at 2 µmol·L$^{-1}$ montelukast, resulting in 70% and 60% mean inhibition of the generation of ROS by FMLP-activated neutrophils with the lucigenin- and luminol-enhanced CL procedures respectively, the IC$_{50}$ for the latter being 1.5 µmol·L$^{-1}$ (confidence intervals 1.1–1.9).

As shown in Table 1, pre-treatment of neutrophils with MK886 (0.5 µmol·L$^{-1}$) did not affect the generation of superoxide by FMLP-activated neutrophils in either the absence or presence of montelukast (0.5 µmol·L$^{-1}$). Treatment of neutrophils with MK886 resulted in almost complete inhibition of the FMLP-activated production of LTB$_4$ by the cells, the values for unstimulated cells and for FMLP-activated cells in the absence and presence of MK886 being 59 ± 8, 332 ± 23 and 22 ± 3 pg LTB$_4$ 10$^7$ cells$^{-1}$ ($n = 5$ with a minimum of two replicates for each system).

The activity of NADPH oxidase in isolated membranes prepared from neutrophils activated with FMLP was markedly attenuated by treatment of the cells with montelukast (2 µmol·L$^{-1}$). The results for membrane fractions prepared from unstimulated neutrophils and those from neutrophils activated with FMLP in the absence and presence of montelukast were 2852 ± 291, 11 543 ± 698 and 6518 ± 407 rlu respectively ($n = 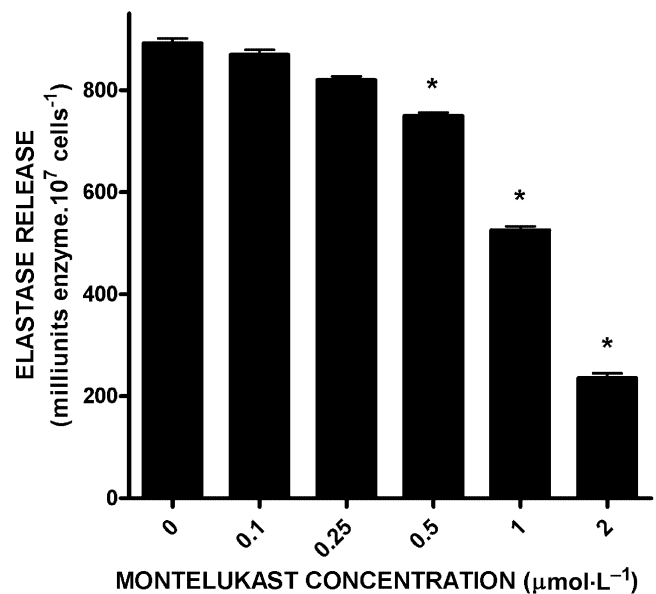5$ with two replicates for each system in each experiment; $P < 0.05$ for comparison of FMLP-activated systems without and with montelukast).

At the maximum concentration of montelukast used in these studies (2 µmol·L$^{-1}$), the drug did not possess detectable superoxide-scavenging activity, with the lucigenin-enhanced CL values of the xanthine oxidase/hypoxanthine superoxide-generating system in the absence and presence of montelukast being 22 939 ± 4850 and 22 271 ± 5413 rlu respectively (data from three separate experiments with three to four replicates for the control and drug-treated systems).

Activation of neutrophils with FMLP (1 µmol·L$^{-1}$) resulted in increased oxygen consumption by the cells that was linear over a 1 min period and was significantly attenuated by pre-treatment of the cells with 1 µmol·L$^{-1}$ montelukast (65 ± 11% of control; $n = 6$ with one to three replicates for each system; $P < 0.05$ for comparison of FMLP-activated systems without and with montelukast).

*Elastase release*
The effects of montelukast on the release of elastase from neutrophils activated with FMLP/cytochalasin B are shown in Figure 2. Treatment of the cells with montelukast resulted in dose-related inhibition of the release of elastase, which achieved statistical significance ($P < 0.001$) at concentrations of 0.5 µmol·L$^{-1}$ and greater, with maximal inhibition (79%) observed at 2 µmol·L$^{-1}$ of this agent. The IC$_{50}$ value for
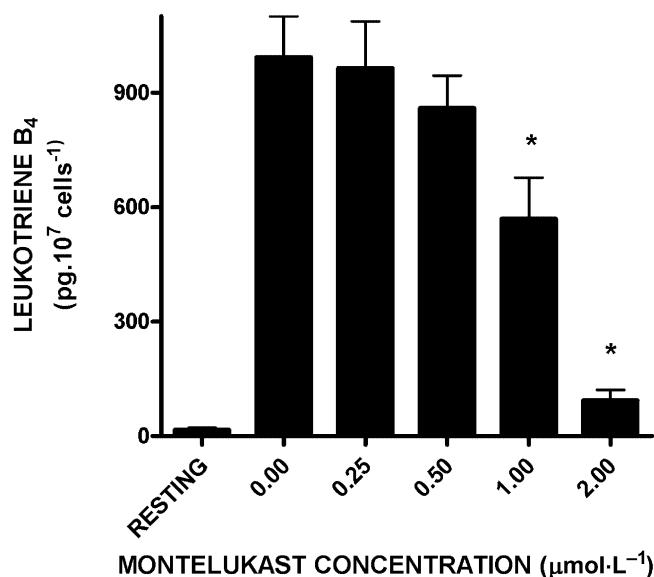
**Figure 3** Effects of montelukast (0.25–2 μmol·L$^{-1}$) on the production of leukotriene B$_4$ (LTB$_4$) by neutrophils activated with platelet-activating factor (200 nmol·L$^{-1}$). The results are presented as the mean values for total extracellular LTB$_4$ (pg 10$^7$ cells$^{-1}$) and vertical lines show SEM ($n = 8$, with two to three replicates for each drug concentration and control system in each experiment). The absolute values for the unstimulated control system and for cells activated with platelet-activating factor in the absence of montelukast were $16 \pm 6$ and $993 \pm 107$ pg LTB$_4$ 10$^7$ cells$^{-1}$ respectively. *$P < 0.001$ for comparison with the drug-free control system.

montelukast-mediated inhibition of elastase release was 1.2 μmol·L$^{-1}$ (95% confidence intervals 0.9–1.4).

*Leukotriene B$_4$*

The effects of montelukast on the production of LTB$_4$ by PAF (200 nmol·L$^{-1}$)-activated neutrophils are shown in Figure 3. Treatment of neutrophils with this agent resulted in dose-related inhibition of the generation of LTB$_4$, which achieved statistical significance ($P < 0.001$) at concentrations of 1 μmol·L$^{-1}$ and greater, with maximal inhibition (89 ± 3%) observed at 2 μmol·L$^{-1}$ montelukast. The IC$_{50}$ value for montelukast-mediated inhibition of LTB$_4$ production was 1.2 μmol·L$^{-1}$ (95% confidence intervals 0.7–1.6).

*Fura-2 fluorescence responses of activated neutrophils*

The results shown in Figure 4 are typical traces of the FMLP- and PAF-activated fluorescence responses of neutrophils in the absence and presence of montelukast at 2 μmol·L$^{-1}$. Addition of FMLP to neutrophils was accompanied by the characteristic, abrupt increase in fura-2 fluorescence intensity, which accompanies increased cytosolic concentrations of Ca$^{2+}$, rising from a basal value of 83 ± 8 nmol·L$^{-1}$ to a peak value of 419 ± 60 nmol·L$^{-1}$. This was followed by a rapid decrease in fluorescence intensity, which slowed after 1–2 min, coincident with influx of Ca$^{2+}$. Although the peak cytosolic Ca$^{2+}$ concentrations were equivalent in control and montelukast-treated neutrophils, the rate of decline in fluorescence intensity was faster in montelukast-treated cells. The time taken for fluorescence intensity to decline to half peak values was
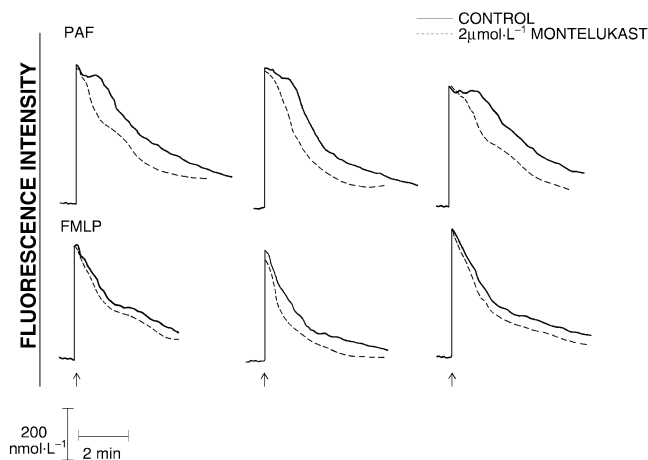


**Figure 4** Chemoattractant-activated fura-2 fluorescence responses of control and montelukast (2 μmol·L$^{-1}$)-treated neutrophils. Platelet-activating factor (PAF) (200 nmol·L$^{-1}$) or N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (1 μmol·L$^{-1}$) were added as indicated ($\uparrow$) when a stable baseline was obtained (±1 min). The traces shown are from three different representative experiments (8 for FMLP and 12 for PAF in the series).

1.3 ± 0.1, 0.9 ± 0.1 and 1.0 ± 0.1 min for FMLP-activated cells in the absence (control system) and presence of 1 and 2 μmol·L$^{-1}$ montelukast respectively ($n = 8$, $P < 0.05$ to $P < 0.001$ for comparison of the control system with each drug-treated system). These observations are compatible with increased efficiency of sequestration/resequestration of cytosolic Ca$^{2+}$ into stores and/or decreased store-operated influx of the cation.

In the case of PAF-activated cells, the peak increases in cytosolic Ca$^{2+}$ were sustained for about 1 min (Fig. 4) as described previously (Steel and Anderson, 2002). Treatment of neutrophils with montelukast (2 μmol·L$^{-1}$) markedly attenuated the duration of the sustained peak elevation in cytosolic Ca$^{2+}$, without affecting the magnitude of the peak response. The mean duration of the peak plateau elevation in cytosolic Ca$^{2+}$ for PAF-activated control cells was 1.13 ± 0.1 min, while the corresponding values for systems treated with 2 μmol·L$^{-1}$ montelukast was 0.3 ± 0.1 min ($n = 12$, $P < 0.001$ for comparison of the control system with each drug-treated system).

*Ca$^{2+}$ influx*

The effects of varying concentrations of montelukast (0.25–2 μmol·L$^{-1}$) on influx of $^{45}$Ca$^{2+}$ following activation of the cells with the chemoattractants are shown in Figure 5. Treatment of the cells with montelukast resulted in a dose-related decrease in the influx of Ca$^{2+}$ activated by both FMLP and PAF, which was statistically significant at concentrations of 1 and 2 μmol·L$^{-1}$.

*Cyclic AMP*

Exposure of neutrophils to montelukast caused a dose-related increase in intracellular cAMP, which achieved statistical significance ($P < 0.05$) at 1 μmol·L$^{-1}$, the values for the control system and systems treated with 0.5, 1 and 2 μmol·L$^{-1}$ montelukast being 4.8 ± 0.3, 6.2 ± 0.2, 7.7 ± 0.3 and

Effects of montelukast on neutrophils
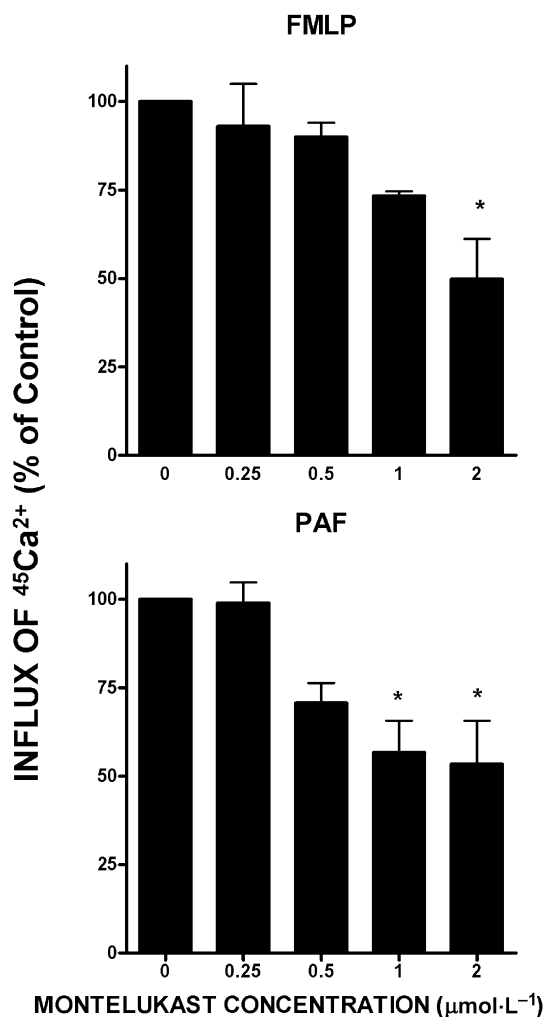R Anderson *et al*
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## FMLP



## PAF



**Figure 5** Effects of montelukast (0.25–2 $\mu mol \cdot L^{-1}$) on the influx of $^{45}Ca^{2+}$ into the neutrophils activated with either N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (1 $\mu mol \cdot L^{-1}$, upper graph) or platelet-activating factor (PAF) (200 nmol·$L^{-1}$, lower graph). The results are expressed as the mean percentages of the drug-free control systems and vertical lines show SEM ($n = 4$–8 with two to four replicates for each drug concentration and control system). The absolute values for uptake of $^{45}Ca^{2+}$ by unstimulated neutrophils and for cells activated with FMLP or PAF were $47 \pm 25$, $150 \pm 34$ and $148 \pm 14$ pmol $^{45}Ca^{2+}$ $10^7$ cells$^{-1}$ respectively. *$P < 0.05$ to $P < 0.01$ for comparison with the corresponding chemoattractant-activated montelukast-free control systems (according to the repeated measures ANOVA, there were significant effects of montelukast at both 1 and 2 $\mu mol \cdot L^{-1}$ for the FMLP system; on *post hoc* testing significance remained at 2 $\mu mol \cdot L^{-1}$).

$7.3 \pm 0.2$ pmol cAMP $10^7$ cells$^{-1}$ respectively. The effects of montelukast alone or in combination with CGS21680, rolipram or salbutamol are shown in Figure 6. Treatment of neutrophils with montelukast (2 $\mu mol \cdot L^{-1}$) in combination with either CGS21680, rolipram or salbutamol resulted in elevations in cAMP, which were significantly ($P < 0.05$) greater than those observed with the individual agents.

### PDE activity
The effects of montelukast, relative to those of rolipram and 3-isobutyl-1-methylxanthine, on cAMP and cGMP PDE
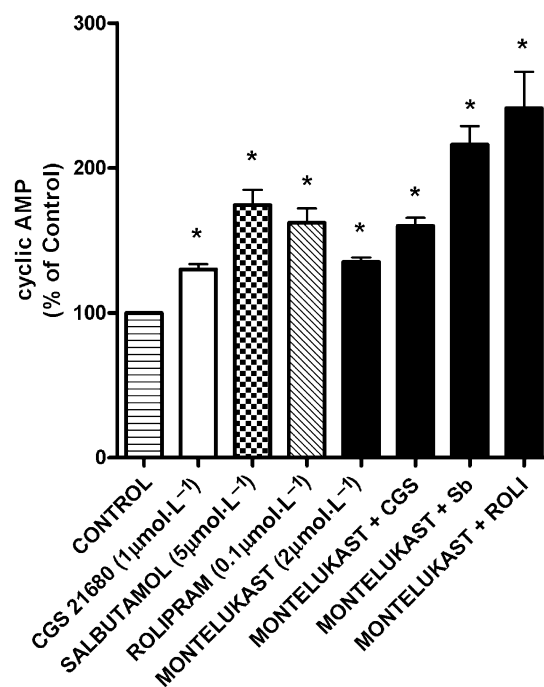


**Figure 6** Effects of CGS21680 (CGS; 1 $\mu mol \cdot L^{-1}$), salbutamol (Sb; 5 $\mu mol \cdot L^{-1}$), rolipram (ROLI; 0.1 $\mu mol \cdot L^{-1}$) and montelukast (2 $\mu mol \cdot L^{-1}$) individually, as well as those of montelukast in combination with the other agents on neutrophil intracellular cAMP. The results are presented as the mean percentages of the drug-free control system and vertical lines show SEM ($n = 6$, with two to three replicates for each drug concentration and control system in each experiment). The absolute value for the drug-free control system was $4.3 \pm 0.3$ pmol cAMP $10^7$ cells$^{-1}$. *$P < 0.05$ for comparison with the drug-free control systems.

activity, when added directly to neutrophil cytosolic extracts, are shown in Figure 7. Montelukast caused dose-related inhibition of both cAMP and cGMP PDE activity, which achieved statistical significance ($P < 0.05$) at concentrations of 1 $\mu mol \cdot L^{-1}$ and higher. Although not shown, similar effects of montelukast were observed using the PDE preparation from bovine heart. Using neutrophil cytosol as the source of PDE activity, the $IC_{50}$ value for montelukast-mediated inhibition of cAMP PDE activity was 3.4 $\mu mol \cdot L^{-1}$ (95% confidence intervals 2.9–3.9).

The cAMP PDE activities of matched cytosol and membrane fractions prepared from montelukast (2 $\mu mol \cdot L^{-1}$)-treated and untreated, unstimulated and FMLP-activated neutrophils are shown in Table 2. Enzyme activity was considerably lower in the membrane fractions, while no redistribution of enzyme activity between the cytosol and membrane compartments was evident following activation of the cells with FMLP. Pre-treatment of the cells with montelukast was accompanied by decreased cAMP PDE activity in the cytosol, and especially the membrane fractions. Given that the cell pellets were diluted approximately 20-fold in sucrose/PMSF following exposure to montelukast ± FMLP, it is likely that the inhibitory effects of montelukast on cAMP PDE were underestimated using this experimental design.

### Inositol triphosphate
The basal $IP_3$ value for unstimulated cells was $45 \pm 2$ pmol $10^7$ cells$^{-1}$, increasing to $63 \pm 2$ pmol $10^7$ cells$^{-1}$ at 10 s following
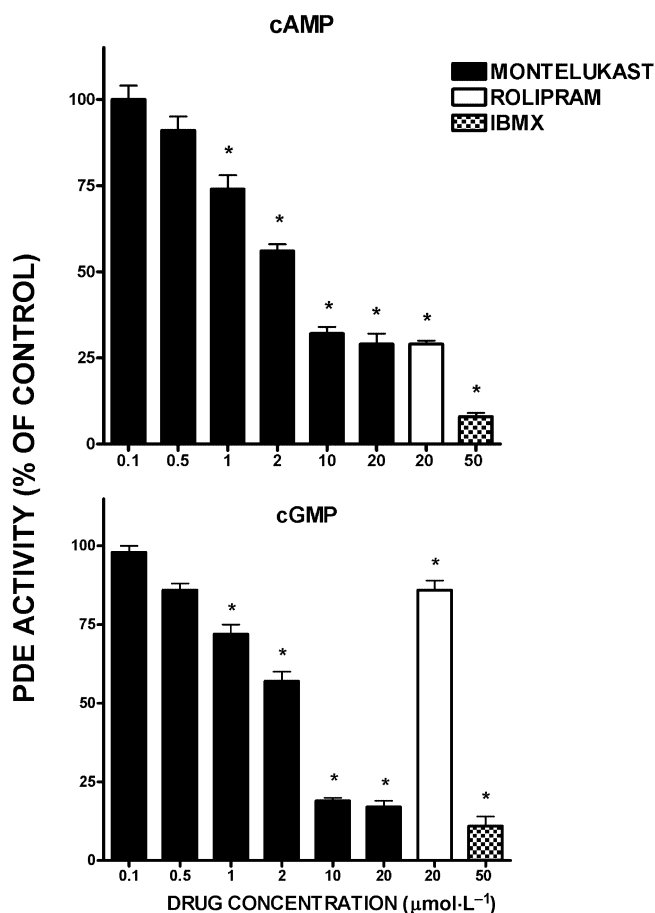
## cAMP



## cGMP

Figure 7 Effects of montelukast (0.5–20 μmol·L⁻¹), rolipram (20 μmol·L⁻¹) and 3-isobutyl-1-methylxanthine (50 μmol·L⁻¹) on cAMP (upper graph) and cGMP (lower graph) phosphodiesterase (PDE) activities in neutrophil cytosol. The results of four to eight and two to four experiments for cAMP and cGMP PDE activity respectively are presented as the mean percentages of the drug-free control systems and vertical lines show SEM. In the case of the cAMP/PDE experiments, the absolute values for the cytosol-free background system and for the cytosol-containing systems in the absence of the drugs were $1213 \pm 53$ and $14\,525 \pm 232$ counts per minute respectively. The corresponding values for the cGMP/PDE experiments were $2031 \pm 206$ and $21\,381 \pm 501$ counts per minute. $*P < 0.05$ for comparison with the corresponding drug-free control system.

Table 2 Cyclic AMP phosphodiesterase (PDE) activities in cytosol and membrane fractions prepared from matched control and montelukast-treated unstimulated and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated neutrophils

| System | PDE activity ($cpm \times 10^{-2}$ $min^{-1}$ $mg$ $protein^{-1}$) | |
| --- | --- | --- |
| | Membranes | Cytosol |
| Control, unstimulated cells | $108 \pm 2$ | $895 \pm 56$ |
| Unstimulated cells + 2 μmol·L⁻¹ montelukast | $34 \pm 10*$ | $718 \pm 57*$ |
| FMLP-activated control cells | $60 \pm 10$ | $938 \pm 38$ |
| FMLP-activated cells + 2 μmol·L⁻¹ montelukast | $21 \pm 6*$ | $609 \pm 34*$ |

Results are expressed as the mean values $\pm$ SEM ($n = 4$, 2 replicates for each system in each experiment).
$*P < 0.05$ for comparison with the corresponding drug-free control systems.

the addition of PAF (200 nmol·L⁻¹) to control neutrophils ($P < 0.05$ for comparison with the basal value), while the corresponding value for PAF-activated, montelukast (2 μmol·L⁻¹)-treated neutrophils was $60 \pm 3$ pmol IP₃ 10⁷ cells⁻¹, which did not differ significantly from the control system ($n = 12$, with two to five replicates for each drug concentration and control system in each experiment).

### ATP levels

Treatment of neutrophils with montelukast (2 μmol·L⁻¹) did not affect neutrophil ATP levels; the values for control and drug-treated cells following a 15 min exposure at 37°C were $62 \pm 2$ and $58 \pm 3$ pmol ATP 10⁷ cells⁻¹ respectively ($n = 2$, with seven replicates for each system in each experiment).

## Discussion and conclusions

Montelukast, a selective antagonist of CysLT₁ receptors, is used primarily in the treatment of allergic conditions such as bronchial asthma and allergic rhinitis (Fox-Spencer, 2006; Nayak and Langdon, 2007; Peters-Golden and Henderson, 2007). The reported pA₂ value for montelukast antagonism of LTD₄-mediated contraction of guinea pig trachea is 9.3 (Jones et al., 1995). Interestingly, beneficial therapeutic effects of this agent have been reported for diverse diseases in which neutrophils play a pathogenetic role, including chronic obstructive pulmonary disease, respiratory bronchiolitis, cystic fibrosis and atherosclerosis (Anonymous, 2004; Rubinstein et al., 2004; Celik et al., 2005; Fitzgerald and Mellis, 2006; Riccioni et al., 2007). The current study was designed to probe potential anti-inflammatory interactions of montelukast with activated human neutrophils in vitro.

Montelukast, at concentrations within the therapeutic range (Cheng et al., 1996; Knorr et al., 2001) and above, caused significant dose-related inhibition of superoxide (lucigenin CL) and hypochlorous acid (luminol CL) generation, as well as production of LTB₄ and release of elastase, by activated neutrophils. In the case of superoxide production, the inhibitory effects of montelukast were found to result from interference with the activation of NADPH oxidase. This conclusion is based on observations that montelukast, at concentrations of up to 2 μmol·L⁻¹, did not possess superoxide-scavenging activity, while treatment of the cells with this agent resulted in decreased oxygen consumption following activation with FMLP, as well as markedly reduced activity of NADPH oxidase in membrane fractions prepared from these cells. MK886, an inhibitor of 5-lipoxygenase-activating protein, was used to probe the possible involvement of LTC₄ and LTD₄ generated by contaminating cells in the neutrophil preparations, in the production of superoxide by these cells. The failure of MK886 to affect the production of superoxide by activated control neutrophils demonstrates that LTC₄ and LTD₄ were not present at high enough concentrations in the cell suspensions to affect neutrophil NADPH oxidase activity. More importantly, however, the failure of MK886 to attenuate montelukast-mediated inhibition of superoxide production by FMLP-activated neutrophils clearly demonstrates that the observed

anti-inflammatory effects of montelukast, in this experimental design, are directed primarily at neutrophils and not at contaminating cells in the cell suspension and, further, the effects were not mediated via antagonism of $CysLT_1$ receptors.

Considering that all the pro-inflammatory activities of neutrophils mentioned above are dependent on elevations in cytosolic $Ca^{2+}$, we also investigated the effects of montelukast on $Ca^{2+}$ fluxes in FMLP/PAF-activated neutrophils. Peak cytosolic $Ca^{2+}$ concentrations in PAF-activated neutrophils were sustained for 60–90 s, followed by a gradual subsidence over a time course of several minutes. The prolonged peak cytosolic $Ca^{2+}$ response observed in PAF-activated neutrophils results from the failure of this chemoattractant to activate both NADPH oxidase and adenylyl cyclase (Nick *et al.*, 1997; Steel and Anderson, 2002), resulting in early store-operated influx of $Ca^{2+}$ and failure of cAMP-dependent protein kinase (PKA)-mediated restoration of $Ca^{2+}$ homeostasis (as described below) respectively. In the case of FMLP-activated cells, NADPH oxidase-mediated membrane depolarization limits influx of $Ca^{2+}$, while activation of adenylyl cyclase favours rapid clearance of cytosolic $Ca^{2+}$ (Iannone *et al.*, 1989; Tintinger *et al.*, 2001). Consequently, the peak cytosolic $Ca^{2+}$ response observed in FMLP-activated neutrophils is of brief duration, declining rapidly for 1–2 min, followed by a levelling-off, coincident with store-dependent influx of $Ca^{2+}$ (Geiszt *et al.*, 1997; Tintinger *et al.*, 2001).

Treatment of neutrophils with montelukast did not affect the magnitudes of the immediate peak increase in cytosolic $Ca^{2+}$ in neutrophils activated with either FMLP or PAF. Taken together with the absence of effects of montelukast on $IP_3$ production by PAF-activated neutrophils, these observations demonstrate that neither phospholipase C nor the $Ca^{2+}$-mobilizing interactions of $IP_3$ with its receptor on intracellular $Ca^{2+}$ stores are affected by this agent. Treatment of neutrophils with montelukast did, however, significantly attenuate the duration of the prolonged peak cytosolic $Ca^{2+}$ response of PAF-activated neutrophils, while hastening the rate of decline in cytosolic $Ca^{2+}$ concentrations in FMLP-activated neutrophils, compatible with decreased store-operated influx of $Ca^{2+}$ in drug-treated cells. Using procedures that selectively measure the influx of $Ca^{2+}$ into chemoattractant-activated neutrophils, we observed that montelukast did indeed cause significant, dose-related inhibition of the uptake of $Ca^{2+}$ by cells activated by both FMLP and PAF, with mean values for inhibition of uptake of 50% and 66%, respectively, for cells treated with 2 μmol·$L^{-1}$ montelukast. Importantly, $Ca^{2+}$ influx is necessary to sustain the $Ca^{2+}$-dependent pro-inflammatory activities of neutrophils (Bréchard and Tschirhart, 2008).

Treatment of neutrophils with montelukast, at the same concentrations that suppressed the $Ca^{2+}$-dependent pro-inflammatory activities of the cells, was found to cause a significant increase in basal cAMP. Basal cAMP is probably maintained by the autocrine interactions of secreted adenosine with adenosine $A_{2A}$ receptors (Mundell *et al.*, 2001). From a mechanistic perspective, the elevation in intracellular cAMP observed in montelukast-treated neutrophils represented the most likely explanation for the $Ca^{2+}$ handling-targeted, anti-inflammatory interactions of this agent with

activated neutrophils. Interestingly, pre-treatment of neutrophils with montelukast followed by addition of CGS21680, rolipram or salbutamol resulted in elevations in neutrophil cAMP, which were significantly greater than those observed with the individual agents. With respect to CGS21680 and salbutamol, these agents were used at concentrations likely to cause saturation of adenosine $A_{2A}$ and $β_2$-adrenoceptors respectively, compatible with lack of agonist interactions of montelukast with either of these G protein/adenylyl cyclase-coupled receptor types. This observation, taken together with the interactive effects of montelukast and rolipram on raising basal cAMP in neutrophils, as well as the findings of a limited series of experiments that revealed an increase in basal cGMP in montelukast-treated cells (data not included), suggested that the drug possessed non-specific PDE inhibitory activity.

The effects of montelukast on cAMP and cGMP PDE activity were measured using cytosolic fractions from isolated neutrophils, as well as a PDE preparation from bovine heart. Addition of montelukast to either of these resulted in striking, dose-related inhibition of the activities of both cAMP and cGMP PDEs in neutrophil cytosol with an $IC_{50}$ value of 3.4 μmol·$L^{-1}$ for the former. The concentrations of montelukast that were found to possess non-specific PDE inhibitory activity therefore closely paralleled those that elevated cAMP and inhibited the $Ca^{2+}$-dependent pro-inflammatory activities of neutrophils, compatible with a causal association between these events. Although the $IC_{50}$ values for montelukast-mediated inhibition of neutrophil PDEs are somewhat higher than those for inhibition of superoxide and $LTB_4$ production and elastase release (1, 1.2 and 1.3 μmol·$L^{-1}$ respectively), this difference may be due to intracellular accumulation of lipophilic montelukast by intact cells, as the drug has an oil : water partition coefficient of $LogK_D = 2.3 \pm 0.2$ (data on file, Merck Research Laboratories).

We also attempted to measure the effects of addition of montelukast to intact neutrophils on cAMP PDE activity in cytosol and membrane fractions prepared from unstimulated and FMLP-activated cells. Activation of neutrophils with FMLP did not result in either increased activity of cytosolic cAMP PDE, or redistribution of enzyme to the membrane, with activity in the membrane fraction being low relative to the cytosol. Treatment of intact neutrophils with montelukast resulted in decreased cAMP PDE activity in the cytosolic and especially the membrane fractions of unstimulated and FMLP-activated cells. In the case of the cytosol, however, this was of lesser magnitude than that observed following direct addition of the drug to the cytosol, due, presumably, to loss and dilution of the drug during cell processing.

As a consequence of activation of PKA, cAMP promotes restoration of $Ca^{2+}$ homeostasis in neutrophils and other cell types by multiple mechanisms, including phosphorylative inactivation of PLCγ (Ali *et al.*, 1998); inactivation of $IP_3$-receptors (Bai and Sanderson, 2006); phosphorylative up-regulation of the $Ca^{2+}$ sequestering/resequestering endomembrane $Ca^{2+}$-ATPase (Anderson *et al.*, 1998; Anderson *et al.*, 2000); inactivation of store-operated $Ca^{2+}$ channels (Binnaz *et al.*, 2006); and inhibition of p38 MAP kinase and consequent interference with the activation of 5-lipoxygenase (Flamand *et al.*, 2002), thereby attenuating an autocrine,

$LTB_4$-mediated secondary wave of $Ca^{2+}$ uptake by the cells (Steel et al., 2007). While the first of these mechanisms does not appear to contribute significantly to the effects of montelukast on $Ca^{2+}$ handling by activated neutrophils observed in the current study, all the other mechanisms may be operative. Moreover, cross-activation of PKA and PKG by cAMP and cGMP may also contribute to restoration of $Ca^{2+}$ homeostasis as PKG has also been reported to restrict store-operated uptake of $Ca^{2+}$ (Ruiz-Velasco et al., 1998).

Although PDE4 subtype B2 appears to be the predominant PDE in human neutrophils (Wang et al., 1999), it is noteworthy that cilostazol, a PDE3 inhibitor, has been reported to attenuate $Ca^{2+}$ fluxes in activated human neutrophils, as well as superoxide generation (Yang et al., 2006), demonstrating, albeit indirectly, the presence of PDE3 in these cells. Using selective inhibitors of PDE3 (cilostamide) and PDE5 (MY5445), we have observed that neutrophils exhibit appreciable activities of both of these PDEs in addition to PDE4 (R. Anderson et al., unpubl. obs.). Given the ability of PDE3 to hydrolyze cAMP, as well as cross-activation of PKAs A and G by cAMP and cGMP, non-specific PDE inhibitors that target PDEs 3, 4 and 5 in neutrophils may be more effective anti-inflammatory agents than those that selectively target PDE4, by preventing compensatory, counteracting increases in the activities of PDEs 3 and 5. The apparent benefits of a combination of PDE3 and PDE4 inhibitors, as opposed to either category of inhibitor alone, have been already described in an animal model of allergen-induced bronchospasm (Underwood et al., 1994).

Although the non-specific PDE inhibitory effects of montelukast described here have not, to our knowledge, been reported previously, several of the early, experimental $CysLT_1$ receptor antagonists such as FPL55712 and LY171883 were documented to possess this property (Fleisch et al., 1984; Hay et al., 1987). More recently, CR3465, a novel $CysLT_1$ receptor antagonist, was reported to possess PDE inhibitory activity (Ferrari et al., 2004). In the case of FPL55712 and LY171883, PDE inhibitory activity appeared to represent a limitation in respect of specificity of pharmacological mode of action and clinical development (Fleisch et al., 1984; Hay et al., 1987), whereas for CR3465 the combination of $CysLT_1$ receptor antagonism and PDE inhibitory activity was considered to be beneficial, because the latter property conferred additional protection by targeting spasmogenic and inflammatory mediators other than CysLTs (Ferrari et al., 2004).

It is noteworthy that PKA also possesses anti-inflammatory activities that are distinct from its effects on $Ca^{2+}$ handling by activated immune and inflammatory cells. These include interference with the activation of NADPH oxidase, and inhibition of p38 MAP kinase (as mentioned above) and phosphatidylinositol 3-kinase (Bengis-Garber and Gruener, 1996; Flamand et al., 2002; Burelout et al., 2007). In addition, montelukast has also been reported to inhibit human recombinant 5-lipoxygenase with a relatively high $IC_{50}$ of 30–50 $\mu mol \cdot L^{-1}$, while synthesis of $LTB_4$ by activated neutrophils was inhibited at drug concentrations of >1 $\mu mol \cdot L^{-1}$ (Ramires et al., 2004). However, effects on $Ca^{2+}$ fluxes and cAMP, which may explain the greater sensitivity of intact cells to the inhibitory effects of montelukast on $LTB_4$ production, were not investigated in this study. Nevertheless, inhibition of 5-lipoxygenase, either directly or indirectly by

the mechanisms described in the current study, together with reported inhibition of signalling via P2Y receptors (Mamedova et al., 2005), suggests that montelukast may be particularly effective in attenuating both the generation and action of autocrine inflammatory mediators.

Antagonism of $CysLT_1$ receptors is clearly the primary mechanism of therapeutic activity of montelukast. However, the PDE-targeted anti-inflammatory activity of this agent described in the current study may contribute to the beneficial effects of this agent, used in addition to inhaled corticosteroids, in some categories of patients with bronchial asthma (Laviolette et al., 1999), possibly by enabling control of the corticosteroid-resistant neutrophil (Barnes, 2007), as well as by countering bronchospasm via direct cyclic nucleotide-mediated effects on airway smooth muscle (Binnaz et al., 2006).

## Acknowledgements

## Conflicts of interest

CF acts on the Speakers' Bureau and Advisory Board of MSD (Pty) Ltd, Johannesburg, South Africa and has received congress travel support from MSD. CG acts on the Speakers' Bureau of MSD (Pty) Ltd, Johannesburg, South Africa.

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