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 μmol·L–1), on Ca2+-dependent pro-inflammatory activities, cytosolic Ca2+ fluxes and intracellular cAMP in isolated human neutrophils activated with the chemoattractants, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (1 μmol·L–1) and platelet-activating factor (200 nmol·L–1).

Experimental approach: Generation of reactive oxygen species was measured by lucigenin- and luminol-enhanced chemiluminescence, elastase release by a colourimetric assay, leukotriene B4 and cAMP by competitive binding ELISA procedures, and Ca2+ fluxes by fura-2/AM-based spectrofluorimetric and radiometric (45Ca2+) procedures.

Key results: Pre-incubation of neutrophils with montelukast resulted in dose-related inhibition of the generation of reactive oxygen species and leukotriene B4 by chemoattractant-activated neutrophils, as well as release of elastase, all of which were maximal at 2 μmol·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 Ca2+ concentrations and store-operated Ca2+ influx. These montelukast-mediated alterations in Ca2+ handling by the cells were associated with a significant elevation in basal cAMP levels, which resulted from inhibition of cyclic nucleotide phosphodiesterases.


Keywords: Adenosine 3′,5′-cyclic monophosphate; calcium; elastase; leukotriene B4; montelukast; neutrophils; phosphodiesterases; reactive oxygen species

Abbreviations: CB, cytochalasin B; CL, chemiluminescence; CysLT, cysteinyl leukotriene; DMSO, dimethylsulphoxide; FLAP, 5-lipoxygenase activating protein; FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; HBSS, Hanks’ balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; IP3, inositol-1,4,5-triphosphate; PAF, platelet-activating factor; PDE, phosphodiesterase; ROS, reactive oxygen species

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 LTC4 and LTD4, 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 Ca2+ by chemoattractant-activated human neutrophils, as well as on the mobilization of stored and extracellular Ca2+. Montelukast, at therapeutically relevant concentrations, on inflammatory activity of montelukast is unclear.

Measurement of reactive oxygen species

These were measured using lucigenin (bis-N-methyl-acridinium 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/H2O2/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 µmol·L⁻¹) in 900 µl of Hanks’ balanced salt solution (HBSS) containing either lucigenin (0.2 mmol·L⁻¹) or luminol (0.1 mmol·L⁻¹), followed by addition of either 100 µl of HBSS (unstimulated control systems) or the chemoattractant, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 µmol·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 LTC4 and LTD4. Neutrophils were pre-incubated with MK886 (0.5 µmol·L⁻¹, final) for 5 min at 37°C followed by addition of montelukast (0.5 µmol·L⁻¹) and a further pre-incubation of 5 min followed by addition of FMLP (1 µmol·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 LTD4 by the cells using the method described below.

The superoxide-scavenging potential of montelukast (2 µmol·L⁻¹) was measured using a cell-free xanthine (1 mmol·L⁻¹)/xanthine oxidase (130 mU ml⁻¹) lucigenin-dependent CL procedure.

Oxygen consumption

This was measured using a three-channel oxygen electrode (Model DW1, Hansatech Ltd, King’s Lynn, Norfolk, UK). Neutrophils (2 x 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 µmol·L⁻¹ followed by addition of FMLP (1 µmol·L⁻¹) and measurement of PO2 over a 5 min time course.
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 x 10⁶ 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 x 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 Ca²⁺
Fura-2/AM was used as the fluorescent, Ca²⁺-sensitive indicator for these experiments (Grynkiewicz et al., 1985). Neutrophils (1 x 10⁶ 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 CaCl₂. The fura-2-loaded cells (2 x 10⁶ 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 Ca²⁺ influx
A radiometric procedure was also used to measure the net influx of ⁴⁶Ca²⁺ 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 Ca²⁺-replete (1.25 mmol·L⁻¹) HBSS to ensure that intracellular Ca²⁺ stores were full to minimize spontaneous uptake of ⁴⁶Ca²⁺ (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 x 10⁶ ml⁻¹ in HBSS containing 25 μmol·L⁻¹ cold, carrier CaCl₂. The Ca²⁺-loaded neutrophils (2 x 10⁶ ml⁻¹) were then incubated for 5 min at 37°C in HBSS containing 25 μmol·L⁻¹ CaCl₂ in the absence or presence of montelukast (2 μmol·L⁻¹) followed by simultaneous addition of FMLP or PAF and 2 μCi ml⁻¹ ⁴⁶Ca²⁺ (as ⁴⁶[Ca]Cl₂, specific activity 24.3 mCi mg⁻¹, Perkin Elmer Life and Analytical Sciences, Boston, MA, USA), or ⁴⁶Ca²⁺ 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 Ca²⁺ is complete (Steel and Anderson, 2002), and the reactions stopped by the addition of 10 ml of ice-cold, Ca²⁺-replete HBSS to the tubes, which were transferred immediately to an ice bath. The cells were then pelleted by centrifugation at 400 x g for 5 min followed by washing with 15 ml of ice-cold, Ca²⁺-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 ⁴⁶Ca²⁺-10⁷ cells⁻¹).

Measurement of LTB₄ and cyclic AMP
Competitive binding enzyme immunoassay procedures (Correlate-EIA™, Assay Designs Inc, Ann Arbor, MI, USA) were used to measure LTB₄ in the supernatants of neutrophils activated with PAF (200 mmol·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 LTB₄, neutrophils (2 x 10⁶ 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 LTB₄ using the enzyme immunoassay procedure. Supernatants from cells activated with PAF were diluted 1:4 prior to assay. These results are expressed as pg 10⁷ cells⁻¹.

In the case of cAMP, neutrophils (2 x 10⁶ 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 10⁷ 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 (β₂-adrenoreceptor agonist, 5 μmol·L⁻¹), CGS21680 (adenosine A₂a 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 x 10⁶ 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 x 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 MgCl₂, 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 [³²P]cAMP or [³²P]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 [³²P]cAMP or [³²P]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 μM ml⁻¹.

In an additional series of experiments, the effects of pre-treatment 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 × 10⁶ 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 (IP₃) 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 KClO₄. The supernatants were assayed for IP₃ using the inositol-1,4,5-triphosphate (H) radioreceptor assay kit (Perkin Elmer Life and Analytical Sciences), which is a competitive ligand binding assay, and the results expressed as pmol 10⁷ 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 × 10⁶ cells ml⁻¹) following exposure of the cells to the drug for 15 min at 37°C, using a luciferin/luciferase CL procedure (Holmsen et al., 1972). These results are expressed as nmol ATP 10⁷ cells⁻¹.

**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-
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 1 μmol·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 2852 ± 291, 11 543 ± 698 and 6518 ± 407 rlu respectively (\(n = 5\) with 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 neutrophils 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
MONTELUKAST CONCENTRATION (µmol-L⁻¹)

Figure 3  Effects of montelukast (0.25–2 µmol-L⁻¹) on the production of leukotriene B₄ (LTB₄) by neutrophils activated with platelet-activating factor (200 nmol-L⁻¹). The results are presented as the mean values for total extracellular LTB₄ (pg 10⁷ 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 ± 6 and 993 ± 107 pg LTB₄ 10⁷ 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 B₄

The effects of montelukast on the production of LTB₄ 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 LTB₄, 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 IC₅₀ value for montelukast-mediated inhibition of LTB₄ 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 Ca²⁺, 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 Ca²⁺. Although the peak cytosolic Ca²⁺ 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

Ca²⁺ influx

The effects of varying concentrations of montelukast (0.25–2 µmol-L⁻¹) on influx of ⁴⁴Ca²⁺ 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 Ca²⁺ 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
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Figure 5 Effects of montelukast (0.25–2 μmol·L⁻¹) on the influx of ⁴⁵Ca²⁺ into the neutrophils activated with either N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (1 μmol·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 ⁴⁵Ca²⁺ by unstimulated neutrophils and for cells activated with FMLP or PAF were 47 ± 25, 150 ± 34 and 148 ± 14 pmol ⁴⁵Ca²⁺ 10⁷ 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 μmol·L⁻¹ for the FMLP system; on post hoc testing significance remained at 2 μmol·L⁻¹).

7.3 ± 0.2 pmol cAMP 10⁷ 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 μmol·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 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 μmol·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 IC₅₀ value for montelukast-mediated inhibition of cAMP PDE activity was 3.4 μmol·L⁻¹ (95% confidence intervals 2.9–3.9).

The cAMP PDE activities of matched cytosol and membrane fractions prepared from montelukast (2 μmol·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. Pretreatment 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 IP₃ value for unstimulated cells was 45 ± 2 pmol 10⁷ cells⁻¹, increasing to 63 ± 2 pmol 10⁷ cells⁻¹ at 10 s following...
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 ± 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 ± 2 and 58 ± 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 LTβ₄ 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 CysLT₁ receptors.

Considering that all the pro-inflammatory activities of neutrophils mentioned above are dependent on elevations in cytosolic Ca²⁺, we also investigated the effects of montelukast on Ca²⁺ fluxes in FMLP/PAF-activated neutrophils. Peak cytosolic Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ and failure of cAMP-dependent protein kinase (PKA)-mediated restoration of Ca²⁺ homeostasis (as described below) respectively. In the case of FMLP-activated cells, NADPH oxidase-mediated membrane depolarization limits influx of Ca²⁺, while activation of adenylyl cyclase favours rapid clearance of cytosolic Ca²⁺ (Iannone et al., 1989; Tintinger et al., 2001). Consequently, the peak cytosolic Ca²⁺ 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 Ca²⁺ (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 Ca²⁺ in neutrophils activated with either FMLP or PAF. Taken together with the absence of effects of montelukast on IP₃ production by PAF-activated neutrophils, these observations demonstrate that neither phospholipase C nor the Ca²⁺-mobilizing interactions of IP₃ with its receptor on intracellular Ca²⁺ stores are affected by this agent. Treatment of neutrophils with montelukast did, however, significantly attenuate the duration of the prolonged peak cytosolic Ca²⁺ response of PAF-activated neutrophils, while hastening the rate of decline in cytosolic Ca²⁺ concentrations in FMLP-activated neutrophils, compatible with decreased store-operated influx of Ca²⁺ in drug-treated cells. Using procedures that selectively measure the influx of Ca²⁺ into chemoattractant-activated neutrophils, we observed that montelukast did indeed cause significant, dose-related inhibition of the uptake of Ca²⁺ 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, Ca²⁺ influx is necessary to sustain the Ca²⁺-dependent pro-inflammatory activities of neutrophils (Bréchard and Tschirhart, 2008).

Treatment of neutrophils with montelukast, at the same concentrations that suppressed the Ca²⁺-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 A₂A 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 Ca²⁺ 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 A₂A and β-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 IC₅₀ 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 Ca²⁺-dependent pro-inflammatory activities of neutrophils, compatible with a causal association between these events. Although the IC₅₀ values for montelukast-mediated inhibition of neutrophil PDEs are somewhat higher than those for inhibition of superoxide and LTB₄ 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 LogK₁₀ = 2.3 ± 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 Ca²⁺ homeostasis in neutrophils and other cell types by multiple mechanisms, including phosphorylative inactivation of PLCγ (Ali et al., 1998); inactivation of IP₃-receptors (Bai and Sanderson, 2006); phosphorylative up-regulation of the Ca²⁺ sequestering/resequestering endomembrane Ca²⁺-ATPase (Anderson et al., 1998; Anderson et al., 2000); inactivation of store-operated Ca²⁺ channels (Binnaz et al., 2006); and inhibition of p38 MAP kinase and consequent interference with the activation of 5-lipooxygenase (Flamand et al., 2002), thereby attenuating an autocrine,
LTB₄-mediated secondary wave of Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ homeostasis as PKG has also been reported to restrict store-operated uptake of Ca²⁺ (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 Ca²⁺ 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 CysLT₁ 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 CysLT₁ 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 CysLT₁ 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 Ca²⁺ 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; Bureau et al., 2007). In addition, montelukast has also been reported to inhibit human recombinant 5-lipoxygenase with a relatively high IC₅₀ of 30–50 μmol·L⁻¹, while synthesis of LTB₄ by activated neutrophils was inhibited at drug concentrations of >1 μmol·L⁻¹ (Ramires et al., 2004). However, effects on Ca²⁺ fluxes and cAMP, which may explain the greater sensitivity of intact cells to the inhibitory effects of montelukast on LTB₄ 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 CysLT₁ 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).

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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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