Montelukast inhibits tumour necrosis factor-α-mediated interleukin-8 expression through inhibition of nuclear factor-κB p65-associated histone acetyltransferase activity

F. Tahan, E. Jazrawi, T. Moodley, G. E. Rovati and I. M. Adcock

Department of Pediatric Allergy, Erciyes University School of Medicine, Kayseri, Turkey; Airways Disease Section, National Heart and Lung Institute, Imperial College, London, UK and Laboratory of Molecular Pharmacology, Department of Pharmaceutical Sciences, University of Milan, Milan, Italy

Clinical and Experimental Allergy

Summary

Background Montelukast is a potent cysteinyi leukotriene-1 receptor antagonist possessing some anti-inflammatory effects although the molecular mechanism of these anti-inflammatory effects is unknown. In this study, we aimed to investigate the effect of montelukast on nuclear factor (NF)-κB-associated histone acetylation activity in phororbol myristate acetate (PMA)-differentiated U937 cells.

Methods We examined the inhibitory effects of montelukast on TNF-α-induced IL-8 production in PMA-differentiated U-937 cells. U-937 cells were exposed to PMA (50 ng/mL) for 48 h to allow differentiation to macrophages. Macrophages were then exposed to TNF-α (10 ng/mL) in the presence or absence of montelukast (0.01–10 μM) for 24 h. After this time, the concentration of IL-8 in the culture supernatant was measured by sandwich-type ELISA kit. The effect of signalling pathways on TNF-α-induced IL-8 release was examined pharmacologically using selective NF-κB/IKK2 (AS602868, 3 μM), (PD98059, 10 μM) and p38 mitogen activated protein kinase (MAPK) (SB203580, 1 μM) inhibitors. NF-κB DNA binding activity was measured by a DNA-binding ELISA-based assay. NF-κB-p65-associated histone acetyltransferase (HAT) activity was measured by immunoprecipitation linked to commercial fluorescent HAT.

Results TNF-α-induced IL-8 release was suppressed by an NF-κB inhibitor but not by MEK or p38 MAPK inhibitors. Montelukast induced a concentration-dependent inhibition of TNF-α-induced IL-8 release and mRNA expression that reached a plateau at 0.1 μM without affecting cell viability. Montelukast did not affect NF-κB p65 activation as measured by DNA binding but suppressed NF-κB p65-associated HAT activity.

Conclusion Montelukast inhibits TNF-α-stimulated IL-8 expression through changes in NF-κB p65-associated HAT activity. Drugs targeting these enzymes may enhance the anti-inflammatory actions of montelukast.

Keywords histone acetylase, IL-8, macrophages, montelukast, NF-κB

Submitted 6 June 2007; revised 21 December 2007; accepted 16 January 2008

Introduction

Montelukast is a potent cysteinyi leukotriene (cysteinyi-LT) receptor antagonist possessing some anti-inflammatory effects, although the molecular mechanisms for these anti-inflammatory actions are unclear [1–3]. All cysteinyi-LT receptor antagonists, act on the CysLT1 receptors, thereby implicating these receptors in the control of airway inflammation, bronchoconstriction and remodeling [4]. Montelukast significantly decreases sputum eosinophil cationic protein, soluble IL-2 receptor, IL-4 and soluble intercellular adhesion molecule (ICAM)-1 levels, reduces eosinophil blood counts, and levels of exhaled nitric oxide in asthmatics [5–7]. Pranlukast reduces RANTES and nuclear factor (NF)-κB production by isolated lung mononuclear cells, suppresses LPS-induced NF-κB-luciferase reporter gene activity and Muc2 expression pro-inflammatory cytokine production via inhibition of NF-κB activation in human epithelial cells and partially represses TNF-α-induced NF-κB activation in peripheral...
blood monocytes, U937 cells and Jurkat T cells and attenuates allergen-specific TNF-α production and NF-κB nuclear translocation in peripheral blood monocytes from atopic [8–11]. The mechanism underlying the inhibitory effect of pranlukast on NF-κB activation is unclear, but does not seems to be due to antagonism of the CysLT₁ receptor [9, 10]. Furthermore, Maeba et al. suggest that high doses of montelukast modulate the production of IL-6, TNF-α from atopic [8–11]. The mechanism underlying the inhibitory effect of pranlukast on NF-κB activity is unclear, but no seems to be due to antagonism of the CysLT₁ receptor [9, 10]. Furthermore, Maeba et al. suggest that high doses of montelukast modulate the production of IL-6, TNF-α and MCP-1 through the inhibition of NF-κB activation [12]. However, the anti-inflammatory effect of cysLT receptor antagonists, such as montelukast at therapeutic doses in patients with asthma, needs to be further investigated. This data suggests that CysLT₁ receptor antagonists, the first new class of asthma drugs to be developed in the past 30 years, may have anti-inflammatory properties in addition to those of corticosteroids [13].

DNA is tightly compacted within the resting cell and along with histone proteins, forms an inaccessible chromatin structure preventing inflammatory gene expression [14]. Post-translational modifications of histone residues have been associated with alterations in the chromatin structure allowing gene expression [14]. Transcriptional co-activator proteins contain histone acetyltransferase (HAT) activity and activation of these co-activators by pro-inflammatory transcription factors such as NF-κB and AP-1 leads to increased gene expression [14]. In addition, these transcription factors are themselves targets for HAT activity increasing their transactivation potential [15, 16].

The effect of HATs on gene expression is counteracted by histone deacetylases (HDACs), a family of 11 related members [17, 18]. HDACs play a critical role in the supression of gene expression by reversing the hyperacetylation of core histones and by targeting acetylated transcription factors. Thus, HDACs are associated with inactive p65 and play a role in the regulation NF-κB-mediated gene transcription [15, 16]. HDAC1 and HDAC2 are able to deacetylate-acetylated NF-κB and terminate the activity of NF-κB [15, 16]. Inhibition of these HDACs results in increased activity of NF-κB and increased expression of inflammatory genes such as IL-8.

Anti-inflammatory agents such as corticosteroids, theophylline and antioxidants decrease inflammatory gene associated HAT activity, at least in part, by recruiting HDAC activity to the activated NF-κB [19]. There is no data available concerning the possible effects of montelukast on histone deacetylation/acetylation status. We, therefore, investigated the effects of montelukast on NF-κB-associated HAT activity.

Materials and methods

Cell culture and stimulation conditions

U-937 cells, a human monocyctic leukaemia cell line, obtained from the American Type Culture Collection, were grown at 37°C under humidified 5% CO₂ in RPMI 1640 medium containing 10% FCS and 5 mL l-glutamine as previously described [20]. The cells were allowed to differentiate for 48 h in the presence of phorbol myristate acetate PMA (50 ng/mL) for 48 h. Macrophages were made quiescent in fresh complete RPMI without PMA and in 0.25% FCS for 24 h before stimulation with TNF-α (10 ng/mL) in the presence or absence of montelukast (10⁻¹⁻¹⁻⁰⁻⁵ M). Cell viability was determined by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) method [20] and was not significantly affected by any of the treatments used. Cell culture medium was collected after 24 h and IL-8 measured by an ELISA. In other experiments, cells were treated for various time-points with TNF-α and montelukast and IL-8 mRNA expression determined by RT-qPCR, NF-κB p65 DNA binding measured by ELISA, HAT and HDAC activities associated with NF-κB p65 determined by immunoprecipitation which is followed by commercially available activity assays. In some experiments, the effects of using selective NF-κB/IKK2 (AS602868, 3 μM), MEK (PD98059, 10 μM) and p38 MAPK (SB203580, 1 μM) inhibitors on TNF-α-induced IL-8 release were determined.

Interleukin-8 enzyme-linked immunosorbent assay

Determination of IL-8 expression was measured by sandwich ELISA (R&D Systems Europe, Abingdon, UK) according to the manufacturer’s instructions.

Reverse transcriptase – real-time polymerase chain detection of interleukin-8 messenger ribonucleic acid

Total RNA was isolated from cells after 4 h stimulation using the RNeasy Mini Kit (QIAGEN, Crawley, Sussex, UK). cDNA was generated by reverse transcription using random hexamers and used as a template in the subsequent PCR analyses. Transcript levels were determined by real-time PCR (Rotor Gene 3000; Corbett Research, Sydney, Australia) using the SyberGreen PCR Master Mix Reagent Kit (Promega, Madison, WI, USA). The sequences of IL-8 PCR primers were sense, 5’-GCCAACACAGAATTTTG-TAAGCTT; antisense, 5’-CTTCGCCAGCCAGTTTCCCT. Primers for GAPDH were sense, 5’-ATTCATTGGACCACGT-CAAGGCT; antisense, 5’-TCAGTTCAACCACTGACACGT. PCR conditions were as previously described [21] and data from the reaction were collected and analysed by the complementary computer software (Corbett Research). Relative quantifications of gene expression were calculated using standard curves and were normalized to GAPDH.
and NF-κB binding to its consensus sequence was determined using a TransAM assay as described previously [22]. Cells were collected and resuspended in mild lysis buffer [10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40] and complete protease inhibitor cocktail (Boehringer–Mannheim, Germany) for 10 min. Nuclei were collected by microcentrifugation (12 000g, 5 min, 4°C) and resuspended in a Tris-based high-salt buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.25 mM EDTA, 10 mM of 2-mercaptoethanol and complete protease inhibitor cocktail). After 30 min, the supernatant was collected and diluted with Tris-based no salt buffer (10 mM Tris-HCl, pH 8.0, 0.25 mM EDTA, 10 mM of 2-mercaptoethanol and complete protease inhibitor cocktail) to 40 mM NaCl final concentration.

Nuclear extracts were evaluated for the presence of NF-κB p65 subunits using Trans-AM NF-κB p65 Transcription Factor Assay kits (Active Motif Europe, Belgium), according to the manufacturer’s instructions. Briefly, an equal amount (5 μg) of nuclear lysate was added to incubation wells precoated with an oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) sequence; the active form of NF-κB contained in the cell extract specifically binds to this oligonucleotide. Bound NF-κB p65 subunit in cell nuclear extracts was detected by a specific anti-p65 antibody (100 μL diluted 1 : 1000 in antibody-binding buffer for 1 h) followed by incubation with an horseradish peroxidase (HRP)-conjugated secondary antibody. The plate was washed again and 100 μL of developing solution was added. The plate was incubated for 2–10 min away from direct light, 100 μL of stop solution was added and the plate was read using a microplate spectrophotometer at 450 nm and results are expressed as OD units. Specificity was confirmed by the lack of binding detected with a non-specific antibody control or in the presence of a 1000-fold excess wild-type oligonucleotide.

**Nuclear factor-κB p65-associated histone acetyltransferase and histone deacetylase activity**

Cell nuclear extracts were prepared using 100 μL RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate) containing complete protease inhibitor mixture following stimulation with TNF-α for 60 min as described previously [23]. The lysis mixture was incubated with 20 μL of anti-NF-κB p65 antibody as previously described [23] before addition of protein A/G agarose beads and washing in 500 μL RIPA buffer. After the last wash, the agarose pellet was stored in 30 μL RIPA at −80°C until analysed.

**Measurement of histone acetyltransferase activity**

HAT activity was measured by ELISA (HAT activity assay kit; Upstate Biotechnology) as previously described [24]. Samples were mixed with acetyl-CoA and incubated for 30 min on a plate precoated with histone H4. Acetylated histones were detected using an anti-acetyl-lysine rabbit polyclonal antibody followed by an HRP-based colorimetric assay.

**Measurement of histone deacetylase activity**

HDAC activity of nuclear extracts was measured with a non-isotopic assay using a fluorescent derivative of epsilon-acetyl lysine (HDAC fluorescent activity assay kit; BIOMOL, Plymouth Meeting, PA, USA). This assay is based on the Fluor de Lys (fluorogenic histone deacetylase lysyl substrate/developer) substrate and developer combination. The assay was performed exactly as recommended by the manufacturer, and emitted light detected at 460 nm in a fluorometric plate reader.

**Statistics**

Statistical analysis was preformed using an ANOVA followed by Bonferroni’s post test analysis. All data shown are mean ± SEM of at least three independent experiments.

**Results**

**Montelukast suppresses tumour necrosis factor-α-induced interleukin-8 production**

TNF-α (10 ng/mL) enhanced basal IL-8 protein expression as determined by ELISA (Fig. 1a). Montelukast induced a concentration-dependent inhibition (IC50 = 4.2 ± 0.7 mM) of TNF-α-induced IL-8 release, which reached a plateau at 0.1 μM (Fig. 1b). Montelukast did not affect cell viability as measured by MTT assay.

We also demonstrated that this effect occurred at the mRNA level as montelukast also caused a complete attenuation of TNF-α-induced IL-8 mRNA production after 4 h in a concentration-dependent manner (IC50 = 3.4 ± 0.3 mM) (Figs 1c and d). Basal expression of IL-8 mRNA was also suppressed by montelukast in a concentration-dependent manner (IC50 = 7.9 ± 0.6 mM).

**Tumour necrosis factor-α-induced interleukin-8 expression is nuclear factor-κB-mediated: no effect of montelukast on nuclear factor-κB DNA binding**

Inhibitor studies indicated that TNF-α-induced IL-8 release was significantly inhibited by AS602868, an IKK2 inhibitor (736 ± 23 vs. 38 ± 26, P < 0.001), whereas inhibitors of the MEK (736 ± 23 vs. 598 ± 81, P > 0.05) and p38 MAPK (736 ± 23 vs. 667 ± 18, P > 0.05) pathways had no effect on IL-8 release (Fig. 2a). TNF-α induced a rapid activation of NF-κB p65 as measured by DNA
binding (Fig. 2b) but this was not affected by the presence of montelukast ($10^{-5}\text{M}$) (Fig. 2b).

**Effect of montelukast on p65-induced histone acetylation and deacetylation activity**

In order to clarify the inhibitory mechanism of montelukast on NF-$\kappa$B activation, we investigated p65-associated histone acetylation and deacetylation in TNF-$\alpha$ and/or montelukast-stimulated cells. We demonstrated a significant induction of NF-$\kappa$B p65-associated HAT activity by TNF-$\alpha$, which was attenuated by the presence of montelukast in a concentration-dependent manner (Fig. 3a). In contrast, p65-associated HDAC activity was not altered after stimulation with TNF-$\alpha$ ($10\text{ ng/mL}$) and montelukast did not affect p65-associated HDAC activity (Fig. 3b).

**Discussion**

Montelukast-inhibited TNF-$\alpha$-induced IL-8 release and mRNA expression in a concentration-dependent manner. We were able to demonstrate that TNF-$\alpha$-induced IL-8 release was regulated by the NF-$\kappa$B pathway as opposed to the MEK/ERK or p38 MAPK pathways. TNF-$\alpha$-induced p65 DNA binding was not affected by the presence of montelukast indicating that this suppressive effect was downstream to p65 DNA binding. We examined the ability of TNF-$\alpha$-stimulated p65 to interact with transcriptional co-activators which contain intrinsic HAT activity and report that pre-treatment of cells with montelukast results in attenuated levels of p65-associated HAT activity. To our knowledge, this is the first study investigating the effect of montelukast on NF-$\kappa$B p65-associated HAT activity.

Previous studies suggest that CysLT1 receptor antagonists may inhibit the NF-$\kappa$B activation [8–12, 25]. The mechanism underlying the inhibitory effect of CysLT1 receptor antagonists on NF-$\kappa$B activation is unclear but it is not due to antagonism of the CysLT1 receptor [9, 10]. In asthmatic airways, there is an increase in NF-$\kappa$B activation and an increase in HAT activity [26, 27]. Histone acetylation is reversed by HDACs, and there is a reduction in HDAC activity in asthmatic airways and in COPD [27–29]. Many of the most frequent lung diseases, including cystic fibrosis, interstitial lung disease and acute respiratory distress syndrome, involve inflammation with the co-ordinate expression of multiple inflammatory genes in the lungs. Modification of core histones plays a critical role in regulating the expression of all genes. Histone acetylation is a major modification that affects gene transcription and is controlled by HATs. Pharmacological manipulation of specific histone acetylation status is a potentially useful approach for the treatment of inflammatory diseases [19]. Montelukast may play an important effect in inflammatory diseases.
Some patients with severe asthma are not adequately controlled even by high doses of inhaler or oral steroids, hence alternative anti-inflammatory strategies are needed [36]. Using anti-TNF-α therapy, Howarth et al. [37] and Berry et al. [38] showed further evidence for a role for TNF-α in severe asthma.

Bacterial and viral infections induce asthma exacerbations. Viruses could induce a neutrophilic inflammation by up-regulation of IL-8 and recruitment of neutrophils [39, 40]. IL-8 is considered to be the most potent chemoattractant cytokine for neutrophils and levels of IL-8 have been related to severity of asthma [41, 42]. Corticosteroids are highly effective as anti-inflammatory therapy in asthma and have the capacity to inhibit multiple activated inflammatory signal transduction pathways. But, unfortunately, corticosteroids fail to down-regulate viral-induced IL-8 secretion in infants. This may explain why steroid therapy is unsuccessful in viral bronchiolitis [43]. Our results suggest that montelukast may modulate inflammation induced by viral or bacterial infection via an effect TNF-α-stimulated IL-8 expression through changes in NF-κB p65-associated HAT activity.

Some anti-inflammatory agents such as corticosteroids and theophylline decrease inflammatory gene associated HAT activity, at least in part, by increasing HDAC activity [19, 44, 45]. The exact mechanism whereby theophylline activates HDAC is not certain although it is likely to be
through distinct signal transduction pathways activated by inflammatory mediators [44, 45]. Theophylline activates HDAC activity and therefore suppresses the expression of inflammatory genes [44]. A significant increase in HDAC activity is seen in bronchial biopsies after treatment of patients with asthma with low doses of theophylline. We show here in contrast that montelukast inhibits p65-associated HAT activity independent from recruitment of HDAC activity. Although we did not see a significant reduction in p65-associated HAT activity in resting cells, this may reflect the sensitivity of the assay. This suggests that the molecular mechanism of the anti-inflammatory actions of montelukast is distinct from those of corticosteroids [19]. Future studies need to explore these events in primary cells from patients.

In summary, montelukast inhibits TNF-α-stimulated IL-8 expression through changes in NF-κB p65-associated HAT activity. This may explain some of the anti-inflammatory effects of montelukast. Manipulation of NF-κB-activated HATs might represent a potentially useful approach for the treatment of many inflammatory diseases.

Acknowledgements

This work was funded in part by the Clinical Research Committee (Royal Brompton Hospital) and Asthma, UK.

References

Inhibition of tumour necrosis factor-α-mediated interleukin-8 expression by Montelukast


This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.