

EDITORIAL

A Tale of Two CysLTs

Introduction

The importance of the cysteinyl-leukotrienes (cysLTs) LTC₄, LTD₄ and LTE₄ as mediators of asthma is now widely recognized [1]. Their biological actions, including bronchoconstriction, microvascular oedema, mucus hypersecretion and eosinophil chemotaxis, are mediated by CysLT type 1 (CysLT₁) and type 2 (CysLT₂) receptors distinct from those for LTB₄, the sole member of a separate branch of the leukotriene family. CysLT₁ is the molecular target of the leukotriene receptor antagonists montelukast, pranlukast and zafirlukast that have entered international asthma treatment guidelines since their introduction in many countries in 1997. These drugs do not block CysLT₂ and the actions of cysLTs mediated by the type 2 receptor remain poorly defined.

Although cys-LT receptor subtypes were first identified pharmacologically, the recent cloning and molecular characterization of both receptors [2–5] represents an important advance in defining their respective roles in airway inflammation. In particular, the ability of the oral CysLT₁ antagonists to reduce eosinophilia in the blood and airways of asthmatic patients over a period of weeks [6] points to an important role of cysLTs and CysLT₁ receptors in regulating eosinophil migration, proliferation, or apoptosis. The current issue of *Clinical & Experimental Allergy* contains an intriguing report by Dr Mita and colleagues from the National Sagamihara Hospital in Kanagawa, Japan, indicating that human eosinophils in fact express greater amounts of mRNA for the CysLT₂ receptor than for CysLT₁ [7]. This raises the interesting possibility that the CysLT₂ receptor may mediate important aspects of eosinophil behaviour. This review will survey current information on the two cysLT receptor subtypes and examine the evidence for their relative contribution to inflammatory processes.

Pharmacological characterization of CysLT receptor subtypes

The existence of cysLT receptors was suggested even before the structures of the leukotrienes were elucidated in 1979. On human and guinea-pig airway preparations, the experimental compound FPL-55712 selectively antagonized the contractile actions of cysLTs, at that time known as slow-reacting substance of anaphylaxis (SRS-A) [8].

Several functional studies of cysLT activity in guinea-pig airway preparations used serine-borate to block the metabolic conversion of LTC₄ to LTD₄. In these models, FPL-55712 blocked the contractions induced by LTD₄ but not those induced by LTC₄, indicating that LTC₄ acts at a second, FPL-55712-insensitive receptor [9,10]. These guinea-pig receptors became known as LTD₄ and LTC₄ receptors, respectively. However, when this experimental approach was performed with human bronchi, FPL-55712 antagonized the effects of both LTC₄ and LTD₄, so in human bronchial smooth muscle, LTC₄ and LTD₄ appeared to act at the same receptor [11], which was termed CysLT₁. Dose–response analysis showed that LTD₄ was the most potent agonist at the human CysLT₁ receptor, with LTC₄ and LTE₄ significantly less active.

Evidence for a second CysLT receptor in humans came from studies of vascular tissue. Labat and colleagues showed that a number of antagonists that block LTC₄- and LTD₄-induced airway smooth muscle contractions, including MK-571, ICI-198615 and SKF 104353, failed to inhibit the cysLT-induced contractile responses of venous preparations from human lung [12]. Unlike airway tissues, LTC₄ and LTD₄ were equally potent in causing vasoconstriction. Contractions of human pulmonary vessels were antagonized by the experimental compound BAYu9773, a non-selective dual antagonist of CysLT₁ and CysLT₂, but not by selective CysLT₁ antagonists [12]. BAYu9773 remains the only compound capable of blocking CysLT₂ receptors. In contrast to vasoconstriction at higher concentrations (10^{-6} – 10^{-5} M), cys-LTs at lower concentrations (10^{-8} – 10^{-7} M) relax many vascular preparations from dogs, guinea-pigs, and humans [13–15]. In pulmonary vessels, this mechanism has been proposed to involve CysLT₂ receptors on the vascular endothelium that produce indirect relaxation via the synthesis of nitric oxide and prostanoids. The possibility that CysLT₁ and CysLT₂ receptors may mediate opposing activities in other tissues is intriguing.

Molecular characterization of CysLT₁ and CysLT₂

The human CysLT₁ and CysLT₂ receptors were both characterized at the molecular level in 1999 [2–5]. Both are classical G-protein-coupled serpentine receptors with seven putative transmembrane regions and their functional activation involves calcium mobilization. The CysLT₂

receptor polypeptide shows 31% amino acid identity to CysLT₁. The molecular studies largely clarified and extended the previous pharmacological observations. At the CysLT₁ receptor, LTD₄ is approximately 10-fold more potent as an agonist of calcium mobilization than LTC₄ or LTE₄. At CysLT₂ receptors, LTC₄ and LTD₄ are roughly equipotent with each other, but both have lower affinity for CysLT₂ than LTD₄ has for the CysLT₁ receptor. The specificity of the 'lukast' drugs for CysLT₁, and the dual antagonism of CysLT₁ and CysLT₂ by BAYu9773 was also confirmed by the molecular studies.

There are striking differences in tissue distribution between CysLT₁ and CysLT₂. Expression of CysLT₁ mRNA is mainly in the spleen, peripheral blood leucocytes and airway smooth muscle [2,3], while large amounts of CysLT₂ mRNA are found in the heart (Purkinje fibres), placenta, spleen and leucocytes, but not in the lung [4,5]. More detailed investigations of the cellular localization of CysLT₁ and CysLT₂ receptors using immunohistochemistry, RT-PCR and *in situ* hybridization in tissues from patients with inflammatory or vascular disease may provide further clues to the pathophysiological roles of these receptor subtypes.

Airway expression of CysLT receptors

The ability of cysLTs to cause contraction of bronchial smooth muscle by a direct action at CysLT₁ receptors is well-recognized. *In situ* hybridization shows expression of CysLT₁R mRNA on airway smooth muscle from normal bronchial biopsies [2], and using flow cytometry our group has shown substantial expression of CysLT₁ protein on cultured human airway smooth muscle cells [16]. *In vivo*, pretreatment of normal or asthmatic subjects with an oral 'lukast' drug shifts the inhaled LTD₄ dose-response curve to the right by a factor of over 100, indicating that CysLT₁ is overwhelmingly responsible for the airway constrictor response to cysLTs [17].

CysLTs also exert pro-inflammatory effects on other cell types. They contribute to tissue oedema by widening endothelial gap junctions in postcapillary venules. In Brown-Norway rats, LTD₄-induced microvascular permeability is reduced to near basal levels by montelukast [18]. CysLTs affect mucociliary clearance by increasing the secretion of mucus glycoproteins and reducing ciliary beat frequency. LTD₄ (but not LTC₄) increases tracheal mucus secretion, an effect that is inhibited by the CysLT₁-specific antagonists pranlukast (83%) and zafirlukast (78%), respectively [19]. Ciliary beat frequency is significantly reduced by LTC₄ and LTD₄, an effect also attenuated by FPL 55712 [20].

However, other cysLT responses may be mediated via CysLT₂ or by other receptors as yet undefined. CysLTs are

highly effective mitogens for airway epithelial cells, but LTC₄ is markedly more potent in inducing their proliferation than either LTD₄ and LTE₄ [21], suggesting that this response is mediated by CysLT₂ receptors. Similarly, LTD₄ synergises powerfully with epidermal growth factor (EGF) to induce proliferation of airway smooth muscle cells; this response is inhibited by pranlukast and pobelukast, but not by zafirlukast, perhaps indicating differential affinities of these drugs for a third, unidentified CysLT receptor [22]. Wang and colleagues showed that airway smooth muscle mass is increased in response to ovalbumin challenge in sensitized Brown-Norway rats. This increase in smooth muscle mass was reduced by about half following pretreatment with the experimental CysLT₁ antagonist MK-571 indicating the prominent involvement of CysLT₁ receptors, but not excluding a contribution of CysLT₂ receptors or of other mediators [23]. In human umbilical vein endothelial cells (HUVECs), LTC₄ and LTD₄ cause a rapid up-regulation of P-selectin, an early adhesion molecule involved in leucocyte rolling prior to diapedesis. The P-selectin response in HUVECs is clearly not mediated by CysLT₁ receptors as it is unaffected by pretreatment with pranlukast or zafirlukast, or by pobelukast (SKF 104,353) at concentrations that effectively blocked cysLT-induced contractions of human bronchial smooth muscle [24]. These data suggest that specific blockade of CysLT₂ receptors may provide anti-inflammatory effects additional to those seen with CysLT₁ antagonists, and even prevent remodelling of bronchial smooth muscle, epithelium, and other airway tissues in chronic asthma.

CysLT receptors and human leucocytes

The ability of cysLTs to increase eosinophils in the airway has been shown in asthmatics after LTE₄ inhalation [25] and reductions in blood and airway eosinophils are observed after oral therapy with CysLT₁ antagonists [6]. In contrast to the immediate bronchodilation seen with these drugs, the anti-eosinophilic effect requires several weeks of treatment [26,27]. In a meta-analysis, the average fall in blood eosinophils was modest, of the order of 20% below pretreatment values, but reductions in biopsy eosinophil counts are more marked, approximately 65% in one study, and accompanied by approximately 50% reductions in T cells and mast cells [27].

The mechanisms by which cysLT cause eosinophilia are unclear, but they may be indirect, such as the CysLT₁-independent induction of P-selectin on vascular endothelium described above [24], or enhanced leucocyte diapedesis following the opening of endothelial gap junctions. Such a mechanism may be relatively non-selective for eosinophils. More specific effects on eosinophil proliferation, adhesion, chemotaxis, or apoptosis must involve direct action at

cysLT receptors on the eosinophil itself. A direct mechanism mediated by CysLT₁ receptors is favoured by evidence that the activities of cysLTs as selective eosinophil chemoattractants *in vitro* and as enhancers of eosinophil survival are both blocked by the CysLT₁ antagonist pobilukast (SKF 104353). Pobilukast reduced eosinophil survival by 50–100% depending on the initial stimulus, and chemotaxis was completely abolished [28,29]. This seems to eliminate the possibility that CysLT₂ receptors also play a role in mediating these eosinophil responses.

Consistent with this view, CysLT₁ receptor mRNA and protein have been quantified in 88% of purified normal human blood eosinophils, and the receptor is also seen on lesser proportions of normal blood monocytes and B cells (36%), granulocytic CD34⁺ precursor cells (100%), and T cells (4%) [30]. CysLT₁ mRNA and protein are also expressed on eosinophil-like cells differentiated from the promyelocytic leukaemia cell line HL-60, and their expression is up-regulated after treatment with interleukin (IL)-5 [31]. IL-5 also induces the expression and activity of leukotriene-synthesizing enzymes in these cells [32,33]. The consequent enhanced capacity of eosinophils both to synthesize and to respond to cysLTs can be seen as part of the general up-regulation of eosinophil activity in the asthmatic airway that is induced at least in part by IL-5.

In this context, the report in the current issue by Dr Mita and colleagues is particularly interesting [7]. This is the first study of both CysLT₁ and CysLT₂ receptors in peripheral blood leucocytes from asthmatic patients [7]. Firstly, using competitive RT-PCR, mRNAs for both CysLT₁ and CysLT₂ were found in neutrophils, monocytes and T lymphocytes; cell-types that are not widely thought to show a wide repertoire of responses to cysLTs. Although mRNA transcription does not indicate that the mRNAs are necessarily translated into protein, it nevertheless suggests that either, or both, receptors may be present on the cell surface and may mediate hitherto-unsuspected activities of cysLTs in these cells. Secondly, in contrast to these leucocytes in which CysLT₁ mRNA predominated over that for CysLT₂, identical experiments in human eosinophils showed the reverse situation, with almost twice as much mRNA for CysLT₂ as for CysLT₁ [7]. With the repeated proviso that the mRNA data need to be confirmed at the protein level, this hints at a prominent role for CysLT₂ receptors in mediating cysLT actions on human eosinophils that may overshadow their CysLT₁-mediated effects and that are not shared to the same extent by other leucocyte types. If confirmed by further experiments, the data may have powerful therapeutic and commercial implications: the current generation of CysLT₁-selective 'lukast' drugs could be less potent or have a narrower spectrum of anti-

eosinophilic activity than novel compounds designed to block both subtypes of cysLT receptor.

The challenge in understanding these data is reconciling the implication that CysLT₂ receptors are the predominant cysLT receptors in human eosinophils, with functional studies that show complete or near-complete inhibition of most cysLT-induced eosinophil responses (survival, chemotaxis) by specific CysLT₁ antagonists [28,29]. This appears to leave no room for a contribution of CysLT₂. Some studies suggest that current 'lukast' drugs vary in their receptor specificity [22], and the concentrations of these drugs used in functional studies need to be optimized carefully to reduce the risk of non-specific antagonism of CysLT₂. However, if these drugs are acting specifically, it seems unlikely that there are major but as yet undiscovered eosinophil functions mediated exclusively by CysLT₂ activation. Alternatively, since the affinity of LTD₄ for CysLT₁ is 10-fold greater than the affinity of LTD₄ (or LTC₄) at CysLT₂, it may be that the receptors are differentially activated by varying agonist concentrations. The relevant analogy is LTB₄, which at low concentrations potently promotes neutrophil chemotaxis by binding to high affinity BLT₁ receptors, and at higher concentrations promotes neutrophil degranulation via a larger population of lower-affinity BLT₂ receptors [34]. Perhaps CysLT₁ similarly mediates eosinophil migration at the low cysLT concentrations found in the periphery of the inflammatory site, while the higher concentrations of cysLTs at the inflammatory focus act via CysLT₂ receptors to promote the release of eosinophil granule proteins, such as MBP, EPO and ECP. This might explain the preponderance of CysLT₂ mRNA in eosinophils and reinforces the need for functional studies to investigate a wide range of agonist concentrations. Experiments to date may have used cysLT concentrations optimal only for CysLT₁ activation, or tended to focus on LTD₄ at the expense of LTC₄ that may be more effective as a CysLT₂ agonist. While combined CysLT₁ and CysLT₂ antagonism has the potential to further reduce the pro-inflammatory effects of cysLTs on eosinophils, more functional experiments are clearly required to elucidate those biological effects mediated at least in part by the CysLT₂ receptor.

An important topic for future research is the mechanism by which CysLT₁ and CysLT₂ receptor expression on eosinophils and other cells is regulated. Both CysLT₁ and CysLT₂ amino acid sequences show potential glycosylation and phosphorylation sites, suggesting there may be post-translational regulation of receptor activity. At the transcriptional level, IL-5 up-regulates CysLT₁R mRNA in eosinophils [31], and factors including asthmatic serum, TGF- β and a combination of IL-1 β , TNF- α and IFN- γ increase CysLT₁ protein expression on cultured human airway smooth muscle cells [16,35]. Genetic polymorphism

in CysLT₁ does not appear to be functionally important [36], but immunological regulation of CysLT₁ or CysLT₂ expression on eosinophils, vascular tissue, or airway smooth muscle cells by the cytokine microenvironment may amplify cysLT effects such as eosinophil migration and bronchoconstriction.

Finally, it is a curiosity of the cysLTs as inflammatory mediators that no normal physiological role has been convincingly ascribed to them other than in rather narrow aspects of inflammatory responses to some non-physiological stimuli in rats [37]. In contrast, their cousins the prostanoids have multiple roles in the regulation of blood flow, tumorigenesis, and other processes in many human organ systems. The prevalence of CysLT₂ receptors on Purkinje fibres in the heart [4] and its activity in other vascular tissues suggest that cysLTs may prove to have normal physiological roles via the CysLT₂ receptor in regulating cardiac contractility and in maintaining vascular tone. The elucidation of CysLT₁ and CysLT₂ receptor structures over the last 18 months is likely to prove invaluable in answering these questions.

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