RBL-2H3 and Primary Mast Cells and Their Use in Screening Novel Anti-inflammatory Compounds

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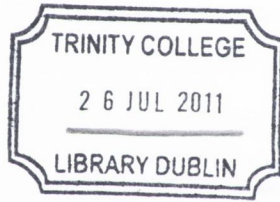
"Life has meaning only in the struggle
Triumph and defeats are in the hands of Gods...
Let's celebrate the struggle!"
Swahili Warrior song

Dedicato alla mia mamma perché mi é stata sempre vicina
ed al mio papá perché mi ha insegnato a ragionare

DECLARATION

This thesis has not been submitted as an exercise for a degree at any other University. The results described are entirely my own work. I agree that the library of Trinity College may lend or copy this thesis upon request.

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Summary

Mast cells (MC) have been mainly studied as key effectors in allergic diseases and inflammatory conditions such hypersensitivity reactions, asthma, atopic dermatitis and multiple sclerosis. Following the crosslinkage of membraneous FcεRI, by antigens, a large number of chemical mediators are secreted. This event leads to the recruitment and activation of basophils and eosinophils that sustain the inflammatory response. The role of mast cells, however, is not limited to the initiation of allergic response but they are also fundamental players in the innate immune response: for example they can be activated directly by pathogens through a family of pattern recognition receptors called ''Toll-like receptors'' (TLRs). In particular, TLR2 and 4 seem to be crucial to the mast cell response to pathogens. In rodents, mast cells respond to lipopolysaccharide through their TLR4s by the release of pro-inflammatory cytokines without concurrent degranulation or they can degranulate following peptidoglycan challenge through a TLR2-mediated pathway.

As part of an ongoing study to identify novel molecules with therapeutic potential, we examined the effect of two diastereoisomers (PH2 and PH5) and their four enantiomers (PH3, PH4, PH21 and PH22) of an indane compound, 2-benzyl-2,3-dihydro-1H-1'H-2,2'-biinden-1-ol, on the degranulation of freshly harvested rat peritoneal mast cells (RPMC) and on the rat basophilic leukaemia mast cell line, RBL-2H3 stimulated with a variety of stimuli (both immunological and non-immunological) .

It is important to underline that MC present different characteristics depending on their anatomical location. In rodent two big mast cell families can be identified: the connective tissue mast cell subset and the mucosal mast cell subset. The features that distinguish different mast cell subsets are morphology, staining properties, lifespan and more importantly different responses to stimuli and modulatory compounds. This represents a crucial aspect of MC physiology during this study as the ability of a therapeutic agent to inhibit exclusively one or both kind of mast cells is crucial in therapies of diseases such the Chron's disease or the ulcerative colitis in which just mucosal mast cells are involved. In this study RPMC represented a model for the connective tissue mast cell subset while RBL-2H3 cells represented a model for the mucosal mast cell subset.

RBL-2H3 cell line, was cloned by the limited dilution technique from leukaemia cells isolated from rats after treatment with the chemical carcinogen, β-chlorethylamine. RBL-2H3 cells have been extensively used for studying IgE-FcεRI interactions, signalling pathways for degranulation and to test novel mast cell stabilisers. Therefore, RBL-2H3 cells could be considered a useful tool for *in vitro* work, as a great number of monoclonal cells can be rapidly obtained by simple cell culture techniques. Despite the large amount of information available on RBL-2H3 cells, a lack of consistency exists between findings from different groups and therefore, factors that influence the experimental

behaviour of RBL-2H3 cells were investigated and the conditions for the most commonly used assay performed with this cell line were also optimised.

Among the tested compounds, only PH22 could uniformly inhibit MC response to a variety of stimuli both in RBL-2H3 cells and in RPMC. The ability of PH22 to effectively stabilise both subfamilies of mast cells gives to this compound an enormous potential as a therapeutic agent. Not only PH22 has the potential to be a treatment in pathologies such as asthma and allergic skin conditions but it also could be particularly significant in diseases in which only MMC are involved such as Chron's disease and ulcerative colitis.

All the other PH compounds were proved to be active on either CTMC or MMC degranulation after at least one of the stimuli tested. similar responses often resulted in different results (in terms of statistical analysis) due to differences in the experimental errors. Further analyses are needed before concluding whether a compound is totally inactive or not on MC after challenge with a particular stimulus.

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Abbreviations

μ	Micro
λem	Emission wavelength
λex	Excitation wavelength
[antigen]	Concentration of the antigen
[IgE]	Concentration of IgE
°C	Degrees centigrade
Ag	Antigen
BMCP	basophil/mast cell progenitor
bp	Base pairs
BS	Basophils
BSA	Bovine serum albumin
BSS	Balanced salt solution
C1P	Ceramide 1-phosphate
Ca^{2+}	Calcium ion
CaCl	Calcium chloride
CAM	Calmodulin
CERK	Ceramide Kinase
CD	Cluster of differentiation
CO_2	Carbon dioxide
CTMC	Connective tissue mast cell
CRAC	Ca^{2+} release-activated Ca^{2+} channels
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenyl
DSCG	Disodium cromoglycate
EBSS	Earl's balanced salt solution
FBS	Foetal bovine serum
gDNA	Genomic DNA
GMP	Granulocyte macrophage progenitor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	Protein G-coupled receptor
h	Hours
HSC	Hematopoietic stem cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High pressure liquid chromatography
HuMC	Human mast cell
IgE	Immunoglobulin E
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motifs
KCl	Potassium chloride
LAT	Linker for activation of T cells
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
M	Molar
MC	Mast cells
MCP	Mast cell committed progenitor
MCDP	Mast cell degranulating peptide
MeOH	Methanol

MFI	Mean fluorescence intensity
mg	Milligrams
MgCl	Magnesium chloride
min	Minutes
ml	Milliliters
mM	Millimolar
MMC	Mucosal mast cells
MMP	Multipotential progenitor
mRNA	Messenger ribonucleic acid
MTS	[3-(4,5-dimethylthiazol-2-yl)- 5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt],
MyD	Myeloid Differentiation Primary Response Protein
NaCl	Sodium chloride
NaH$_2$PO$_4$	Sodium di-hydrogen phosphate
NaHCO$_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
ng	Nanograms
OPT	*o*-phthalaldehyde
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PIP$_3$	Phosphatidylinoditol triphosphate
PKC	Protein kinase C
PRR	Pattern recognition receptors
rLPS	Rough LPS
RPMC	Rat peritoneal mast cells
S.E.M.	Standard error of the mean
sLPS	Smooth LPS
SOCC	Store operated calcium channels
TLR	Toll like receptors
TNF-α	Tumour necrosis factor alpha

1 Introduction

1.1 Mast cells, a brief historical overview

Mast cells were described for the first time in 1878 in a doctoral thesis of a 24 year old German student, Paul Ehrlich (1854–1915). The thesis "Contributions to the Theory and Practice of Histological Staining" was presented at the Medical faculty of Leipzig University. It represents a milestone as, for the first time, a histological study was based not only on the mere observation of cellular morphology, but also considering the reactivity of cellular components with specific dyes; this approach was extremely innovative for that time. His foresight lead Ehrlich to observe '*the behaviour of aniline dyes towards protoplasmic deposit of certain cells*"; he proved that an "*undetermined chemical substance*" contained in granular cells, typically located around blood vessels and in connective tissues, could change the nuance of aniline dyes (phenomenon called metachromasia) [1].

Figure 1

Paul Ehrlich (1854-1915) father of haematology, revolutionary immunologist, and creator of the field of chemotherapy. In 1878 he described for the first time a "new" cell type that he called mast cells.

This pioneering work made it possible to identify a new type of cell that Ehrlich called "**Mastzellen**" (Mast = breast (from the greek word μαστός) and Zellen = cells). Ehrlich was driven to choose this name to underline the "*well-fed*"' aspect of this cells and the thought that they were providing nutrients to tissues [2].

After more than a century, mast cells still attract the attention of scientists as they are involved in multiple aspects of immunology. The initial view of mast cells as key elements during allergic and inflammatory diseases is widening; nowadays it is known that mast cells play an important role during host defence (see paragraph 1.6), tissue remodelling, fibrosis and autoimmunity [3]. Mast cells are involved in virtually all pathological conditions and the understanding of their physiological and pathological role is becoming more clear.

1.2 Mast cells development

Mast cells derive from a CD34$^+$/c-kit$^+$ hematopoietic stem cell (HSC) progenitor in the bone marrow [4-6]. Unlike other figurative elements such as erythrocytes, platelets and neutrophils, MC do not complete their development within the bone marrow but reach their maturity in tissues [7]. The development of mast cells from a hypothetical committed progenitor is not fully understood and is still under debate [8-10]; in fact, a MC committed progenitor (MCP) with "lymphocyte-li" characteristic was isolated only in the fetal blood [11] and its presence in adult blood was never proved. Such MCP (called also promastocytes) was isolated by Rodewald and coworkers in 1996, and expressed high levels of c-kit and low levels of Thy-1 (c-kit^{hi}/Thy-1^{lo}) and did not expressed the FcεRI and was insensitive to cytokines that normally lead the differentiation of white cells. However promastocytes cultured in presence of IL-3 and FCS could differentiate in complete and functional mast cells. In addition to this if injected in mast cell-deficient mice, such MCP could give rise to MC population in the peritoneal cavity.

The main current opinions regarding mast cell development identifies mastocytes as a progeny of CMP (*common progenitor for the myeloid lineage*). Hovewer, Cheng and coworkers [12] identified in the bone marrow a *Lin$^-$/c-kit$^-$/Sca-1$^-$/Ly6c$^-$/FcεRIα$^-$/CD27$^-$/β7$^+$/T1-ST2$^+$* mast cell committed progenitor (MCP) that descends from a multipotential progenitor (MPP) without any round of cell division; this characteristic is reflected in the high proliferation potential of mast cells [8]. This finding suggests that mast cells derive from a different lineage rather than the one that leads, though CMP, to the production of monocytes/macrophages (Mφ), granulocytes (G), erythrocytes (E) and platelets (PL) (Figure 2). The fact that MC derive directly from MPP might also explain how mast cells can revert their morphology to the resting phenotype after degranulation; on the other hand, cells derived from CMP, normally die after accomplishing their function. Cheng and co-workers suggest that MCP might generate both connective tissue and

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mucosal mast cells (see paragraph 1.3) depending on factors present in the particular tissue where differentiation occur.
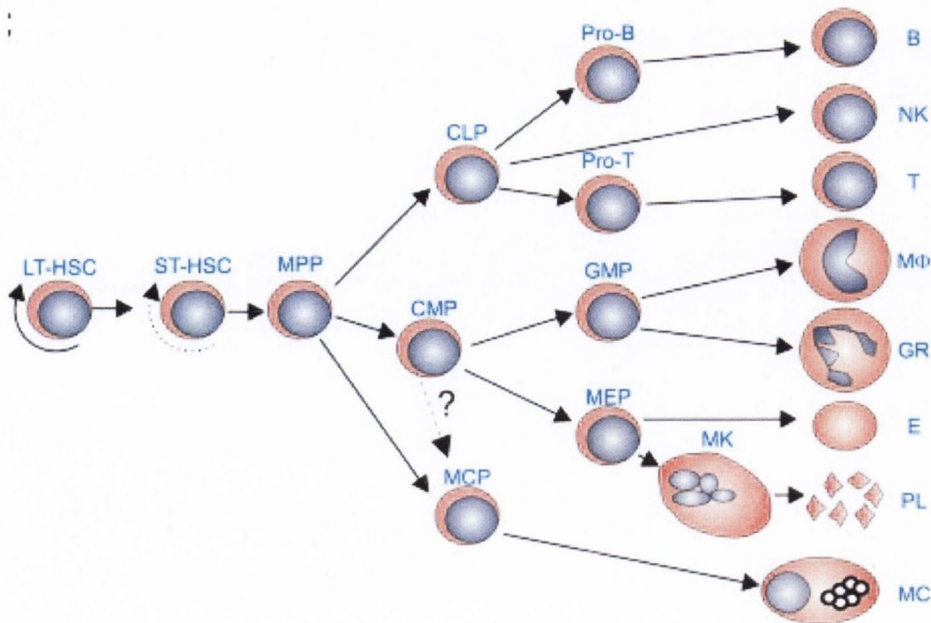
Figure 2

Lineage model of haematopoiesis according to Chen and co-workers [12]. According to the authors, mast cells derive from a separate lineage rather than the one that produces monocytes though CMP. MC derive from a mast cell committed progenitor that rise directly from a multipotent progenitor in the bone marrow.

CMP: common myeloid progenitor, MCP: mast cell committed progenitor, Mφ: monocytes/macrophages, GR: granulocytes, E: erythrocytes, PL: platelets, MC: mast cells.

In contrast to the work of Cheng and co-workers, Arinobu's group re-examines the hypothesis of a common progenitor for both mast cells and their circulating relatives, the basophils [9]. The authors report to have isolated such a common progenitor **(BMCP)** and proved that its development is crucially regulated by the granulocyte-related transcription factor CCAAT/enhancer-binding protein α (C/EBPα); in particular BMCP will differentiate into mast cells when C/EBPα is absent and it will differentiate into basophils if C/EBPα is present.

Arinobu's data also contradicts Cheng's work proving that the β7[*] CMP and granulocyte macrophage progenitor **(GMP)** conserves a high potential to generate MC. There is other evidence [13] that basophils and mast cells might be generated from the same lineage and the

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[*] β7 is an integrin expressed in BMMC and it is an essential molecule for tissue specific homing of putative precursors for intestinal mast cells

identification of the committed progenitor, by Arinobu's group, represents an important step in elucidating the mechanism of development of this lineage and therefore in the understanding of the basophil/mast cell disorders such allergies and other autoimmune diseases.
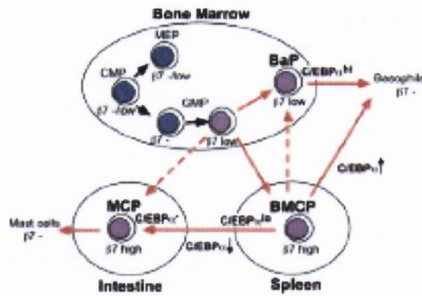
Figure 3

Lineage model of haematopoiesis according to Arinobu and co-workers [9]. The authors re-examines the hypothesis that mast cells and basophils share a common progenitor (BMCP); they also demonstrated how β7 CMP/GMP possesses high potential to differentiate into MC. These data are in contrast with those reported by Cheng and co-workers [12].

CMP: common myeloid progenitor, MCP: mast cell committed progenitor, GMP: granulocyte macrophage progenitor, BaP: basophils progenitor

1.3 Mast cells heterogeneity and classification

Since mast cells were discovered, it appeared clear that several mast cell types exist. Not only do MC present different characteristics between species, but even within the same organism mast cells from various parts of the body show different morphology, histochemistry and functions. In rodents, the common classification divides MC into two big groups: the connective tissue mast cells (CTMC) such as those from the skin and peritoneal cavity and the mucosal mast cells (MMC), typically from anatomical locations like bone marrow or intestinal lamina propria [14]. Despite the fact that this classification is widely accepted, it is not always possible to assign a mast cell population to one of the two categories simply on the basis of the anatomical site of origin. Sometimes, in fact, mastocytes show intermediate properties between the two classes [15]. CTMC and MMC differ in several aspects of their phenotype such as: a) size (with CTMC being more uniform in shape and larger than MMC that also show variable-sized

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granules), b) MMC are the only ones to show migratory capacity, c) protease content (CTMC contain mast cell protease (MCP) I while MMC contain MCPII) d) different staining properties due to their different proteoglycans matrix [16, 17] e) histamine content and f) lifespan [18].

Also human mast cells (HuMC) show different subpopulations: the common marker to identify different HuMC types is the content of neutral proteases. MC with a high content of tryptase are designated MC_T while mast cells that, in addition to tryptase, have also shown a high content of chymase are designated MC_{TC}. However, due to their characteristics and localisation, MC_{TC} closely correspond to the rodent connective tissue mast cells, while MC_T resemble the rodent mucosal mast cells.

Even though MMC and CTMC show striking differences in their phenotype, it has been postulated that they arise from the same bone marrow-derived progenitor. In the light of this, it must be clarified that the mast cell heterogeneity, does not correspond to different phases of a single mast cell lineage but it is due to microenvironmental factors that can divert mast cell progenitors toward one or the other subtype [14]; in particular, despite the fact that the MC progenitor needs growth factors such as SCF and c-kit to give rise to both MC subtypes, T cell-derived growth factors (IL-4,IL-3) are necessary to obtain intestinal and bronchial MMC*.

The observation that the final stage of MC maturation is conditioned by micro-environmental factors is supported by the fact that, under normal conditions, only MC precursors can be found in the circulation and that they reach their maturation in the tissue of residence (vascularized connective tissue or serosal cavities) [19]. Moreover, CTMC and MMC can mutually switch phenotype: it has been proven that bone marrow-derived cultured mast cells injected in various locations of mast cells-deficient mice showed different phenotype

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* The observation that MMC need T cell-derived growth factors is supported by the fact that T_H2-type inflammatory responses are accompanied with an increase in MC count (reactive mastocytosis). Moreover, in atymic mice just the MMC subpopulation results absent while the CTMC population seems unaffected.

depending on the site of injection [20] and MMC can switch to CTMC-like phenotype depending on culture conditions [7, 21, 22].

The most important feature that distinguishes CTMC from MMC is, without doubt, their different response to stimuli and modulatory compounds. For example, while CTMC release histamine after challenging with polybasic compounds such as compound 48/80 and peptide 401, MMC are refractory to these stimuli [23]. The response to many pharmacological mediators, not also differs between the two classes of MC but also within the same subtype from different species. For example, DSCG is inactive on rat MMC, is a potent stabiliser of rat peritoneal mast cells (example of CTMC), but it is totally ineffective on mouse peritoneal mast cell and also inactive on cutaneous mast cells of humans, cows, dogs and monkeys [18, 23]. Also Nedocromil and theophylline have been reported to be active on peritoneal, pulmonary and cutaneous mast cells of rat, pig, man and monkey but ineffective on MMC of these species [18].

The species-specificity of an agent to promote or suppress mast cell activation underlines the limit of an *in-vitro* model to test potential histamine liberators or mast cell stabilisers. Moreover, the ability of a therapeutic agent to inhibit exclusively one or both types of mast cells is crucial in therapies of diseases such ulcerative colitis and Crohn's diseases where only MMC are involved.

1.4 Mast cells mediators

1.4.1 Tryptase

Tryptase is a serine proteinase expressed by all MC subtypes and it is released during mast cell activation; two families of tryptase are known (α and β tryptase); each family of tryptase has, in its turn different subtypes of tryptases (αI, αII and βI, βII and βIII) [24]. When a tryptase is released, it acts mainly in the proximity of activated mast cells and presents a restricted range of substrates (casoactive intestinal peptide, kininogens and fibronectin) [25-27]. The optimal condition for the enzymatic activity of tryptases is at low pH (5.5-6.5) [28],

characteristic of tissues during inflammation. Due to its powerful effect on the growth of epithelial cells, airway smooth muscle cells and fibroblasts, tryptase plays an important role during tissue destruction and remodelling, it can also stimulate surrounding mast cells to degranulate, representing an amplification signal for the inflammatory response [29]. Tryptase can also influence the recruitment and activation of neutrophils and basophils; this phenomenon is apparently mediated by the activation of PARs (protease-activated receptors) [30]. Clinically, tryptases are important as markers of various pathological conditions such as systemic mastocytosis and myelodisplastic syndrome [24, 31].

1.4.2 *Chymase*

Chymases are chymotryptic serine proteases that differ for preference of substrate, solubility and susceptibility to inhibitors; they are present exclusively in the granules of the CTMC (or MC_{TC} in humans) subset. In rodents, a huge variety of chymases are known, while in humans, chymase heterogeneity seems to be simpler. Even though the role of chymases during inflammation is not fully understood, it is known that these enzymes have a broad range of effects such as activation of angiotensin, potentiation of the effect of histamine (see paragraph 1.4.3) and matrix degradation [16, 32]; they can also influence the bioavailability of certain cytokines such as IL-1β and IL-4 and they can contribute to tissue destruction around the mast cell activation site and influence the microvascular permeability [33]. Another important function of mast cell chymase, is the ability to recruit monocytes and neutrophils and to induce the production of IL-8 (CXCL-8) that represents a further activating signal for chemotaxis [34]. All the above effects, however, present interspecies variations, making troublesome the understanding of the role of chymases in inflammation.

1.4.3 *Histamine*

Histamine (2-(4-imidazoyl)ethylamine) is certainly the most studied among the mast cells' mediators. In mast cells and basophils histamine

is synthesised in the Golgi apparatus through decarboxylation of the amino acid histidine by the enzyme histidine decarboxylase. It is stored in granules in ionic association with residues of glycosaminoglicanes (GAG) or heparin or proteoglycans. Once released, its half life is quite short as it is degraded within a minute by two different enzymatic pathways. The first one is ubiquitous and involves the histamine methylation by histamine N-methyltranferase and oxidation by a monoaminoxidase (MAO-B) to give N-methylimidazole acetic acid. The second pathway, mainly located in the periphery, [33, 35] involves an histaminase that oxidise, histamine to give imidazole acetic acid, followed by condensation with phosphorybosyldiphosphate to give riboside-N-3-imidazole acetic acid.

Histamine is the most important mediator of the acute allergic response; when injected intradermally histamine evokes the so called ''*Lewis triple response*'' characterised by erythema, oedema and hyperaemia [36]. The erythema and the oedema are caused by the action of histamine on the vessels of the microcirculation; the hyperaemia, instead, is the effect of arteriolar dilatation mediated by an axonal reflex that involves the sensory afferent nerve terminals [37].

Histamine is not only involved in allergy but plays important roles in many (patho)physiological processes: for example, it stimulates gastric acid secretion or it is involved in the regulation (by neurons located in the tuberomammilary nucleus) of several brain functions as sleep/wakefulness, thermoregulation, food intake and so on [38].

Histamine exerts its potent effects through a family of G-protein coupled receptors designated as "H" receptors. Four distinct receptors have been characterised (H_1 to H_4) encoded by different genes. The discussion of the histamine receptors' characteristic outside the scope of this thesis but Table 1 briefly summarises their distribution, mediated responses and pathophysiological relevance.

Table 1 Distribution and functional characteristics of histamine receptors [35]

Receptor	Distribution	Mediated response	Pathophysiological relevance
H₁	CNS	attention, sleep regulation, convulsions	Allergic reaction
	Smooth muscles	Typical immediate response of allergic reactions typ I (triple response), rhinitis asthma, anaphylaxis urticaria	
H₂	Brain	Increase working memory	Gastric secretion
	Gastric parietal cells	Stimulation of acid secretion	
	Cardiac tissue	Positive ionotropic effect	
H₃	CNS	Autoreceptor controlling the synthesis and release of histamine.	Involved in cognition, sleep-wake status inflammation
H₄	Bone marrow and leukocytes	Suggested role in inflammatory processes and autoimmune responses	
	brain (low density)		

1.4.4 β-hexosaminidase

This granule-associated enzyme is an exoglycosidase* that has been extensively used to monitor mast cell degranulation as it is released in parallel with histamine. Its role during the inflammation process is not known, but it has been hypothesised that it could act in concert with tryptases and chymases for the degradation of glycoproteins and proteoglycans† of the extracellular matrix (important event during the remodelling of the inflamed tissue) [33]. The enzyme β-hexosaminidase shows optimal activity at low pH (pH = 4.5), a typical condition during inflammatory processes.

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* exoglycosidases (also called glycoside hydrolases) are enzymes that catalyze the hydrolisis of the glycoside bound
† Proteoglycans are a special class of glycoproteins that show one or more covalently attached glycosaminoglycane (long unbranched polysaccharides) chains instead of common oligosaccharides.

1.4.5   *Interleukins (IL-)*

Interleukins are small soluble, non-antigen-specific proteins that cell secrete to influence the behavior of other cells in close proximity.

1.4.5.a   IL-4 and IL-13

Interleukin 4 and interleukin 13 are closely related and have similar functions. However, IL-13 is more important during allergy and inflammatory conditions.

Both cytokines are important to induce T helper 2 (T_{H2}) cells and to increase the production of IgE from activated B cells. Interestingly IL-4 is stored in mast cell granules and it promptly released upon MC activation, while IL-13, has not been found intracellularly even if its mRNA is constitutively expressed. IL-13 mRNA has been found to be overexpressed after anti-IgE challenge with consequent IL-13 release after 8 hours from the stimulus.

1.4.5.b   IL-5

Interleukin 5 is produced mainly by MC_T and its production can be challenged by anti-IgE activation of the MC. Its main role is to stimulate B cell growth and to increase immunoglobulin secretion. It is also a key mediator in eosinophil activation.

1.4.5.c   IL-8 (CXCL-8)

IL-8 (renamed CXCL-8) is a potent chemoactrant and activator for neutrophils and granulocytes and hence it enhances the inflammatory response. Interleukin 8 also triggers integrins to change their conformation in order to allow leukocytes (neutrophils and naïve T cells) to penetrate the endothelium (process called extravasion). IL-8 can direct the recruited leukocytes toward the site of infection by forming a chemical gradient in the inflammation site.

1.4.5.d Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF can attract monocytes to the site of inflammation/infection and stimulate them to mature as macrophages, it can also regulate many of the functions of neutrophils, monocytes macrophages and dendritic cells (a type of antigen presenting cells).

1.4.6 Tumour necrosis factor alpha

Tumour necrosis factor-α (TNF-α) plays an important role during inflammation, allergic diseases and gram negative infections. The TNF-α production by activated mast cells represents one of the early events in the initiation of the inflammatory process. Once released in the extracellular matrix, TNF-α starts an extremely complicated series of events that lead to the initiation and amplification of the inflammatory/immune response. Firstly TNF-α can activate the endothelium to express P-selectin, E-selectin ICAM-1 and ICAM-2; these proteins represent cell-adhesion molecules that mediate the recruitment of circulating monocytes from the bloodstream to the inflamed tissue. TNF-α also coadjuvates this process as itself represents a chemoattractant for white cells. TNF-α induces blood clotting in small vessels near the site of inflammation or infection in order to contain it *in loco*. The production of other pro-inflammatory cytokines such as IL-2, IL-8 (CXCL-8) and IL-6 can also be stimulated by the released TNF-α through the activation of NF-κB.

1.5 Mast cells degranulation

1.5.1 Mast cells degranulation after IgE sensitisation

During allergic reactions, mast cells are triggered following the crosslinkage of IgE-linked FCϵRIs (see paragraph 1.5.1.a) by an antigen. After receptor aggregation, a complex downstream pathway (paragraph 1.5.1.b) is activated leading to the final response represented by the release of granular mediators (histamine, tryptase, chymase,

heparin etc), both newly (TNF-α, IL-13) and preformed (IL-4, IL-5, IL-13, TNF-α etc.) cytokines and lipid mediators (PGD$_2$, LTB$_4$, LTC$_4$). All the mast cells' products mediate the inflammatory response in conjunction with macrophage and T-cell-derived cytokines.

1.5.1.a   The FcεRI, an introduction

Receptors for the Fc region of immunoglobulins are called FcR; these membranous receptors have a crucial role in the immune inflammatory response as they mediate the phagocytosis of pathogens by macrophages, dentritic cells and neutrophils. FcRs also play a pivotal role for the release of pro-inflammatory mediators by mast cells and basophils that express a subtype of FcR (FcεRI) characterised by the ability to bind IgE with high affinity ($1 \times 10^{10} M^{-1}$) [39, 40].

In rodents, the FcεRI presents a tetrameric structure composed by one α, one β and two γ subunits ($\alpha\beta\gamma_2$) and it is expressed exclusively on MC and BS (Figure 4). The α subunit has the only function of binding the ligand through its extracellular domain. The β chain is essential for the receptor's translocation to the membrane and it gives stability to the receptor itself. The β chain can also amplify the activation signal that subsequently will be transducted by the γ chain (paragraph 1.5.1.b).

In humans, the FcεRI is constituted by one α and two γ subunits [39]. Despite the fact that $\alpha\gamma_2$ complexes lack of the β chain, they can still be translocated to the membrane but their stability is sensibly reduced.
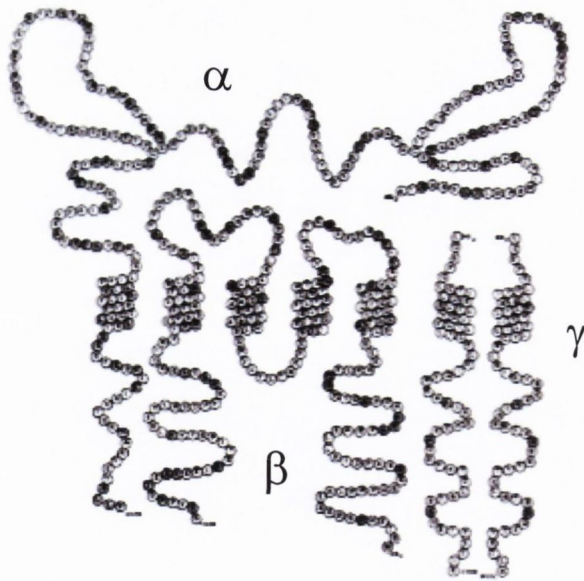
Figure 4

Model of the mouse high affinity receptor for IgE (FcεRI) as described by Kinet and co-workers [41].

1.5.1.b The IgE-dependent degranulation pathway

The initial event of the cascade that leads to MC degranulation is the aggregation of crosslinked FcεRIs in specific regions of the plasma membrane called *lipid rafts.* These microdomains are detergent resistant portions of the cellular membrane that contain sphingolipids, cholesterol and glycosylphosphatidylinositol-anchored proteins. The composition of Lipid rafts is heterogeneous and can change in response to various stimuli; the particular constituents concentrated in these microdomains play a crucial role for the protein-protein interaction during signaling [42].

Once aggregated, FcεRIs migrate into lipid rafts, they associate with a component of the SRC-family kinases called **LYN** that phosphorylates the β and the γ chain of FcεRIs, in particular domains called ITAMs (immunoreceptor tyrosine-based activation motifs). The phosphorylated receptors can interact with SH2* regions of other adaptor molecules

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* SH2 is the acronym for ''SRC homology region 2''. SH2 are specific modules contained in adaptor molecules that bind tyr-phosphorylated receptors with high affinity. The specificity of the binding is guaranteed by the sequence of 3-5 aminoacids after the pTyr. The selectivity of this interaction determines with downstream pathway will be activated 43 **Comoglio, M. P., Boccaccio, C., I**

such **LAT** (linker for activation of T cells) and **SYK** (spleen tyrosine kinase). Once activated, LAT plays a crucial role in recruiting a multitude of other adaptor molecules (GRB2, GADS, SLP76, VAV etc.) and signaling enzymes (PLCγ1, PLCγ2, PLD). Due to the great number of proteins involved during these early stages of mast cell activation, the signaling process can be finely regulated and diversified leading to the release of a complex array of pro-inflammatory mediators [44]. To complicate this, aggregated FcεRI can activate complementary signaling cascades that amplify the signal for the MC activation (Figure 5). These pathways involve the adaptor molecule **NTAL** and another member of the SRC-family kinases, **FYN** [45] (Figure 5).

As it can be seen in Figure 5 the phosphorylation of LAT and NTAL after the initiation of the intracellular cascade, leads to the activation of PLCγ and PI3K that can break the phosphatidylinositol(4,5)diphosphate in inosytol triphosphate ($InsP_3$) and diacylglycerol (DAG). The interaction of $InsP_3$ with its receptors promotes the release of calcium from intracellular storages. The released calcium plays an important role as second messenger during MC signaling. The increase of the free cytosolic calcium concentration has a biphasic trend: the initial rise due to the release of the ion from intracellular compartments is only transient and after reaching a plateau (after approximately 30s) it has to be sustained by the influx of the ion from the extracellular environment [14]. The extracellular influx of Ca^{2+} is stimulated by the ion released from the endoplasmic reticulum that activates a store operated calcium channels (SOCC) subfamily localised on the cellular membrane. These particular SOCCs are called CRAC channels (Ca^{2+} release-activated Ca^{2+} channels) and once activated they allow entry of calcium from the extracellular environment to the cytosol. The mechanisms by which the calcium can act as second messenger are not fully understood but its role during MC degranulation is unanimously accepted. It is thought that once in the cytosol Ca^{2+} can bind to a ubiquitous protein called calmodulin (CAM) that works as calcium sensor for many downstream pathways; once the concentrationum of intracellular Ca^{2+} rises, CAM

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recettori per i fattori di crescita *Farmacologia generale e molecolare*, 2nd Edn. UTET 1999.

binds four ions and then it interacts with target proteins initiating the signalling cascade.

It has been suggested that the rise of Ca^{2+} and Ca^{2+}/CAM complex in the mast cell can lead to various downstream events such as:

- the activation of various protein kinases C (PKC) (that have an important role during the activation and termination of the secretory process). In particular, PKCs have been shown to increase the phosphorolation of the light and heavy chain of myosin during degranulation. Myosin associates with actin to form motile transitory protrusions on the cell surface called *"membrane ruffles"* that are essential cytoskeletal rearrangements during MC degranulation [14, 46, 47]

- the regulation of the granule-plasma membrane fusion [8]

- the activation of the ceramide kinase (CERK) to convert ceramide to ceramide 1-phosphate (C1P), that can act both intracellularly (reinforcing the rise in cytosolic calcium) and extracellularly (interacting with specific G-protein receptors) to amplify the degranulative signal [48, 49].

Many efforts have been made to produce efficient chemotherapeutics that can affect calcium homeostasis and lead to a decrease in mediator release by triggered mast cells. Such molecules might inhibit the rise of the free cytosolic calcium concentration at different levels such as interfering with the conductance of calcium channels or interfering with the binding of the ion to calmodulin.
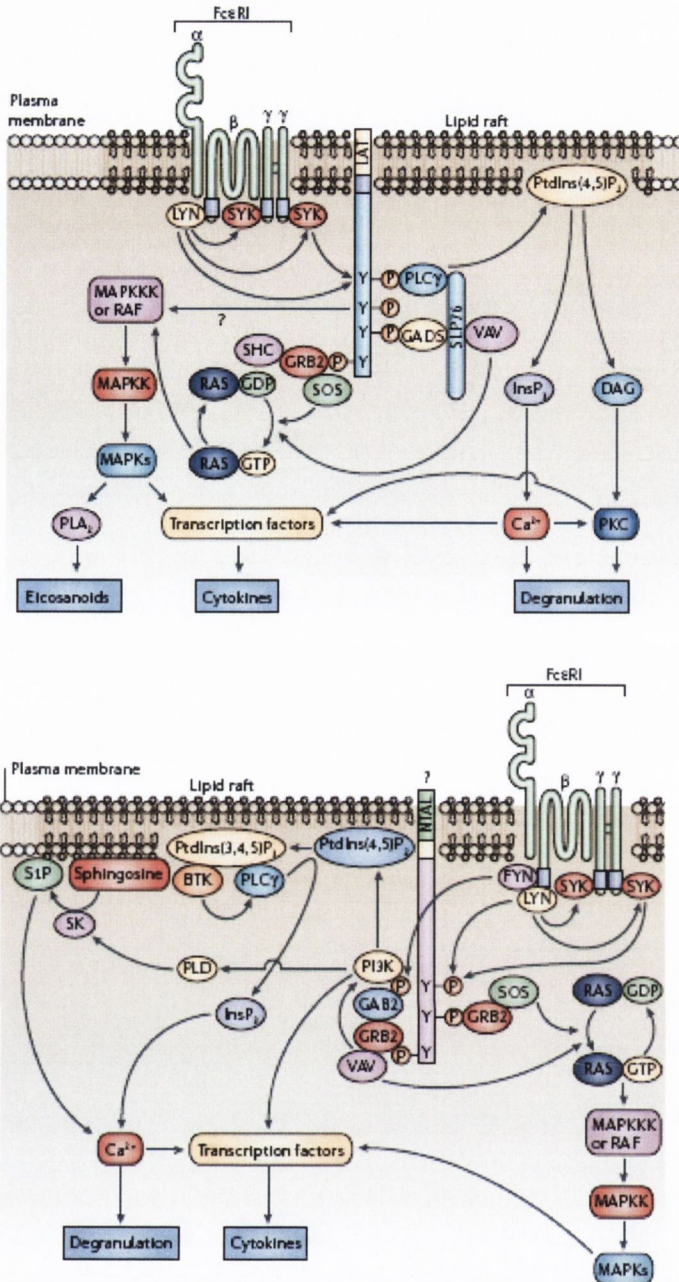
Figure 5

Upper panel: schematic representation of the events that follow the crosslinkage of FcεRs (one receptor is shown in the picture for practical reasons), refer to text for further details.

Lower panel: schematic representation of one of the complementary pathways that are activated by crosslinkage of FcεRs. The function of these accessory pathways is to amplify and regulate the signal for the degranulation.

Picture extracted from [44].

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1.5.1.c *The protein-G-coupled (complementary) pathway for degranulation*

The protein G-coupled receptors (GPCR) are a large family of membranous receptors that can be activated by a multitude of molecules such as complement, chemokines, amines and neurotranmitters. The protein G-coupled receptors are characterized by seven membrane spanning domains with an intracellular C-terminus and an extracellular N-terminus. GPCR can transduce extracellular signals through the interaction with heterotrimeric (Figure 7) GTP-regulated signaling proteins. GPRC are present on mast cells and, once activated, they can lead the mastocyte directly to degranulation (e.g. receptor for the C3a fragment of the complement cascade) or they can amplify and sustain other degranulative pathways initiated by other concurrent stimuli [44, 50]. Whether GPCR mediate co-stimulatory signals or not, a pivotal role during the intracellular cascade is played by the phosphatidylinositol 3-kinase (PI3K) that eventually leads to the increase of intracellular calcium and to degranulation. GPCR can also activate PLC to produce DAG and InsP$_3$; the interaction of InsP$_3$ with its receptors promotes the release of calcium from intracellular storages (Figure 6), [44].
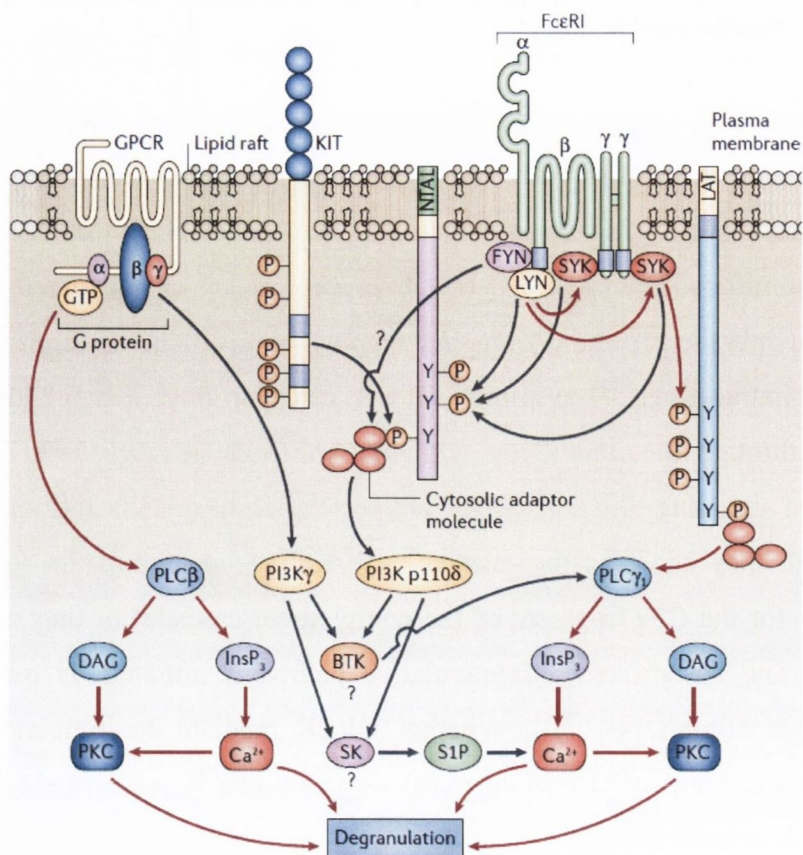
Figure 6

Schematic representation of the protein G-coupled (complementary) pathway for MC degranulation. Refer to text for further details.

Picture extracted from [44]

1.5.2 *Mast cells degranulation after compound 48/80 treatment*

Compound 48/80 is a mixture of polymers derived from N-methyl-p-methoxy-phenylethylamine) and belongs to the large family of polybasic compounds that are known to stimulate mast cell degranulation.

Compound 48/80 can penetrate the lipid bilayer of the cellular membrane and it has been proven to interact with the G_i and G_0 subfamilies of GPCRs and to promote the early events of the GCPR-coupled signal (dissociation of the $\alpha\beta\gamma$ heterotrimer in its constituents, α and $\beta\gamma$ and increase of the GTPase activity of the α subunit, step ③ and ⑤ in Figure 7) [51-53]. Interestingly, compound 48/80 and all the other polybasic compounds are not active on all the MC subfamilies,

MMCs being insensitive to such molecules (see paragraph 1.3). The mechanism by which MMC do not respond to compound 48/80 it is not fully understood but it is known that MMC can acquire sensitivity to it depending on the culture conditions [54].
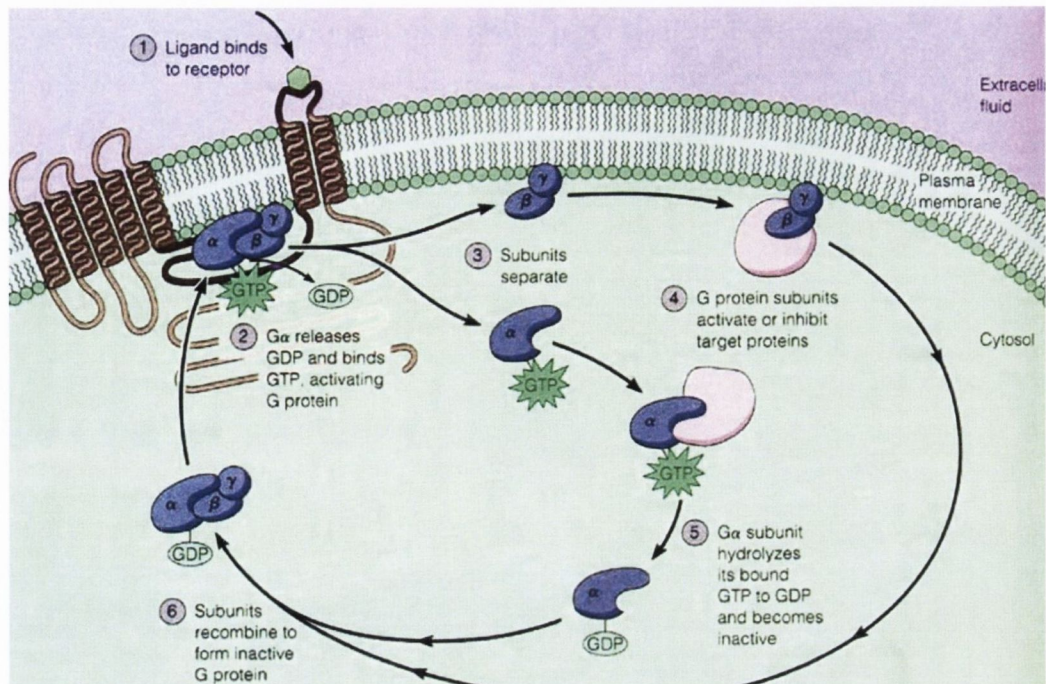
Figure 7

Schematic representation of the activation/inactivation cycle of GPCRs. Compound 48/80 and other polybasic compounds are known to interact with GCPR in mast cells facilitating step ③ and ⑤ of the cycle.

Picture extracted from: http://219.221.200.61/ywwy/zbsw(E)/edetail6.htm

1.5.3 Mast cells degranulation after calcium ionophore A23187 treatment

The exact mechanism by which the calcium ionophore A23187 can induce mast cell degranulation is not fully understood. It is commonly thought that A23187 can form aggregates in lipid matrices, forming channels or pores in the cellular membrane and therefore facilitating the transport of cations across the cellular membrane. Another hypothesis is that the A23187 molecule itself could act as a mobile carrier and exchanging one Ca^{2+} (in) with $2H^+$ (out) across the lipid bilayer [55]. However Balasubramian and co-workers [56] suggested that the mechanism of action of A23187 might not be only a mere reflection of its ability to aggregate in hydrophobic environments but also that

additional components might be responsible for the cell response. According to the authors, calcium ionophore A23187 can increase the cytosolic calcium concentration activating the Ca^{2+} influx through native Ca^{2+} channels or mobilising the ion from intracellular storages in a phospholipase C (PLC)-dependent fashion. Once activated, PLC can cleave the phosphatidylinositol(4,5)diphosphate into $InsP_3$ and DAG. $InsP_3$ interacting with its receptors on the sarcoplasmic reticulum promotes the release of calcium from intracellular storages.

1.6   Mast cells in innate immunity

Innate immunity represents the first line of defense of an organism and it is characterised by a fast response (0-4 hours). It is distinguished from the adaptative immune response as it does not contribute to the immunological memory and it does not rely on expansion of lymphocyte clones specific for antigens already encountered by the body. The innate immunity makes use of the ability of a certain class of receptors, called PRRs (pattern recognition receptors) to recognise specific molecular patterns/structures expressed only by invading microorganisms but not by host cells. These pathogen-associated molecular patterns (PAMPs) are highly conserved motifs in microbial cellular products essential for the life of the microorganism. Although the overall microbial products differ between different strains and species, the highly conserved patterns confer to the molecule the "virulence factor" that represents the alarm bell to trigger the early (innate) immune response [57]. PPRs are a group of different families of receptors located in various sites of the body such the as cell surface, the intracellular compartment or the blood stream. Their activation can evoke downstream events, like opsonisation and activation of complement with the subsequent production of cytokines and chemokines that promote and sustain the recruitment of effector cells (neutrophils, macrophages etc.).

Due to their strategical locations (mainly structures that interface with external environment such as lungs and skin) MC have a crucial role during host defense; after pathogen activation (see paragraph 1.6.2), mast cells can release pro-inflammatory cytokines (IL-13, TNF-α, tryptases, chymases etc, see paragraph 1.4) and chemoattractants such CCL-5, CCL-11[*] and LTB$_4$ to recruit eosinophils and monocytes to the site of infection. CXCL-8 (IL-8) and TNF-α enhance the expression of integrins and adhesion molecules (p-selectin, E-selectin, ICAM I etc) to

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[*] Chemokine designed with CC present the two first amino terminal cysteine residues adjacent while, those in those designed with CXC the cysteine residues are separated by one aminoacid.

allow the recruited cells to infiltrate through the endothelium. Moreover CXCL-8 can direct eosinophils and other leukocytes through the cytokine gradient to the site of infection. Mast cells produce also GM-CSF that enhance the survival of effector cells [58] (Table 2).

The role of mast cells during host defense is attracting the attention of researchers as it represents an important aspect of innate immunity. It has been shown, in fact, that not only do mast cells intervene during bacterial infections *(Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, [59, 60])*, but also they have a crucial role during parasite infections *(Trichinella spiralis, Nippostrongylus brasiliensis [61, 62])* and viral infections (Sendal virus, Respiratory syncytial virus [63, 64]). Moreover, the response of mast cells (and hence the array of mediators released) varies with the particular PAMP presented by the invading pathogen with the subsequent selective modulation of the immune   response.

Table 2 Properties of some chemokines and pro-inflammatory mediators released by mast cells during pathogen invasion. Mast cells play a crucial role to initiate and sustain the innate immune response recruiting effector cells to the site of infection and promoting their survival during the inflammation process.

Mediator	Properties[58]
CCL-5	It promotes the infiltration and activation into tissues of eosinophils and other leukocytes
CCL-11	Chemoattractant for eosinophils and other leukocytes
IL-8 (CXC-8)	It triggers the change of conformation of integrins in order to allow leukocytes (neutrophils and naïve T cells) to penetrate the endothelium (process called extravasion) and it also directs leukocytes toward the site of infection and it activates target cells (e.g. neutrophils)
LTB$_4$	It recruits effector cells and it regulates the immunoresponse
TNF-α	It increases blood flow and vascular permeability. It also increases the adhesiveness of the endothelial cells for leukocytes enhancing the expression of P-selectin, E-selectin, ICAM-1
GM-CSF	It enhances survival of recruited cells
IL-13	Function of T helper 2-type cytokine
Histamine	It increases vascular permeability
Tryptase/Chymase	They contribute in tissue remodeling, it enhances CXC-8 production
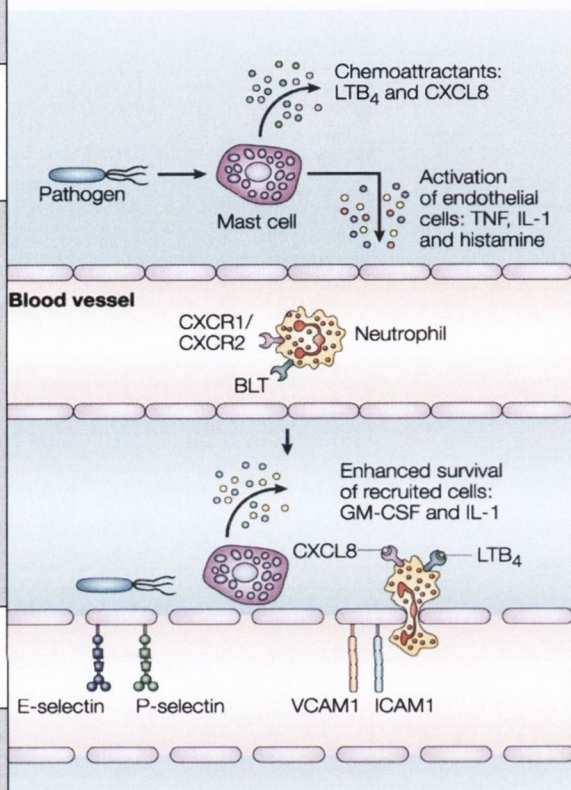
From: Marshall J.S. (2004) [30]

1.6.1   Toll like receptors, a brief introduction with a particular focus on TLR2 and 4

Toll receptors were originally discovered in the fruitfly *Drosophila Toll*. At the beginning, they were thought to be involved only during embryonic development but their similarity with the Interleukin-1 receptor (IL-1R) suggested a possible involvement in innate immunity. This was proved by the fact that *Drosophila* presenting mutant toll receptors were killed by fungal infections. In *Drosophila*, the toll receptors are associated with the production of antimicrobial peptides and do not present the PPR function (function that is carried out by other components situated upstream in the cascade of events, [65]). In mammals, toll-like receptors possess the PPR function and therefore they can directly recognize pathogens. Many members of the TLR family, however, need accessory proteins to recognise ligands (see below). Several TLRs have been discovered during the past years and they have been assigned to different subfamilies on the basis of the ability to recognise specific classes of PAMPs. Toll-like receptors are one of the most primitive pathogen-recognition system and this reflects the difficulty for microorganisms to mutate their PAMPs; mutations of PAMPs, in fact, lead to the loss of the ability of the microorganism to adapt. The TLR1 family, (whose recognised PAMPS are lipopeptides such as zymosan and PGN) is the one that has received the strongest evolutionary push and shows the biggest specie-specific adaptations.

Among all the TLRs discovered (10 in humans), TLR2 and TLR4 are prominent in mast cell biology and they will be described in the paragraphs 1.6.1.b and 1.6.1.c.

1.6.1.a   TLR structure and signaling

Toll-like receptors present a horse-shoe-shaped extracellular domain showing several (16-18) leucine-rich repeats that are responsible for the interaction of the receptor with its ligands. In particular TLR1, 2 and 4 bind hydrophobic ligands (such as LPS) that are known to interact with the internal pocket of the horse-shoe-shaped domain.

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As previously anticipated, the intracellular tail of toll-like receptors shows high similarity, in a conserved region, called TIR (Toll/IL-1R) domain, with the intracellular tail of the IL-1R. Following ligand binding, TLRs dimerise in homo and heterodimers; this event evokes a conformational change that promotes the recruitment of adaptor proteins to the TIR domain of the intracellular tail. The first adaptor to be recruited is the myeloid differentiation primary response protein 88 (MyD88), that, in turn, recruits (through another protein-protein interaction domain called "death domain") a serine/threonine innate immunity kinase called IRAK. IRAK starts an intracellular cascade that leads to the activation of the transcription factor NFκB that activates the genes necessary for the production of pro-inflammatory cytokines [66].
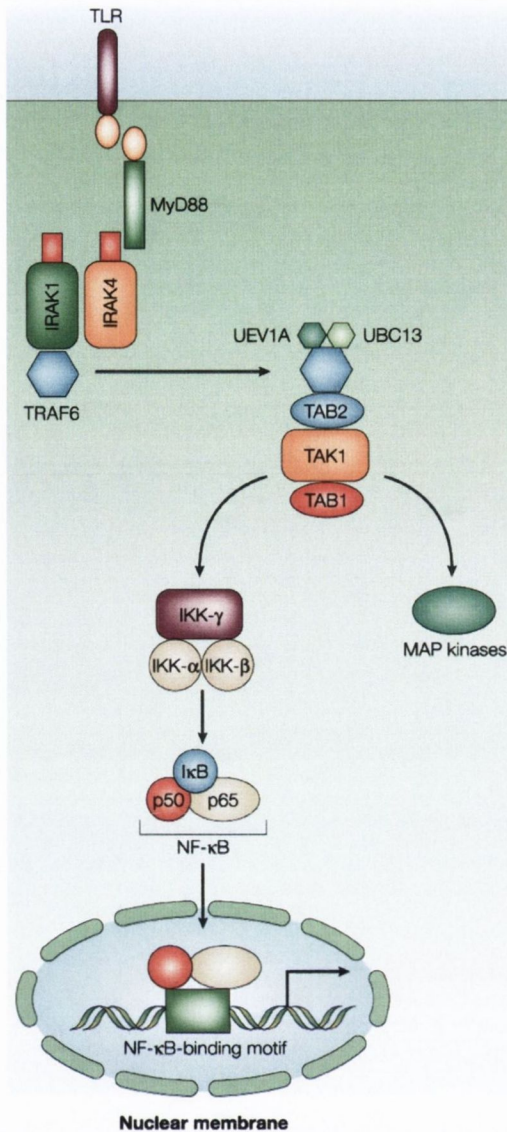
Figure 8

Schematic representation of the events that follow the ligand recognition and the dimerisation of TLRs. The first event is the recruitment of MyD88, that activates IRAK that in its turn starts an intracellular cascade that leads to the transcription of genes for the production of pro-inflammatory cytokine.

Picture extracted from [66]

1.6.1.b TLR2

TLR2 mediates the response to lipoproteins and lipopeptides, components of the outer wall of **Gram positive** bacteria (e.g. peptidoglycan, PGN). In order to transmit the signal, TLR2 has to form heterodimers with TLR1 and/or 6. Dimers are stabilised by the ligand that forms a bridge between the two horse-shoed-shaped pockets of the receptors, creating in such way a long hydrophobic region. Other

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protein-protein interactions, localised close the ligand binding pocket, can further stabilise the dimer [67].

1.6.1.c TLR4

TLR4 mediates the response to hydrophobic ligands like Lipopolysaccharide (LPS). LPS is a component of the outer wall of **Gram-negative** bacteria and evokes a potent immune response. LPS structure varies between different strains of bacteria but the basic skeleton is composed of Lipid A (dimer of N-acetylglucosamine linked to fatty acid chains) linked to a polysaccharide component and to an highly variable antigen portion containing oligosaccharides (O-core sugars). The pathogenic properties of various type of LPS crucially depends from composition, length and phosphorylation of the O-core sugars; for example LPS from *Escherichia Coli* presenting five or seven acyl-chains is 100 times less potent than the LPS that possess the ideal number of six acyl-chains, [67]. LPS that lacks of the antigen portion is called "rough" [*] LPS (rLPS), while LPS that presents a complete structure is called smooth LPS (sLPS). The ligand's recognition by TLR4 is strictly dependant on the type of LPS involved.

Rough LPS can directly bind the TLR4 while sLPS needs the presence of the co-receptor CD14 to activate the intracellular pathway. The exact role of CD14 in TLR4/MD2 signaling is still unclear but as this receptor does not present any intracellular domain, it is thought that its role must be "limited" to the presentation of the ligand to the TLR4; it has been reported that CD14 can form a horse-shoe-shaped structure with a hydrophobic pocket that could hold the lipid portion of LPS and present its carbohydrate chains to the TLR4 [68]. The presence of a CD14-independent and a CD14-dependent pathway for LPS adds complexity to the role of TLR4/MD2 in innate immunity.

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[*] the terms "rough" and "smooth" are due to the aspect of the colonies.
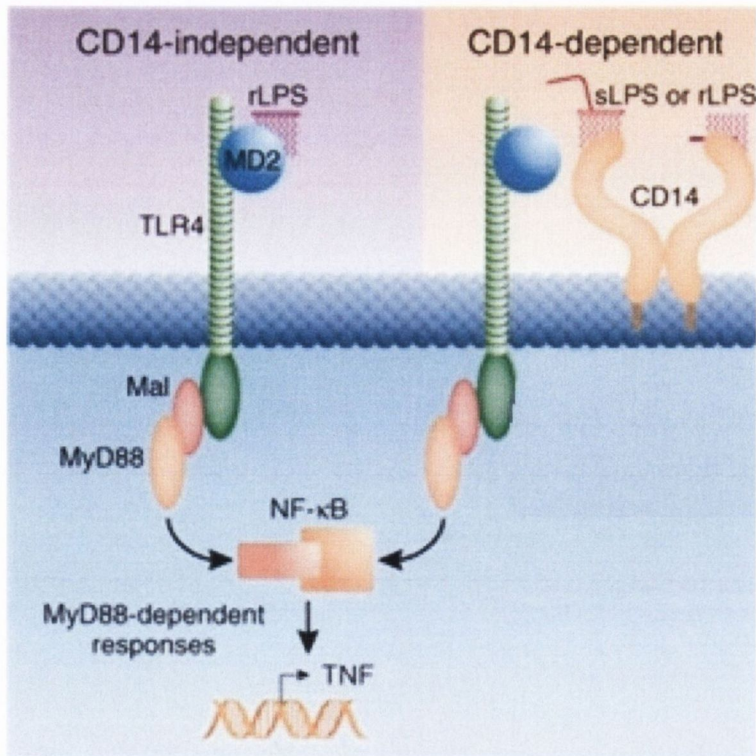
Figure 9

LPS recognition by TLR4 is strictly dependant on the type of LPS involved. Rough LPS can directly bind the TLR4/MD2 complex while sLPS needs the presence of the co-receptor CD14 to activate the intracellular pathway. Refer to text for further details

Picture modified from [68]

1.6.2 *TLR2 and 4 in mast cells*

It has been reported that mast cells produce different patterns of pro-inflammatory cytokines when stimulated with TLR2 or TLR4 ligands. Mastocytes stimulated through their TLR2 dependent pathway produce TNF-α, IL-4, IL-5, IL-6 and IL-13; furthermore they have been reported to degranulate when TLR2 ligands were tested. After TLR4 stimulation, on the other hand, mast cell produce TNF-α, IL-1β, IL-6 and IL-13 but not IL-4 or IL-5 and they do not degranulate [69].

1.7 *RBL-2H3 cells*
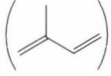
The continuous rat cell line, RBL-2H3, was cloned by the limited dilution technique from leukaemia cells originally isolated from rats

after treatment with the chemical carcinogen, β-chlorethylamine[*] [70]. RBL-2H3 cells have been extensively used for studying IgE-FcεRI interactions [71], signalling pathways for degranulation [72] and to test novel mast cell stabilisers. RBL-2H3 cells could be considered a useful tool for *in vitro* work, as a great number of monoclonal cells can be rapidly obtained by simple cell culture techniques.

1.8   *History of a novel indane analogue of pterosins Z*

Pterosins are a large family of sesquiterpenes[†] that can be isolated from the fern *Pteridium aquilinium*. The biosynthetic pterosin precursor, ptaquiloside, is thought to be responsible for the carcinogenicity of the pterosin containing ferns. Pterosins, and in particular pterosin Z, posses smooth muscle relaxant properties [73] and they can inhibit the calcium induced contraction of guinea-pig ileum. This suggests that the mechanism of action of these molecules might involve the inhibition of the Ca^{2+} influx from extracellular environment or interfere with the calcium/calmodulin $(CaM)^{‡}$ cascade of reactions within the cell [75].

Due to the crucial role of Ca^{2+} during MC degranulation (see paragraph 1.5.1.b),, the ability of these molecules to interfere with the calcium signalling, suggested a possible activity as mast cell stabilisers. Many pterosin related compounds have been synthesised and tested both as smooth muscle relaxant and mast cell stabilisers and a number of these compounds have been shown to inhibit 48/80-induced histamine release from rat peritoneal mast cell (RPMC) [76]. In particular, in this thesis two diastereoisomers (PH2 and PH5) and their four enantiomers (PH3, PH4, PH21 and PH22) (see paragraph 2.3) of a novel indane derivative of pterosin Z have been tested as mast cells stabilisers. Moreover in

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[*] For further information regarding the genesis of the RBL-2H3 cell line refer to Appendix 4

[†] Sesquiterpenes are molecules constituted by three isoprene units and have the molecular formula $C_{15}H_{24}$

[‡] Calmodulin (Cam) is a calcium sensor that interacts with multiple protein target [74
    Rhoads, A. R. and Friedberg, F., Sequence motifs for calmodulin recognition. *Faseb J* 1997. **11**: 331-340.]

preliminary studies some of the pterosin Z analogues synthesised in our laboratories, have shown to partially inhibit the production of cytokines from Jurkat [*] cells (Frankish, Sheridan, unpublished data). For this reason, PH2 and PH5 were tested also as potential inhibitors on the IL-13 and TNF-α production and release.

1.9 Aims

The original aim of this work was to investigate the effect of PH2, PH5 and their resolved enantiomers on the degranulation of mast cells induced both by non immunological stimuli (compound 48/80, calcium ionophore A23187, PGN, pam2CSK4) and by immunological stimuli (IgE/anti-IgE). Furthermore, PH2 and PH5 were also tested as inhibitors of the production and release of interleukin such as TNF-α and IL-13 on RPMC. Peritoneal MC can be easily obtained by peritoneal lavage of small animals (e.g., rats), however, they need to be purified, which can, often, impede their ability to react to stimuli [78]. In addition, it is impossible to maintain them in primary culture over prolonged period of time. From this, the necessity to have, in our laboratory, an *in vitro* tool that would have allowed us to screen, not only the compounds of interest for this thesis, but also the other pterosin Z-derived compounds that showed interesting properties during preliminary studies. The ideal model should have been a fast growing, easy to cultivate mast cell line with a long lifespan and, more important thing, it had to show high affinity receptors for IgE and high histamine content.

RBL-2H3 had already been extensively used for studying degranulation pathways, test novel mast cell stabilisers [79] and also it is possible to obtain a large number of homogeneous cells with simply cell culture techniques. Unfortunately, since the early stages of the

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[*] Jurkat (Human T cell lymphoblast-like) cell line: stable tumor cell line capable of producing human interleukin-2, established by John Hansen (Fred Hutchinson Cancer Research Center, Seattle). [77   **Abraham, R. T. and Weiss, A.,** Jurkat T cells and development of the T-cell receptor signalling paradigm. *Nat Rev Immunol* 2004. **4**: 301-308.

optimization of the experimental procedures, it was evident that the huge amount of information present in the literature was often inconsistent and inaccurate. Therefore, in addition to the investigation of the mast cell stabilizing property of PH2 and PH5, this thesis concerns the characterization of RBL-2H3 as a model for degranulation studies. RBL-2H3 cell line represents also a system that resembles the particular MC subtype involved in the pathogenesis of diseases such as ulcerative colitis and Chron's disease. This is important as it is not possible to anticipate the effect of a therapeutic agent on a particular type of MC based on information obtained with other subtypes (CTMC vs MMC, see paragraph 1.3),

Summarising the specific aims of this study were:

- To validate RBL-2H3 cells as an *in* vitro model to study mast cell degranulation and mast cell response to LPS
- To set up an *ex vivo* model using freshly harvested RPMC to study mast cell degranulation.
- To investigate the effect of PH2, PH5 and their resolved enantiomers (PH3, PH4, PH21 and PH22) on RBL-2H3 degranulation after immunological stimulation (IgE/antigen)
- To investigate the effect of PH2, PH5 and the resolved enantiomers on RPMC degranulation induced by immunological (IgE/antigen) and non immunological stimuli (compound 48/80, calcium ionophore A23187, PGN, pam2csk4)
- To compare the results obtained with RBL-2H3 cells (example of MMC) and RPMC (example of CTMC)
- To investigate the effects of PH2, PH5 and the resolved enantiomers on the production and release of TNF-α and IL-13 after LPS and PGN treatment of both RBL-2H3 and RPMC

2 Materials and Methods

2.1 Materials and Methods used during experiments with RBL-2H3 cells

2.1.1 Cell culture

RBL-2H3 cells were purchased from LCG Promochem[*], partner of ATCC (Teddington, UK) and were cultured in α-MEM (GIBCO, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), (Biosera, Ringmer, UK) and 100 U/ml penicillin-streptomycin (GIBCO) at 5% CO_2 and 37°C. Cells were subcultured using either trypsin-EDTA (GIBCO) or Accutase® (Bioquote, York, UK) when reaching 80% confluency and were plated at 1- 2.6×10^5 cells/cm^2 in 24 or 6-well plates for experiments.

2.1.2 RBL-2H3 cells degranulation studies after anti-DNP IgE/DNP-BSA treatment

RBL-2H3 cells (1×10^5 cells/cm^2) were seeded in 24-well plates and they were allowed let to adhere for at least 3 hours. Then the supernatant was removed and replaced with fresh medium containing anti-dinitrophenyl (anti-DNP)-IgE (Sigma, Dublin, Ireland) (serial dilutions to obtain final concentrations from 5000 to 4.88 ng/ml). Each concentration was tested in triplicate. Plates were incubated for 16 hours at 5% CO_2 and 37°C. After incubation, cells were washed twice with PBS and subsequently 500 μl of Earle's balanced salt solution (EBSS, GIBCO), with 0.1% bovine serum albumin (pH 7.4), added to the wells. Cells were triggered with 33 μl of DNP-BSA (5 μg/ml) and incubated for 30 min at 37°C. Supernatants were analysed as follows:

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[*] RBL-2H3 cell line was deposited at an unknown passage number by Reuben P. Siraganian in 1978. It was isolated and cloned in 1978 in the Laboratory of Immunology at the National Institute of Dental Research from Wistar rat basophilic cells. No other strains have been registered since then.
[70 **Eccleston, E., Leonard, B. J., Lowe, J. S. and Welford, H. J.,** Basophilic leukaemia in the albino rat and a demonstration of the basopoietin. *Nat New Biol* 1973. **244**: 73-76.]

prepared dissolving commercially available histamine (Sigma) in releasing media.

To obtain the amount of histamine in each sample, the absolute fluorescence values obtained were interpolated with the standard curve. The amount of histamine released was then converted into percentage of total cellular histamine content by comparison with the value produced by a Triton X-100 (Sigma) lysate of the same cells according to Equation 1.

2.1.3 Degranulation studies after calcium ionophore, MCDP, compound 48/80, PGN treatment

RBL-2H3 cells (1×10^5 cells/cm^2) were seeded in 24-well plates and they were let to adhere for at least 3 hours. Then the supernatant was removed and replaced with fresh medium containing calcium ionophore, mast cell degranulating peptide (MCDP), compound 48/80 and peptidoglycan (PGN) at the desired concentration. Plates were incubated for 30 min at 5% CO_2 and 37°C. Supernatants were analysed as previously described (see paragraphs 2.1.2.a and 2.1.2.b). The absorption was converted into the percentage of total cellular β-hexosaminidase activity by comparison with the absorption produced by a Triton X-100 (Sigma) lysate of the same cells according to Equation 1. Each treatment was performed in triplicate.

2.1.4 RBL-2H3 mast cell degranulation in presence of test compounds

During experiments performed in presence of test compounds, 0.5% of the supernatant volume was substituted with the vehicle or the vehicle charged with the appropriate amount of the test compound. There was a minimum of ten incubations (spontaneous release, basal release, total content, positive control, vehicle pretreated (DMSO) and test compound pretreated (PH3, PH4, PH5, PH21 and PH22, see paragraph 2.3 for additional information on test compounds) where:

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- the spontaneous release is the amount of released mediator when no stimulus is applied
- the basal release is the amount of mediator released after stimulation
- the total content is the amount of histamine found in the supernatant after cell lysis obtained with TritonX-100, it therefore represents the amount of mediator contained in the cells.

Each incubation was performed overnight (16 hours) and in triplicate. Supernatants were analysed as previously described (see paragraph 2.1.2.a and 2.1.2.b). The percentage of the vehicle was always maintained at 0.5% of the volume of the supernatant.

The inhibition percentage of the mediator release was calculated using the following equation:

% inhibition=(secreted w/o drug – secreted with drug)/secreted w/o drug]*100

Equation 2

2.1.5 *Cytotoxicity evaluation of test compounds*

2.1.5.a *LHD assay*

Cytotoxicity of test compounds was analysed using a colorimetric assay based on the measurement of the lactate dehydrogenase (LDH) in the supernatants. LDH is a stable cytoplasmic enzyme present in most cells, which is rapidly released into cell culture supernatant upon damage of the cytoplasmic membrane. The LDH assay is based on the ability of LDH to reduce NAD^+ to $NADH + H^+$ by oxidation of lactate to pyruvate; subsequently, during a coupled reaction helped by the catalyst diaphorase, 2H are transferred from $NADH/H^+$ to a tetrazolium salt (yellow) to form a formazan salt (red) that presents a maximum of absorption at 500nm. An increase of the LDH released by damaged cells correlates with the formation of the coloured formazan salts. The cytotoxicity of the test compounds, disodium cromoglycate (DSCG),

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terfenadine and quercetin was tested using an LDH detection kit (Roche, Mannheim, Germany) containing all the reagents for the coupled reaction.

Terfenadine was used as positive control because of its potent cytotoxic effect [82], on the other hand DSCG was used as negative control due to its negligible cytotoxic effect [83]. In order to reproduce the experimental condition during the degranulation experiments, RBL-2H3 cells (1×10^5 cells/cm^2) were seeded in 24-well plates and incubated overnight with test compounds (PH2, PH3, PH4, PH5, PH21 and PH22) and controls (DSCG, terfenadine, quercetin). Each compound was tested in triplicate.

To determine the percentage cytotoxicity, the average of the triplicate was calculated and the value obtained for the background control was subtracted. Subtracting the background value was necessary because the reaction medium, in which the cells were incubated during the experiment, contained 1% of FBS that has an intrinsic LDH activity. To the values obtained, the following equation was, then, applied:

$$\% \text{ Cytotox} \frac{(\text{exp. value } - \text{spontaneous LDH}}{(\text{maximum LDH release- spontaneous LDH release})} *100$$

Equation 3

where:

- ► "exp. value" is the absorbance obtained for the treated well (subtracted of the background value).
- ► "spontaneous LDH release" is the absorbance obtained from untreated cells (subtracted of the background value)
- ► "maximum LDH release" is the absorbance obtained from TritonX-100 (2% in assay medium) lysed cells (subtracted of the background value).

2.1.5.b MTS assay

Cell viability was tested using the CellTiter96Aqueous Proliferation assay (Promega, Southampton, UK). This assay is based on the bioreduction by living cells of the MTS tetrazolium compound (Owen's reagent) to a coloured formazan salt with a maximum of absorbance at 490 nm. The amount of formazan salt produced is directly proportional to the number of living cells in the sample. In order to reproduce the experimental condition during the degranulation experiments, RBL-2H3 cells (1×10^5 cells/cm^2) were seeded in 24-well plate and incubated overnight with test compounds (PH2, PH3, PH4, PH5, PH21 and PH22) and controls (DSCG, terfenadine, quercetin). Each compound was tested in triplicate.

In order to find the percentage of viable cells, values obtained for untreated wells were considered as 100% viable while values obtained for the intrinsic medium activity (no cells present) were considered as 0% viability. According to the manufacturer's instructions, the relationship between absorbance and the number of viable cells is linear; by writing the equation of a line passing through those two points (0 and 100% of viability) is possible to obtain the values of viability corresponding to the absorbance values obtained with the samples.

In other words as, 0% viability (X_0) and 100% viability (X_{100}) have known absorbances (Y_0 and Y_{100}), the percentage of viability of treated samples (X_x) with experimental absorbance Y_x can be obtained with the following equation:

$$X_x - Y_0 = [(Y_{100} - Y_0)/(X_{100} - X_0)] * (X_x - X_0)$$

Equation 4

2.1.6 LPS activation of RBL-2H3

RBL-2H3 cells have been reported to respond to components of the outer wall of gram negative bacteria such as LPS, resulting in the production and release of Th2-associated cytokines [84]. To test the amount of tumour necrosis factor-α (TNF-α) and interleukin (IL)-13 released, RBL-2H3 cells were seeded at a density 1×10^5 cells/cm^2 in

24-well plates. Cells were incubated overnight in the presence of 0.1-1 μg/ml of either smooth lipopolysaccharide (sLPS) (*Escherichia coli* 0111:B4 or *Escherichia coli* 055:B5, Sigma) or rough lipopolysaccharide (rLPS) (*Salmonella enterica* serotype Minnesota Re 595, Sigma). Supernatants were analysed as follows:

2.1.6.a Measurement of cytokine concentration (TNF-α, IL-13)

Levels of TNF-α and IL-13 were measured in RBL-2H3 cells' supernatants using ELISA kits (Biosource, Nivelles, Belgium) according to the manufacturer's instructions. The study was performed in triplicate using cells from 3 different passages.

2.1.7 TLRs expression in RBL-2H3

mRNA expression for TLRs was investigated through RT-PCR; subsequently protein expression of TLR4 was analysed through flow cytometry.
CD14 and MyD88 protein expression was tested in parallel with the same protocol.

2.1.7.a Qualitative RT-PCR

Total RNA was isolated from RBL-2H3 using an RNeasy mini kit (spin column technology based method) (Qiagen, Crawley, UK) according to the manufacturer's instruction. RBL-2H3 cells were seeded at a density of 2.6×10^5 cells/cm^2 in 6-wells plates and cultivated for two days prior to RNA isolation. During the purification of the total RNA, DNase/RNase-free labware was utilized and all the surfaces (bench, pipettors, glassware and gloves) were pretreated with RNase AWAY (Molecular bioproduct, Sandiego, CA) to avoid RNase and DNA contamination.

The choice of using a nitrocellulose spin column was made in order to avoid heparin contamination of the RNA specimens. It has been postulated that the presence of heparin can inhibit the reverse

transcription polymerase chain reaction (RT-PCR), [85, 86]. mRNA purity and integrity was tested spectrophotometrically using a nanodrop ND-100 (Labtech International, UK) and by electrophoresis in denaturing conditions on 1.2% agarose gels (1.2% agarose in TBE buffer) (Bioline, London, UK), the running buffer was prepared diluting a 5X stock of TBE buffer (54 g TRIS, 27.5 g boric acid, 3.72 EDTA/L of DEPC-treated water). All the reagents were purchased from Sigma. Gels were visualised by ethidium bromide staining using a ChemiDoc gel documentation system (Bio-Rad, Hemel Hempstead, UK).

After RNA quality control, 4 μg samples were treated with a TURBO DNA-free kit (Ambion, London, UK) to remove contaminating genomic DNA (gDNA). The RNA preparation was reverse transcribed to cDNA using a cDNA synthesis kit (Bioline, London, UK) following the manufacturer's instruction. Primers were designed on *Rattus norvegicus* genes using Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm), synthesised by MWG-Biotech (Ebersberg, Germany) and optimized using rat gDNA (Bioline). Sequences for PCR primer pairs, annealing temperatures, size of the transcripts and the GeneBank accession numbers of the correspondent nucleotide sequences for TLRs are shown in Table 3. The PCR reaction (25 μl) contained 0.5 μl of cDNA, 0.4 μM of each primer, 2 mM $MgCl_2$, 2.5 mM dNTPs and 15 U *BioTaq* in NH_4 buffer.

The PCR cycling conditions were: 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 30 s, annealing temperature for 45 s, 72°C for 45 s and a final cycle at 72°C for 10 min. Ten microlitres of the product were run on a 2% agarose gel (Bioline), the running buffer was prepared diluting a 5X stock of TBE buffer and visualised by ethidium bromide staining using a ChemiDoc gel documentation system (Bio-Rad). β-actin was amplified as internal control.

Optimisation for TLR7 primers failed to lead to significant results. At all the conditions tested*, the TLR7 transcript was not detected in the agarose gel. One of the two primers tested (sense: 5'-AGCTCTGTTCTCCTCCACCA-3', antisense: 5'-CATGGGTGTTTGTGCTATCG-3) has been reported to be optimal for PCR analysis [87]. Other designed primers than the two reported in Table 3 presented not suitable characteristic (inadequate primer length, GC content, wrong size of transcript etc.) for PCR and were discarded.

---

* Primers were optimised using different annealing temperatures (from 56 to 62 °C), different MgCl₂ concentrations (from 1 to 2.5 mM), different primers concentrations (from 0.4 to 0.8 μM) and occasionally DMSO was added to the master mix (DMSO helps in amplifying products)

Table 3 Primer sequences for RT-PCR

	GenBank accession no.	Forward primer	Reverse primer	Annealing temperature	Product size
TLR1	XM_223421	5'-TACCCTGAACAACGTGGACA-3'	5'-ATCGACAAAGCCCTCAGAGA-3'	58°C	165 bp
TLR2	NM_198769	5'-GGAGACTCTGGAAGCAGGTG-3'	5'-CGCCTAAGAGCAGGATCAAC-3	58°C	245 bp
TLR3	NM_198791	5'-AGCCTTCAACGACTGATGCT-3'	5'-GTTGGCAATGTTGTTGTTGC-3'	58°C	120 bp
TLR4	NM_019178	5'-TGCTCAGACATGGCAGTTTC-3'	5'-TCAAGGCTTTTCCATCCAAC-3'	58°C	206 bp
TLR5	XM_223016	5'-GCCAGAGCCAGATTGAAGTC-3'	5'-TGTGAATCTCGTTGGCAGAG-3'	58°C	168 bp
TLR6	NM_207604	5'-GTCTCCCCACTTCATCCAGA-3'	5'-AGAGCCCTCAGCTTGTGGTA-3'	56°C	156 bp
TLR7*	EF_032637	5'-AGCTCTGTTCTCCTCCACCA-3'	5'-CATGGGTGTTTGTGCTATCG-3'	//	194 bp
		5'-ATTGGACCTCAGCCACAAAC-3'	5'-CCACCAGACAAACCACACAG-3'	//	231 bp
TLR8	EF_032638	5'-CTGTGGAATGCAAATGATGG-3'	5'-TCATTTCTCCCCAAGTCCAG-3'	62°C	230 bp
TLR9	AY_59725	5'-TCAACAAGAACACGCTCAGG-3'	5'-GAGAGCTGGGGTGAGACTTG-3'	62°C	176 bp
TLR10	XM_223422	5'-TGCAGGAGCTGAACATGAAC-3'	5'-ATTGGACAGGTCCACAAAGC-3'	60°C	235 bp
β-Actin	NM_031144	5'-GTCGTACCACTGGCATTGTG-3'	5'-CTCTCAGCTGTGGTGGTGAA-3	59°C	181 bp
* Optimisation for TLR7 did not lead to satisfactory results. For this reason, no annealing temperature is reported in this table, see text.					

2.1.7.b Flow cytometry (for protein expression of TLR4, CD14 and MyD88)

Indirect staining for cell surface TLR4, CD14 and intracellular MyD88 proteins was obtained with unlabelled polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), followed with ALEXAFluor647 -conjugated IgG antibody (Biosciences, Dublin, Ireland). Briefly, one million cells were suspended in 100 µl of PBS charged with 0.1% of BSA. Subsequently cells were incubated with the primary antibody (20 µg/ml) for 15 min and fixed with reagent A from Fix and Perm kit (Biosciences, Dublin, Ireland) for an additional 15 min. Cells were then washed three times in 3 ml of PBS supplemented with 0.1 % NaN_3 and 5% FBS. After washing, cells were resuspended in 300 µl of PBS/1% BSA and incubated in the dark for 15 min with the secondary antibody (20ng/mg). After incubation, samples were treated with reagent B (permeabilisation reagent) from Fix and Perm kit for additional 15 min. Cells were washed three times as previously described and resuspended in 300 µl of PBS; samples were transferred in a 96 well plate and analysed through flow cytometry for single-colour staining. The analysis was carried out using a BD FACS Array (BD Biosciences, Oxford, UK).

Table 4

Target protein / Antibody	TLR4	CD14	MyD88
Primary Antibody	Rabbit Polyclonal IgG (20 ng/ml)	Goat Polyclonal IgG (20 ng/ml)	Goat Polyclonal IgG (20 ng/ml)
Secondary Antibody	Alexa Fluor 647 Goat Anti-rabbit IgG (20 ng/ml)	Alexa Fluor 647 Rabbit Anti-goat IgG (20 ng/ml)	Alexa Fluor 647 Rabbit Anti-goat IgG (20 ng/ml)

2.2 Material and Methods used during experiments with RPMC

2.2.1 Peritoneal lavage

Animals (male Wistar rats, 250-300g) were sacrificed according to guidelines laid down by the working party report (Laboratory Animals (1996) 30, 293-316, Laboratory Animals (1997) 31, 1- 32), on Directive 86/609/EEC (No. L 358, ISSN 0378-6978), which is endorsed by the Bioresources Ethical Review Committee of the University.

Peritoneal exudate was isolated by peritoneal lavage with pre-warmed buffered salt solution (BSS, NaCl 137 mM; KCl 2.7mM; $MgCl_2$ 1.0 mM; $CaCl_2$ 0.5mM; NaH_2PO_4 0.4mM; Glucose 5.6mM; HEPES 10mM) from male Wistar rats (Bioresources Unit in TCD, Dublin, Ireland). The buffered solution was prepared and then sterilised through filtration using Nalgene surfactant-free cellulose acetate bottle filters with 0.2 μm pores (Nalgene, Hereford, UK). Animals were sacrificed in an atmosphere saturated with CO_2. Then the pre-warmed (37°C) BSS (15 - 20ml) was injected i.p. and the abdomen was gently massaged for 3 min. Usually three animals were used per experiment (typically $\approx$ 48 samples). All the

The crude cell suspension was aspirated following a mid-line incision and centrifuged for 5 min at 400 $\times g$ at room temperature. Cells were washed three times with BSS and then resuspended in the appropriate volume of complete alpha-MEM or releasing media. All the procedures were performed in a sterile cabinet. No mast cell purification procedures were applied to the peritoneal exudate when degranulation had to be tested; it was proven [78], in fact, that purification through density media (e.g. Ficoll, metrizamide) leads to a loss of 50-80 % of the high affinity receptors for IgE undermining the mast cells' ability to degranulate. Moreover, following the density gradient purification, mast cells can degranulate after triggering with anti-IgE even in absence of sensitization [78]. Loeffler and coworkers [88] proved that

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consistent and uniform responses to compound 48/80 and other releasing agents were achieved without purifying cell procedure.

2.2.2 *Purification of RPMC by density gradient*

When needed (as during PCR analysis), Rat peritoneal mast cells (RPMC) were purified using a Percoll/BSA density gradient. Briefly, the peritoneal exudate was resuspended in 4 ml of BSS and layered onto 10 ml of Percoll solution (1.5 ml of 1.54 M NaCl, 27 mM KCl, 3.8 M CaCl$_2$; 0.75 ml of distilled water; 0.21 ml of 35% BSA solution; 10.5 ml of Percoll; pH = 7.2). After centrifugation at 225 x g for 20 min in a swing out centrifuge, RPMC could be easily isolated as they formed a pure mast cell pellet at the bottom of the centrifuge vial. After purification, mast cells were resuspended in 10 ml BSS and washed twice.

2.2.3 *RPMC degranulation studies in presence after anti-DNP IgE/DNP-BSA treatment*

After the peritoneal lavage, the cell suspension was aliquoted and the appropriate amount of anti-DNP IgE (to reach final concentrations from 5000 to 4.88 ng/ml) was added. Each concentration was tested in triplicate. Samples were incubated for 16 hours at 5% CO$_2$ and 37°C. After incubation, cells were washed twice with PBS and subsequently 500 μl of EBSS, with 0.1% BSA (pH 7.4), was added to the wells. Cells were triggered with 33 μl of DNP-BSA (5 μg/ml) and incubated for 30 min at 37°C. Supernatants were analysed as described in paragraphs 2.1.2.a and 2.1.2.b.

2.2.4 *Degranulation studies after calcium ionophore, compound 48/80, Pam2CSK4 and PGN treatment*

After peritoneal lavage, the cell suspension was re-suspended in the appropriate amount of releasing media (EBSS with 0.1% BSA, pH 7.4)

containing calcium ionophore A23187, compound 48/80, pam2CSK4 and PGN at the desired concentration. Samples were incubated for 30 min at 5% CO_2 and 37°C. Supernatants were analysed as described in paragraphs 2.1.2.a and 2.1.2.b.

2.2.5 *RPMC degranulation in presence of test compounds*

During experiments performed in presence of test compounds, 0.5% of the supernatant volume was substituted with the vehicle or the vehicle charged with the appropriate amount of the test compound. There was a minimum of ten incubations (spontaneous release, basal release, total content, positive control, vehicle pretreated (DMSO) and test compound pretreated (PH3, PH4, PH5, PH21 and PH22) where:

- the spontaneous release is the amount of released mediator when no stimulus is applied
- the basal release is the amount of mediator released after stimulation
- the total content is the amount of histamine found in the supernatant after cell lysis obtained with TritonX-100, it therefore represents the amount of mediator contained in the cells.

Each incubation was performed in triplicate. Supernatants were analysed as previously described (see paragraph 2.1.2.a and2.1.2.b). The percentage of the vehicle was always maintained at 0.5% of the volume of the supernatant.

The inhibition percentage of the mediator release was calculated using Equation 2

2.2.6 *LPS activation of RPMC/peritoneal lavage*

Cells from the peritoneal lavage were resuspended in the necessary amount of complete media and they were incubated overnight in the

presence of 0.1-1 μg/ml of sLPS (*Escherichia coli* 0111: Sigma). Supernatants were analysed as follows.

2.2.6.a.1 Measurement of cytokine concentration (TNF-α, IL-13)

Levels of TNF-α and IL-13 were measured in the supernatants using ELISA kits (Biosource, Nivelles, Belgium) according to the manufacturer's instructions. The study was performed in triplicate using cells from 3 different passages.

2.2.7   TLRs in RPMC

To investigate whether purified RPMC possess TLRs, mRNA expression was investigated through RT-PCR.

2.2.7.a.1 Qualitative RT-PCR

mRNA isolation from purified mast cells (see paragraph 2.2.2), purification and PCR analysis were performed as already described for RBL-2H3 (see paragraph 2.1.7.a).

2.3 Chemistry of the test compounds (PH2, PH3, PH4, PH21 and PH22)

The chemical structures of PH2 and PH5 are shown in Figure 10 and Figure 11. The structures are accompanied by their compound name, their chemical name, their molecular weight and their melting point. PH2 and PH5 are diastereoisomers of the **2-benzyl-2,3-dihydro-1H,1'H-2,2'-biinden-1-ol** compound and their relative stereochemistry has been established by X-ray studies. Moreover, each distereoisomer is a mixture of two different enantiomers; PH3 and PH4 are the resolved enantiomers of PH2 with a molecular rotation (α) of +0.09 and -0.1 respectively, while PH21 and PH22 are the resolved enantiomers of PH5, α =-1.608 and 1.896 respectively (see Table 5). No information about the stereochemistry of the different enantiomers is available to date. Test compounds were synthesised by TopChem laboratories (Dublin, Ireland) and stored at -20°C.
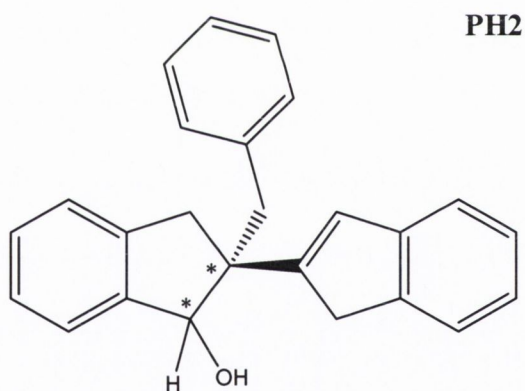
PH2

$C_{25}H_{22}O$: 2-benzyl-2.3-dihydro-1H,1'H-2,2'-biinden-1-ol
Molecular Weight: 338.44. Melting point: 609.19K

Figure 10: Chemical structure and physical properties of PH2

PH5
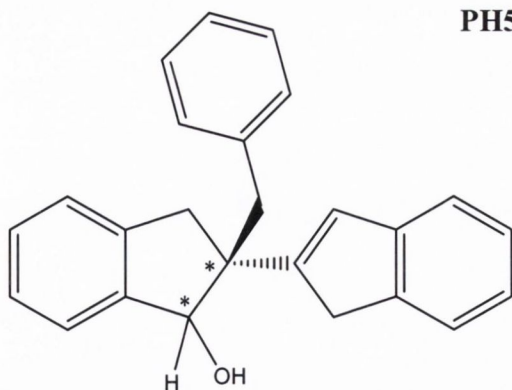
C$_{25}$H$_{22}$O: 2-benzyl-2.3-dihydro-1H,1'H-2,2'-biinden-1-ol
Molecular Weight: 338.44. Melting point: 609.19K

Figure 11: Chemical structure and physical properties of PH5

Table 5

PH2		PH5	
Enantiomer1: **PH3** α = +0.09	*Enantiomer2:* **PH4** α = - 0.1	*Enantiomer1:* **PH21** α = -1.608	*Enantiomer2:* **PH22** α = +1.896

Statistical analysis

For statistical studies of results discussed in this thesis, a Gaussian distribution of the obtained data was assumed when the number of the samples *(n)* was above 3. In the light of this, a series of parametric test were used to perform statistical comparison between groups. In particular the Student's t-test, the one-way ANOVA and the two-way ANOVA were applied:

- the **Student's t-test** was used when statistical comparison was performed between two groups
- in case of statistical comparisons between three or more groups the Student's t-test can not be used (it is strongly suggested to avoid to use a series of t-test to compare several groups). In that case, statistical comparison was carried out using the **one-way ANOVA** test followed by Bonferroni's[*] post test
  The one-way ANOVA compares three or more groups that differ in just ONE characteristic (e.g. presence of the treatment)
- in some experiments, groups differed in more than one characteristic (e.g. different compounds in different concentrations); in those cases a **two-way ANOVA** was used for statistical comparison.

The disadvantage of using the Bonferroni's test is that sometimes (especially if many groups are compared) it leads to p values[†] that are too high and confidence intervals that are too wide. A $p<0.05$ was considered as statistically different[‡] [89].

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[*] The Bonferroni post test is a mathematical correction to the statistical analysis to reduce falsely significant results due to multiple statistical analysis.
[†] The p value expresses the probability that two populations show different means due to a true difference or as a coincidence due to the random sampling.
[‡] The term "statistical significant" simply means that a given result is unlikely to have occurred by chance.

As it will be underlined in the results and discussion, after statistical comparison, groups that showed similar results resulted in different p values and consequent different statistical significance. These results must be considered with care before assuming that test compounds evoke different responses from target cells.

When the n value was equal to 3, being the Gaussian distribution of the values excluded, the non parametric Kruskal-Wallis test was used to perform statistical comparison between groups.

2.4 Buffers composition

Table 6 through Table 9 show the composition of buffers used during experiments.

Table 6

Earle's Balanced Salt Solution (EBSS), (GIBCO), pH = 7.4	
Components	**Millimolarity (mM)**
INORGANIC SALTS:	
$CaCl_2$	1.8
KCl	5.3
$MgSO_4 \cdot 7H_2O$	
NaCl	0.81
$NaHCO_3$	
$NaH_2PO_4 \cdot 2H_2O$	116
$MgCl_2$	30
OTHER COMPONENTS	
D-Glucose	5

Table 7

Balanced Salt Solution (BSS), pH = 7.4	
Components	**Millimolarity (mM)**
INORGANIC SALTS:	
$CaCl_2$	0.5
KCl	2.7
$MgSO_4 \cdot 7H_2O$	
NaCl	137.0
$NaHCO_3$	
$NaH_2PO_4 \cdot 2H_2O$	0.4
$MgCl_2$	1.0
OTHER COMPONENTS	
D-Glucose	5.6
HEPES	10.0

Table 8

Citrate Buffer, pH = 4.5	
Components	**Millimolarity (mM)**
INORGANIC SALTS:	
$Na_3C_6H_5O_7 \cdot 2H_2O$	50
$C_6H_6O_7 \cdot 2H_2O$	50

Table 9

TBE buffer (5x)	
Components	**Molarity (M)**
INORGANIC SALTS:	
TRIS	0.44
H_3BO_3	0.44
EDTA	10×10^{-3}
OTHER COMPONENTS	
DEPC WATER	1 liter

3 Results

3.1.1 *RBL-2H3 degranulation following immunological stimulation (anti-DNP IgE/DNP-BSA)*

RBL-2H3 cells have the ability to degranulate after crosslinking of the IgE-linked FcεRIs by a multivalent antigen. In order to assess the optimal concentration of IgE (to maximise the degranulation), RBL-2H3 cells were incubated with serial dilutions of antibody (from 5000 ng/ml to approximately 5 ng/ml). The dose response obtained presented a significant bell-shaped trend starting with a lower release (7±5% of the total content) corresponding to the smallest IgE concentration, reaching the maximal release (20±1%) at approximately of 78 ng/ml of IgE and then decreasing to reach a minimal release (3±1%) at the highest IgE concentration used (Figure 12).
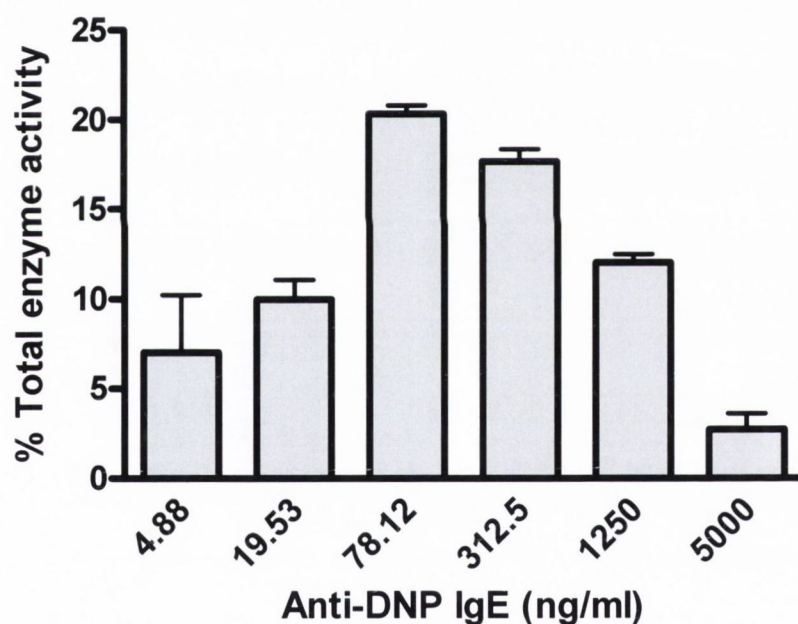
Figure 12

RBL-2H3 degranulation expressed as percentage of the total β-hexosaminidase activity (see Equation 1). Cells were sensitised with serial doubling dilutions of anti-DNP IgE and triggered with 330 ng/ml of DNP-BSA. The dose response presented a bell-shaped trend. Values represent mean±S.E.M. (n=3)

As it will be discussed later see paragraph 4.1.1 the bell-shaped trend might be due to an excess of bridging of the FcεRI by the multivalent allergen, according to this, one would expect that the response might be altered in the case of an increased number of surface receptors on the cell membrane (more receptors means less probability of bridging). To achieve this condition, RBL-2H3 were detached using Accutase instead of trypsin/EDTA. Accutase is a mixture of several proteolytic and collagenolytic enzymes that has been reported to preserve receptors and surface markers otherwise digested during trypsination [90].

When Accutase was used to detach cells prior the experiment, the bell shaped trend, as predicted, shifted toward a plateau (Figure 13).
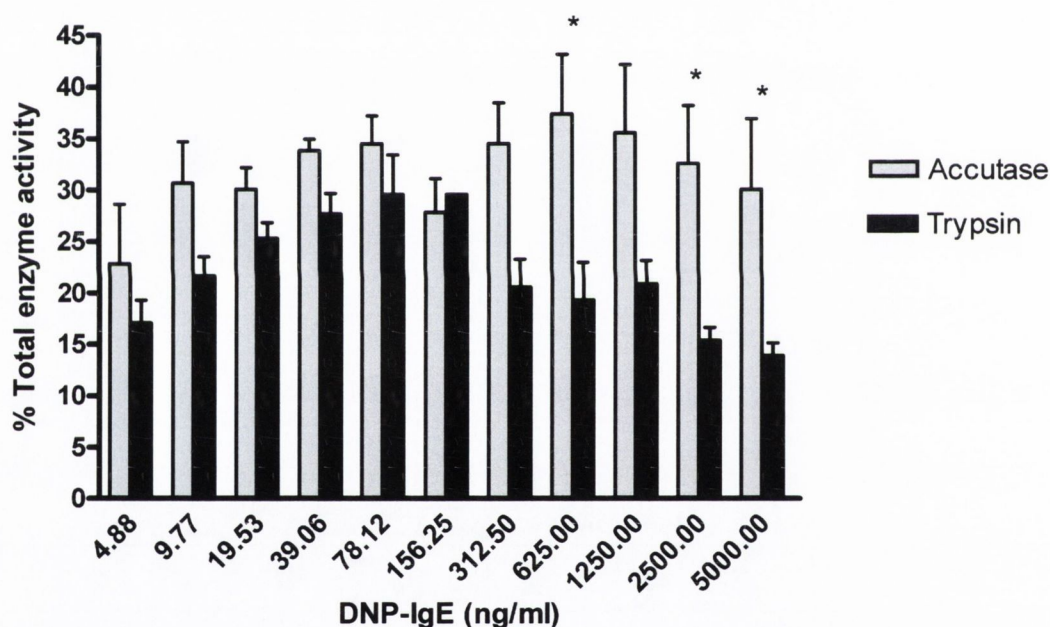
Figure 13

RBL-2H3 degranulation expressed as percentage of the total β-hexosaminidase activity (see Equation 1). Cells were detached using trypsin (■) and Accutase (■) prior the experiment. When Accutase was used the bell-shaped trend shifted toward a plateau, this might be due to a greater number of FcεRI receptor on the cell surface avoiding an excess of crosslinking when antigen is applied (see text and Table 13).

Cells were sensitised with serial doubling dilution of anti-DNP IgE and triggered with 330 ng/ml of DNP-BSA. Values represent mean±S.E.M., n=6.

* indicates statistical significance (p<0.05) between groups (trypsin vs. Accutase)

Statistical comparison was carried out using two-way ANOVA.

Once the optimal conditions, in terms of IgE concentration, for degranulation was assessed, we wanted to establish the time course from stimulation to complete degranulation. Figure 14 shows that the maximal release of the enzyme β-hexosaminidase was complete after 10 minute from the addition of the trigger (DNP-BSA) [92].
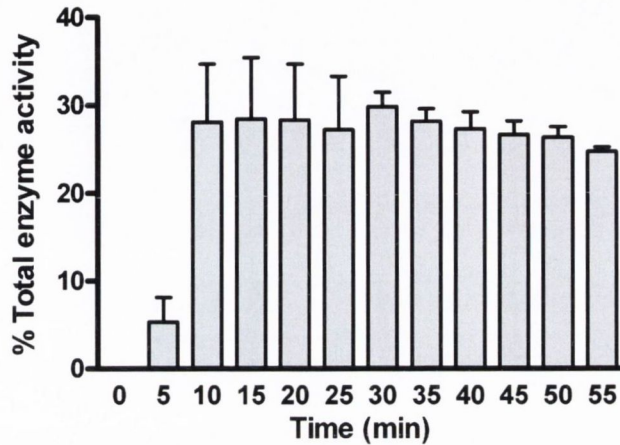
Figure 14

Time dependency of the β-hexosaminidase release induced by anti-DNP IgE sensitisation, followed by antigen stimulation (DNP- BSA) in RBL-2H3 cells. The release of the enzyme is complete 10 min after the stimulation with antigen. Each value represents the mean ± S.E.M., n=3.

3.1.1.a   Effect of media composition on IgE-mediated degranulation of RBL-2H3 cells

Figure 15 shows the effect of different media on the RBL-2H3 degranulation followed monitoring the release of histamine and β-hexosaminidase. The release of the two mediators showed the same trend, confirming that histamine and β-hexosaminidase are released in parallel. The releasing media composition was found to be crucial for an optimal degranulation. Maximal degranulation (36±1% of the total enzyme activity / 43±1% of the total histamine content) was obtained when EBSS charged with 0.1% of BSA was used; the percentage of mediator released crucially dropped when BSS charged with 0.1% of BSA was used (14±2% of the total enzyme activity / 20±1% the total

histamine content) and it was completely suppressed when BSA was removed from the balanced salt solution.
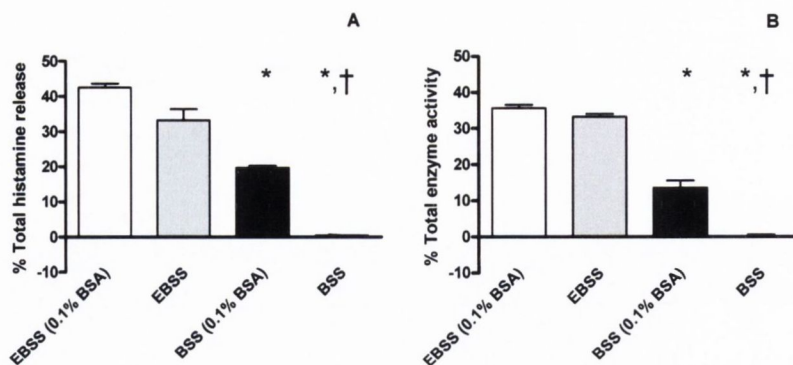
Figure 15

A) Effect of the releasing media composition on the histamine release from RBL-2H3. (□) Earle's balanced salt solutions (EBSS) +0.1% of bovine serum albumin (BSA), (■) EBSS, (■) Balanced salt solution (BSS) +0.1% BSA. B) Effect of the releasing media composition on the β-hexosaminidase release from RBL-2H3. (□) Earle's balanced salt solutions (EBSS) +0.1% of bovine serum albumin (BSA), (■) EBSS (■) Balanced salt solution (BSS) +0.1% BSA . Each value represents the mean ± SEM, n=3. The release of β-hexosaminidase and histamine followed the same trend.

* indicates significantly different (p<0.05) from EBSS (with and without BSA);

† indicates significantly different (p<0.05) from BSS without BSA.

3.1.1.b Effect of common mast cell stabilisers on IgE-mediated degranulation of RBL-2H3 cells

As part of the preliminary study focused on assessing the experimental protocol to test our novel compounds (see paragraphs 1.8 and 2.3), three mast cell stabilisers (quercetin, ketotifen and DSCG) were tested on IgE induced degranulation.
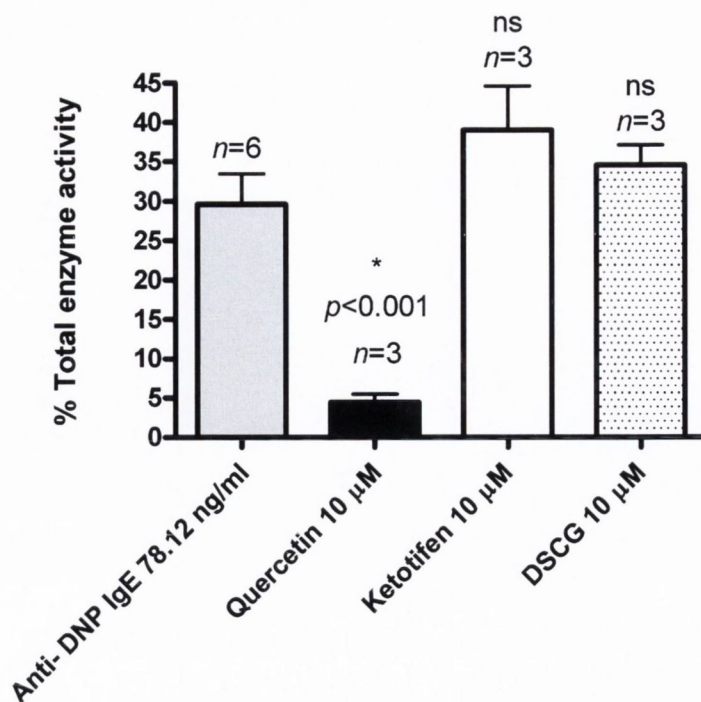
Figure 16

Effect of quercetin, ketotifen, DSCG (all at 10 μM) on IgE/Ag-triggered RBL-2H3 degranulation. Cells were sensitised with 78 ng/ml of anti-DNPIgE and triggered with 330 ng/ml of DNP-BSA. Values represent mean±S.E.M.

* indicates statistical significance (p<0.001) compared with baseline.

Statistical comparison was carried out using one-way ANOVA.

3.1.1.b.1 Effect of quercetin on IgE-mediated degranulation of RBL-2H3 cells

Quercetin reduced the amount of total enzyme release from 30±2% to 4±2% in RBL-2H3 giving a percentage of inhibition of 70±7% (Figure 16).

3.1.1.b.2 Effect of DSCG on IgE-mediated degranulation of RBL-2H3 cells

The RBL-2H3 cell line failed to be stabilised by DSCG. As previously stated, RBL-2H3 cells are traditionally referred as MMC and their insensitivity to DSCG confirms their similarity to this mast cell subtype.

3.1.1.b.3 Effect of ketotifen on IgE-mediated degranulation of RBL-2H3 cells

As shown in Figure 17, when ketotifen was tested on the IgE-mediated degranulation of RBL-2H3 cells, it did not inhibit the release of the granular enzyme β-hexosaminidase. For concentrations in between 0.2 and 0.65 μg/ml the amount of enzyme released was not statistically different ($p>0.05$, n=6) from control. Moreover for concentrations in between 12.5 and 100 μg/ml ketotifen significantly enhanced the release of β-hexosaminidase ($p<0.05$, $n=6$). At concentration of 200 μg/ml the percentage of the total enzyme activity was significantly decreased compared with the untreated control ($p<0.001$, $n=3$).
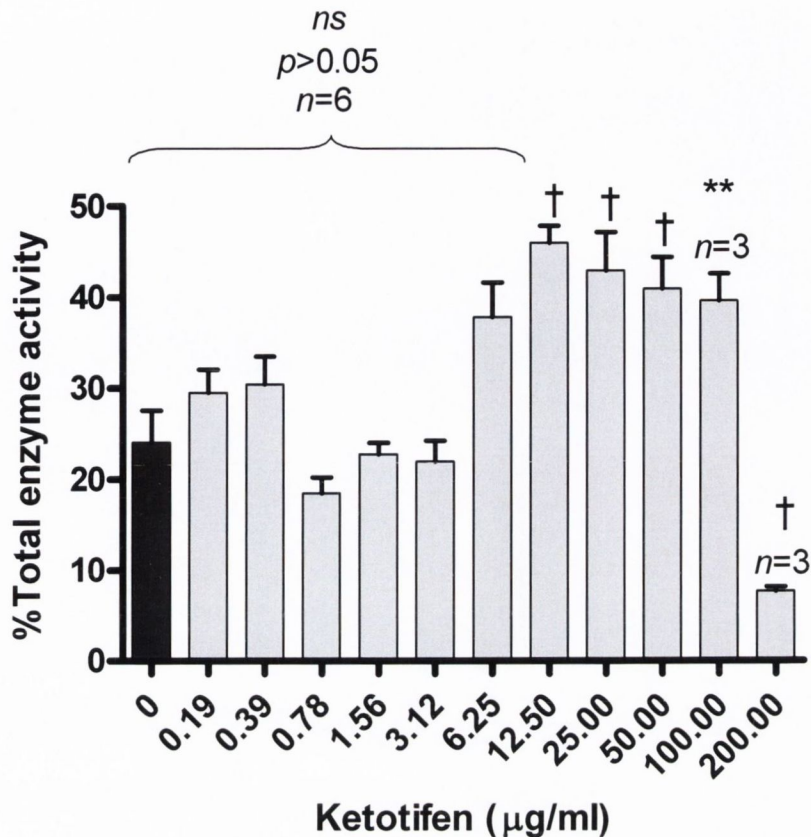
Figure 17

Effect of various concentrations of ketotifen (from 0.2 to 200 µg/ml) on β-hexosaminidase release from RBL-2H3 after anti-DNP IgE/DNP-BSA treatment. For concentrations in between 0.2 and 0.65 µg/ml the amount of enzyme released was not statistically different (p> 0.05, n=6) from control. For concentrations in between 12.5 and 100 µg/ml ketotifen significantly enhanced the release of β-hexosaminidase (p<0.05, n=6). At concentration of 200 µg/ml the percentage of the total enzyme activity was significantly decreased compared with the untreated control (p<0.001, n=3). Values represent means±S.E.M., n=3-6)

Concentrations above 100 µg/ml of ketotifen have been reported [94] toxic and this was confirmed after microscopic analysis of the treated cultures. Figure 18 shows a phase contrast micrograph of RBL-2H3 cell layers with or without treatment. It appears evident how ketotifen affected the cell survival at concentrations of 100-200 µg/ml. RBL-2H3 cells were radically reduced in number after 16 hours in culture in presence of the drug. They also showed morphological changes: instead of the normal oblong shape, cells appeared spherical, with numerous blebs and black precipitates (most likely apoptotic bodies). Cell morphology and cell count was still affected, even at concentrations of 50 µg/ml.

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Control

Vehicle (MeOH)
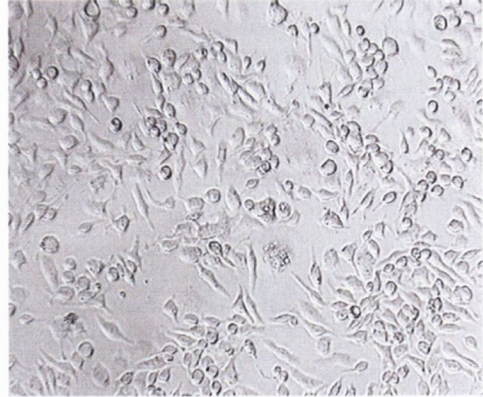
Ketotifen 200 μg/ml
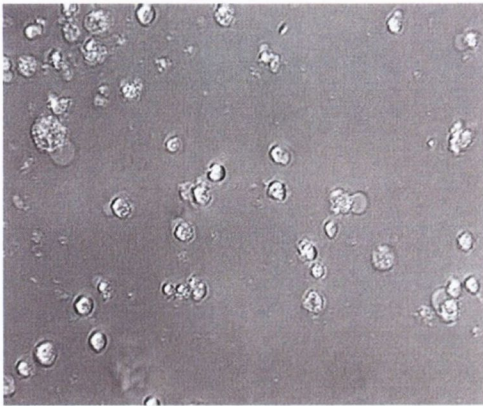
Ketotifen 100 μg/ml
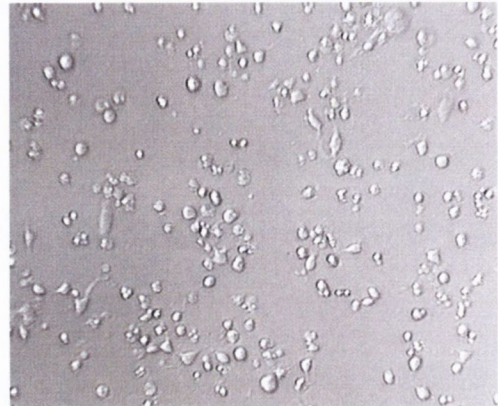
Ketotifen 50 μg/ml
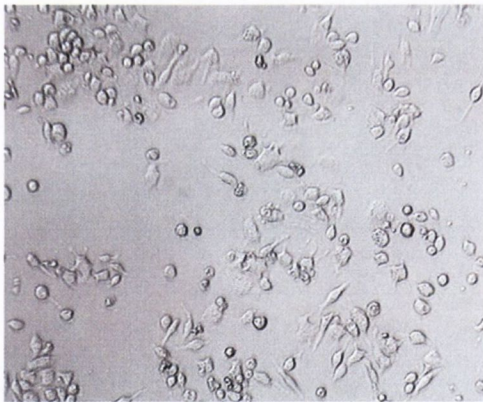
Figure 18

Phase contrast micrographs of RBL-2H3 cells incubated overnight (16 hours) in presence of ketotifen at various concentrations (200, 100, 50 μg/ml) or in presence of vehicle (Methanol). Ketotifen affected cell survival at 200 and 100 μg/ml. Moreover, survived cells presented morphological changes (spherical shape with blebs and precipitates)

3.1.2 *RBL-2H3 degranulation following non-immunological stimulation (calcium ionophore A23187, MCDP, compound 48/80, PGN)*

The effect of calcium ionophore A23187, MCDP, compound 48/80 and PGN on RBL-2H3 cells was examined and compared with data obtained after stimulation with anti-DNP IgE/DNP-BSA (Figure 19)
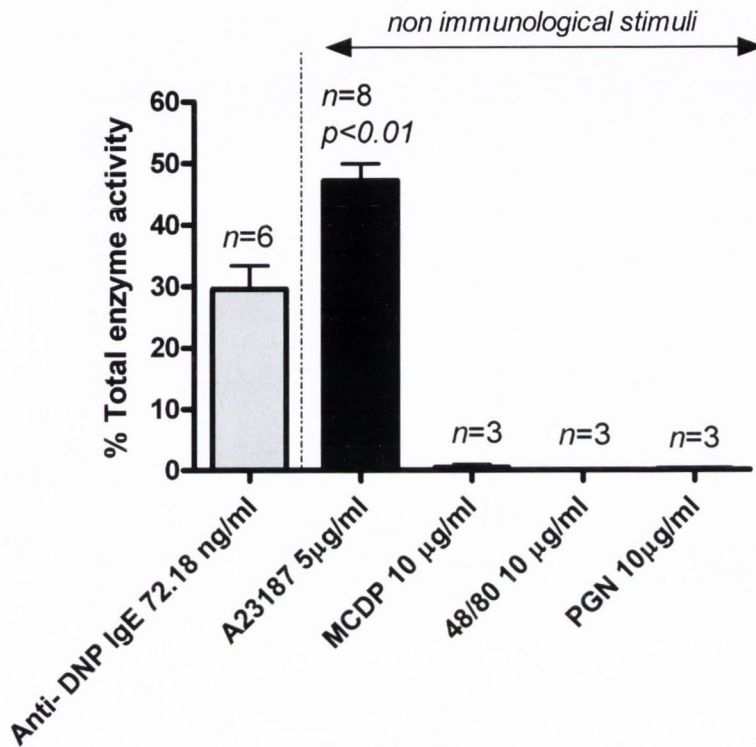
Figure 19

The effect of various non-immunological stimuli on RBL-2H3 cells was tested and compared with data obtained using IgE/Ag (78 ngml^{-1}/300 ngml^{-1}). Among the stimuli used, only calcium ionophore evoked a degranulative response from RBL-2H3 cells. Values represent mean±S.E.M.

* indicates statistical significance (p<0.001) compared with IgE/Ag. Statistical comparison was carried out using one-way ANOVA test.

3.1.2.a *Calcium ionophore-triggered degranulation*

We tested the ability of calcium ionophore A23187 to trigger degranulation in RBL-2H3 cells.

As shown in Figure 19, when RBL-2H3 were exposed to A23187 (5 µg/ml) their response was 1.65 times that obtained with IgE/BSA: the degranulation reached 47±8% of the total β-hexosaminidase content

and it was statistically different (p<0.01, n=6-8) from the IgE/Ag-stimulated release (30±4%).

3.1.2.a.1 Effect of quercetin on calcium ionophore A23187-induced degranulation of RBL-2H3 cells

Quercetin reduced the amount of total enzyme release from 49±7% to 26±10% in RBL-2H3, giving a percentage of inhibition of 48±11% (Figure 20).
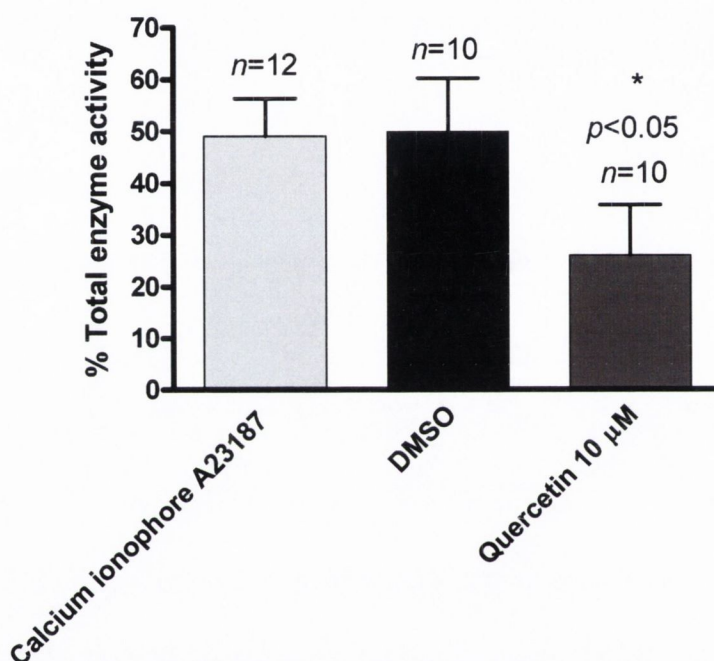
Figure 20

Effect of quercetin (10 μM) on calcium ionophore A23187-triggered RBL-2H3 degranulation. Cells were stimulated with 5 μg/ml of ionophore. Values represent mean±S.E.M. n=10-12.

* indicates statistical significance (p<0.05) compared with baseline.

Statistical comparison was carried out using Student's t-test

3.1.2.b MCDP- triggered degranulation

In our laboratory, we tested the release of β-hexosaminidase after MCDP treatment; by doing this we bypassed the problem of the low histamine content in RBL-2H3 and we overcame the limitations of the spectrofluorometric assay (such as interference by other amines and

fluorescence quenching by DMSO, see Appendix 1 and Appendix 2). Under our experimental conditions MCDP (0.3-10 µg/ml) failed to produce a response from RBL-2H3 (for simplicity Figure 19 shows the effect of MCDP only at 10 µg/ml, the effect of MCDP was negligible at all the concentrations tested).

3.1.2.c   Compound 48/80- triggered degranulation

Compound 48/80 is one of the most powerful mast cell secretagogues. Under our experimental conditions Compound 48/80 failed to produce a response from RBL-2H3.

3.1.2.d   PGN- triggered degranulation

PGN is a component of the outer wall of Gram-positive bacteria and in mast cells it activates a TLR2-dependent pathway that leads to degranulation (see paragraph 1.6.1.b).

Under our experimental conditions PGN (0.1-100 µg/ml) failed to induce degranulation in RBL-2H3 (for simplicity Figure 19 shows the effect of PGN only at 10 µg/ml, the effect of PGN was negligible at all the concentrations tested).

3.2   Investigation of the stabilising activity of novel indane analogues of pterosins Z on RBL-2H3 cells

Compounds PH2, PH5 and their resolved enantiomers (see materials and methods) were tested on RBL-2H3 cells triggered with both immunological and non-immunological stimuli.

3.2.1   Inhibition of IgE-mediated degranulation of RBL-2H3 cells by PH2, PH3, PH4, PH5, PH21 and PH22

Figure 21 shows the effect of our test compounds and quercetin (used as positive control, see paragraph 3.1.1.b) on the IgE-mediated release of histamine and β-hexosaminidase in RBL-2H3 cells. When histamine

is considered (columns on the right for each entry, in darker colours), a reduction of the release of histamine is observed in samples treated with the vehicle alone (DMSO), this solvent effect masks the activity of test compounds. When β-hexosaminidase is considered (columns on the left for each entry, in lighter colours) the percentage of the total release was not affected by the presence of the vehicle (DMSO). As histamine and β-hexosaminidase are released in parallalel, the solvent effect observed **only** for the histamine values must be attributed to a possible interference of DMSO with the assay.
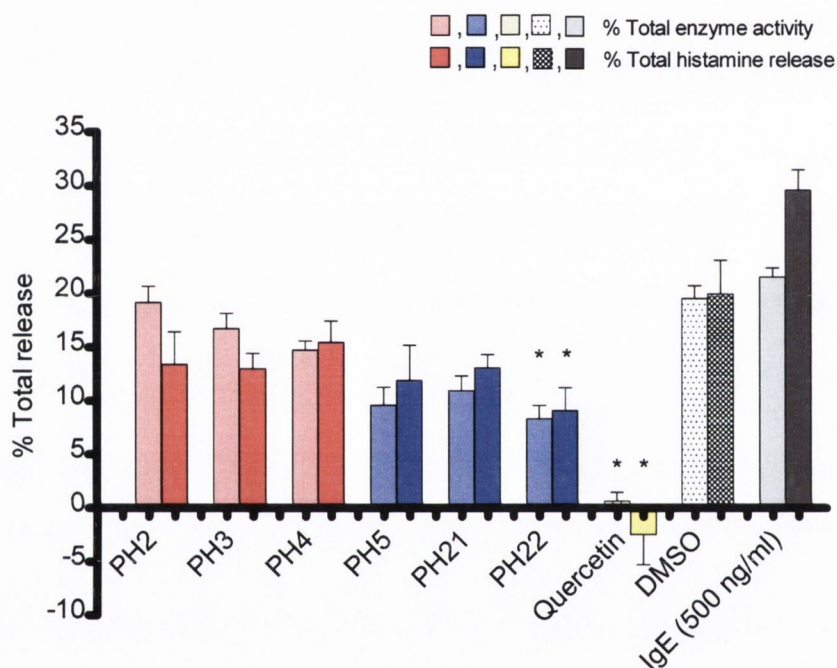
Figure 21

Effect of PH2, PH3, PH4, PH5, PH21, PH22 and quercetin (positive control) on the RBL-2H3 degranulation monitored following the release of β-hexosaminidase (lighter colours) and histamine (darker colours). Values represent mean±S.E.M., n=6

* indicates statistical significance (p<0.001) compared with baseline (DMSO).

Statistical comparison was carried out using one-way ANOVA test.

As shown in Figure 21, PH2 and its resolved enantiomers (PH3 and PH4) showed a negligible activity on IgE-mediated release of both histamine and β-hexosaminidase on RBL-2H3 cells. Values were not statistically different ($p > 0.1$, n=6) from the baseline (DMSO).

PH5 and its enantiomers (PH21 and PH22), on the other hand, showed some activity but the statistical analysis underlined that only the results

obtained with PH22 were statistically different from baseline ($p<0.05$, n=6). However the values of PH5 and PH21 do not significantly differ from PH22 ($p> 0.05$, n=6). Despite this similarity, due the experimental error, the statistical comparison between values obtained for PH5 and PH22 and the vehicle control was not significant. To conclude that PH5 and PH21 are ineffective as RBL-2H3 stabilisers just on the basis of the statistical analysis of this data could be rushed. The effect of various concentrations of PH22 on RBL-2H3 cells is shown in Figure 22.

Table 10 reports the effect of test compounds shown in Figure 21 expressed as percentage of inhibition (calculated according to Equation 2). As previously highlighted PH2, PH3 and PH4 did not show any significant effect on the degranulation.

Table 10 Percentage of inhibition of β-hexosaminidase and histamine by PH compounds from anti-DNP IgE/DNP-BSA-triggered RPMC

* indicates statistical significance (p<0.05) compared with vehicle (DMSO)

Statistical comparison was carried out using one-way ANOVA

	Enzyme, % Inhibition ± SEM	Histamine, % Inhibition ± SEM
PH5	37 ± 5	18 ± 9
PH21	34 ± 5	11 ± 3
PH22 (*)	45 ± 6	27 ± 4
Quercetin (**)	72 ± 1	83 ± 4
DMSO	12 ± 8	1 ± 5 †

† The inhibition value was calculated using control values obtained for the β-hexosaminidase. Control values obtained for histamine could not be used as DMSO quenches the fluorescence of the OPT adduct, leading to an apparent decrease of the released histamine, see text for further details.

PH22 showed a dose-dependant inhibition of the degranulation (Figure 22).

A degranulation of approximately 20% was progressively suppressed by increasing concentrations of the drug, reaching negative values in correspondence of 30 and 50 μM. A negative value of the percentage of total release could indicate a potential decrease of the spontaneous release of (usually ≅ 5-10% of the total) (see equation Equation 1). At

30 and 50 μM, however, cells appeared of irregular shape with numerous blebs and black precipitates suggesting a possible toxic effect of the test compound leading to a loss of cells (and granular mediators) during the washing step (see materials and methods).
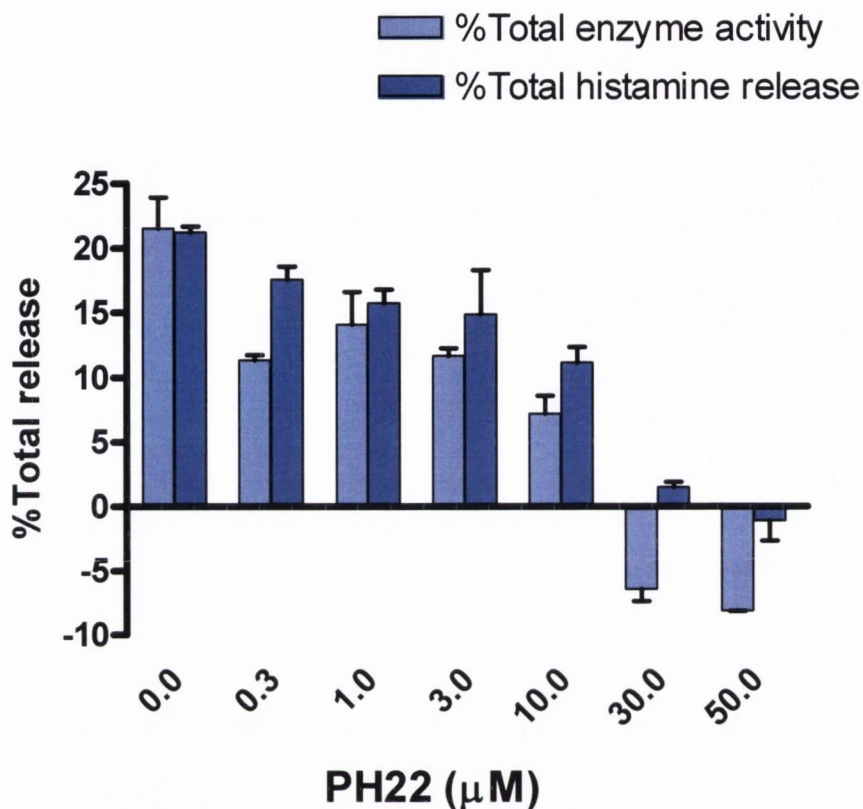
Figure 22

Dose response of PH22 on β-hexosaminidase and histamine release from RBL-2H3 cells. Values represent mean±S.E.M., n=3.

3.2.2 Inhibition of Calcium ionophore A23187-induced degranulation of RBL-2H3 by PH2, PH3, PH4, PH5, PH21 and PH22

Figure 23 shows the effect of our test compounds and quercetin (used as positive control) on the calcium ionophore A23187-induced degranulation of RBL-2H3, monitored following the release of the granular enzyme β-hexosaminidase.

In contrast to the results obtained with the IgE-mediated degranulation (Figure 21), in this case both PH2, PH5 and their resolved enantiomers showed a significant ($p<0.05$) activity from samples treated with the

vehicle alone (DMSO). The solvent effect, in this case, was negligible (Figure 23).
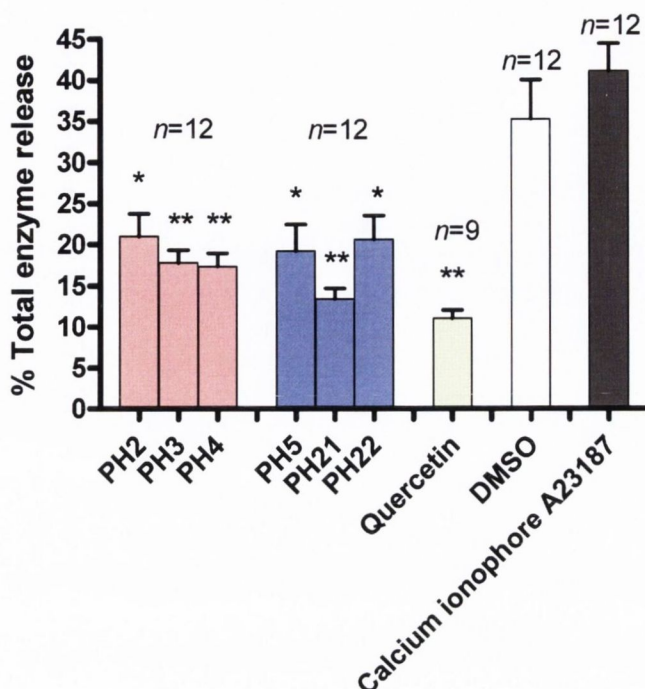
Figure 23

Effect of PH2, PH3, PH4, PH5, PH21, PH22 and quercetin (positive control) on the calcium ionophore A23187-triggered RBL-2H3 degranulation (monitored following the release of β-hexosaminidase). All the compounds were tested at 10 μM. Values represent mean±S.E.M., n=9-12

	Enzyme, % Inhibition ± SEM
PH2 (*)	49 ± 7
PH3 (*)	57 ± 4
PH4 (*)	58 ± 4
PH5 (*)	53 ± 8
PH21 (*)	67 ± 3
PH22 (*)	50 ± 7
Quercetin (*)	73 ± 2
DMSO	14 ± 11

Table 11. Percentage of inhibition of β-hexosaminidase by PH compounds from calcium ionophore A23187-triggered RBL-2H3 cells

* indicates statistical significance (p<0.05) compared with vehicle (DMSO)

Statistical comparison was carried out using one-way ANOVA

PH5 and PH22 showed a dose dependant inhibition of the calcium ionophore-triggered degranulation (Figure 24). Quercetin was used as positive control

A degranulation of approximately 80% (vehicle control) was progressively suppressed by increasing concentrations of PH2 and PH5. In particular PH5 could significantly inhibit the degranulation from concentrations as low as 5 μM (p<0.001, n=3). Increasing concentrations of the drug had more accentuated effect with a maximal effect at 50μM. PH5, on the other hand, showed significant activity only from concentrations of 10 μM or above. Statistical comparison (carried out using two-way ANOVA test) of the two sets of results reported in Figure 24 (upper and lower panel) showed that PH2 was more effective than PH5 at concentrations of 5 and 10 μM. For concentrations above 10 μM the two molecules showed the same potency in inhibiting RBL-2H3 degranulation after calcium ionophore A23187 treatment.
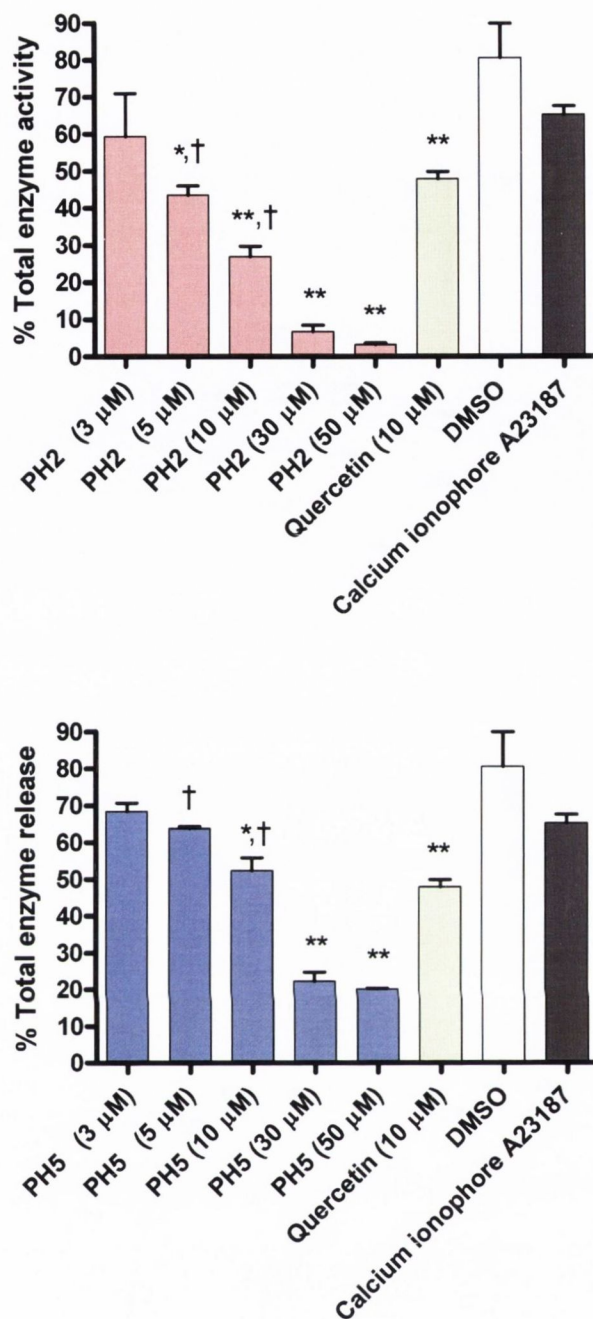
Figure 24

Dose response of PH2 (upper panel) and PH5 (lower panel) on β-hexosaminidase from RBL-2H3 cells after calcium ionophore A23187 treatment. Values represent mean±S.E.M., n=3.

*indicates statistical significance (p<0.001, n=3) compared with baseline (DMSO).

** indicates statistical significance (p<0.01, n=3) compared with baseline (DMSO). Statistical comparison was carried out using one-way ANOVA test.

† indicates statistical significance (p<0.5, n=3) compared with the corresponding entry in the other panel. Statistical comparison was carried out using two-way ANOVA .

3.3 Evaluation of PH2, PH5, quercetin, DSCG and terfenadine cytotoxicity on RBL-2H3 cells

The Cytotoxocity profile of PH2 and PH5 was investigated and compared with that of terfenadine, DSCG and quercetin. The cytotoxicity of the examined compounds was tested using two different assays aimed to assess cell death (LHD assay) and cell viability (MTS assay).

3.3.1 *Cytotoxicity of quercetin on RBL-2H3 cells*

Figure 25 shows the effect of various concentrations of quercetin on both the percentage of cell viability (black curve, MTS assay) and on the percentage of damaged/death cells (curve in red, LDH assay. Quercetin did not show any marked cytotoxic effect on RBL-2H3 cells. The percentage of damaged/dead cells resembled zero for concentrations in between 0.3 and 3 μM, raised to 19±1 at 10 μM to reach a value of 44±2 at 100 μM. Values of cell viability at concentrations between 0.3 and 30 μM were 100% or above; quercetin, in fact, can promote cell survival by blocking JNR- ERK-mediated apoptosis and also activating survival signalling proteins such as phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (AKT) [126-128].
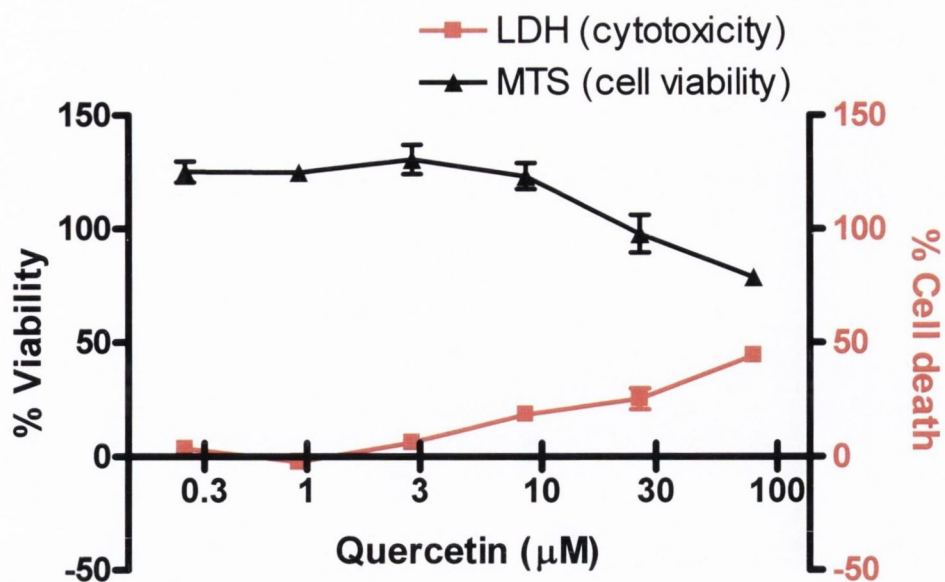
Figure 25

Cytotoxicity profile of quercetin (from 0.3 to 100 µM) on RBL-2H3 cells. Cytotoxic effect was determined using LDH and MTS assay aimed to assess respectively the cell damage and the cell viability (see text). Quercetin did not show any marked cytotoxic effect at all the concentration used. Values represent means±S.E.M., n=6.

3.3.2   Cytotoxicity of DSCG on RBL-2H3 cells

DSCG did not show any cytotoxic effect on RBL-2H3. At all the concentrations tested the cell viability (curve in black) was above 100% and the "release" of LDH was negligible (curve in red). Whether DSCG can enhance the cell survival or not is controversial and it depends on the type of cell investigated.
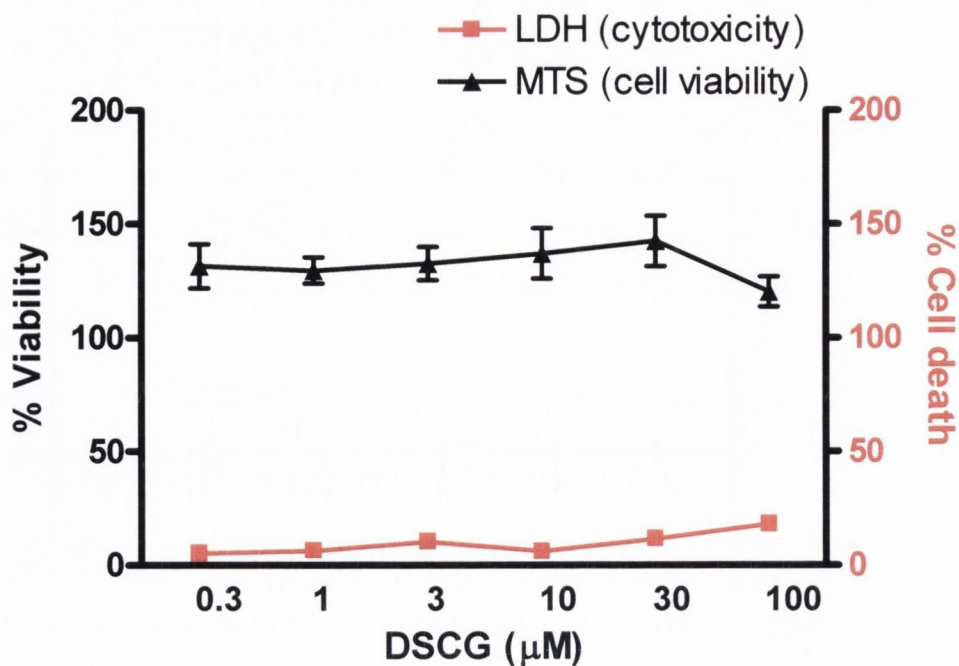
Figure 26

Cytotoxicity profile of DSCG (from 0.3 to 100 μM) on RBL-2H3 cells. Cytotoxic effect was determined using LDH and MTS assay aimed to assess respectively the cell damage and the cell viability (see text). DSCG did not show any marked cytotoxic effect at all the concentration used. Values represents mean±S.E.M., n=6

3.3.3   *Cytotoxicity of terfenadine on RBL-2H3 cells*

Unlike quercetin and DSCG, terfenadine showed a marked toxic effect on RBL-2H3 (Figure 27). No metabolic activity (measured through the concentration of the reduced MTS salt) was detected even at 3 μM. Moreover the LDH activity (index of cell death) jumped from 14±10% at 0.3 μM to 36±7% at 1 μM to reach 56±5% at 3 μM. At concentrations above 1 μM the percentage dead cells remained practically constant at about 50% while the cell viability was zero. This result can suggest a possible pro-apoptotic effect of terfenadine on RBL-2H3 cells. This result is in accordance with the reported pro-apoptotic effect of Terfenadine on other cell types [129].
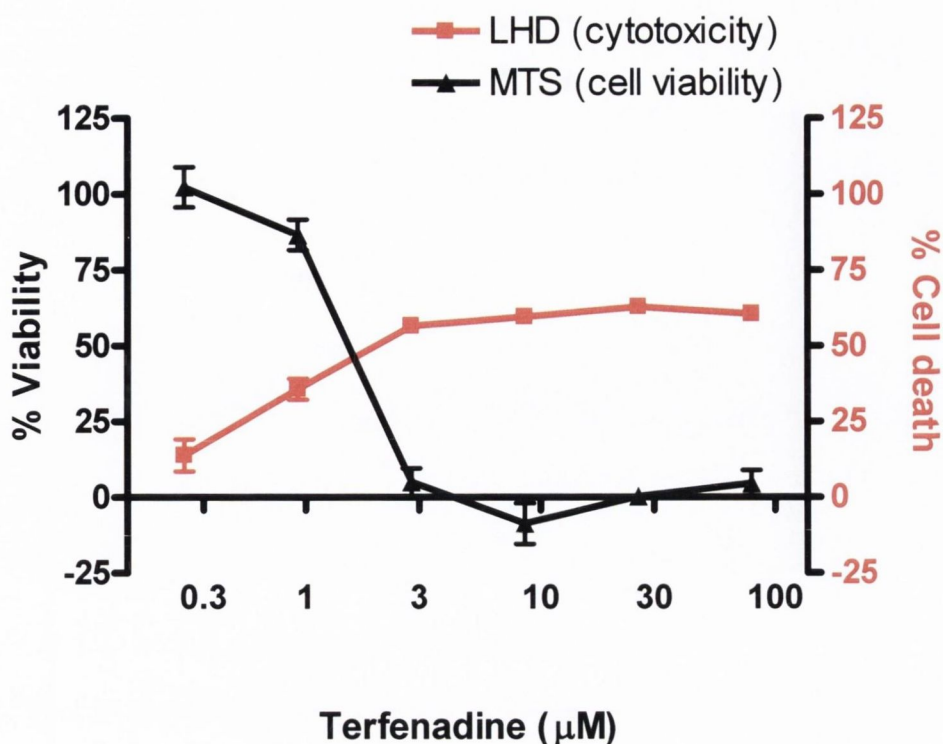
Terfenadine (μM)

Figure 27

Cytotoxicity profile of terfenadine (from 0.3 to 100 μM) on RBL-2H3 cells. Cytotoxic effect was determined using LDH and MTS assay aimed to assess respectively the cell damage and the cell viability (see text). Terfenadine suppressed completely the cell viability even at low concentrations (3 μM). On the other hand, the cell death reached a plateau (set at about 50%). This discrepancy between the two set of results might suggest a possible pro-apoptotic effect of terfenadine on RBL-2H3 cells (see text). Values represent mean±S.E.M., n=6.

3.3.4 *Cytotoxicity of PH2 and PH5 on RBL-2H3 cells*

PH2 at 100 and 30 μM almost completely suppressed the metabolic activity of RBL-2H3 cells (Figure 28). The percentage of death cells at the same concentrations, on the other hand, had 59.3±1.0% as higher value. The discrepancy between the two results might suggest a possible pro-apoptotic effect of PH2 on RBL-2H3 cells.

At 10 μM, the cell viability presented a value of 86±40%, while the cell death was 43±8%. The former value is higher of what could be predicted following the trend of the viability values obtained for the other concentrations; this is because of two (out of six) inexplicably high values. This inconsistency is even more evident if Figure 28 is compared with Figure 29 that gives information regarding the cytotoxicity of PH5 under the same experimental conditions. PH5

caused a progressive decrease of cell viability with a trend similar to PH2 (except for the value at 10 µM). At a concentration of 3 µM, 86±20% were viable and 28±1 were dead. The PH2 cytotoxicity was reduced to zero at concentrations of 1µM and below.
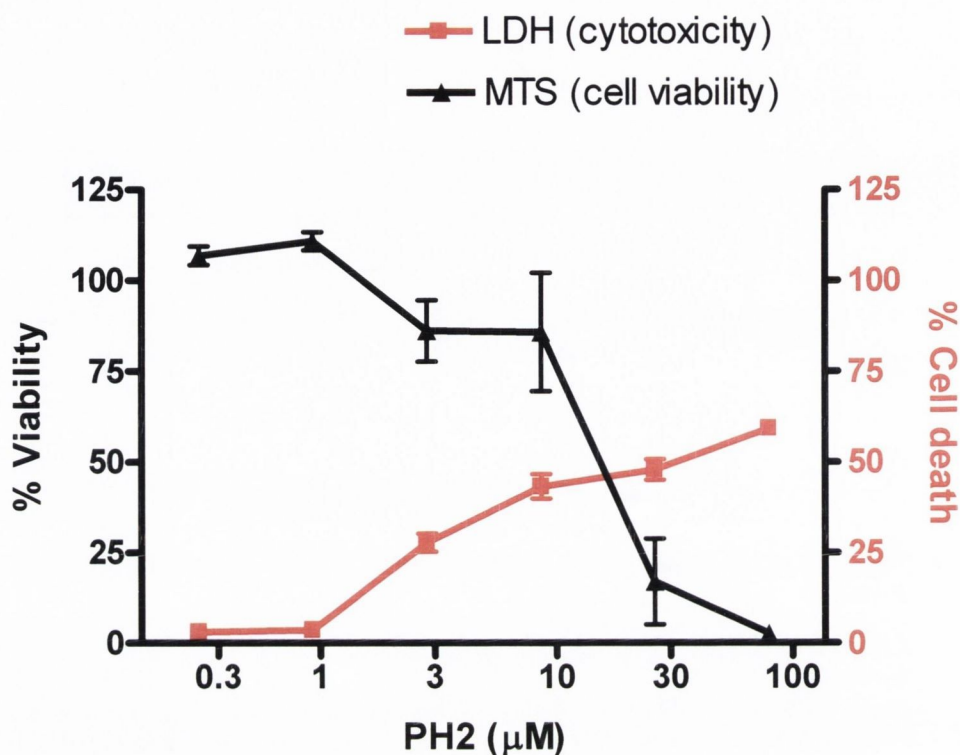
Figure 28

Cytotoxicity profile of PH2 (from 0.3 to 100 µM) on RBL-2H3 cells. Cytotoxic effect was determined using LDH and MTS assay aimed to assess respectively the cell damage and the cell viability (see text). While PH2 suppressed the cell viability at higher concentrations (100 and 30 µM), the cell death did not exceed the value of 60%. This discrepancy between the two set of results might suggest a possible pro-apoptotic effect of terfenadine on RBL-2H3 cells (see text).Values represent means±S.E.M., n=6.

As previously anticipated, PH5 reflected what was found for PH2 (Figure 28 and Figure 29). At 100 and 30 µM the RBL-2H3 cell viability was almost completely suppressed (-1±1 and 22±9% respectively) but the cell death showed a plateau set at about 55%. This result, similar to the one obtained for PH2 might suggest a possible pro-apoptotic effect of PH5 on RBL-2H3 cells. Figure 29 shows how cell viability was not affected for concentrations below 10 µM; the death cells showed a constant value of approximately 25%.
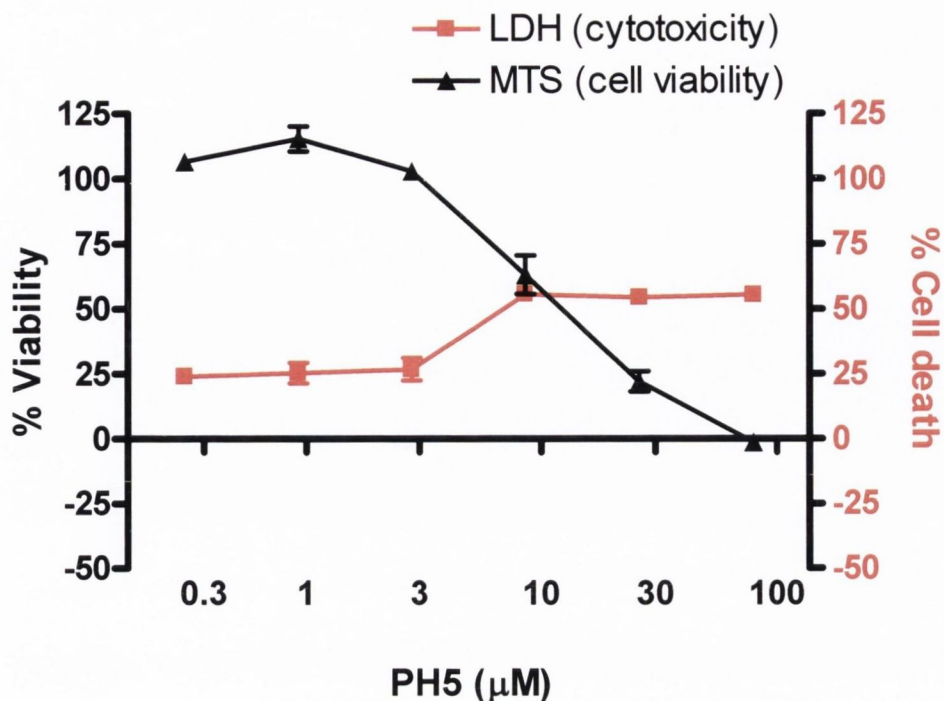
Figure 29

Cytotoxicity profile of PH5 (from 0.3 to 100 µM) on RBL-2H3 cells. Cytotoxic effect was investigated using LDH and MTS assay aimed to assess respectively the cell damage and the cell viability (see text). While PH5 suppressed the cell viability at higher concentrations (100 and 30 µM), the cell death reached a plateau (set at about 50%). This discrepancy between the two set of results might suggest a possible pro-apoptotic effect of PH5 on RBL-2H3 cells (see text). Values represent means±S.E.M., n=6.

The 50% cytotoxicity/cell viability obtained for PH5 at 10µM, might suggest that the inhibitory effect of our test compounds on the histamine/β-hexosaminidase release observed in Figure 21 might be due to the cell death rather than to a pharmacological effect of the drug. On the other hand, PH2 and its resolved enantiomers did not show any significant effect on the RBL-2H3 degranulation despite the similar cytotoxicity profile with PH5. If the inhibition of the degranulation would be due to cell death, this would influence the results obtained for both compounds but PH2 was not statistically different from the baseline (DMSO) (Figure 21); this consideration tips the scales in favour of a pharmacological effect that, in my opinion, should be further investigated.

3.4 RBL-2H3 cell line as model to study the MMC response to toll-like receptors' ligands

3.4.1 RBL-2H3 activation after LPS sensitisation

Mast cells have been mainly studied as key effectors in allergic diseases but in recent years they have attracted the attention of scientists as they seem to play a crucial role during bacterial infections. Sharing many similarities with MMC, the RBL-2H3 cell line was a good candidate as an *in vitro* model to study the effect of our test compounds on TLR-mediated responses. Responses to sLPS (serotype O111:B4 and O55:B5) and rLPS (Re 595 *S. Enterica*) were tested.

3.4.1.a TNF-α release after stimulation with sLPS and rLPS of RBL-2H3 cells

RBL-2H3 have been reported to produce TNF-α after sLPS (serotype 055:B5) stimulation in a time and dose dependent manner [84]. Under our experimental conditions, however, RBL-2H3 failed to produce any response (Figure 30. Figure 31 and Figure 32).

Initially, a concentration of 1 μg/ml of sLPS (serotype O111:B4) was tested and supernatants were analysed at different time points (3, 6, 16 hours). Figure 30 shows that the amount of TNF-α released after 16 hours (5 ± 0 pg/ml) of incubation was not statistically different ($p>0.05$) from the control (4 ± 1 pg/ml). The use of an ultra-sensitive kit for the detection of TNF-α (see materials and methods) and the analysis of a control sample supplemented with commercially available TNF-α, excluded a possible inadequacy of the protocol. Subsequently, different concentrations of sLPS were tested; the dose dependency of the TNF-α production was measured in a period of 16 hours in order to observe the maximal response. RBL-2H3 failed to respond at any the concentrations used; the amount of TNF-α produced was below the detection limit of the kit (Figure 31).
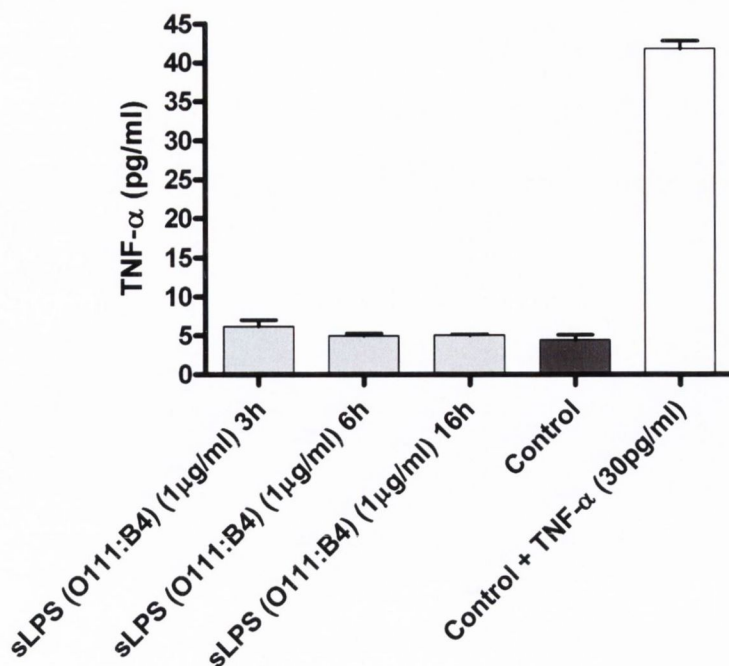
Figure 30:

Effect of sLPS (O111:B4, 1 μg/ml) on RBL-2H3 cells. Smooth LPS did not increase the release of TNF-α from RBL-2H3 after 3, 6, 16 hours of treatment. Values represent the mean±S.E.M., n=3.
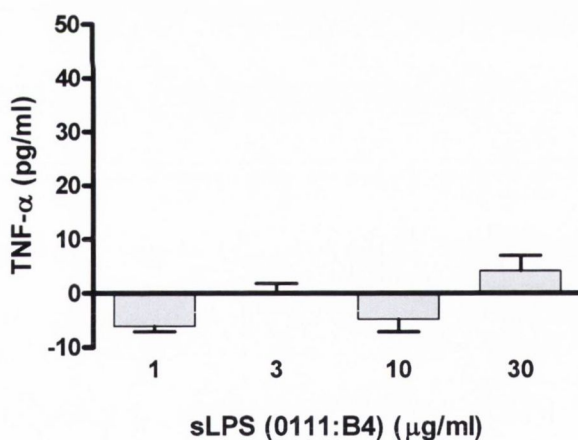
Figure 31:

Effect of various concentrations (from 1 to 30 μg/ml) of sLPS (O111:B4) on the RBL-2H3 release of TNF-α. In a period of 16 hours, sLPS did not increase the release of TNF-α at any of the concentrations tested. The amount of TNF-α produced was below the detection limit of the kit (4 pg/ml). Values represent the mean±S.E.M. n=3.

In rodents, the ligand's recognition by TLR4 depends on the particular structure/antigenic properties of LPS. It was therefore hypothesised that the observed lack of response of RBL-2H3 cells was due to the particular strain of sLPS used in our experiments. Two other strains of

LPS were tested, namely the smooth serotype O55:B5 and a rough variant of LPS (*Salmonella enterica,* serotype minnesota, Re 595). None of the concentrations used of rLPS and sLPS (O55:B5) elicited a response from RBL-2H3 cells in a period of 16 hours (Figure 32). The lack of response to all the LPS strains tested raised the question whether the TLR4 pathway in RBL-2H3 cells is operative or not. We, therefore, wanted to investigate whether TLR4, CD14 and MyD88 were expressed by RBL-2H3 cells.
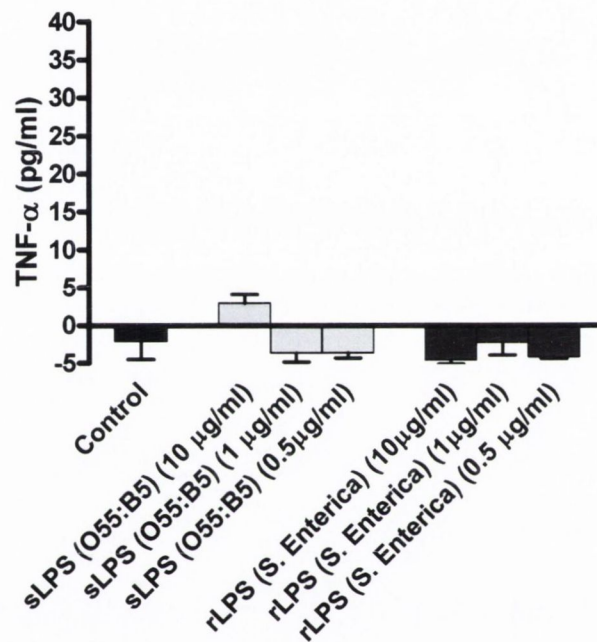
Figure 32:

Effect of three (10, 1 and 0.5 µg/ml) concentrations of sLPS (O55:B5) and rLPS (S. Enterica, serotype minnesota) on the RBL-2H3 release of TNF-α. At all the concentrations tested, in a period of 16 hours, both sLPS and rLPS did not increase the release of TNF-α from RBL-2H3. The amount of TNF-α produced was below the detection limit of the kit (4 pg/ml). Values represent the mean±S.E.M. n=3.

3.4.1.b IL-13 release after stimulation with LPS of RBL-2H3 cells

Due to its crucial role in allergy and inflammation, the level of IL-13 released after sLPS (0111:B4) stimulation was tested in parallel with the level of TNF-α (see paragraph 4.4.1.a).

The result obtained reflected what was previously found for TNF-α. Smooth LPS at concentrations elsewhere reported effective [84], failed to produce a response from RBL-2H3.

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The amount of IL-13 released in a period of 16 hours was not statistically different (p> 0.05, *n*=6) from the untreated control; the amount of IL-13 released was equal to 35.1±12 pg/ml for the untreated sample and 39±25 pg/ml for the untreated.
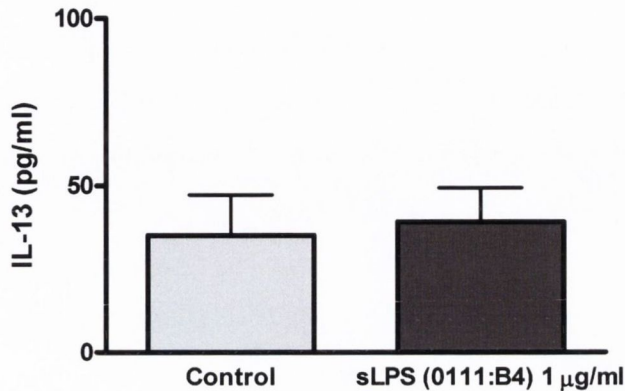
Figure 33:

Effect of sLPS (O111:B4, 1 μg/ml) on the RBL-2H3 production of IL-13. In a period of 16 hours sLPS did not increase the release IL-13 from RBL-2H3. Values represent the mean±S.E.M. n=6.

3.4.2   *TLRs in RBL-2H3 cells*

The lack of response of RBL-2H3 cells to LPS and PGN raised the question whether these cells express the toll-like receptors or not. mRNA expression of TLR1-6 and 8-10 was investigated through RT-PCR; subsequently, the expression of the TLR4 protein was assessed by FACS analysis as the signalling through this particular receptor is the pathway that leads to the cellular response to LPS. As the TLR4 signalling pathway is conditioned by the presence of both the TLR4 co-receptor CD14 and the adaptor protein MYD88, expression for this two proteins was also tested. The information obtained as allowed a comparison of RBL-2H3 with both RPMC (example of CTMC), MMC and BS.

3.4.2.a   *TLRs mRNA expression in RBL-2H3 cells*

As shown in Figure 34, mRNA transcripts TLR1, 2, 8, 9 and 10 were absent while transcripts for TLR3, 4, 5 and 6 were detected. TLR7 could not be investigated as none of the designed primers lead to

positive results during the optimisation stage using genomic DNA (see materials and methods). The band corresponding to TLR3, due to the small size of the transcript (120 bp), is localised close to the region of the primer dimers and it can be confused for smear. Results obtained with gDNA, during primer optimisation, show that the TLR3 band is affected by some smear but the band is still clearly visible and localised right above 100 bp (as suggested by comparison with the marker on the left). Gels of products from RBL-2H3 cultures presented an high incidence of smear at the bottom of the gel, possibly due to unspecific binding; however the TLR3 band, although large and smeared, presents a brightness higher than that of usual smear and it is correctly localised right above 100 bp and it is definitely separated from the primer dimer region; therefore it is possible to confirm the presence of the mRNA transcript for TLR3.

The band for the TLR4 transcripts is clearly visible both in the gDNA gel and in the RBL-2H3 gel and falls at 200 bp, in accordance with what was expected (206 bp).

While the signal for TLR5 appears barely visible right below the 200 bp, confirming the presence of the transcript, the band corresponding to TLR6 is evident, clear and easily distinguishable.

TLRs

M 1 2 3 4 5 6 8 9 10 β-actin

300 bp
200 bp
100 bp

gDNA

300 bp
200 bp
100 bp
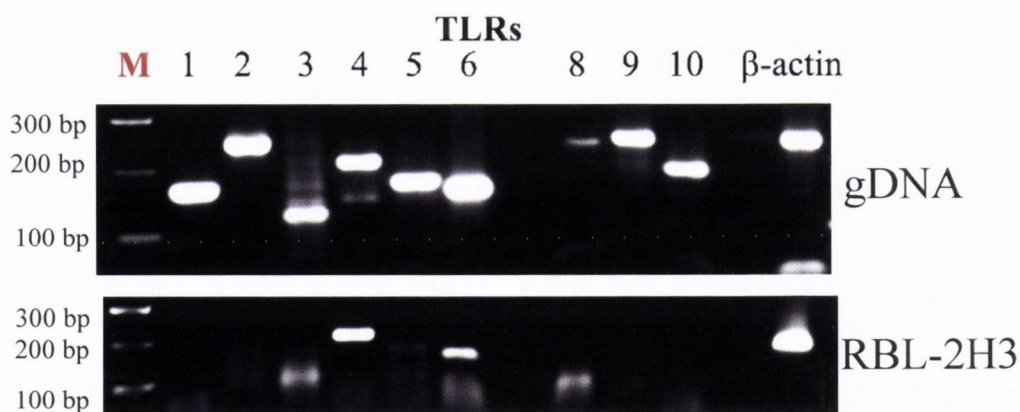
RBL-2H3

Figure 34:

Representative image photographs of agarose gels showing RT-PCR products for TLR1 trough TLR10 for RBL-2H3 and rat gDNA (positive control). β-actin was amplified as internal control. Transcripts encoding TLR 3, 4, 5 and 6 were detected in RBL-2H3 cells. TLR7 could not be investigated as none of the designed primers lead to positive results during the optimisation stage using genomic DNA (see materials and methods)

While the absence of the transcript for the TLR4 would have excluded the expression of the protein, explaining the lack of response to LPS, the positive result obtained is not conclusive since the presence of the mRNA is not necessarily synonymous with protein expression. TLR4 surface expression was investigated through flow cytometry.

3.4.2.b TLR4, CD14 and MyD88 surface expression in RBL-2H3 cells

FACS analysis of RBL-2H3 confirmed previous results obtained through RT-PCR.

Figure 35 is a representative flow cytometry showing a neat distinction between fluorescence intensity obtained with RBL-2H3 samples treated with both primary and secondary antibodies (red peak) and the background fluorescence intensity obtained treating the samples only with the secondary antibody (green peak). The plot on the right represents, for both the background (control) and the fluorescent samples (TLR4), the mean of the "mean fluorescence values". A $p < 0.05$ indicates a statistical difference among the two results; this confirms the expression of TLR4 by RBL-2H3.
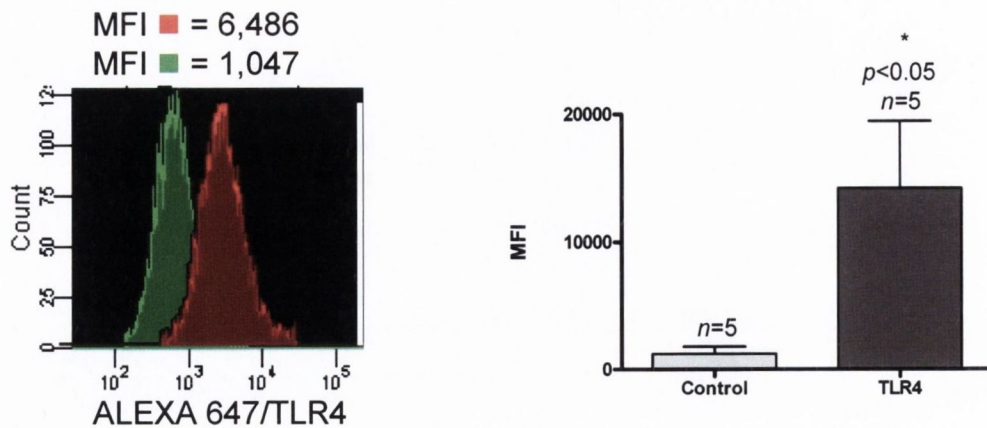
Figure 35:

Presence of TLR4 protein on RBL-2H3 cells. Panel on the left: representative flow cytometry peaks showing mean fluorescence intensity (MFI) for TLR4 (red peak) vs negative control (green peak). Panel on the right: histogram columns represent the mean values and S.E.M. of MFIs from n=5 experiments.

* indicates statistical significance (p<0.05) compared with control.

Statistical comparison was carried out using Student's t-test

This finding pushed us to question why RBL-2H3 do not respond to sLPS despite the surface expression of TLR4. It has been reported [132] that granulocyte basophils show the same behaviour. Bieneman and co-workers. [132] suggested that the lack of expression of the accessory protein CD14 (see paragraph 1.6.1.c) could be the reason of this unresponsiveness. Due to the high similarity between basophils and RBL-2H3 cells we hypothesised that a similar explanation could be applied to this cell line. CD-14 surface expression was therefore investigated. FACS analysis highlighted a lack of the accessory protein, (Figure 36). The representative flow cytometry shows how the two fluorescence peaks (Control ■ and CD14 ■) overlapped perfectly.
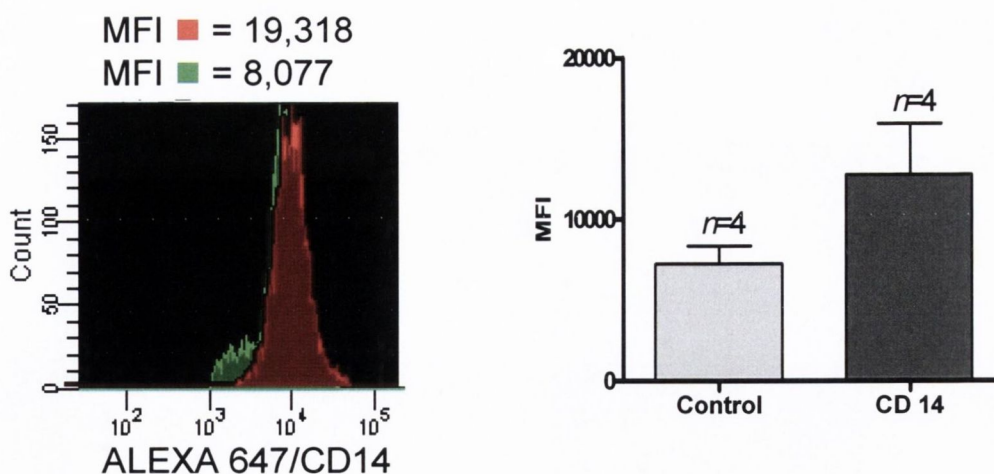
Figure 36

Presence of CD14 protein on RBL-2H3 cells. Panel on the left: representative flow cytometry peaks showing mean fluorescence intensity (MFI) for CD14 (red peak) vs negative control (green peak). Panel on the right: histogram columns represent the mean values and S.E.M. of MFIs from n=4 experiments.

The plot on the right shows the means±S.E.M. of the MFI (mean fluoresce intensity) of negative control (background fluorescence) and sample treated with anti CD14/labelled secondary antibody. After statistical comparison, a $p>0.05$ indicates a non-statistical difference between the two results; this confirms the lack of CD14 and explains the unresponsiveness of RBL-2H3 cells to sLPS (O111:B4 and O55:B5).

However, the previous results do not explain why RBL-2H3 cells did not respond after rLPS stimulation. As explained in paragraph 1.6.1.c, rLPS is not dependent on CD14 to elicit the release of mediators (TNF-α) but the intracellular signalling pathway requires the adaptor protein MyD88 to activate NF-kB and initiate the transcription of TNF-α. FACS analysis of resting and LPS-treated RBL-2H3 cells showed that MyD88 seems to be deficient in this cell line (Figure 37 and Figure 38). In Figure 37 the peak corresponding to the fluorescence of the control sample is slightly shifted on the left compared to test samples indicating a possible low expression of MyD88 protein. However, statistical analysis of the obtained values of five independent experiments reported a p value higher than 0.05, suggesting a lack of the protein. Moreover, in experiments performed with LPS-treated cells, the test

and the control peak perfectly overlapped confirming the previous finding.
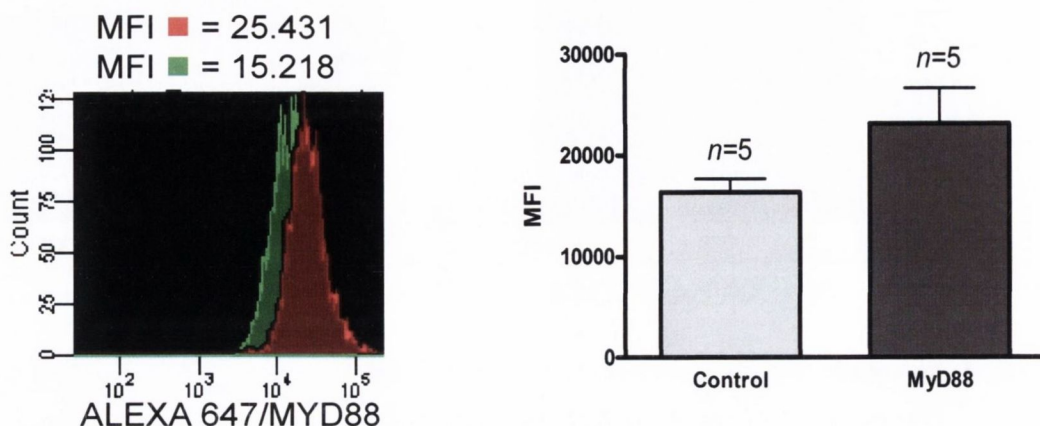
Figure 37

Presence of MyD88 protein in resting RBL-2H3 cells. Panel on the left: representative flow cytometry peaks showing mean fluorescence intensity (MFI) for MyD88 (red peak) vs negative control (green peak). Panel on the right: histogram columns represent the mean values and S.E.M. of MFIs from n=5 experiments.
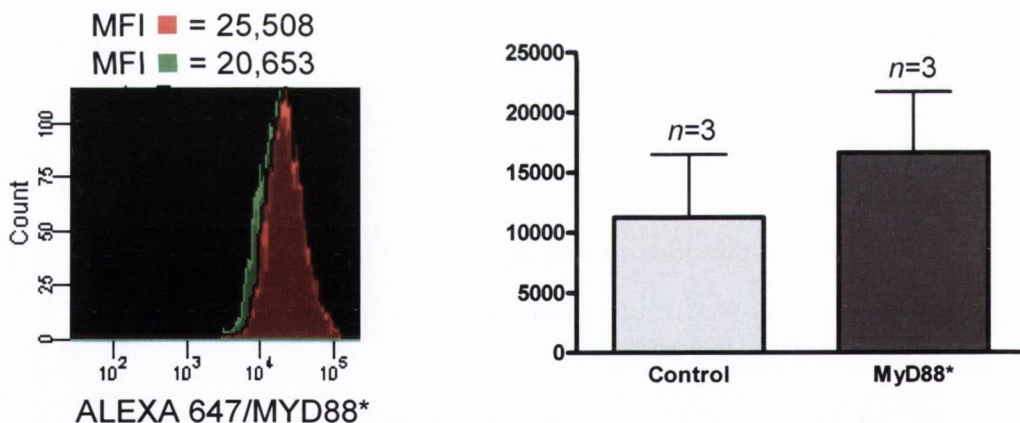
Figure 38

Presence of MyD88 protein in LPS-sensitised RBL-2H3 cells. Panel on the left: representative flow cytometry peaks showing mean fluorescence intensity (MFI) for MyD88 (red) vs negative control (green). Panel on the right: histogram columns represent the mean values and S.E.M. of MFIs from n=3 experiments

The unresponsiveness of RBL-2H3 cells to LPS can be attributed to a deficient TLR4 cascade. This aspect of RBL-2H3 cells' biochemistry has been previously reported in basophils [132] and is one further characteristic that differentiate this cell line and MMC. Supajatura and

co-workers [69] in fact reported that BMMC (example of MMC) can be challenged with LPS to produce both TNF-α and IL-13.

It must be underlined, however, that RBL-2H3 cells have been reported to be sensitive to sLPS [84]. The only explanation for this controversy is the possible change of phenotype of this cell line under different culture conditions. A study performed by Froese and co-workers [133, 134] proved that RBL cell lines which originated from the same tumour [70] and maintained in different laboratories, presented a changed phenotype in terms of number, kind and molecular weight of FcεRs. As different culture conditions may lead to an alteration of the FcεRs, they could also lead to a different TLR/CD14/MyD88 expression pattern and hence conferring to the cells different abilities to respond to stimuli.

3.5 Methods assessments for the use of RPMC as model for degranulation

Rat peritoneal mast cells belong to the subset of MC defined as connective tissue mast cell (see paragraph 1.3). They have the advantage of being easy to obtain as they can be isolated by a simple peritoneal lavage. However, cells from peritoneal lavage represent a heterogeneous population of which approximately 3% is composed of MC [136]. RPMC can be purified by density gradient purification without loss of cell viability. However, such a procedure might compromise their response to stimuli [78]. RPMC, over the years, have been widely used to test various mast cell stabilisers. In the present study RPMC were used to investigate the mast cell stabilising properties of our novel compounds and the results obtained were compared with those obtained with RBL-2H3 that are classified as MMC.

3.5.1 RPMC degranulation following immunological stimulation (anti-DNP IgE/DNP-BSA)

In the light of what has been found for the IgE-mediated RBL-2H3 degranulation (see paragraph 4.1.1) we wanted to investigate the effect of different concentrations (from 5000 ng/ml to approximately 5ng/ml)

of anti-DNP IgE on RPMC degranulation. The RPMC response to IgE/antigen presented huge inter-experimental variation, showing a percentage of degranulation from negligible values to approximately 35% of the total histamine content. Moreover, a considerable experimental error was often obtained due to a substantial dispersion of values obtained between the triplicates. This discrepancy between results from different experiments is a limitation for the study of IgE-mediated degranulation.

Due to this discrepancy between results, the IgE/antigen stimulus was not used to further studies regarding the mast cell stabiliser properties of di-indane analogues on RPMC (see paragraph 4.6).
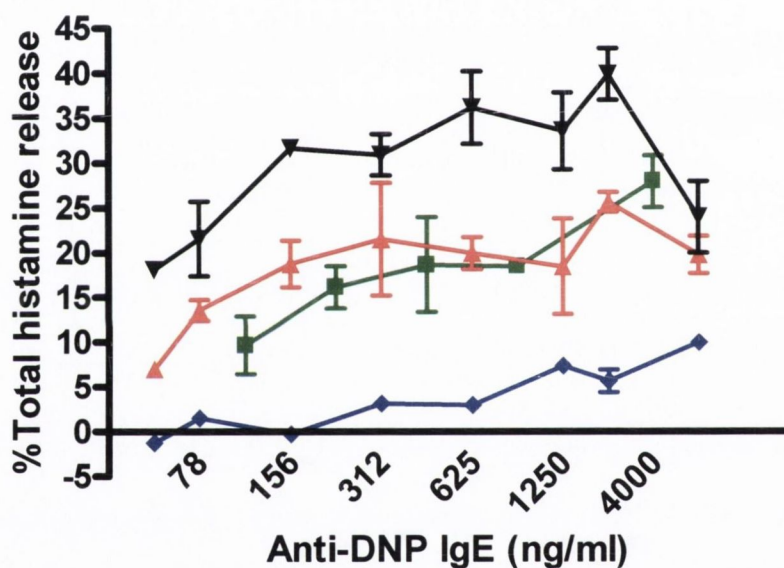
Figure 39

Dose response curves, for four independent experiments, of RPMC degranulation to different concentrations of anti-DNP-IgE (from 39 ng/ml to 5000 ng/ml). Values represent means±S.E.M., n=3.

3.5.2 RPMC degranulation following non-immunological stimulation (calcium ionophore A23187 compound 48/80, pam2CSK4, PGN)

In order to assess the optimal conditions for the RPMC degranulation, the effect various concentrations of: calcium ionophore A23187, compound 48/80, pam2CSK4 and PGN was tested.

3.5.2.a Calcium ionophore-triggered degranulation

As previously described, calcium ionophore A23187 can trigger mast cells to degranulate due to its ability to increase the free cytosolic calcium concentration into the cell (see paragraph 4.1.2.a). As shown in Figure 40, calcium ionophore A23187 could evoke a potent response even at low concentrations (0.63 μg/ml). The RPMC response to the ionophore was dose-dependent. The percentage of degranulation at 5 μg/ml of ionophore was 79±12%. This concentration was used during subsequent assays where calcium ionophore was used as a stimulus.
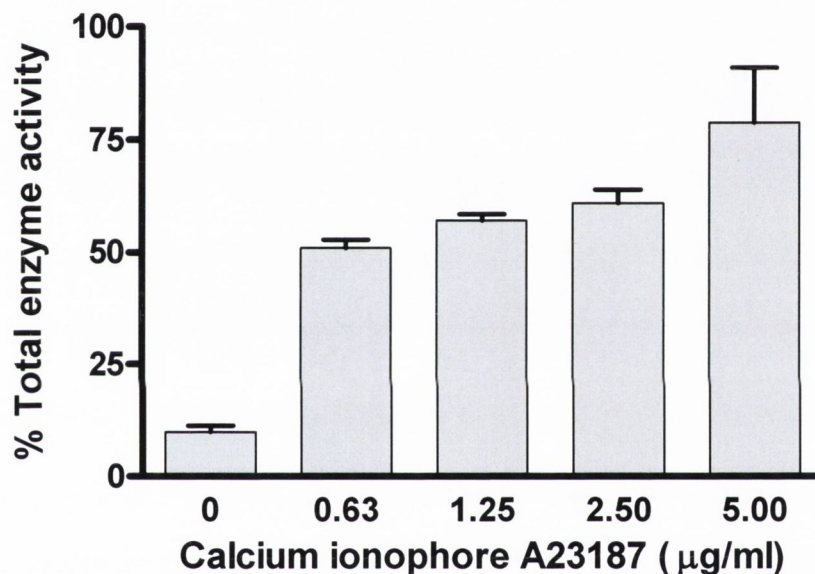
Figure 40

Calcium ionophore A23187 evoked a dose dependent degranulative response from RPMC.

Statistical analysis confirmed the linear trend of the dose response (p<0.05, n=3)

Values represent means±S.E.M.

3.5.2.b Compound 48/80- triggered degranulation

Cells from peritoneal lavage were incubated with serial dilutions of compound 48/80 (from 20 to 1.25 μg/ml). The dose response presented an increasing linear trend in function with the 48/80 concentration. Compound 48/80 could significantly trigger the degranulation even at low concentrations (at 1.25 μg/ml the degranulation was 6±2% of the

total enzyme activity), the higher obtained level of degranulation was observed at 20 µg/ml (38±8% of the total enzyme activity). Higher concentrations of compound 48/80 were not tested to avoid possible cytotoxic release of the granular mediator (Figure 41) A concentration of 10 µg/ml was used during subsequent assays were compound 48/80 was used as a stimulus.
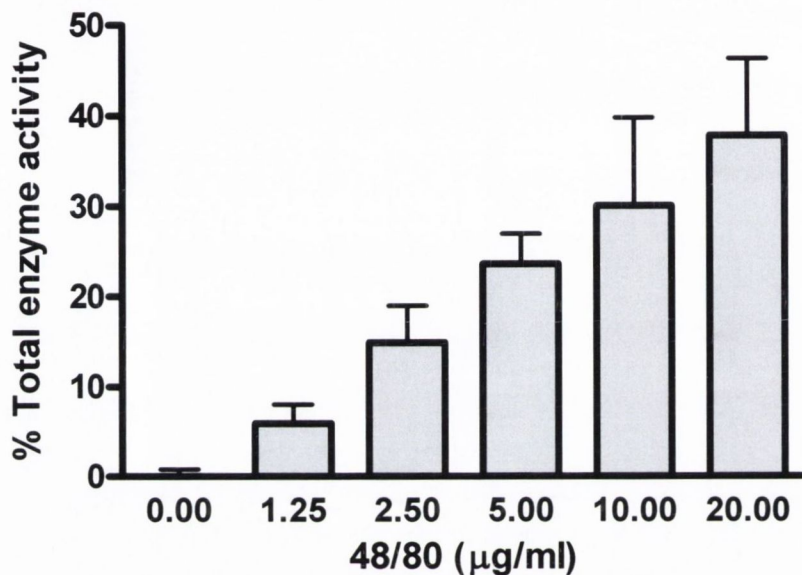
Figure 41

Compound 48/80 evoked a dose dependent degranulative response from RPMC.
Statistical analysis confirmed the linear trend of the dose response (p<0.05, n=3).
Values represent means±S.E.M.

3.5.2.c Pam2CSK4-triggered degranulation

Cells from the peritoneal lavage were incubated with 1, 5 and 10 µg/ml of pam2CSK4. The dose response was dose related and at 10 µg/ml it reached a value of 38±1% of the total release (Figure 42). The concentration of 10 µg/ml was used during subsequent assays were compounds pam2CSK4 was used as a stimulus.
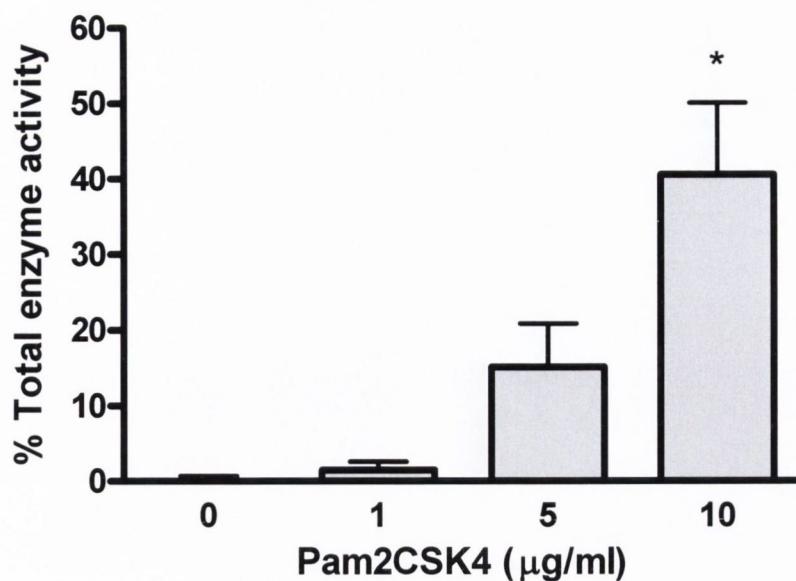
Figure 42

Ppam2CSK4 evoked a dose dependent degranulative response from RPMC cells. Maximal release was obtained at 10 μg/ml. Values represent mean±S.E.M., n=9.

* indicates statistical significance compared with untreated sample* indicates statistical significance (p<0.05) compared with untreated samples.

Statistical comparison was carried out using one-way ANOVA test

3.5.2.d PGN-triggered degranulation

Cells from the peritoneal lavage were incubated with 1, 10 and 100 μg/ml of PGN. The response to PGN was very limited; maximal degranulation (19±1%) was obtained at 10 μg/ml (Figure 43). Due to the low percentage of MC degranulation after treatment, PGN was not used for further studies involving our novel mast cell stabilisers.
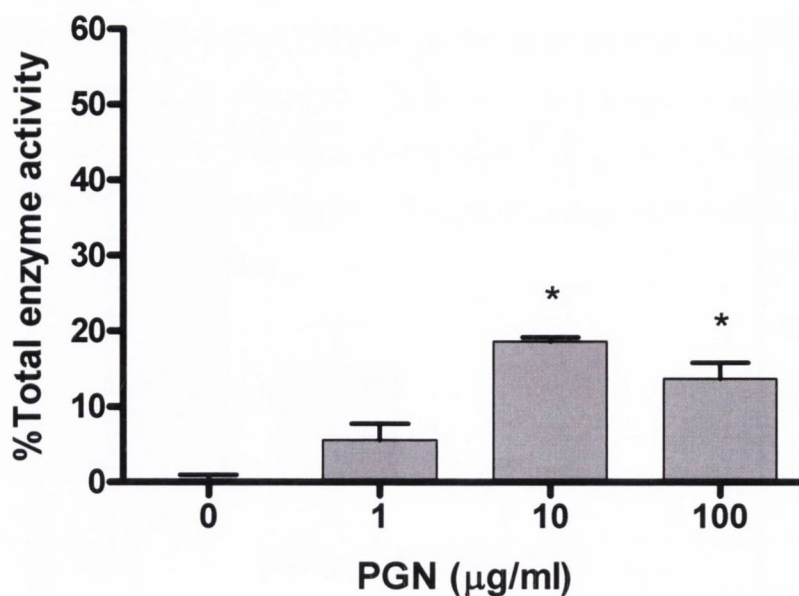
Figure 43

PGN evoked a very limited response from RPMC. Maximal release was obtained at 10 μg/ml. Values represent mean±S.E.M., n=3.

* indicates statistical significance compared with untreated sample* indicates statistical significance (p<0.05) compared with untreated samples.

Statistical comparison was carried out using one-way ANOVA test

3.6 Investigation of the stabilising activity of novel indane analogues of pterosin Z on RPMC/peritoneal lavage

Compounds PH2, PH5 and their resolved enantiomers (see materials and methods) were tested on RPMC (triggered with both immunological and non-immunological stimuli). RPMC were not purified as it has been shown that isolation might compromise the ability of cells to respond to stimuli. Since MC are the only cell type in the peritoneal lavage that can respond (with the release of histamine/β-hexosaminidase) to IgE and other degranulative agents, the results obtained can be attributed exclusively to RPMC. However, cell types such as macrophages and neutrophils are present as well (2.5 and 6.5 x 10^6 cell/ml respectively), and also express toll like receptors and produce TNF-α after LPS stimulation. Therefore, results obtained after LPS treatment of the peritoneal lavage can not be attributed exclusively to the mast cell response. In purified RPMC, even the untreated controls

showed high values of TNF-α released, suggesting that the purification step might have introduced unknown "activating factors" to the system. Cells from the peritoneal lavage were used during the experiments in order to provide general information regarding the effects of PH compounds on the LPS-stimulated cytokine production.

3.6.1 *Inhibition of 48/80-induced degranulation of RPMC by PH2, PH3, PH4, PH5, PH21 and PH22*

Figure 44 shows the effect of our test compounds and DSCG (used as positive control) on the 48/80-induced release of histamine from RPMC. Initially, just one animal was used during experiments and just histamine was used as indicator of degranulation as the amount of β-hexosaminidase was negligible. While DSCG significantly decreased the RPMC degranulation from 82.5±6% to 53.6±7% (p>0.05, *n=12*), PH2 and PH5 showed negligible activity on the histamine release; values were not statistically different (p> 0.05, *n*=13-15) from vehicle (DMSO). DMSO produced a slight decrease of the degranulation that masked the effect of the compounds. In an attempt to find a more suitable vehicle that could not interfere with the degranulation process, other two solvents were tested on the 48/80-triggered RPMC degranulation: propanol and the diethylene glycol monoethyl ether (Transutol). Both solvents showed a marked effect on MC degranulation, proving themselves inadequate as vehicles for our test compounds (Figure 45).
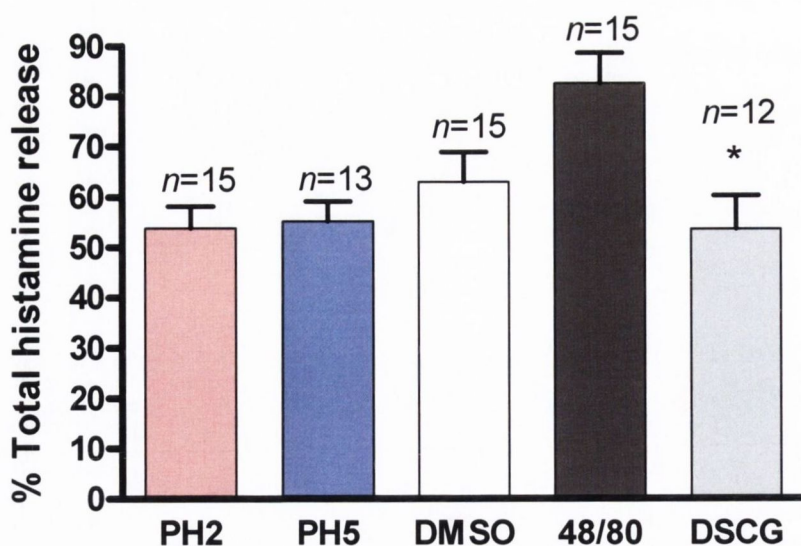
Figure 44

Effect of PH2, PH5 and of the positive control DSCG (all at 10 μM) on the compound 48/80-triggered degranulation of RPMC. The degranulation was monitored following the release of histamine. A pronounced solvent effect (DMSO) masked the effect of compounds. DSCG significantly inhibited the release of histamine.

Values represent mean±S.E.M., n=12-15

* indicates statistical significance (p<0.05) compared with untreated samples.

Statistical comparison was carried out using one-way ANOVA test
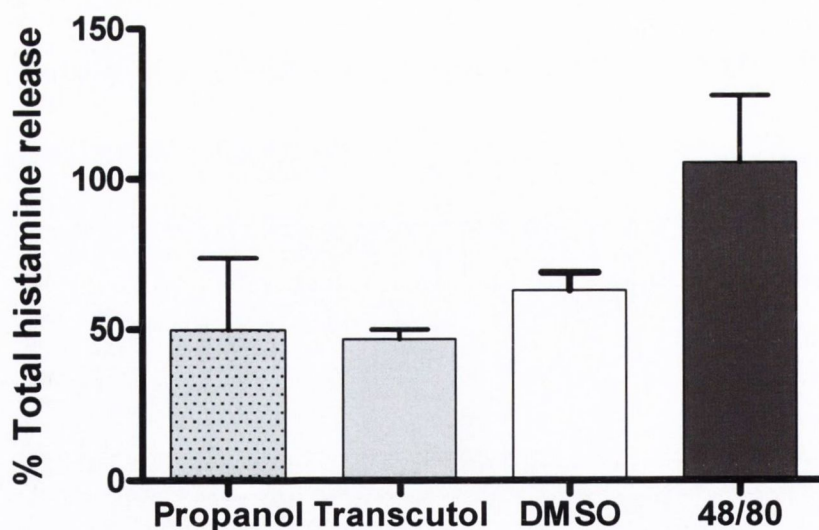
Figure 45

Effect of various solvents on the compound 48/80-triggered degranulation of RPMC. All the solvent tested inhibited RPMC degranulation. Values represent mean±S.E.M., n=6.

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When lavaged cells from three animals were pooled, the β-hexosaminidase activity was measurable.

The effect of PH compounds on compound 48/80-treated samples reflected what had already been found for the release of histamine (Figure 46). DMSO caused a decrease in degranulation that masked the effect of test compounds; the comparison of the values obtained for PH compounds and quercetin and with the vehicle control was not statistically different.
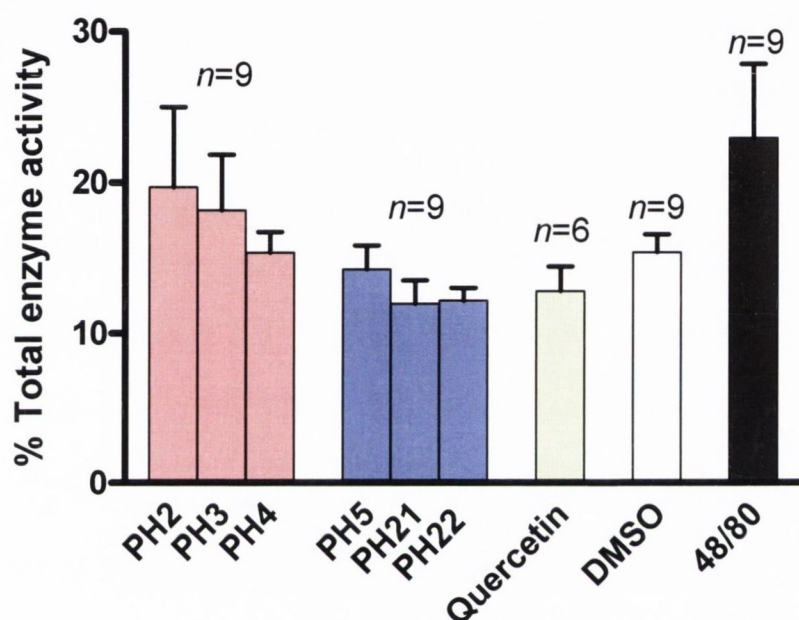
Figure 46

Effect of PH2, PH3, PH4, PH5, PH21, PH22 and of the positive control quercetin (all at 10 μM) on the compound 48/80 triggered degranulation of RPMC. The degranulation was monitored following the release of β-hexosaminidase. A pronounced solvent effect (DMSO) masked the effect of compounds. Values represent mean±S.E.M., n=6

3.6.2 *Inhibition of calcium ionophore A23187-induced degranulation of RPMC by PH2, PH3, PH4, PH5, PH21 and PH22*

Figure 47 shows the effect of our test compounds and quercetin (used as positive control) on the calcium ionophore A23187-induced degranulation of RPMC, monitored following the release of the granular enzyme β-hexosaminidase. All the compounds tested showed similar activity; however the statistical analysis underlined that only results obtained with PH21 and PH22 were statistically different

($p<0.05$) from vehicle (DMSO). In particular PH21 and PH22 reduced the amount of total enzyme release from 30±5% to 14±3% and 12±3% respectively. The values of PH2, PH3, PH4 and PH5 did not significantly differ from PH21 and PH22 ($p>0.05$, $n=12$). Despite this similarity, due to the experimental error, the statistical comparison between values obtained for the latter and the vehicle control was not significant. To conclude that PH2, PH3, PH4 and pH5 are ineffective as RBL-2H3 stabilisers just on the basis of the statistical analysis of this data could be rushed. Quercetin reduced the amount of total enzyme release from 30±5% to 9±1% giving a percentage of inhibition of 71±3%.
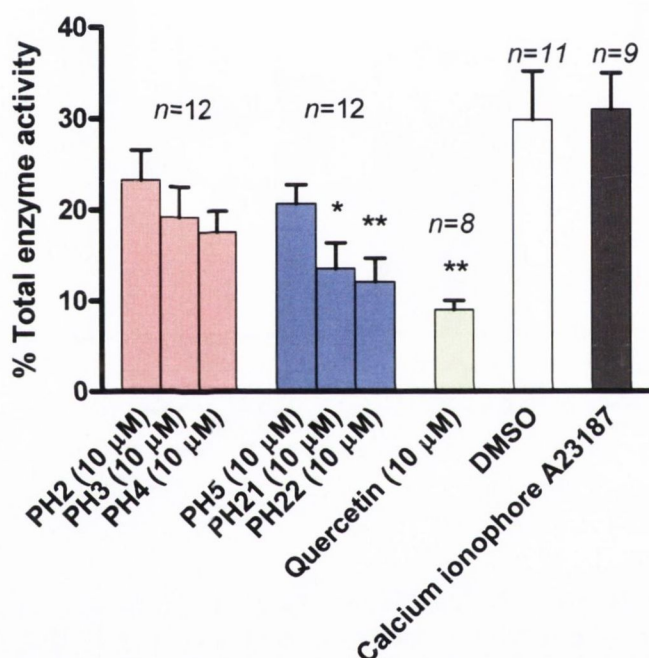
Figure 47

Effect of PH2, PH3, PH4, PH5, PH21, PH22 and quercetin (positive control) on the calcium ionophore A23187-triggered RPMC degranulation. Values represent mean±S.E.M., n=8-12.

* indicates statistical significance (p<0.05) compared with vehicle (DMSO).

** indicates statistical significance (p<0.01) compared with vehicle (DMSO).

Statistical comparison was carried out using one-way ANOVA.

Table 12 shows the effect of test compounds shown in Figure 47, expressed as percentage of inhibition (calculated according equation Equation 2). As previously highlighted PH2, PH3, PH4 and PH5 did not show any significant effect on the degranulation.

	Enzyme, % Inhibition±SEM
PH2	25±1
PH3	38±1
PH4	43±7
PH5	33±7
PH21 (*)	56±9
PH22 (*)	57±8
Quercetin (**)	71±3
DMSO	4±2

Table 12: Percentage of inhibition of β-hexosaminidase by PH compounds from calcium ionophore A23187-triggered RPMC

* indicates statistical significance (p<0.05) compared with vehicle (DMSO)

**indicates statistical significance (p<0.01) compared with vehicle (DMSO).

Statistical comparison was carried out using one-way ANOVA.

Figure 48 shows the effect of increasing concentrations of PH21 (upper panel) and PH22 (lower panel) on RPMC degranulation triggered by calcium ionophore A23187. The response of MC did not appear dose related and was constant across the all range of concentrations used (1-50 µM). While PH21 appeared to significantly inhibit the RPMC degranulation at all the concentration used, PH22 was active at concentrations of 10 µM and above.
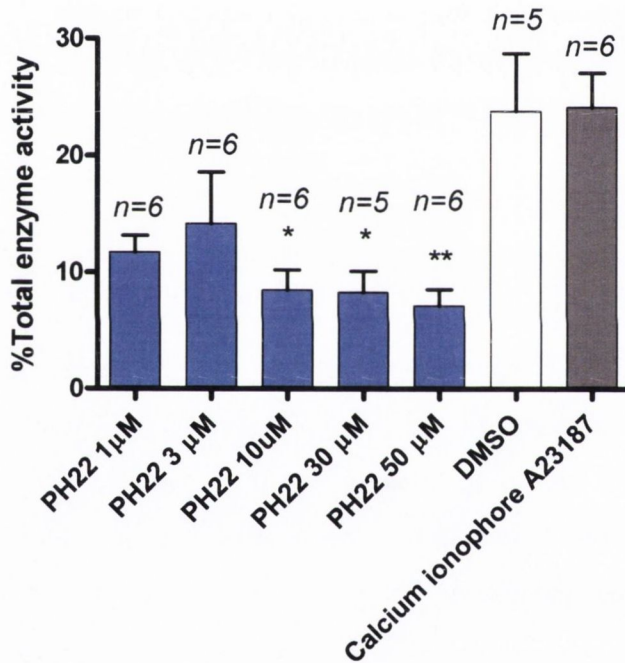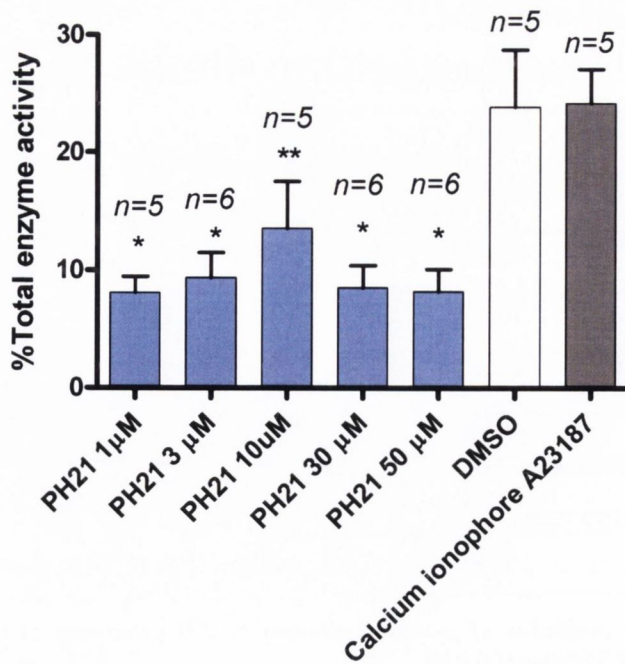
Figure 48

*Dose response of PH21 (upper panel) and PH22 (lower panel) on the β-hexosaminidase from RPMC after calcium ionophore A23187 treatment. Values represent mean±S.E.M., n=6. * indicates statistical significance (p<0.05) compared with baseline (DMSO).*

* indicates statistical significance (p<0.05) compared with baseline (DMSO).

** indicates statistical significance (p<0.01) compared with baseline (DMSO).

Statistical comparison was carried out using one-way ANOVA test

3.6.3 *Inhibition of Pam2CSK4-induced degranulation of RPMC/peritoneal exudate by PH2, PH3, PH4, PH5, PH21 and PH22*

Figure 49 shows the effect of our test compounds on the pam2CSK4-induced release of β-hexosaminidase in RPMC. All the compounds tested showed similar activity; however, the statistical comparison with the vehicle control showed that PH22 was the only active molecule ($p<0.05$, $n=6$). In samples treated with the vehicle alone (DMSO), a decrease of the degranulation was observed and this partially masked the effect of the test compounds. Moreover, the substantial experimental error obtained in the DMSO results makes the statistical difference with some of the PH compounds not significant. Unfortunately, these results are based on a single experiment in which each compound was tested on three samples ($n=3$). Additional experiments might lead to reduce the magnitude of the error in the DMSO result. Even though such experiments were desirable, they could not be performed owing to reasons that are beyond the scientific interest.
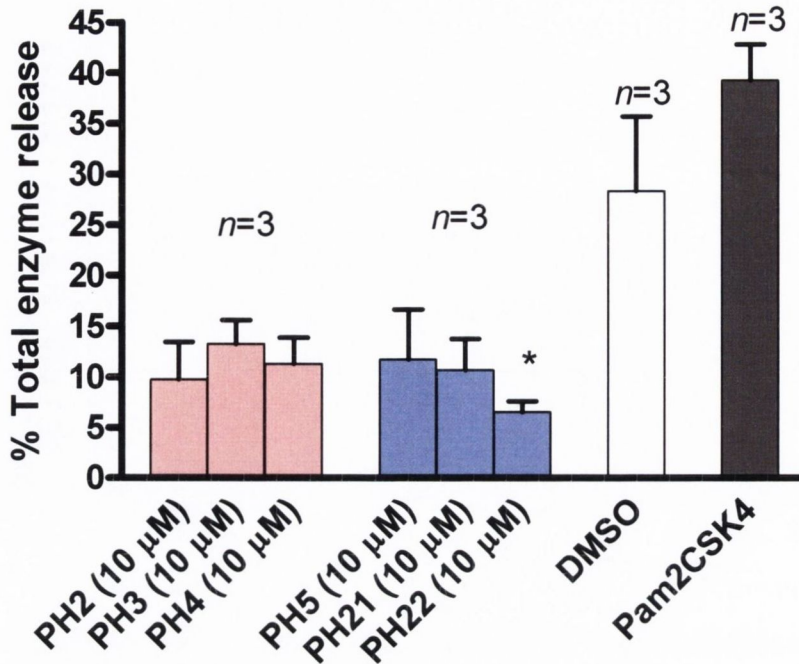
Figure 49

Effect of PH2, PH3, PH4, PH5, PH21, and PH22 on the pam2CSK4-triggered RPMC degranulation monitored following the release of β-hexosaminidase. Valued represent mean±S.E.M., n=3.

*indicates statistical significance (p<0.05) compared with vehicle (DMSO). Statistical comparison was carried out using one-way ANOVA test

3.6.4 *Activation of cells from the peritoneal lavage after LPS treatment*

As done for RBL-2H3 cells (see paragraph 4.4.1), cells from the peritoneal cavity were treated with sLPS (O111:B4) and the amount of IL-13 and TNF-α released was measured. As it has been explained more in detail in paragraph 4.6, in this case the response to LPS can not be attributed only to RPMC as other cells present in the peritoneal exudate (neutrophils, macrophages etc) respond to LPS with the production of TNF-α and IL-13. Several attempts of separating RPMC from other cells were performed but purified mastocytes resulted initiated to TNF-α release even in absence of LPS.

While LPS failed to trigger the production of IL-13, the amount of TNF-α released increased to sixfold; from a baseline of 155±1 pg/ml it

reached a maximal level of 915±1 pg/ml when cells were treated with 1µg/ml of sLPS.

It can be assumed that the TNF-α released was newly synthesised as the lysate of untreated cells showed a comparable amount to the control (95±2 and 155±1 pg/ml respectively).

Figure 50

Effect of different concentrations (from 0.01 to 10 µg/ml) of sLPS (O111:B4) on the production of IL-13 (panel on the left) and TNF-α (panel on the right) from peritoneal cells. While LPS failed to trigger the production of IL-13, the amount of TNF-α released sixfold increased. The TNF-α released was totally new synthesised as the lysate of untreated cells showed comparable TNF-α amount to the control. Values represent the mean±S.E.M., n=6.

* indicates statistical significance (p<0.01) compared with control.

3.6.5 *TLRs in RPMC*

As shown in Figure 51 mRNA transcripts for TLR1, 2, 3, 4, 5, 6 and 8 were detected while transcripts for TLR9 and 10 were absent. TLR7 could not be investigated as none of the designed primers lead to positive results during the optimisation stage using gDNA (see materials and methods). Except for the band corresponding to TLR2, which was barely visible, all the other bands are clearly distinguishable and of the correct size (see Table 3). In the light of the TLR pattern expressed by purified peritoneal mast cells, one could expect a positive response to LPS and to TLR2 ligands.

Previous results (Figure 42 showed the ability of RPMC to degranulate after treatment with pam2CSK4 but the response to PGN was limited. The responsiveness to pam2CSK4 and the presence of TLR2, TLR1 and TLR6 (with which TLR2 forms heterodimers) suggests that the

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signalling pathway associated with these receptors has to be operative. The TLR2 ability to bind PGN has been part of a heated dispute and it has been proposed that another PPR might be involved in PGN recognition rather than TLR2. Girardin and co-workers [141] suggested that NOD-2 can be implicated in this process[*]. It is known that highly purified preparations of PGN loose the ability to stimulate cytokine production from target cells and this might be due to the loss of other TLR2 ligands such as lipoteichoic acid [96].

Figure 51

Representative image photographs of agarose gels showing RT-PCR products for TLR1 trough TLR10 for RPMC and rat gDNA (positive control). β-actin was amplified as internal control. Transcripts encoding TLR1, 2, 3, 4, 5, 6 and 8 were detected in RPMC cells. TLR7 could not be investigated as none of the designed primers lead to positive results during the optimisation stage using genomic DNA (see materials and methods).

3.6.6 Effect of PH3, PH4, PH21 and PH22 on the production TNF-α from cells from the peritoneal lavage after LPS treatment

When cells from the peritoneal lavage were stimulated with 1 μg/ml of sLPS for 16 hours, the release of TNF-α was tenfold increased: a spontaneous release of 125 ± 2 pg/ml increased to 1231 ± 2 pg/ml (Figure 52). All the TNF-α released was newly synthesised as supernatants of untreated samples, lysed with Triton-X (TX in Figure 52), showed the same TNF-α content of the control (see Figure 52). None of the

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[*] The nucleotide-binding oligomerisation domain-2 (NOD-2) is the intracellular receptor for the muramyl dipeptide (MDP) component of PGN.

compounds under investigation showed an inhibitory effect on TNF-α production ($p>0.05$, $n=9$). Luteolin was used as positive control and at the concentration of 50 μM it completely inhibited the release of TNF-α. It also confirmed the previous observation that the TNF-α released after LPS stimulation was newly synthesised as, the lysate of untreated cells showed a comparable amount to the control (126±2 and 114±1 pg/ml respectively).
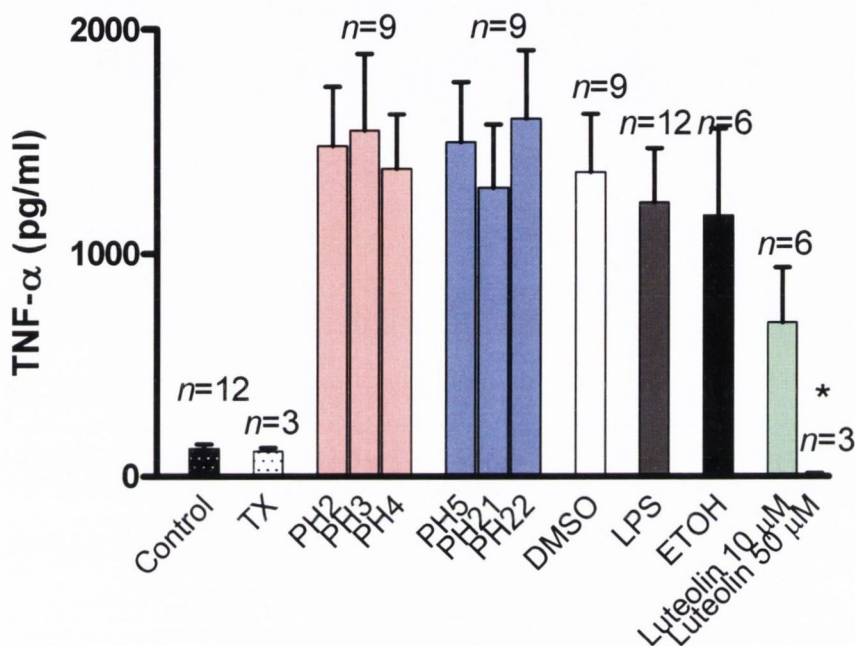
Figure 52

Effect of PH2, PH3, PH5, PH21, PH22 and luteolin (positive control) on the LPS-stimulated release of TNF-α from cells from the peritoneal lavage. Any of the compounds tested inhibited the production/release of TNF-α. All the TNF-α released after LPS treatment is newly synthesised as a Triton-X lysate of unstimulated samples presented the same cytokine levels of control samples. Luteolin at 50 μM completely suppressed the release of TNF-α. Values represent mean±S.E.M., n=3-12.

* indicates statistical significance (p<0.001) compared with non treated samples

Statistical comparison was carried out using a Kruskal-Wallis non parametric test.

4 Discussion

4.1 Methods assessment for the use of RBL-2H3 cell line as model for degranulation studies, a brief discussion

RBL-2H3 cells were initially chosen as they have been widely used as a model to study mast cell biology, with particular emphasis on the degranulation process after IgE sensitisation. Several groups have used RBL-2H3 to study physical-chemical aspects of mast cell degranulation, intracellular pathways involved during mast cell degranulation and novel mast cell stabilisers [71, 79, 97-101]; what made this cell line even more suitable for our study was the similarity with MMCs that are known to be involved in diseases such as colitis and Chron's disease. Mucosal mast cells are difficult to isolate and they can not be cultivated for a long period of time; having the opportunity to take advantage of an *in vitro* model that could bypass such difficulties was a rather attractive prospect. Unfortunately, during the optimisation of our experimental protocols, it was evident that a lack of consistency exits among the large amount of data published over the years regarding this cell line; it follows that it was necessary to optimise the conditions for the most commonly used assays. This brought us to: a) assess the optimal conditions for RBL-2H3 degranulation following both immunological and non-immunological stimulation, b) identify a suitable stabiliser for this cell line to be used as a positive control, c) define the pattern of expression of toll-like receptors and compare it with that of RPMC and with what has been reported for MMC and basophils d) investigate the expression of CD14 and MYD88 in the attempt to clarify the observed behaviour after LPS stimulation. As it will be discussed, while the RBL-2H3 cell line presents some unquestioned advantages, it showed some limitations and the results obtained with this cell line must be considered with care before drawing conclusions.

4.1.1 *Comments on the RBL-2H3 degranulation following immunological stimulation (anti-DNP IgE/DNP-BSA), a brief discussion of the observed bell-shaped response*

As shown in paragraph 3.1.1 the RBL-2H3 response to increasing concentration of DNP-IgE is bell-shaped. This phenomenon has been reported previously, not only for RBL-2H3 cells and mast cells in general [102, 103], but also for their circulating relatives: the granulocyte basophils [91]. The same characteristic trend can also be obtained by increasing the concentration of anti-IgE while keeping the concentration of IgE at a optimal levels [104]. The bell-shaped response to IgE has intrigued scientists for several decades and many theories have been put forward. Initially, to explain the descending portion of the curve, it was suggested that:

a) an excess of multivalent antigen could saturate the binding sites on the IgE molecules, leading to non-crosslinkable monovalent antigen/IgE-FcεRI complexes

b) at high concentrations of IgE, the ability of the antibody to pre-cluster the FcεRIs might lower the minimum concentration of antigen necessary to trigger the degranulation [104].

However, several objections to this theory have been put forward: Magro and Alexander [105], confirmed that increasing concentrations of IgE, when suboptimal/optimal concentrations of antigen were used, lead to an increased degranulation (case 1 in Table 13); they also proved that if a **monovalent monomer** anti IgE (Fab) is added to the system, the effect of increasing concentrations of IgE is antagonised, resulting in less bridging of receptors and, hence, a decrease in degranulation (case 1a in Table 13). However, in the case of supraoptimal concentrations of antigen, which should lead to a decrease in degranulation (case 3), when the monomer is added the overall effect is actually an increase in degranulation (case 3a). The authors interpreted this result as the ability of the monomer to inhibit the

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formation of an excess of bridging caused by the increased antigen concentrations.

In other words, they concluded that the descending portion of the bell-shaped curve might be regulated by a *"turn off"* mechanism activated by an excess of FcεRI bridging. This result can be supported by the finding that, to obtain a maximal secretory response, just a small portion (around 5%) of the FcεRIs has to be crosslinked [71]. Furthermore, it has been shown that only a few hundreds or less of chemically prepared oligomers of IgEs are necessary to trigger the RBL-2H3 cells to degranulate [97].
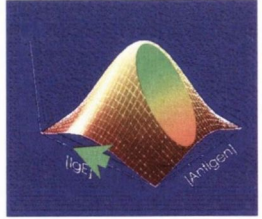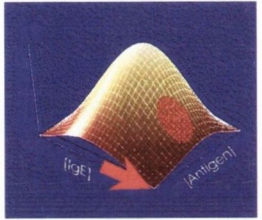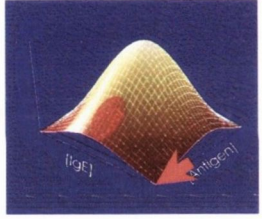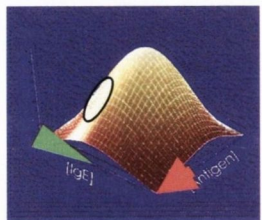
Case 1	⇑ [IgE] within optimal range + optimal [antigen]	⇒	⇑ degranulation	
Case 1a	⇑ [IgE] within optimal range + optimal [antigen] + ⇑ monomer anti IgE (Fab)	⇒	⇓degranulation	*The Fab fragment, due to its monomeric nature, is unable to crosslink the FcεRIs and, acting as "antigen antagonist", decreases the probability of bridging leading to a decrease of degranulation [105]*
Case 2	⇑ [IgE] above optimal range + optimal [antigen]	⇒	⇓degranulation	
Case 3	optimal [IgE] + ⇑ [antigen] above the optimal range	⇒	⇓ degranulation	
Case 3a	optimal [IgE] + ⇑ [antigen] above the optimal range + ⇑ monomer anti IgE (Fab)	⇒	⇑ degranulation	*According to the theory that the excess of bridging is a switch to suppress the degranulation, the presence of the Fab fragment inhibiting the crosslinkage of FcεRIs leads to an increaset of degranulation [105]*
Case 4	⇑ [IgE] within optimal range + ⇑ [antigen] above the optimal range	⇒	No decrease in degranulation	
	⇓	⇓		⇓
Hypothesis ⇒ Decrease of degranulation due to excess of bridging?				

Table 13

Explanatory table of the effects of fixed and/or increasing concentration of IgE vs increasing and/or fixed concentrations of antigen. Green colour indicates an alteration of conditions that positively affect the degranulation process, red colour indicates actions that negatively affect the degranulation. Steady conditions are indicated in black. Of particular importance is that a negative effect can be masked by a positive one (Case 4). [105]

According to what has been stated, one would expect that the bell-shaped response might be altered in the case of an increased number of surface receptors on the cell membrane (more receptors means less probability of bridging). To achieve this condition, RBL-2H3 were detached using Accutase instead of trypsin/EDTA. When Accutase was used to detach cells prior the experiment, the bell shaped trend, as predicted, shifted toward a plateau (Figure 13).

It is still not known what the intracellular mechanisms are that act as "switch" for the degranulation in conditions of supraoptimal stimulation. It has been suggested [104], that the src homology 2-containing[*] inositol phosphatase (SHIP) might be involved in the inhibitory mechanism that leads to the inhibition of degranulation; SHIP can suppress mast cell degranulation by limiting, through the hydrolysis of PIP_3, the influx of calcium from the extracellular compartment and it is crucial for the phosphorylation (and activation) of the adaptor protein Shc (that, in its turn, phosphorylates PKC-δ, a negative regulator of mast cell degranulation) [106-108].
It is probable that the ability of mast cells (and basophils) to control degranulation after massive antigen sensitisation, might represent a protective mechanism against exaggerated allergic reactions.

4.1.1.a.1 Discusison on the effect of quercetin on IgE-mediated degranulation of RBL-2H3 cells

Quercetin is a natural occurring product belonging to the family of phenolic phytochemicals called flavonoids. Flavonoids have been reported to possess antioxidant [109], anticarcinogenic [110] and anti-inflammatory properties [111] and, in particular, they have been reported to inhibit mast cell degranulation [112]. Different flavonoids show different mast cell stabiliser properties in terms of potency and

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[*] SRC-homology-2-domain, (SH2 domain). A protein-protein interaction domain that is commonly found in signal-transduction molecules. It specifically interacts with phosphotyrosine-containing sequences [44 **Gilfillan, A. M. and Tkaczyk, C.,** Integrated signalling pathways for mast-cell activation. *Nat Rev Immunol* 2006. **6:** 218-230.].

spectrum of activity. The effect of flavonoids, in fact, varies with the nature of the stimulus and the mast cell subtype. Quercetin is the only flavonoid that uniformly inhibits secretion stimulated by a variety of agonists both in mucosal mast cells and connective tissue mast cells (e.g. PMC), [93, 113].

In our experimental condition, quercetin reduced the amount of total enzyme release (Figure 16).

The mechanism of action by which quercetin and the other flavonoids inhibit mast cell secretion is still under debate. However the most credited theory suggests that flavonoids interfere with the receptor-directed Ca^{2+} intake through calcium channels in the membrane [93]

4.1.1.a.2 Discussion on the effect of DSCG on IgE-mediated degranulation of RBL-2H3 cells

DSCG is structurally related to quercetin and has been reported effective as a connective tissue mast cell stabiliser. DSCG has been proven ineffective on mucosal mast cells and basophils. The exact mechanism of action is still unknown, but it is thought that it acts at a membrane level and it affects the calcium influx across the cell membrane. The reason why DCSG lacks activity in MMC has not been clarified.

The RBL-2H3 cell line failed to be stabilised by DSCG. As previously stated, RBL-2H3 cells are traditionally referred as MMC and their insensitivity to DSCG confirms their similarity to this mast cell subtype.

4.1.1.a.3 Discussion on the effect of ketotifen on IgE-mediated degranulation of RBL-2H3 cells

Ketotifen was initially chosen because of its well known mast cell stabiliser properties. Moreover it has been proven effective on RBL-2H3 cells at concentrations in between 40 and 100 μg/ml [80, 114]. As shown in Figure 17, except for the concentration equivalent to 200 μg/ml when ketotifen was tested on the IgE-mediated degranulation of

RBL-2H3 cells, it did not inhibit the release of the granular enzyme β-hexosaminidase.

Concentrations above 100 μg/ml of ketotifen have been reported [94] toxic and this was confirmed after microscopic analysis of the treated cultures (Figure 18).

The results obtained at concentrations above 200 μg/ml, therefore, constitute a false positive; the reduction of the β-hexosaminidase released is consequent to the loss, during the washing step, of the released enzyme by dead cells (see materials and methods). Except a group that, despite the known cytotoxic effect, surprisingly used 100 μg/ml of ketotifen [80], the most commonly used concentration to reduce mast cell degranulation is between 20 and 40 μg/ml [114-116]. In particular Kim and co-workers [114] reported that ketotifen (42 μg/ml) significantly inhibited RBL-2H3 degranulation. The concern about the meaningfulness of this result is that the author does not specify if results obtained with treated samples are statistically different from vehicle control (DMSO), that in addition to that, showed a pronounced and highly variable effect on the degranulation. Another perplexity is raised as Kim and co-workers do not report the number of samples on which the statistical analysis was performed and they used the student's t-test instead of the one-way ANOVA to compare groups. As shown in Figure 18, ketotifen 50 μg/ml affected in some extent RBL-2H3 cultures and I would consider with care results obtained with any concentration close to that value. In my hands ketotifen increased the degranulation at concentrations elsewhere reported effective as a mast cell stabiliser (20 μg/ml [94] and 40 μg/ml [114, 117]). Together with the ability to inhibit IgE-mediated MC degranulation, ketotifen itself has been reported to increase the release of mediator from MC (and possibly basophils) through a non-cytotoxic mechanism [117]. It is not clear if this property might have increased the release of β-hexosaminidase under our experimental conditions despite the presence of IgE. However, ketotifen failed to suppress RBL-2H3 degranulation and therefore it could not be used as positive control.

4.1.2 *Comments on the RBL-2H3 degranulation following non-immunological stimulation (calcium ionophore A23187, MCDP, compound 48/80, PGN)*

As previously stated, the effect of calcium ionophore A23187, MCDP, compound 48/80 and PGN on RBL-2H3 cells was examined and compared with data obtained after stimulation with anti-DNP IgE/DNP-BSA (Figure 19). The following paragraphs are a brief comment of the data shown in section 3.1.1.b.

4.1.2.a *Calcium ionophore-triggered degranulation*

We tested the ability of calcium ionophore A23187 to trigger degranulation in RBL-2H3 cells. Calcium ionophore A23187 facilitates the transport of calcium from the external environment into the cytoplasm through an unknown mechanism (a controversial theory suggests the possibility of the formation of channels in the cell membrane [55, 118, 119]). It has been reported that the increase in free cytosolic calcium concentration plays a pivotal role during degranulation, and it is sufficient to trigger cell secretion [120]..

4.1.2.a.1 *Discussion on the effect of quercetin on calcium ionophore A23187-induced degranulation of RBL-2H3 cells*

The mechanism (see paragraph 4.1.1.a.1) by which quercetin can interfere with the receptor-directed Ca^{2+} intake, can still be considered valid. The increase of free cytosolic Ca^{2+} concentration during stimulation with A23187 has, in fact, at least three components[*], one of which depends by Ca^{2+} influx through membrane channels [56].

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[*] other than the influx of Ca^{2+} through native channels, two other components play a role in the rise of the cytosolic concentration of the ion: A) the PLC-dependent mobilisation of the ion from intracellular stores (with the subsequent activation of the store-operated calcium channels) and B) the influx of Ca^{2+} through pores created by the ionophore 56 **Dedkova, E. N., Sigova, A. A. and Zinchenko, V. P.,** Mechanism of action of calcium ionophores on intact cells: ionophore-resistant cells. *Membr Cell Biol* 2000. **13**: 357-368..

4.1.2.b MCDP- triggered degranulation

MCDP is a polybasic polypeptide, a component of the bee venom, and it is well established that at concentrations between 0.1-10 µg/ml it can evoke a potent response from mast cells. The mechanism by which MCDP leads to mast cell degranulation is not fully understood. At higher concentrations (45-50 µg/ml), MCDP shows anti-inflammatory properties and can inhibit mast cell degranulation [121]. This double activity is thought to be related to the fact that MCDP shows both IgE-independent effects on mast cells as well as IgE-mediated actions; MCDP can, in fact:

a) either mimic IgE molecules[*] or it can act as antigen and cross-link the IgE-bound FcεRI *(pro-inflammatory effect)* [121, 122].

b) interact, through its two disulfide bridges, with the disulfide bonds at the hinge region of IgE[†] molecules; the "hinge" S-S bonds are reduced leading to a less flexible conformation of the IgE molecule that results in an inhibition of the signal transmission when the antigen binds *(anti-inflammatory effect)* [121].

Buku and co-workers [121] used RBL-2H3 cells for binding studies of MCDP to FcεRI proving that this peptide can bind the receptors and act as an "IgE antagonist". No data regarding the degranulation of this cell line after MCDP treatment are available. Buku and co-workers, in fact, in the same publication used RPMC instead of RBL-2H3 for the

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[*] The ability of MCDP to mimic the IgE is due to its basic and hydrophobic clusters of aminoacids that resemble the portion of IgE that binds the FcεRI.

[†] IgE molecules are roughly Y-shaped. They consist of three equal sized portion connected by a flexible hinge region that contains two disulfide bonds.

HINGE
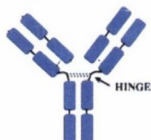
degranulation assay; this choice was justified because of the higher histamine content and greater responsiveness of RPMC to a wide range of stimuli [121].

In our laboratory, we tested the release of β-hexosaminidase after MCDP treatment. Mast cell degranulating peptide failed to produce a response from RBL-2H3 This result, however, is not surprising as RBL-2H3 and MMC in general, are reported to be insensitive to polybasic peptides (such as peptide 401) [23]. It can be assumed that the mechanism of action of MCDP on mast cells has to involve a pathway that takes into account the different effects on different subfamilies of mast cells. Polybasic compounds (compound 48/80, mastoparan, substance P and some peptides) can activate G proteins (in particular they can directly stimulate the GTPase activity of pertussis toxin-sensitive G_{i-3}) in a receptor-independent fashion [53]][123] (see paragraph 1.5.1.c for the role of G protein in mast cell activation).

RBL-2H3, MMC and basophils, do not express G_{i-3} under normal conditions and this might explain the lack of response. The unresponsiveness of RBL-2H3 to compound MCDP and other polybasic compounds is not the only characteristic that this cell line shares with MMC.

4.1.2.c Compound 48/80- triggered degranulation

Compound 48/80 is one of the most powerful mast cell secretagogues. To accomplish this function the aromatic ring of compound 48/80 has to penetrate deep into the plasma membrane, interact with the COOH terminus of G proteins and activate them [15]. As anticipated (see paragraph 4.1.2.b), possibly because of the lack of G_{i-3}, compound 48/80 does not evoke a response in RBL-2H3. Intriguingly, the prolonged exposure to the kinase inhibitor quercetin can induce a substantial increase in the α subunit expression of G_{i-3}, sensitising RBL-2H3 to 48/80 [95].

4.1.2.d PGN- triggered degranulation

PGN is a component of the outer wall of Gram-positive bacteria and in mast cells it activates a TLR2-dependent pathway that leads to degranulation (see paragraph 1.6.1.b).

Under our experimental conditions PGN (0.1-100 μg/ml) failed to induce degranulation in RBL-2H3; effect of PGN was negligible at all the concentrations tested). Other than the possibility that our PGN preparation had reduced potency[*], the only possible explanation to this result was that RBL-2H3 might present a defective TLR2 signalling pathway. TLRs mRNA expression was therefore assessed

4.2 Comments on the stabilising activity of novel indane analogues of pterosins Z on RBL-2H3 cells

Compounds PH2, PH5 and their resolved enantiomers (see materials and methods) were tested on RBL-2H3 cells triggered with both immunological and non-immunological stimuli.

4.2.1 General conclusion on the effect of PH2, PH3, PH4, PH5, PH21 and PH22 on RBL-2H3 degranulation

The effect of PH compounds on RBL-2H3 degranulation can be summarised as follows:

> ➥ when anti-DNP IgE/DNP-BSA were used to trigger RBL-2H3 cell degranulation, PH22 was the only compound that inhibited MC degranulation. The RBL-2H3 response to different concentration of PH22 was dose-dependant.

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[*] there has been a dispute about the effect of commercially available PGN on TLR2 signalling. The contaminant present in the preparation (such lipothecoic acid, TLR2 ligand itself) might confer to the preparation higher potency than purified PGN [96
Netea, M. G., Ferwerda, G., de Jong, D. J., Jansen, T., Jacobs, L., Kramer, M., Naber, T. H., Drenth, J. P., Girardin, S. E., Kullberg, B. J., Adema, G. J. and Van der Meer, J. W., Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. *J Immunol* 2005. **174**: 6518-6523..

- when calcium ionophore A23187 was used to trigger the RBL-2H3 degranulation, all the compounds could effectively inhibit MC degranulation. The response to test compounds was dose dependant.

The mechanism of action of PH2, PH5 and their enantiomers is not known; however it has been previously shown that similar compounds can inhibit the $CaCl_2$-induced contraction of guinea pig ileum [76], suggesting a possible effect on the calcium movement within the cell. We have demonstrated that calcium is important during RBL-2H3 degranulation as the use of a releasing media in which the concentration of the ion was reduced, affected the cell response.

Test compounds were proved effective even when RBL-2H3 cells were stimulated with calcium ionophore A23187 that has been reported to work as a carrier (in lipid bilayers) exchanging one Ca^{2+} with $2H^+$ [55] and it can also form channels or pores due to the ability to aggregate in lipid matrices. The inhibition of the degranulation, despite the presence of a Ca^{2+} carrier, indicates that test compounds might interfere with the formation of the Ca^{2+}/CaM complex rather than as Ca^{2+} influx inhibitors.

However Balasubramian and co-workers [56] demonstrated that additional components other than the ion exchange properties of the calcium ionophore are, in some cases, responsible for the cell response. Calcium ionophore A23187 can increase the cytosolic calcium concentration activating the Ca^{2+} influx through native Ca^{2+} channels or mobilising the ion from intracellular storage in a phospholipase C-dependent fashion with the subsequent influx of the ion into the cells through membranous channels activated by a store-regulated mechanism (SOCC). It would be interesting, therefore, to measure how the test compounds affect the increase of free cytosolic calcium concentration during the degranulation process, to exclude a possible action of our compounds on calcium channels. The intracellular calcium concentration can be measured using a fluorescent indicator that binds to free calcium; one of the most popular indicator is Fura-2 and it has been widely used for both suspended and attached cells.

4.3 Comments of the cytotoxicity of PH2, PH5, quercetin, DSCG and terfenadine on RBL-2H3 cells

The Cytotoxocity profile of PH2 and PH5 was investigated and compared with that of terfenadine, DSCG and quercetin. Terfenadine is a well known H_1 receptor blocker and it presents also mast cell stabiliser properties, it is currently commercialised with the name of Allerzil® to treat the symptoms of allergic rhinoconjunctivitis. DSCG, works exclusively as mast cell stabiliser and is marketed as treatment of asthma, colitis and mastocytosis other than common allergic conditions. Quercetin is a natural product that belongs to the large family of secondary plant metabolites called flavonoids that show antiallergic and anti-inflammatory activities with an unknown mechanism. Quercetin is marketed in conjunction with vitamins (Natrol®) as a food supplement to relieve allergy and inflammatory conditions.

The cytotoxicity of the examined compounds was tested using two different assays aimed to assess cell death (LHD assay) and cell viability (MTS assay). The crucial difference between the two methods is that the LDH cytotoxicity assay quantifies the cytotoxicity/cytolysis through the detection of the LDH* activity released from damaged/dead cells. While the second (MTS assay) is a cell viability assay based on the bioreduction, by living cells, of the MTS tetrazolium compound (Owen's reagent) to a colored formazan. The choice to investigate these two antithetical aspects might seem redundant as, at a first glance, both assays lead to complementary results. Unfortunately, this is not the case, as a cell, after an insult, can undergo a form of programmed death called *apoptosis* that is characterised by a progressive shrinkage of the cell, followed by condensation and fragmentation of the cytosol. Apoptosis is a slow, complex and strictly orchestrated process that maintains the membrane integrity avoiding the leakage of the intracellular content. The death through apoptosis is opposite to the other type of cellular death identified as *necrosis*, in which (usually after a severe physical cellular damage or metabolic poison) cellular

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* LDH is the acronym for **Lactate dehydrogenase.** LDH is a widely distributed intracellular enzyme. It catalyses the transformation of the pyruvate in lactate during hypoxia.

constituents disintegrate in a uncontrolled fashion; the cell, therefore, rapidly dies and its contents leak out* [125]. It appears obvious how, in the event of an apoptotic death, the analysis of "leaked" intracellular enzymes (e.g. LDH) can lead to an erroneous conclusion (low level of indicator but massive cell death). On the other hand, the measurement of the cell viability gives a better idea on the metabolic condition of the cells. Only a direct comparison of the two indices of cell death, however, can suggest a possible apoptotic or necrotic process (see below). The results obtained with these two techniques are only indicative. More focused assays based on the detection of specific caspases† can give more reliable results. Caspases might be detected by immunocytochemistry or immunofluorescence (in which caspases' substrates are linked to tag molecules that give, after cleavage, either coloured or fluorescent products)

4.3.1 Cytotoxicity of quercetin on RBL-2H3 cells

The percentage of damaged/dead RBL-2H3 cells after quercetin for almost all the concentrations used. Moreover, at some of the concentrations tested quercetin, could promote cell viability. It has been reported that quesrcetin might promote cell survival by blocking JNR-ERK-mediated apoptosis and also activating survival signalling proteins such as phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (AKT) [126-128].

4.3.2 Cytotoxicity of DSCG on RBL-2H3 cells

DSCG did not show any cytotoxic effect on RBL-2H3. Moreover the cell viability after treatment was above the test control. Whether DSCG

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* The reason why a cell "chooses" to die by programmed death or not has been subject of study for many years and still today many questions have not been answered; however a dissertation on this topic is behind the scope of the preset thesis; for further reading reference [125   **Potten C., W. J.**, *Apoptosis, the life and death of cells.* Cambridge University Press: 2004.

† Caspases are protease enzymes that are activated during apoptosis. They take part in the cascade of reactions that orchestrate the cell disassembly.

can enhance the cell survival or not is controversial and it depends on the type of cell investigated.

4.3.3 *Cytotoxicity of terfenadine on RBL-2H3 cells*

Unlike quercetin and DSCG, terfenadine showed a marked toxic effect on RBL-2H3(Figure 27). No metabolic activity was detected even at 3 µM. Moreover the LDH activity (index of cell death) markedly increased from 1 to 3 µM. At concentrations above 1 µM the percentage dead cells remained practically constant at about 50% while the cell viability was zero. This result can suggest a possible pro-apoptotic effect of terfenadine on RBL-2H3 cells. This result is in accordance with the reported pro-apoptotic effect of Terfenadine on other cell types [129].

4.3.4 *Cytotoxicity of PH2 and PH5 on RBL-2H3 cells*

As previously stated, results obtained with PH2 and PH5 on RBL-2H3 cell viability/cell damage were very similar to each other (Figure 28 and Figure 29). At 100 and 30 µM the RBL-2H3 cell viability was almost completely suppressed but the cell death showed a plateau. This result, similar to the one obtained for PH2, might suggest a possible pro-apoptotic effect of PH5 on RBL-2H3 cells. Figure 29 shows how cell viability was not affected for concentrations below 10 µM.

The 50% cytotoxicity/cell viability obtained for PH5 at 10µM, might suggest that the inhibitory effect of our test compounds on the histamine/β-hexosaminidase release observed in Figure 21 might be due to the cell death rather than to a pharmacological effect of the drug. On the other hand, PH2 and its resolved enantiomers did not show any significant effect on the RBL-2H3 degranulation despite the similar cytotoxicity profile with PH5. If the inhibition of the degranulation would be due to cell death, this would influence the results obtained for both compounds but PH2 was not statistically different from the baseline (DMSO) (Figure 21); this consideration tips the scales in

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favour of a pharmacological effect that, in my opinion, should be further investigated.

4.4 RBL-2H3 cell line as model to study the MMC response to toll-like receptors' ligands, a brief discussion

4.4.1 *RBL-2H3 activation after LPS sensitisation*

Mast cells have been mainly studied as key effectors in allergic diseases but in recent years they have attracted the attention of scientists as they seem to play a crucial role during bacterial infections. Mast cells are mainly located in areas that interface with the external environment and therefore they can act as sentinels toward invading pathogens; they have been proven to respond to components of the bacterial wall through a family of pattern recognition receptors called *"Toll-like receptors"* (TLRs).

As previously described (paragraph 1.6.1.b) mast cells can degranulate through a TLR2 dependent pathway or they can be challenged, though their TLR4s, to release newly synthesised mediators such TNF-α, IL-13 etc. Both MMC and CTMC have been reported to respond to TLR2 and TLR4 ligands. Sharing many similarities with MMC, the RBL-2H3 cell line was a good candidate as an *in vitro* model to study the effect of our test compounds on TLR-mediated responses. Responses to sLPS (serotype O111:B4 and O55:B5) and rLPS (Re 595 *S. Enterica*) were tested.

4.4.1.a *TNF-α release after stimulation with sLPS and rLPS of RBL-2H3 cells*

RBL-2H3 have been reported to produce TNF-α after sLPS (serotype 055:B5) stimulation in a time and dose dependent manner [84]. Under our experimental conditions, however, RBL-2H3 failed to produce any response (Figure 19, Figure 20, Figure 30).

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In rodents, the ligand's recognition by TLR4 depends on the particular structure/antigenic properties of LPS. It was therefore hypothesised that the observed lack of response of RBL-2H3 cells was due to the particular strain of sLPS used in our experiments. Two other strains of LPS were tested, namely the smooth serotype O55:B5 and a rough variant of LPS (*Salmonella enterica,* serotype minnesota, Re 595). The choice of using both smooth and rough type of LPS was suggested by the fact that these two kinds of LPS have different ways of interaction with the TLR4. While rLPS can bind directly TLR4, the LPS serotype O55:B5, being a smooth type, requires the presence of the TLR co-receptor CD14 to activate the pathway that, via MyD88, leads to the activation of NF-κB and the subsequent transcription of genes involved in the production of TNF-α, (see paragraph 1.6.1.c). None of the concentrations used of rLPS and sLPS (O55:B5) elicited a response from RBL-2H3 cells in a period of 16 hours (Figure 32). The lack of response to all the LPS strains tested raised the question whether the TLR4 pathway in RBL-2H3 cells is operative or not. We, therefore, wanted to investigate whether TLR4, CD14 and MyD88 were expressed by RBL-2H3 cells.

4.4.1.b *IL-13 release after stimulation with LPS of RBL-2H3 cells*

Due to its crucial role in allergy and inflammation, the level of IL-13 released after sLPS (0111:B4) stimulation was tested in parallel with the level of TNF-α (see paragraph 4.4.1.a).

The result obtained reflected what was previously found for TNF-α. Smooth LPS at concentrations elsewhere reported effective [84], failed to produce a response from RBL-2H3.

The amount of IL-13 released in a period of 16 hours was not statistically different from the untreated control.

4.4.2 *TLRs in RBL-2H3 cells*

The lack of response of RBL-2H3 cells to LPS and PGN raised the question whether these cells express the toll-like receptors or not.

mRNA expression of TLR1-6 and 8-10 was investigated through RT-PCR; subsequently, the expression of the TLR4 protein was assessed by FACS analysis as the signalling through this particular receptor is the pathway that leads to the cellular response to LPS. As the TLR4 signalling pathway is conditioned by the presence of both the TLR4 co-receptor CD14 and the adaptor protein MYD88, expression for this two proteins was also tested. The information obtained as allowed a comparison of RBL-2H3 with both RPMC (example of CTMC), MMC and BS.

The TLR mRNA expression profile in RBL-2H3 cells (Figure 34) was compared to the one from RPMC (example of CTMC) (Figure 51), MMC and BS (see Table 14). TLR pattern in RBL-2H3 cell line differs significantly from all the mentioned cell types.

While the difference between RPMC (CTMC) and RBL-2H3 can be interpreted as a further feature that underlines mast cell heterogeneity, attention must be put on the differences between RBL-2H3, MMC and BS: as briefly explained in the introduction and reviewed in Appendix I, MMC and BS are in some ways similar to each other and each cellular type share some features with RBL-2H3 whose origin and identity is uncertain (see Appendix 4). Differently from both basophils and MMC, RBL-2H3 do not express TLR2, compromising the response to components of the Gram-positive bacteria outer membrane such as PGN (see paragraph 4.1.2.d) and lipoteichoic acid or to soluble synthetic ligands such as pam2CSK4[*]. This difference underlines how RBL-2H3 cells are far from the phenotype of both MMC and BS.

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[*] In the light of this result the study of the effect of Pam2CSK4 on RBL-2H3 cells was not performed.

Table 14:

TLRs expression in CTMC (RPMC), MMC, basophils and RBL-2H3. Information regarding the TLRs expression in MMC and basophils refers to murine and human cells respectively. Eventual interspecies differences must be considered. These information, hence, are just as an indication.

	CTMC, (Figure 51),	MMC,[69, 130] (mouse)	Basophils, [131] (human)	RBL-2H3 cells. (Figure 34)
TLR expression				
TLR1	+	+	ND	-
TLR2	+	+	+	-
TLR3	+	+	ND	+
TLR4	+	+	+	+
TLR5	+	-	ND	+
TLR6	+	+	ND	+
TLR7	+	+	ND	ND
TLR8	+	+	ND	-
TLR9	-	-	+	-
TLR10	-	ND	+	-

FACS analysis of RBL-2H3 cells confirmed the presence of TLR4. This finding pushed us to question why RBL-2H3 do not respond to sLPS despite the surface expression of TLR4. It has been reported [132] that granulocyte basophils show the same behaviour. Bieneman and co-workers. [132] suggested that the lack of expression of the accessory protein CD14 (see paragraph 1.6.1.c) could be the reason of this unresponsiveness. Due to the high similarity between basophils and RBL-2H3 cells we hypothesised that a similar explanation could be applied to this cell line. CD-14 surface expression was therefore

investigated. FACS analysis highlighted a lack of the accessory protein, (Figure 36).

However, the previous results do not explain why RBL-2H3 cells did not respond after rLPS stimulation. As explained in paragraph 1.6.1.c, rLPS is not dependent on CD14 to elicit the release of mediators (TNF-α) but the intracellular signalling pathway requires the adaptor protein MyD88 to activate NF-kB and initiate the transcription of TNF-α. FACS analysis of resting and LPS-treated RBL-2H3 cells showed that MyD88 seems to be deficient in this cell line (Figure 37, Figure 38).

The unresponsiveness of RBL-2H3 cells to LPS can be attributed to a deficient TLR4 cascade. This aspect of RBL-2H3 cells' biochemistry has been previously reported in basophils [132] and is one further characteristic that differentiate this cell line and MMC. Supajatura and co-workers [69] in fact reported that BMMC (example of MMC) can be challenged with LPS to produce both TNF-α and IL-13.

It must be underlined, however, that RBL-2H3 cells have been reported to be sensitive to sLPS [84]. The only explanation for this controversy is the possible change of phenotype of this cell line under different culture conditions. A study performed by Froese and co-workers [133, 134] proved that RBL cell lines which originated from the same tumour [70] and maintained in different laboratories, presented a changed phenotype in terms of number, kind and molecular weight of FcεRs. As different culture conditions may lead to an alteration of the FcεRs, they could also lead to a different TLR/CD14/MyD88 expression pattern and hence conferring to the cells different abilities to respond to stimuli.

4.4.3 *General conclusions on RBL-2H3 cell line as a mast cell model*

When the RBL-2H3 cell line was established, it effectively accomplished the task to help researchers to investigate the basic cellular mechanisms correlated with the mast cell/basophil degranulation and the physical-chemical interaction between IgE and Fc receptors and between IgE/FcεRI complexes and the cytoskeleton [71, 97, 99, 135]. While the adequacy of this in vitro tool was optimal

in a scientific context where the knowledge of mast cells and in particular of basophils was limited, today the use of RBL-2H3 is less justified. The RBL-2H3 cell line, in fact, shows some limitations: in first instance, it is a tumorous cell line and it obviously presents aberrant characteristic and abnormalities and also culture conditions can deeply affect its phenotype [133, 134]. Moreover, as it will be explained in Appendix 4, the nature (mastocytes or basophils) of RBL-2H3 cells is uncertain and therefore, results obtained with this cell line have to be analysed and interpreted with care. However, RBL-2H3 cells have the advantage to possess a functional downstream pathway of the FcεRI that has been widely studied. Furthermore, a great number of homogeneous cells can be easily obtained with simple cell culture techniques. For these reasons, RBL-2H3 can still be used in preliminary studies to elucidate elementary characteristics of mast/cells basophils biochemistry. It being understood that the obtained results have to be interpreted with the necessary care and validated with other models. Perhaps more meaningful results can be obtained with primary MMC and basophils, which are now easier to isolate, and therefore delivering a greater benefit/cost ratio.

4.5 Methods assessments for the use of RPMC as model for degranulation

Rat peritoneal mast cells belong to the subset of MC defined as connective tissue mast cell (see paragraph 1.3). They have the advantage of being easy to obtain as they can be isolated by a simple peritoneal lavage. However, cells from peritoneal lavage represent a heterogeneous population of which approximately 3% is composed of MC [136]. RPMC can be purified by density gradient purification without loss of cell viability. However, such a procedure might compromise their response to stimuli [78]. RPMC, over the years, have been widely used to test various mast cell stabilisers. In the present study RPMC were used to investigate the mast cell stabilising properties of our novel compounds and the results obtained were compared with those obtained with RBL-2H3 that are classified as MMC.

4.5.1 RPMC degranulation following immunological stimulation (anti-DNP IgE/DNP-BSA)

The RPMC response to IgE/antigen presented huge inter-experimental variation, showing a percentage of degranulation from negligible values to approximately 35% of the total histamine content. Moreover, a considerable experimental error was often obtained due to a substantial dispersion of values obtained between the triplicates. This discrepancy between results from different experiments is a limitation for the study of IgE-mediated degranulation.

When results from single experiments are analysed (Figure 39), however, the dose-response curve obtained did not show the characteristic bell-shaped trend observed for RBL-2H3 cells (see paragraph 4.1.1). Whether the bell shaped-trend is just an in-vitro phenomenon or not is still under debate, but it presents an intriguing matter as there are obvious implications about the legitimacy of using this *in vitro* model to study mast cell biology. Due to this discrepancy between results, the IgE/antigen stimulus was not used to further studies

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regarding the mast cell stabiliser properties of di-indane analogues on RPMC (see paragraph 4.6).

4.5.2 *RPMC degranulation following non-immunological stimulation (calcium ionophore A23187 compound 48/80, pam2CSK4, PGN)*

In order to assess the optimal conditions for the RPMC degranulation, the effect various concentrations of: calcium ionophore A23187, compound 48/80, pam2CSK4 and PGN was tested.

4.5.2.a Calcium ionophore-triggered degranulation

As previously described, calcium ionophore A23187 can trigger mast cells to degranulate due to its ability to increase the free cytosolic calcium concentration into the cell (see paragraph 4.1.2.a). Calcium ionophore A23187 could evoke a potent response even at low concentrations. The RPMC response to the ionophore was dose-dependent (Figure 40).

4.5.2.b Compound 48/80- triggered degranulation

Cells from peritoneal lavage were incubated with serial dilutions of compound 48/80. The dose response presented an increasing linear trend in function with the 48/80 concentration. Compound 48/80 could significantly trigger the degranulation even at low concentrations, the higher obtained level of degranulation was observed at 20 µg/ml. Higher concentrations of compound 48/80 were not tested to avoid possible cytotoxic release of the granular mediator Figure 41 concentration of 10 µg/ml was used during subsequent assays were compound 48/80 was used as a stimulus.

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4.5.2.c Pam2CSK4-triggered degranulation

Cells from the peritoneal lavage were incubated with 1, 5 and 10 µg/ml of pam2CSK4. The dose response was dose related and at 10 µg/ml it reached a value of 38±1% of the total release (Figure 42). The concentration of 10 µg/ml was used during subsequent assays were compounds pam2CSK4 was used as a stimulus.

4.5.2.d PGN-triggered degranulation

The response to PGN was very limited; maximal degranulation was obtained at 10 µg/ml Figure 42. PGN is known to trigger mast cell degranulation through a TLR2-dependent pathway and previous results obtained with the soluble ligand of TLR2, pam2CSK4, suggest that the signalling pathway in RPMC is operative (paragraph 4.5.2.c). This limited response, however, can be explained with the hypothesis that PGN might not be a TLR2 ligand. As it will be explained in paragraph 4.6.3, another PPR other than TLR2 seems to be responsible for the cellular response to PGN. Other components of the outer wall of Gram-negative bacteria (TLR2 ligands themselves) often represent impurities of PGN preparations and just these chemical entities might be responsible for the observed response. Clearly, as these impurities represent a minimal percentage of the preparation, the cellular response is highly variable and limited. Due to the low percentage of MC degranulation after treatment, PGN was not used for further studies involving our novel mast cell stabilisers.

4.6 Investigation of the stabilising activity of novel indane analogues of pterosin Z on RPMC/peritoneal lavage

Compounds PH2, PH5 and their resolved enantiomers (see materials and methods) were tested on RPMC (triggered with both immunological and non-immunological stimuli). RPMC were not purified as it has been shown that isolation might compromise the ability of cells to respond to stimuli [78]. The peritoneal lavage,

however, contains other leukocytes in addition to mast cells (e.g. eosinophils, neutrophils, macrophages). The total leukocyte count of the rat peritoneal cavity is approximately 12.5×10^6 cells/(ml of exudate), of which just 0.4×10^6 are mast cells [136]. Since MC are the only cell type in the peritoneal lavage that can respond (with the release of histamine/β-hexosaminidase) to IgE and other degranulative agents, the results obtained can be attributed exclusively to RPMC. However, cell types such as macrophages and neutrophils are present as well (2.5 and 6.5×10^6 cell/ml respectively), and also express toll like receptors and produce TNF-α after LPS stimulation. Therefore, results obtained after LPS treatment of the peritoneal lavage can not be attributed exclusively to the mast cell response. In purified RPMC, even the untreated controls showed high values of TNF-α released, suggesting that the purification step might have introduced unknown "activating factors" to the system. Cells from the peritoneal lavage were used during the experiments in order to provide general information regarding the effects of PH compounds on the LPS-stimulated cytokine production.

4.6.1 *General conclusion on the effect of PH2, PH3, PH4, PH5, PH21 and PH22 on RPMC degranulation*

The effect of PH compounds on RPMC degranulation can be summarised as follows:

- all the test compounds failed to inhibit the RPMC degranulation induced by compound 48/80, their effect was not statistically different from vehicle control. DMSO inhibited the MC degranulation masking the effect of PH compounds.

- when calcium ionophore A23187 was used to trigger the RPMC degranulation, PH21 and PH22 (resolved enantiomers of PH5) inhibited the RPMC degranulation at concentrations equal to 1 μM and above. Such inhibition was not dose-related.

- When pam2CSK4 (soluble ligand of the TLR2 receptor) was used to trigger the RPMC degranulation, PH22 was

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the only compound that could effectively inhibit the release of granular mediators.

The lack of efficacy of PH compounds on compound 48/80-induced degranulation represented an unexpected result; as previously stated, structurally related compounds were proved effective in reducing RPMC response to compound 48/80 [137]. The compound 48/80-induced degranulation is a process that requires calcium as second messenger; however, secretion during this process is dependent mainly by intracellular calcium [138]. As previously shown, deprivation of extracellular Ca^{2+} did not inhibit RPMC degranulation.

In the light of this and according to what was observed for RBL-2H3 (see paragraph 4.2.1), it can be assumed that PH compounds might act on the calcium influx from the external environment, in other word they could interact with calcium channels on the membrane. This assumption is not in contrast with the observation that PH compounds inhibited RPMC degranulation in presence of calcium ionophore A23187. In fact, as previously stated (see paragraph 4.2.1) the mechanism of action of calcium ionophore A23187 involves different components, one of which acts through the activation native Ca^{2+} channels on the cellular membrane [55]. In mast cells, the calcium influx from the extracellular environment is dependant on a particular family of store operated calcium channels (SOCC). This channels, namely CRAC channels (Ca^{2+} release-activated Ca^{2+} channels), are membraneous calcium selective channels and play a crucial role during several allergic and autoimmune diseases [139]. Further studies regarding intracellular $[Ca^{2+}]$ fluctuation and CRAC conductivity in mast cells might help to elucidate the mechanism of PH compounds.

During compound 48/80-triggered degranulation, as for all the other degranulative processes in mast cells, the calcium liberated from intracellular storage or the calcium from the external environment has to bind the ubiquitous protein calmodulin (CAM) to act as second messenger [140]. As PH compounds did not work only during the

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compound 48/80-triggered degranulation, it seems possible to exclude CAM among the possible target of PH compounds.

4.6.2 *Activation of cells from the peritoneal lavage after LPS treatment*

As done for RBL-2H3 cells (see paragraph 4.4.1), cells from the peritoneal cavity were treated with sLPS (O111:B4) and the amount of IL-13 and TNF-α released was measured. As it has been explained more in detail in paragraph 4.6, in this case the response to LPS can not be attributed only to RPMC as other cells present in the peritoneal exudate (neutrophils, macrophages etc) respond to LPS with the production of TNF-α and IL-13. Several attempts of separating RPMC from other cells were performed but purified mastocytes resulted initiated to TNF-α release even in absence of LPS.

While LPS failed to trigger the production of IL-13, the amount of TNF-α released increased to sixfold; from a baseline of 155 ± 1 pg/ml it reached a maximal level of 915 ± 1 pg/ml when cells were treated with 1μg/ml of sLPS.

It can be assumed that the TNF-α released was newly synthesised as the lysate of untreated cells showed a comparable amount to the control (95 ± 2 and 155 ± 1 pg/ml respectively).

4.6.3 *TLRs in RPMC*

As shown in Figure 51, mRNA transcripts for TLR1, 2, 3, 4, 5, 6 and 8 were detected while transcripts for TLR9 and 10 were absent. TLR7 could not be investigated as none of the designed primers lead to positive results during the optimisation stage using gDNA (see materials and methods). Except for the band corresponding to TLR2, which was barely visible, all the other bands are clearly distinguishable and of the correct size (see Table 3). In the light of the TLR pattern expressed by purified peritoneal mast cells, one could expect a positive response to LPS and to TLR2 ligands.

Previous results (Figure 42) showed the ability of RPMC to degranulate after treatment with pam2CSK4 but the response to PGN was limited. The responsiveness to pam2CSK4 and the presence of TLR2, TLR1 and TLR6 (with which TLR2 forms heterodimers) suggests that the signalling pathway associated with these receptors has to be operative. The TLR2 ability to bind PGN has been part of a heated dispute and it has been proposed that another PPR might be involved in PGN recognition rather than TLR2. Girardin and co-workers [141] suggested that NOD-2 can be implicated in this process[*]. It is known that highly purified preparations of PGN loose the ability to stimulate cytokine production from target cells and this might be due to the loss of other TLR2 ligands such as lipoteichoic acid [96].

4.6.4 *Effect of PH3, PH4, PH21 and PH22 on the production TNF-α from cells from the peritoneal lavage after LPS treatment*

When cells from the peritoneal lavage were stimulated with 1 μg/ml of sLPS for 16 hours, the release of TNF-α was tenfold increased: a spontaneous release of 125 ± 2 pg/ml increased to 1231 ± 2 pg/ml . All the TNF-α released was newly synthesised as supernatants of untreated samples, lysed with Triton-X (TX in Figure 52), showed the same TNF-α content of the control (see Figure 52). None of the compounds under investigation showed an inhibitory effect on TNF-α production ($p>0.05$, $n=9$). Luteolin was used as positive control and at the concentration of 50 μM it completely inhibited the release of TNF-α. It also confirmed the previous observation(Figure 50) that the TNF-α released after LPS stimulation was newly synthesised as, the lysate of untreated cells showed a comparable amount to the control (126 ± 2 and 114 ± 1 pg/ml respectively).

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[*] The nucleotide-binding oligomerisation domain-2 (NOD-2) is the intracellular receptor for the muramyl dipeptide (MDP) component of PGN.

4.6.5 *General conclusions on the effect of PH2, PH3, PH4, PH5, PH21 and PH22 on the production of TNF-α from cells from the peritoneal lavage after LPS treatment*

The effect of PH compounds on the production of TNF-α from cells from the peritoneal lavage after LPS treatment can be summarised as follows:

+ all the test compounds failed to inhibit the TNF-α production induced by LPS treatment; their effect was not statistically different from vehicle control.

The response to LPS, at least in macrophages, is dependant on the rise of the cytosolic calcium concentration and CRAC channels are involved in the process [142, 143]. According to what was postulated in paragraph 4.6.1, if PH compounds were interfering with the CRAC conductivity to the bivalent cation, the previous result would be a neglection of such hypothesis. However, preliminary studies undertaken in our laboratory showed that PH compounds were effective in inhibiting cytokine release from jurkat cells. The above result has to be considered with care as it was obtained treating cells from a peritoneal lavage that therefore represents a heterogeneous population with different responsiveness and different intracellular pathways. Further studies regarding intracellular $[Ca^{2+}]$ fluctuation and CRAC conductivity in mast cells might help to elucidate the mechanism of PH compounds.

4.7 Comparison of the stabilising properties of PH2, PH3, PH4, PH5, PH21 and PH22 on RBL-2H3 cells and RPMC

Among the tested compounds, only PH22 could uniformly inhibit MC response to a variety of stimuli both in RBL-2H3 cells and in RPMC. As already explained, RBL-2H3 cells and RPMC belong to two different subsets of mast cells. The former are designed as MMC and the latter are addressed as CTMC (see paragraph 1.3). CTMC and MMC respond in different ways to a variety of MC stabilisers with the most known example represented by the lack of effect of DSCG on MMC. The ability of PH22 to effectively stabilise both subfamilies of mast cells gives to this compound an enormous potential as therapeutic agent. Not only PH22 has the potentiality to be a treatment in pathologies such as asthma and allergic skin conditions but also it could be particularly significant in diseases in which only MMC are involved (Chron's disease, colitis etc.). The difference between CTMC and MMC might give account of the different dose-dependency of RBL-2H3 cells and RPMC degranulation after PH21 and PH22. All the other PH compounds were proved active on either CTMC or MMC degranulation after at least one of the tested stimuli. As previously stated, similar responses often resulted in different results (in terms of statistical analysis) due to differences in the experimental errors. Further analyses are needed before concluding whether a compound is totally inactive or not on MC after challenge with a particular stimulus.

5 Conclusion and future Studies

It can be concluded that the original aim of this work was fully achieved. The effect of PH2, PH5 and their resolved enantiomers on the degranulation of mast cells induced both by non immunological stimuli (compound 48/80, calcium ionophore A23187, PGN, pam2CSK4) and by immunological stimuli (IgE/anti-IgE) was tested and interesting results were obtained. However, some protocols need further optimisation and therefore some experiment will be repeated in order to clarify the molecular mechanism of action of our novel molecules.

PH2 and PH5 were also tested as inhibitors of the production and release of interleukin such as TNF-α and IL-13 on RPMC.

RBL-2H3 was employed as model of the MMC. Unfortunately, since the early stages of the optimization of the experimental procedures, it was evident that the huge amount of information present in the literature was often inconsistent and inaccurate. Therefore, in addition to the investigation of the mast cell stabilizing property of PH2 and PH5, the aim of this thesis was extended; part of the work was dedicated to the characterization of RBL-2H3 as a model for degranulation studies.

Summarising the specific aims of this study were:

- To validate RBL-2H3 cells as an *in* vitro model to study mast cell degranulation and mast cell response to LPS
- To set up an *ex vivo* model using freshly harvested RPMC to study mast cell degranulation.
- To investigate the effect of PH2, PH5 and their resolved enantiomers (PH3, PH4, PH21 and PH22) on RBL-2H3 degranulation after immunological stimulation (IgE/antigen)
- To investigate the effect of PH2, PH5 and the resolved enantiomers on RPMC degranulation induced by immunological (IgE/antigen) and non immunological

stimuli (compound 48/80, calcium ionophore A23187, PGN, pam2csk4)

→ To compare the results obtained with RBL-2H3 cells (example of MMC) and RPMC (example of CTMC)

→ To investigate the effects of PH2, PH5 and the resolved enantiomers on the production and release of TNF-α and IL-13 after LPS and PGN treatment

The preliminary results obtained in this thesis give information regarding the effect of our molecules on rodent MC; mast cells from different species can show differential responses to stimuli and stabilisers [144]: for this reason, PH compounds should also be tested on human mast cells triggered by both immunological and non immunological stimuli. There are several protocols that describe how to obtain cultures of human mast cells from peripheral blood progenitors and umbilical cord blood with a relatively small effort [145, 146].

→ As some of the compounds tested were active on RBL-2H3 cells whose features are those of a MMC/basophil-like cell line; it would be useful to test their ability to inhibit human basophil activation triggered by both immunological and non-immunological stimuli. Basophils represent less than 1% of the white cells in the blood stream and their isolation has been hampered by many difficulties for many years. However, a three step procedure for human basophils purification has recently been developed making it easier to obtain enriched preparations of functionally intact cells [147].

→ Given the fact that the supposed mechanism of PH compounds might be to interfere with the calcium movement in the mastocyte, the analysis of how the free cytosolic calcium concentration varies when compounds are applied might give useful information. The use of ratiometric fluorescent dye, like Fura-2, could be a valid approach. Fura-2 is a polyamino

carboxylic acid which binds to free intracellular calcium; it is a widely used dye for calcium imaging for both suspended and attached cells. Fura-2 is excited at 340 nm and 380 nm of light, and the ratio of the emissions at those wavelengths is directly correlated to the amount of intracellular calcium [148].

6 Appendices

6.1 Appendix 1: DMSO quenches the fluorescence of the OPT/histamine adduct

6.1.1 Appendix 1: Introduction

DMSO can quench the fluorescence of the OPT adduct with histamine. This can result in a false positive effect of DMSO on the inhibition of the MC degranulation. Before testing the effect of our compounds, we therefore wanted to quantify the effect of DMSO on the fluorescence of the formed fluorophore.

6.1.2 Appendix 1: Materials and methods

6.1.2.a Histamine assay

The amount of histamine was assayed using a previously described fluorometric assay, [81]. Briefly, histamine reacts with o-phthalaldehyde (OPT) to form a fluorophore. To 0.5 ml of supernatant from each well, 0.1 ml of 1M NaOH and 25 µl of OPT (1% (w/v) in methanol) were added. The supernatant was, then, incubated at room temperature for 4 min. The reaction was stopped by the addition of 50 µl of 3M HCl. Precipitated proteins were removed by centrifugation and 200 µl aliquots of the supernatant were transferred into black 96-well plates. The concentration of the fluorescent product of the reaction was measured using a FLUOstar OPTIMA microplate reader set at: λ_{ex} = 360 nm and λ_{em}= 450 nm, respectively. The quenching percentage of the fluorescence was calculated according the following equation:

% quenching = (fluorescence with DMSO/fluorescence w/o DMSO) * 100

Equation A 1

6.1.3 *Appendix 1: Results and discussion*

Figure A I shows the effect of various percentages (1, 2 and 4 %) of DMSO on the fluorescence produced by a sample of 625 ng/ml of histamine treated with a solution of OPT (1% (w/v) in methanol) in basic condition (see material and methods). The fluorescence obtained in absence of DMSO (1667 A.U.) was reduced by the 17.4% when 1% of DMSO was present in the reaction mixture showing a value of 1377 A.U. The increase in the percentage of DMSO lead to a progressive decrease of the fluorescence; at 4% of DMSO the fluorescence value was of 906 A.U.
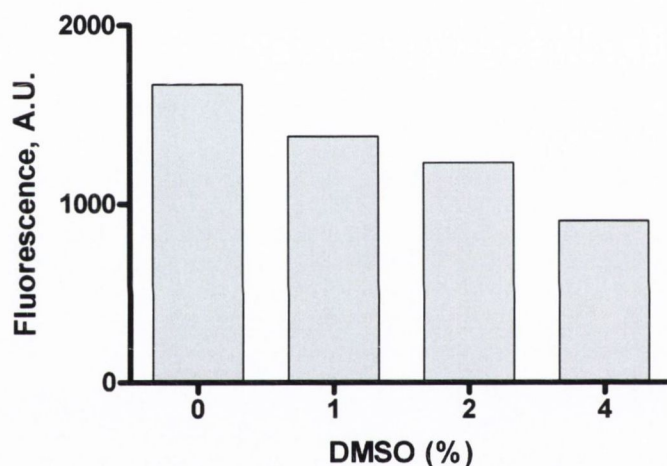
Figure A I

Effect of three different concentrations (1, 2 and 4%) of DMSO on the fluorescence of an OPT-treated sample of histamine (625 ng/ml). DMSO quenched the fluorescence of the OPT adduct in a dose-dependent manner.

	DMSO (%)	% of quenching
	4	45.6
of		
OPT	2	26.1
	1	17.4

Table A I

Percentage of quenching the fluorescence of the adduct by different percentages of DMSO.

In the experiments described in the text the concentration of DMSO was maintained at 0.5% to minimise this effect; however it appears

clear how the pronounced solvent effect shown in Figure 16 is not due to a suppressed histamine release by the vehicle but to a decreased fluorescence.

6.2 Appendix 2: Histamine content in RBL-2H3

6.2.1 Appendix 2: Introduction

Histamine is usually detected thanks to its ability to react with ortho-phthalaldehyde to give a fluorescent adduct. Unfortunately, this method suffers interference by many other substances such as other amines, ions and solvents. To avoid this limitation, the use of an enzyme-linked immunosorbent assay (ELISA) kit specific for histamine was used in parallel and results obtained with the two different techniques were compared

6.2.2 Appendix 2: Materials and Methods

6.2.2.a Cell culture

RBL-2H3 cells (1×10^5 cells/cm^2) were seeded in 24-well plates and they were incubated overnight. The day after cells were washed twice with PBS (GIBCO) and subsequently 500 µl of EBSS (GIBCO), with 0.1% bovine serum albumin (Sigma) (pH 7.4), was added to the wells. According to what has been previously reported, the total amount of histamine in samples from RBL-2H3 cultures (maintained in similar conditions), should exceed the limit of the detection of the kit used during our experiments; for this reason, samples were diluted 2.5 times (or more) before analysis. When needed cells were lysed with a solution of 10% Triton-X (Sigma). Histamine content was assayed as follows.

6.2.2.b Histamine assay

The amount of histamine was measured using a histamine enzyme-linked immunosorbent assay (ELISA) kit (Labor Diagnostika Nord, Nordhorn, Germany) according to the manufacturer's directions or by using a previously described fluorometric assay [81] (see Appendix 1).

6.2.3 *Appendix 2: Results and Discussion*

In an attempt to avoid the DMSO interference during the histamine detection (see Appendix 1), an ELISA kit was initially used to test the amount of histamine released. The total amount of histamine detected from the Triton-X lysate of RBL-2H3 cells was approximately 30 ng/ml. While this value was in accordance with the one obtained under similar experimental conditions by Nakatani and co-workers (20-45 ng/well) [149], it seemed to be far too low compared to the result obtained by Kobayashi and co-workers (0.5 mg/ml) [80].

On the other hand, Buku underlined how RBL-2H3 cells contain such a small amount of histamine which required them to use RPMC for degranulation studies using MCDP [150] and Barsumian [151] and co-workers reported a huge range (from 365 to 1276 ng/10^6 cells) of histamine content for RBL-2H3[*]. In the light of this, as all the values obtained during experiments were extremely close to the limit of detection of the kit we hypothesised that the samples were too concentrated. Supernatants were then diluted (from 2.5 to 320 times). Results obtained were inconsistent: the amount of histamine varied from 17-10 ng/ml, when supernatants were diluted 10 and 20 times respectively, to 128-300 ng/ml when supernatants were diluted 40 and 80 times. All the values obtained fell into the range of the calibration curve. Experiments were repeated several times and results were always inconsistent. There are no reasonable hypotheses to explain why the analysis of serial dilutions of the same samples lead to such different results.

When the total amount of histamine contained in the lysate was measured using the OPT method (see paragraph 2.1.2.b), the result was much higher (1494±53 ng/ml) than that obtained using the ELISA kit. The discrepancies between results obtained with the two different techniques (ELISA and OPT) made us consider the eventuality that the OPT method could suffer from interference by other analytes (for example other amines) able to react with the *o*-phthalaldehyde. Mast

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[*] RBL-2H3 were initially identified with the name RBL-IV HR[+] see Appendix 4.

cells and RBL-2H3 cells in particular, have been reported to contain serotonin [152, 153] that could react with OPT. This eventuality has an obvious implication as the kinetics of the serotonin release might differ from the one of histamine; other authors hypothesised that, not only the release of serotonin has a biphasic trend but that this amine might also be stored in other cellular compartments and that its release might be dependant on stimuli that do not affect the release of histamine [152, 154-156]. If the serotonin and histamine release is differentially regulated, test compounds might affect the release of these two amines in different ways and a selective detection technique is essential to test the stabilising properties (in terms of suppression of the degranulation) of test compounds on MC.

6.3 Appendix 3: Serotonin content in RBL-2H3 cells; is the OPT method selective for histamine detection?

6.3.1 Appendix 3: Introduction

There are reports in the literature describing the RBL-2H3 cell line as a serotonin-releasing cell line. Whether histamine and serotonin are secreted in parallel or not and if there are different pools of 5-HT has been subject of many publications [92, 152-155].

Reports show that the release of histamine can be inhibited by amitriptyline while the release of serotonin is unaffected, suggesting that two different pools are present in mast cells [154]. In the same year the same author published a paper (following the discovery of two different serotonin binding proteins that require different conditions for extraction) in which he confirmed the existence of separate 5-HT pools. However, the electron microscope radioautographic analysis of the intracellular distribution of [^{3}H]serotonin proved that all the labelled serotonin was accumulated within cytoplasmatic granules [152]. A possible explanation for this observation could be that both pools might be intragranular; one pool might be associated with the granule membrane and the other might be localised within the granular core.

This hypothesis, however, is not universally accepted as it has been proposed that the release of histamine is in parallel to the one of serotonin but the reuptake of the latter by mast cells is preferred to the one of histamine [92]. The possibility that the release of histamine/serotonin could not be in parallel and/or that our compounds could affect the release of the two mediators to different extents (like the above-mentioned amitriptyline) lead us to run the opt assay for both the amines to test the selectivity of the method/methodology for histamine.

6.3.2 *Appendix 3: Materials and Methods*

6.3.2.a *Appendix 3: Serotonin detection through HPLC*

6.3.2.a.1 *RBL-2H3 sample preparation*

RBL-2H3 cells were cultured for two days in α-MEM as previously described (see paragraph 2.1.1); after two days in culture RBL-2H3 cells were detached using trypsin-EDTA and counted using an emocytometric chamber. Aliquots containing 1×10^6 cells were washed twice in PBS. Cells were resuspended in 0.5 ml of the mobile phase used for the HPLC analysis (see paragraph 6.3.2.a.1.1) and they were sonicated using a Branson sonifier 150 (Fisher scientific, Dublin, Ireland). The lysate was then filtered using Phenex-NY 4mm syringe filtes with 0.45 μm pores (Phenomenex, Cheshire, UK) and analysed through HPLC to detect the amount of serotonin.

6.3.2.a.1.1 *HPLC protocol*

The HPLC analysis was conducted with an automated HPLC system (Shimadzu ADVP module). Injection volumes were 20 μl into a reverse phase analytical column (C18 column, specific area surface 250mm x 4mm, particle size 5.0 μm, Phenomenex) heated to 30 °C and protected with a guard column (Lichrosorb RP18, specific surface area 30 x 4mm, Phenomenex). An electrochemical detector (Digital Electrochemical Amperometric Detector, Mason Technology Ltd.) was connected to the automated HPLC 28 system. The flow rate was 1.2ml/min (LC-10AT pump, Shimadzu) and the acquisition time was 50 minutes. CLASS-VP software (Shimadzu) was used in the acquisition and integration of the chromatographs. The signal to noise ratio was 3 to 1.

Peak heights compared with internal and external standards allowed quantification of concentrations.

The mobile phase consisted of 0.1M citric acid monohydrate (Sigma), 0.1M sodium di-hydrogen phosphate monohydrate (Merck), 1.4mM octane-1 sulfonic acid (BDH) and 0.1M EDTA disodium salt dehydrate

(Sigma) dissolved in double-distilled NANO-pure HPLC grade water (Sigma). The pH was adjusted on pH meter (Jenway) to 2.8 by the addition of 5M NaOH. Monoamine standards (dihydroxyphenylacetic acid, 5-hidroxyindoleacetic acid, 5-hydroxytryptamine and N-methyl-5-hydroxytryptamine) were all obtained from Sigma Aldrich. Standard solutions of 10mg/10ml were prepared by dissolving standards in 10ml of HPLC mobile phase and stored refrigerated in glass volumetric flasks. A 10ml solution of each standard and a standard mix containing 5ng/20 μl of each standard was also prepared.

6.3.2.b *Appendix 3: Histamine/serotonin detection through spectrofluorometric method (OPT)*

Serial dilutions (from 5000 to approximately 78 ng/ml) of samples containing either histamine or serotonin were prepared. The amount of histamine/serotonin was assayed using a previously described fluorometric assay, [81] (see Appendix 1).

6.3.3 *Appendix 3: Results and discussion*

The HPLC analysis confirmed that RBL-2H3 is a serotonin containing cell line. The total amount of serotonin detected from cell lysates (1 x 10^6 cells/sample) was 93 ± 15 μg/10^6 cells.

To ensure that the serotonin released from RBL-2H3 during degranulation does not interfere with the detection of histamine during the spectrofluorometric analysis, samples prepared diluting a standard solution of commercially available serotonin or histamine were analysed with the OPT method.

Figure A II shows how the fluorescence of histamine samples (■) treated with OPT had a linear trend with the concentration of the amine. Serotonin, on the other hand did not react with OPT, the fluorescence obtained did not vary with the concentration of the amine and its value was the same of the background (■).

The discrepancy found for the histamine level in RBL-2H3 cells among the ELISA and the OPT method (see Appendix 2) can not to be

attributed to the interference of serotonin during the spectrofluorometric analysis. Comparison between results obtained measuring the percentage of degranulation through the detection of histamine (OPT) and the detection of β-hexosaminidase leads to overlapping results suggesting that the fluorometric analysis of the samples is a reliable tool to monitor MC degranulation. At this time it is not possible to give an explanation for the different results obtained with the ELISA kit and the OPT. However the incongruence found when different dilutions of the same samples were tested with the ELISA kit (see appendix 3) rise doubts on the reliability of the kit.
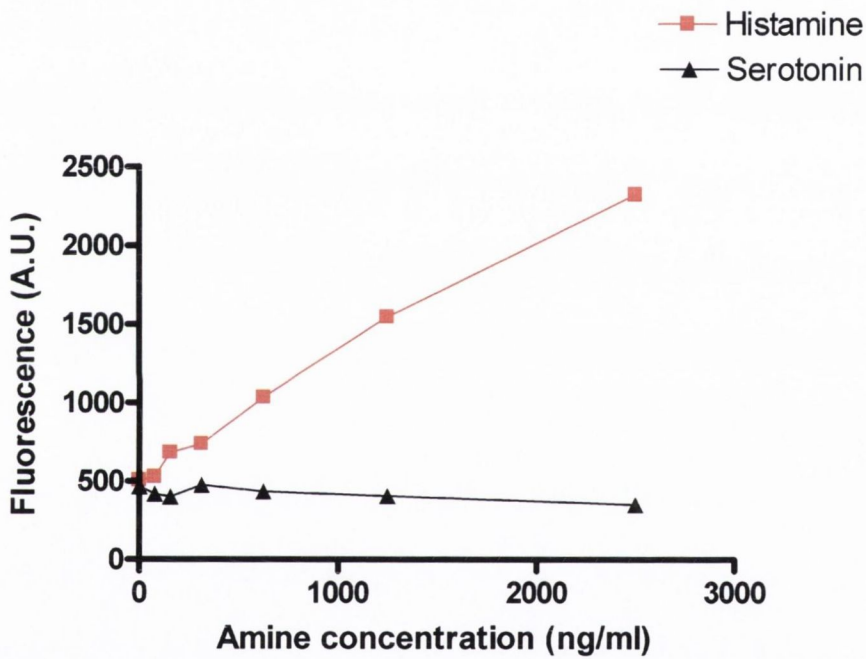
Figure A II

6.4 Appendix 4: RBL-2H3 cells: really mast cells or basophils?

6.4.1 Appendix 4: Introduction

As anticipated in the text, for many years investigation into mast cell biology was restricted to CTMC because they were easy to obtain particularly from the peritoneal cavity; on the other hand, mucosal mast cells and basophils were disregarded due to the difficulties to isolate these two cell types. It is not surprising if during this stalemate in research, the sudden availability of a cell line, namely RBL, presenting typical characteristics of both MMC and basophils kindled the enthusiasm of researchers. The most commonly used variants of RBL cells are the RBL-2H3 cells. This cell line is generally described in the literature as a mast cell line. RBL-2H3 cell line has been commonly used to model mast cell physiology, in particular, studies on binding of IgE to FcεRI receptors and subsequent downstream events. However, since its introduction, there has been a rapid proliferation of often contradictory and sometimes misleading data and the purpose of this review is to analyse the various reports in the literature and to make an assessment of this cell line as a potential model for the mast cell.

6.4.2 Appendix 4: The relationship between mast cell and basophils

Basophils (BS) and mast cells (MC) are two functionally similar but distinct cell types that play a pivotal role in the initiation and development of type I hypersensitivity reactions. Granulocyte basophils are circulating cells representing less than 1% of the white cell population while mature mast cells can be found exclusively in tissues and in particular in regions located at interfaces with the external environment such as lungs, skin and mucosal surfaces. Despite their similarity for many years it was postulated that these two cell types did not derive from common progenitors but they descended from different lineages [6, 157]; subsequently various objections to this hypothesis

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have been put forward suggesting a possible common origin [6, 9, 13, 158] (see paragraph 1.2)

After crosslinking of their IgE-bound FcεRI by allergens, both BS and MC release a range of preformed and newly synthesised mediators that evoke a potent immune allergic response. While preformed mediators such as histamine, heparin, chemotactic factors, tryptases and chymases, are stored in granules and released upon exocytosis, newly synthesised mediators like prostaglandin D2 (PGD2), the leukotrienes C4 and D4 (LTC4, LTD4) and platelet activating factor (PAF) are produced and secreted after cell stimulation [159].

It is also well established that both mast cells and basophils take part in the adaptative immune response to pathogens. MC due to their "sentinel locations", at the interface of the body with its external environment such as the lungs and intestine, are the first activators of the inflammatory response, not only releasing cytokines and chemoattractants for other immune cells such as neutrophils and macrophages but also phagocytosing invading pathogens and presenting the antigen to T cells. The role of basophils during host defence is less well known but they are thought to sustain the inflammatory process with the release of immunoregulatory cytokines such as IL-4, IL-13 and TNF-α [160, 161].

6.4.3 *Appendix 4: the provenance of the RBL-2H3 cell line*

Rat basophilic cells were originally obtained by Eccleston and co-workers in 1973 when one of the rats treated with the potent carcinogen [2-(α-chlor-β-isopropylamine)ethylnaphtalene] * developed a rare (at that time unknown) form of granulocytic leukaemia characterised by a considerable peripheral blood basophilia [162]. This basophilic leukaemia progressed rapidly and death resulted from pulmonary haemorrhages caused by the release of heparin from infiltrated basophils. Such infiltration also occurred in the liver, spleen, lymph

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* [2-(α-chlor-β-isopropylamine)ethylnaphtalene] (ICI 42464) also known as β-chlorethylamine can induce myeloid or lymphatic leukaemia in newborn rats, when 0.1% of the compound is added to the diet.

nodes and bone marrow, the latter producing a reduction of other marrow elements. The cytochemical analysis of the infiltrated granulocytes confirmed that the features of the cells were those of basophils (BS); These leukaemic cells showed basophilic granules containing histamine, serotonin and chemotactic factors, possessed cytological features that were different from those typical of mastocytes and the Harada's method for the specific staining of mast cell resulted negative. Moreover mast cell count was reduced [70].

The basophilic leukaemia was serially transplanted for 20 generations by i.p injection of heparinised whole blood without a change of phenotype. On the other hand, sub-cutaneous injection of leukaemic cells induced solid tumours consisting almost entirely of basophils [70]. The opportunity to transplant the tumours and to obtain a large number of basophils, signalled a turning point in the cytochemical and biochemical study of this particular rare type of granulocyte. Cells from the transplanted tumours were adapted to culture by Kulczycky and co-workers [163]; cultured cells maintained the ability to initiate basophilic tumours after subcutaneous injection into newborn rats. Cells from this new cell line, called RBL-1, presented unquestionable advantages like manipulability, higher percentage of viability and homogeneity and they were also easier to obtain than cells from tumours and for these reasons, RBL cells were extensively used for routine studies to investigate the interaction of IgE with receptors for the Fc portion of immunoglobulin-E, today known as FcεR [164]. The first attempt in this direction was carried out by Kulczycki and co-workers [163, 164]. They started their study comparing the capacity of RBL-1 and mouse mastocytoma cells to deplete the rat immune sera of PCA[*] activity. Results showed that RBL-1 cells were more effective than mastocytoma cells to deplete the reagenic activity of rat sera. On the other hand, RBL-1 cells had approximately a similar effect to that reported for normal rat peritoneal mast cells. Furthermore it was highlighted, for the first time, that RBL could bind IgE with high affinity. By 1974, due to their similarity with basophils and with mast cells (mucosal), rat

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[*] Passive cutaneous anaphylaxis

basophilic leukaemia cells were an important *in vitro* tool to try to delineate some of the *in vivo* phenomena related to IgE sensitisation. The key feature of this cell line was to provide a flexible tool to build the mainstay of an entire stream of research that lead to elucidate the early events of cell secretion.

Unfortunately RBL-1 failed to give a response after IgE/antigen sensitisation; this particular cell line was therefore disregarded for further studies regarding exocytosis [165]. However, the potential of such an *in vitro* model pushed researchers, between 1973 and 1975, to develop various cloned sublines with different releasing characteristics; cell lines started at different times during the tumour development not only differed in chromosome number but also in their ability to release histamine by IgE-mediated stimulation. In particular, a cell line called RBL-HR$^+$, derived from RBL-IV, showed a percentage of total histamine release of $39 \pm 13\%$ [151]. The development of RBL-HR$^+$, however, was clouded by a misleading interpretation: in 1977 Metzger and co-workers reported to have established a new mastocytoma cell line while attempting to induce myeloma tumors with Abelson virus and pristine in mice. This cell line, called AB-CBF$_1$-MCT-1 (or MCT-1) was found to release histamine after IgE/antigen sensitisation and attracted the interest of the group due the possibility of having a robust *in vitro* model to study IgE-mediated mast cell exocytosis [166]. Later, the same group reported that the MCT-1 cell line did not arise from mouse but had its origins in the rat [167]. The observation that i.p. inoculation of "MCT-1" cells in Wistar rats did not result in mastocytomas but in basophilic tumors, that the electrophoresis mobility of lactic dehydrogenase of "MCT-1'' was typical of the rat enzyme and that the chromosomal analysis had similar pattern to RBL-1 proved that "MCT-1" was a subline of the rat basophilic leukaemia cell line, at that time the object of investigation by that very same lab. Since it presented the important characteristic of being a histamine releasing cell line, it was rebaptised RBL-HR$^+$. Subsequently, cells from RBL-HR$^+$ were cloned by the limiting dilution method in an attempt to reduce cell heterogeneity and the RBL-2H3 cell line was then isolated. While the origin of RBL-HR$^+$ was clarified, the

misleading conclusion that RBL are mast cell-like cells remained. The original concept that these rat basophilic leukaemia cells originated from a basophilic tumour and their features were those of basophils [70, 168] seems to have been largely forgotten.

Basophils and mast cells are functionally similar and share many similarities: for example both cell types stain metacromatically with alcian blue, have similar granule content and a route of degranulation ([10, 169]); moreover, mature basophils and basophil myelocytes can often be confused with mast cells [170]. However BS and MC present some ultrastructural differences that allow the differentiation of the two cell types [171]. In particular basophils are reported to resemble the class of MC called ''mucosal mast cells'' (MMC): both cell types (BS and MMC) have been reported to be insensitive to compound 48/80 and to the common mast cell stabiliser DSCG [172, 173], to degranulate after calcium ionophore A23187 challenge and to be stabilised by ketotifen and quercetin after IgE/antigen sensitisation [115, 174]. On the other hand, they are different in many aspects such as expression of membrane glycoproteins [170], expression of chemotactic receptors, different pathways of recruitment [175], distinct differentiation patterns [9], presence of crystal granules and other ultrastructural characteristics [171]. In recent years it has been shown that MMC and BS respond in different ways to LPS (via TLR4) [132, 176] and show a different expression pattern of other TLRs [131].

6.4.4 *Appendix 4: functional characteristics of RBL-2H3 cells*

RBL-2H3 have been widely used by many groups in an extremely broad range of applications, from degranulation studies and investigation of many mast cell stabilisers to physical-chemical properties of the FcεR and its interaction with the cytoskeleton [79, 94, 97, 99, 135, 163, 164]. RBL-2H3 cells present the unquestionable advantage of being an easy to cultivate cell line and a large number of homogeneous cells can be easily obtained. However, different laboratories have reported conflicting results and data obtained with this

cell line have to be interpreted with the necessary care. The discrepancies between results from different laboratories might be related to the fact that RBL cell lines from different laboratories but derived from the same basophilic leukaemia, for example, have been shown to express different densities of receptors for IgE with different molecular weights [133, 134]. This has obvious implication for the extent of degranulation; as the degree of FcεRI aggregation by IgE/antigen complexes and the manner of clustering of such complexes play a fundamental role in inducing or inhibiting secretion from this cells [71, 97, 105].

6.4.5 *Appendix 4: conclusion*

Since RBL-2H3 cells are tumorous cells, they obviously present aberrant characteristics and abnormalities. Additionally, culture conditions can affect their phenotype leading to different strains, and even within the same strain, experimental conditions can have a profound effect on their function. RBL-2H3 cells show many common characteristics to both MMC and basophils; they present similar responses to LPS to basophils but diverge from both cell types in their TLR expression [177]. While RBL-2H3 cells resemble mucosal mast cells in some respects, and display their basophilic lineage in others, their true nature lies somewhere in between. This, coupled with the fact that their functionality may differ in different laboratories suggests that results obtained from studies with this cell line may not reflect primary cell physiology.

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