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Investigations into the Anti Allergy Properties of Novel Benzocycloalkanone Based Compounds

Orla Woods B.A. (Mod.)

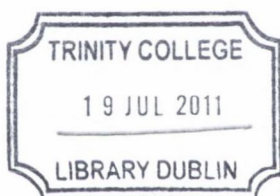
A Thesis Presented to the University of Dublin for the
Degree of Doctor of Philosophy



Based on Research Carried Out Under the Supervision of
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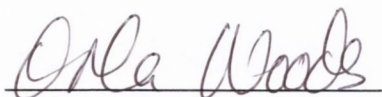
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A handwritten signature in dark ink, appearing to read 'Orla Woods', is written over a horizontal line.

Orla Woods

Summary

An allergic response occurs because of a hypersensitive response by mast cells to an otherwise innocuous environmental substance. Allergen challenge causes mast cell degranulation, resulting in the release of mast cell mediators, including histamine, which interact with their receptors to induce the symptoms of allergy. Mast cell stabilising drugs inhibit the release of these mediators by preventing the activation of mast cells upon allergen challenge, while histamine receptor 1 (H1R) antagonists act at the H1R to prevent histamine binding.

The primary aim of this project was to evaluate the mast cell stabilising activity of novel benzocycloalkanone-based compounds. Accordingly, these compounds were assessed *in vitro* using the rat peritoneal mast cell assay on Percoll-purified mast cells. Histamine release, elicited by incubating mast cells with calcium ionophore A23187 or compound 48/80, was quantified by fluorescence detection using *o*-phthaldialdehyde (OPT). Results indicate that several novel compounds assessed possess *in vitro* mast cell stabilising activity superior to that of disodium cromoglycate (DSCG), a commonly prescribed mast cell stabiliser.

Compounds which demonstrate potent *in vitro* mast cell stabilising activity were then evaluated for possible *in vivo* activity using passive cutaneous anaphylaxis (PCA), which quantifies IgE-mediated mast cell degranulation by measuring histamine-induced vascular permeability following its release from mast cells. Several novel compounds demonstrate potent PCA activity which is equivalent to that of DSCG. Two representative compounds, TZ2.01 and TZ2.04, which demonstrate potent PCA activity, were assessed for systemic toxicity using the SHIRPA behavioural observation protocol. These compounds did not exhibit any evidence of neurotoxicity, central nervous system stimulating or depressing, or autonomic nervous system modulating activity at the dose administered.

When several test compounds, which demonstrated potent PCA activity, were subsequently assessed for *in vitro* mast cell stabilising activity they surprisingly appeared to induce histamine release. We initially hypothesised that these compounds may be interfering with the fluorescence method of histamine analysis and therefore decided to devise a novel liquid chromatography/mass spectrometry (LCMS) method to quantify histamine release from RPMCs. This method was fully validated according to European Medicines Agency guidelines and complies with validation criteria of lower limit of quantitation, accuracy, precision, calibration

curve, selectivity, carry-over, dilution integrity and stability. Histamine release from mast cells was quantified following derivatisation using dansyl chloride. The data obtained using this method of analysis confirmed that this particular series of test compounds stimulate histamine release from mast cells. TZ2.13, designed specifically as a more hydrophilic derivative of this series, was subsequently evaluated and shown to be a potent mast cell stabiliser.

As several compounds which inhibited the PCA response *in vivo* elicited histamine *in vitro* they were assessed for H1R antagonist activity using the guinea pig ileum model for H1R antagonist activity. This was carried out to investigate a possible alternative mechanism of action for these compounds. In the guinea pig ileum model several compounds demonstrated non-competitive H1R antagonist activity. As a result of this observation TZ2.05, a non-competitive H1R antagonist, was also investigated for *in vivo* anti-pruritic behaviour using compound 48/80 induced scratching behaviour. TZ2.05 demonstrated anti-pruritic activity which may be useful in the treatment of conditions characterised by allergic pruritis such as atopic dermatitis.

In conclusion, this work has identified novel benzocycloalkanone-based compounds which display anti-allergic activity. Of the several series of compounds evaluated, many display superior *in vitro* activity than that of the mast cell stabiliser DSCG, while their activity *in vivo* is comparable to this drug. Compounds which display PCA activity but do not stabilise mast cells *in vitro* have been shown to demonstrate non-competitive H1R antagonist activity, while at least one of these agents also possesses anti-pruritic activity. The emergence of several potent anti allergy compounds from these studies is encouraging and indicates further investigations must be carried out to determine their true potential as anti-allergy agents.

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This thesis is dedicated to my parents and to my Nana

Abbreviations

Å	Angstrom
μA	Microamperes
A.D.	Anno Domini
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
B.C.	Before Christ
BCR	B Cell Receptor
BMI	Body Mass Index
<i>B. Pertussis</i>	<i>Bordetella pertussis</i>
BSA	Bovine Serum Albumin
BSSA	BSA Wash Buffer
°C	Degrees Celsius
Ca ²⁺	Free Calcium
cAMP	Cyclic Adenosine Monophosphate
CCL	Chemokine Ligand
CCR	Chemokine Receptor
CD	Cluster of Differentiation
CD117	c-Kit Receptor (SCF Receptor)
CD40L	CD40 Ligand
CEA	Chicken Egg Albumin
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane
	Conductance Regulator
CHO-K1	Chinese Hamster Ovary Cell Line
Cl ⁻	Free Chloride
cLogP	Computed Log(Partition Coefficient)
cm	Centimetre
CNS	Central Nervous System
COX	Cyclooxygenase
CRAC	Calcium Release-Activated Calcium
	Channels
CV	Coefficient of Variation
CXC	Chemokine Ligand
1,7-DAH	1,7-Diaminoheptane
D-1,7-DAH	Dansyl 1,7-Diaminohepatane

2D	Two Dimensional
DAG	Diacylglycerol
DC	Dansyl Chloride
DCs	Dendritic Cells
DH	Dansyl Histamine
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSCG	Disodium Cromoglycate
EAE	Experimental Autoimmune Encephalomyelitis
ELAM-1	Endothelial-Leukocyte Adhesion Molecule-1
ELISA	Enzyme Linked Immunosorbent Assay
EMA	European Medicines Agency
ER	Endoplasmic Reticulum
ESI	Electrospray Ionisation
FcεRI	High Affinity IgE Receptor
FcεRIα	High Affinity IgE Receptor α Subunit
FcεRIβ	High Affinity IgE Receptor β Subunit
FcεRII	Low Affinity IgE Receptor
FcγRII	Low Affinity IgG Receptor
FDA	U.S. Food and Drug Administration
g	Average Acceleration Produced by Gravity
g	Gram
GC	Gas Chromatography
GDP	Guanosine Diphosphate
GM-CSF	Granulocyte-Macrophage Colony- Stimulating Factor
GP	Guinea Pig
GPCR	G Protein Coupled Receptors
GTP	Guanosine Triphosphate
H+	Hydrogen Bond
HBSS	Hank's Balanced Salt Solution
HMC-1	Human Mast Cell Line-1
H1R	Histamine Receptor 1
H2R	Histamine Receptor 2
H3R	Histamine Receptor 3
H4R	Histamine Receptor 4

HPLC	High Performance Liquid Chromatography
IC ₅₀	Concentration at which 50% Inhibition of Maximum Response Occurs
ID ₅₀	Dose at which 50% Inhibition of Maximum Response Occurs
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMB	Irish Medicines Board
I.P.	Intraperitoneal
IP3	Inositol-1,4,5-triphosphate
IS	Internal Standard
ISAAC	International Study on Asthma and Allergic in Childhood
ITAM	Immunoreceptor Tyrosine Based Activation Motif
I.V.	Intravenous
Kg	Kilogram
KL	Kit Ligand
LCMS	Liquid Chromatography Mass Spectrometry
LLOQ	Lower Limit of Quantitation
LogD _{7.4}	Distribution Coefficient at pH 7.4
LogP	Log(Partition Coefficient)
LT	Leukotrienes
M	Molar
M	Molecular Ions
M+H	Protonated Molecular Ions
MAPK	Mitogen Activated Protein Kinase
MCM	Mast Cell Medium
MCP-1	Monocyte Chemotactic Protein-1
MC _T	Mucosal Mast Cells
MC _{TC}	Connective Tissue Mast Cell
mg	Milligram
MGF	Mast Cell Growth Factor
MHCI	Major Histocompatibility Complex Class I
MHCII	Major Histocompatibility Complex Class II
µg	Microgram

μl	Microlitre
ml	Millilitre
mm	Millimetre
μM	Micromolar
mM	Millimolar
mol	Mole
MS	Multiple Sclerosis
MS	Mass Spectrometry
MS/MS	Selected Ion Monitoring
m/z	Mass to Charge Ratio
n	n Number; number of experiments/ subjects
ng	Nanogram
NK	Natural Killer Cell
nM	Nanomolar
nm	Nanometre
NSAIDs	Non-steroidal Anti-Inflammatory Drugs
OPT	o-Phthaldialdehyde
P	P value
PAF	Platelet Activating Factor
PCA	Passive Cutaneous Anaphylaxis
pg	Picogram
PG	Prostaglandins
pH	The measure of Acidity or Alkalinity
PI3K	Phosphatidylinositol-3-OH Kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKC	Protein Kinase C
pKa	Acid Dissociation Constant
PLC	Phospholipase C
P.O.	Per Oral
PSA	Two Dimensional Polar Surface Area
PSI	Pound Force per Square Inch
QC	Quality Control
QSAR	Qualitative Structure Activity Relationship
r ²	Coefficient of Determination; Goodness of Fit
RA	Rheumatoid Arthritis

RBL-2H3	Rat Basophilic Leukemia Cell Line
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
rpm	Rotations per Minute
RPMC	Rat Peritoneal Mast Cell
SAR	Structure Activity Relationship
SCF	Stem Cell Factor
SD	Standard Deviation
SEM	Standard Error of the Mean
SHIRPA	The SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; Imperial College School of Medicine at St. Marys'; Royal London Hospital, St. Bartholomew's and the Royal London School of Medicine; Phenotype Assessment
SNAP	Soluble N-ethyl-Maleimide-Sensitive Factor-Attachment Proteins
SNARE	Soluble N-ethyl-Maleimide-Sensitive Factor-Attachment Protein Receptor
SNP	Single Nucleotide Polymorphism
t	Time
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th0	Naïve T Helper Cells
Th1	T Helper 1 Cells
Th2	T Helper 2 Cells
TLC	Thin Layer Chromatography
TNF	Tumor Necrosis Factor
TOF	Time of Flight
TRPC	Transient Receptor Potential Channels
TX	Thromboxanes
UV	Ultraviolet
VAMP	Vesicle Associated Membrane Protein
VEGF	Vascular Endothelial Growth Factor
V	Volts
ω 6	Omega 6
WAO	World Allergy Organisation

WFI
w/v

Water for Injection
Mass/Volume

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Chapter 1

Introduction

1.1 Allergy Throughout History

Although allergy is considered to be a twentieth century phenomenon, by all accounts it has affected humans for millennia. What seems to be the first documented case of allergy occurred in Egypt, where it is said King Menses (the first Egyptian Pharaoh) was killed by a wasp sting sometime between 3640 BC and 3300 B.C. (Ader 2007). In the first century A.D. Emperor Augustus, Emperor Claudius and Britannicus, all of the Roman Julio-Claudian family, were thought to have suffered from asthma with seasonal allergic rhinitis, with atopic eczema and perennial rhinoconjunctivitis and with allergy to horses respectively (Ring 1985). Thomas More wrote in his biography "History of King Richard III" that in Tudor England King Richard III was allergic to strawberries (More 2005).

1.2 The Modern Era of Allergy

The modern era of allergy began in the 19th century when in 1819 Dr. John Bostock first presented the clinical symptoms of hay fever to the Royal Medical and Chirurgical Society of London. He described it as a disease of the upper respiratory tract and although he re-named the condition "catarrhus aestivus" some years later, the term hay-fever was already in common usage and therefore it remained (Bostock 1819). Since then much research has been conducted in the area of allergy and atopic disease; in 1913 Charles Robert Richet was presented with the Nobel Prize for Medicine in recognition of his work on anaphylaxis and in 1982 Sune K. Bergstrom, Bengt I. Samuelsson and John R. Vane were awarded the Nobel Prize in Physiology and Medicine for their work in discovering prostaglandins, a subclass of which, leukotrienes, act as the "slow reacting substance of anaphylaxis" (Nobel 2009).

1.3 Worldwide Incidence of Allergy

Allergic diseases include asthma, allergic rhinitis (hay fever), allergic conjunctivitis, atopic dermatitis (eczema), allergic sinusitis, urticaria (hives), food, drug and insect allergies and anaphylaxis. Despite the incidence of allergy and atopic disease throughout history, it is apparent that the numbers of people suffering with allergic diseases have increased dramatically in recent times; in Europe (WHO 2003) as in the United States of America (AAFA 2009), the prevalence of asthma, allergic rhinitis and eczema have increased during the second half of the 20th century. In America this increase is seen across all age, sex and racial groups (AAFA 2009). The World Allergy Organisation estimates that in some parts of the industrialised world up to 50% of the population are affected by IgE-mediated allergic diseases

(WAO 2010c). In the United States of America alone over 50 million people suffer from allergy (total population 309 million people (USCB 2010)) (NIAID 2003).

1.3.1 Asthma

It is estimated that the number of people affected by asthma worldwide has doubled since 2002, currently over 300 million people are affected and it is the most common chronic disease among children (WHO 2009). In Western Europe, the incidence of asthma has doubled in ten years; around 8% of the Swiss population suffers from asthma compared to 2% 25-30 years ago (WAO 2009). In America alone 16.4 million adults and 7 million children suffer with asthma (CDC 2008) and in 2005 255,000 people died of asthma worldwide (WHO 2009). It is a common misconception that diseases of allergy are "Western" diseases when in fact it is in low- and lower- middle income countries that the highest number of asthma related deaths occur each year (WHO 2009).

1.3.2 Allergic Rhinitis

According to the World Allergy Organisation (WAO) 115 million people worldwide suffer with allergic rhino-conjunctivitis (a co-occurrence of allergic conjunctivitis with allergic rhinitis) (WAO 2009). The International Study on Asthma and Allergies in Childhood (ISAAC) found that between 0.8% and 14.9% of 6-7 year olds and between 1.4% and 39.7% of 13-14 year olds suffer with allergic rhinitis (WAO 2009). It is difficult to establish the worldwide prevalence of allergic rhinitis in adults because an international study on the topic has not yet been conducted. However, currently 5.9% of adults in France, 29% of adults in the United Kingdom (WAO 2009) and up to 30% of adults in the United States of America (AAAAI 2009) suffer with allergic rhinitis. Other countries with a high prevalence of the disease include Australia and New Zealand while countries such as Greece, Romania and Indonesia have a very low incidence of this disease (WAO 2009).

1.3.3 Atopic Dermatitis, Food Allergy and Drug Allergy

The prevalence of atopic dermatitis in the general population is 2-5% although this rises to 10% in children and young adults (WAO 2010a). It currently affects approximately 9% of Americans (AAAAI 2009) and in England eczema, hay fever and asthma are among the most common chronic diseases of childhood; in 1996 24% of children between 2-15 years old were diagnosed with eczema, 21% with asthma and 9% with hay fever (ONS 2009).

Food hypersensitivity reactions affect up to 2.5% of the general population, 8% of children under 3 years and up to 30% of children who suffer from atopic dermatitis

(WAO 2010b). In 90% of cases these allergies accounted for by only eight foods; milk, eggs, peanuts, tree nuts, wheat, soy, fish and shellfish (AAAAI 2009). Up to 10% of adverse drug reactions are allergic in nature and penicillin is the most common cause of drug-induced anaphylaxis (AAAAI 2009).

1.3.4 The Incidence of Allergy in Ireland

Ireland, with a total population of 4.5 million people (CSO 2010), has an extremely high incidence of allergy; in fact Ireland has the fourth highest prevalence of asthma in the world (470,000 people), behind Australia, New Zealand and the United Kingdom (ASI 2009). In Ireland around 80 people die each year from asthma and 30% of these deaths occur in asthmatics who are under 40 years old (ASI 2009).

It is estimated that overall up to 15% of people in Ireland suffer with allergic rhinitis (Jackman 2009) with 12% of children suffering from the condition (rising to 36% in those with asthma) and it is more prevalent in urban than rural areas. Eczema has an incidence of 7% in Irish children, rising to 21% in those with asthma (Taylor, Holland *et al.* 1996).

It is clear that the spectra of allergy and atopic diseases are major world health problems and that the numbers of people affected by these diseases and conditions are increasing. It is vital the mechanisms of these diseases are understood so they may be manipulated, properly treated and eliminated in the future. Until that time it is essential that they are controlled with the use of drugs and other treatments.

1.4 Mast Cells

Mast cells play a crucial role in the onset of the allergic response, which occurs when mast cells undergo a hypersensitive response to certain environmental antigens or allergen; this is known as type I, or immediate, hypersensitivity, the mechanisms of which will be discussed later.

Mast cells were first described by Paul Ehrlich in 1878 in his doctoral thesis which was entitled "Contributions to the Theory and Practice of Histological Staining" (Ehrlich 1878). Here he described "mastzelle" as cells which contain granules that respond in a characteristic manner to aniline dyes, resulting in metachromasia of these granules. He wrongly assumed that mast cells exist to nourish other cells, but otherwise his description of them was remarkably accurate. He subsequently won the Nobel Prize for physiology or medicine in 1908 in recognition of his work on immunity (Nobel, 2009).

1.4.1 Mast Cell Development

Mast cells arise from CD34+ (Kirshenbaum, Kessler *et al.* 1991), CD117+ (c-kit) (Nocka, Buck *et al.* 1990), CD13+ (Ochi, Hirani *et al.* 1999) pluripotent haematopoietic bone marrow progenitor cells (Kitamura, Shimada *et al.* 1979). Mast cell precursors express low levels of FC ϵ RI α (Ochi, Hirani *et al.* 1999). Mast cell precursors are present at embryonic stages of development (Kitamura, Shimada *et al.* 1979) as well as in adult bone marrow (Kitamura, Shimada *et al.* 1977), skin (Matsuda, Kitamura *et al.* 1981) and peripheral blood (Kitamura, Hatanaka *et al.* 1979) indicating their circulation in the blood before their migration to peripheral tissues where they undergo maturation. Mast cell precursors express chemokine receptors CXCR2, CCR3, CXCR4 and CCR5 and interaction with their respective substrates results in their chemotaxis (Ochi, Hirani *et al.* 1999). Mast cell precursors as well as mature mast cells adhere to membranes by binding to the tissue matrix component laminin, this allows their localisation in specific tissues such as the heart, blood vessels, muscosa and lungs etc. (Patella, Marino *et al.* 1995).

1.4.2 The effect of Cytokines on the Development of Mast Cells

Cytokines are crucially involved in the modulation of mast cell differentiation, proliferation and growth, survival, migration and mediator release. These cytokines include stem cell factor (SCF), interleukin (IL)-3, IL-4, IL-6, IL-9 and IL-10; SCF is considered one of the most important.

SCF (also known as kit ligand (KL), mast cell growth factor (MGF) and steel factor (Huang, Nocka *et al.* 1990; Williams, Eisenman *et al.* 1990; Zsebo, Williams *et al.* 1990), is a 30 kd protein which is produced by fibroblasts (Nocka, Buck *et al.* 1990) and is expressed as membrane bound protein or as soluble growth factor (Anderson, Lyman *et al.* 1990). Its receptor is c-kit (CD117), a member of the transmembrane tyrosine kinase III receptor family (Lemura, Tsai *et al.* 1994) which is expressed on immature and mature mast cells (Nocka, Buck *et al.* 1990).

1.4.2.1 Cytokines and Mast Cell Differentiation

SCF is required for the maturation of CD34+ mast cell progenitors to mature mast cells (Kirshenbaum, Goff *et al.* 1992) although other growth factors such as IL-3, regulates the maturation of mast cells (Ihle, Keller *et al.* 1983), are also necessary (Grabbe, Welker *et al.* 1994). IL-6 and tumor necrosis factor (TNF)- α induce mast cell development from spleen cells while IL-1, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), transforming growth factor (TGF)- β and mast cell growth factors IL-9 and SCF fail to do so. IL-9 interacts with and promotes the

growth and differentiation of haematopoietic progenitor cells (Yang, Ricciardi *et al.* 1989; Donahue, Yang *et al.* 1990; Birner, Hultner *et al.* 1992) while interferon (IFN)- γ suppresses SCF and IL-3-mediated mast cell differentiation (Rottem, Hull *et al.* 1994). IL-3 also has a role in host defence against infection, by expanding populations of haematopoietic progenitor cells leading to increased numbers of mast cells and basophils and increased immunity against parasites (Lantz, Boesiger *et al.* 1998).

1.4.2.2 Cytokines and Mast Cell Chemotaxis

SCF critically regulates the migration of mast cell precursors during their proliferation and differentiation and acts as a potent chemoattractant for mature mast cells (Keshet, Lyman *et al.* 1991); an effect which is potentiated by IL-3 (Meininger, Yano *et al.* 1992).

1.4.2.3 Cytokines and Mast Cell Growth and Proliferation

SCF stimulates the proliferation of mast cells (Anderson, Lyman *et al.* 1990; Huang, Nocka *et al.* 1990; Nocka, Buck *et al.* 1990). IL-3 (Ihle, Keller *et al.* 1983), as well as IL-4 (Hamaguchi, Kanakura *et al.* 1987), regulates the growth and proliferation of mast cells; IL-4 and IL-3 synergistically trigger and support the proliferation of connective tissue type mast cells (Tsuji, Nakahata *et al.* 1990). SCF, in combination with IL-4, strongly increases the proliferation rate in mature human mast cell culture (Bischoff, Sellge *et al.* 1999) and IL-9 enhances the proliferation of mast cells induced by IL-3 or IL-4 (Hultner, Moeller *et al.* 1989; Moeller, Hultner *et al.* 1989 ; Hultner, Druez *et al.* 1990). IL-3, IL-5, IL-6, IL-9 and granulocyte/macrophage colony stimulating factor (Ochi, Hirani *et al.* 1999) as well as IL-10 (Thompson-Snipes, Dhar *et al.* 1991) influence mast cell and mast cell precursor proliferation. IFN- γ , the prototypical Th1 cytokine, also influences mast cell growth and development by suppressing SCF-mediated progenitor and mature mast cell proliferation (Ochi, Hirani *et al.* 1999) as well as SCF and IL-3-mediated mast cell growth (Rottem, Hull *et al.* 1994).

1.4.2.4 Cytokines and Mast Cell Survival

SCF, IL-3 and IL-6 are crucial for mast cell survival and their withdrawal causes mast cells to rapidly undergo apoptosis (Tsuji, Nakahata *et al.* 1990; Lemura, Tsai *et al.* 1994; Mekori, Gilfillan *et al.* 2001). IL-4 (Lorentz, Wilke *et al.* 2005) and intracellular IL-15 (Mirghomizadeh, Winoto-Morbach *et al.* 2009) also promote mast cell survival.

1.4.3 Mast Cell Classification

Mature mast cells are classified according to their location as well as the presence of mast cell mediators tryptase and chymase in their granules. MC_T contain tryptase and are predominantly located in the small bowel mucosa and other mucosal surfaces; they are also known as mucosal mast cells. MC_{TC} contain tryptase and chymase and are predominantly located in the skin and small bowel submucosa; they are also known as connective tissue mast cells (these include peritoneal mast cells) (McEuen and Walls 2008). Both MC_T and MC_{TC} mast cells mature from the CD34+ mast cell progenitor (Kobayashi, Nakano *et al.* 1986). MC_T are distinguished from MC_{TC} by the predominance of chondroitin sulphate proteoglycan in their granules while MC_{TC} contain heparin proteoglycan in their secretory granules, which mediates their preferential staining with metachromatic dyes such as toluidine blue (Hamaguchi, Kanakura *et al.* 1987; Meininger, Yano *et al.* 1992). Mature MC_T granules have a characteristic scroll morphology whereas mature MC_{TC} granules have a characteristic grating and lattice morphology (Craig, Schechter *et al.* 1989; Gebhardt, Sellge *et al.* 2002).

1.4.4 Mast Cell Receptors

Mast cells express many receptors on their cell surface; mature mast cells express CD13 and CCR3 chemokine receptor (Ochi, Hirani *et al.* 1999), FcεRI (high affinity immunoglobulin (Ig) E receptor) (Repetto, Bandara *et al.* 1996), FcεRII (CD23; low affinity IgE receptor) (Zhang, Murphy *et al.* 2007) and FcγRI (CD64; high affinity IgG receptor) (Woolhiser, Okayama *et al.* 2001). They also express FcγRII (CD32; low affinity IgG receptor) and FcγRIII (CD16; low affinity IgG receptor) (Okayama, Hagaman *et al.* 2001). Mast cells express receptors for cytokines such as interleukin (IL)-3 (Gebhardt, Sellge *et al.* 2002), IL-4, IL-13, IL-6, IL-9 and IL-10 (Kaur, Hollins *et al.* 2006; Prussin and Metcalfe 2006) and interferon-γ (IFN-γ). As already discussed mast cells express high levels of SCF receptor c-kit (CD117) (Nocka, Buck *et al.* 1990). High affinity IgE receptor FcεRI plays a pivotal role in the onset of an allergic reaction; this will be discussed later. FcεRI is expressed during early stages of mast cell development, but only when lineage-commitment is complete.

1.5 The Adaptive Immune System

The immune system is composed of both the innate and the adaptive (or humoral) immune systems. The adaptive immune system generates antibodies specific to invading pathogen/antigen; cells of the adaptive immune system include B-cells and Dendritic cells (DCs). Although mast cells are the central players in allergy and

atopic diseases, cells of the adaptive immune system such as T cells and B cells are crucially involved in the onset and maintenance of allergy.

1.5.1 T Cells

T cells are the primary lymphocytes of the adaptive immune system. The development of T cells from pluripotent stem cells occurs in the bone marrow followed by the thymus. T cells express T cell receptors (TCRs) (Huppa, Axmann *et al.* 2010) which recognise MHC bound antigens and major histocompatibility complex (MHC) (Schnabl, Stockinger *et al.* 1990; Holling, van der Stoep *et al.* 2002) which presents processed antigen to other immune cells.

Immature T cells express both CD4 (CD4+) and CD8 (CD8+) receptors on their cell surface. CD4+ T cells are selected via interaction with the MHC class II (MHCII) molecule (Holling, van der Stoep *et al.* 2002); while CD8+ T cells are selected via interaction with MHC class I (MHCI) molecule during their development (Marusic-Galesic, Stephany *et al.* 1988); these interactions determine which MHC class they will interact with as mature T cells. A tiny fraction of T cells are negative for both CD4 and CD8 (CD4- CD8-) (Cowley, Hamilton *et al.* 2005).

T cells interact with antigen presenting cells (APCs) such as DCs, B cells, monocytes and macrophages. MHCI expressing APCs present, after intracellular processing, endogenously-generated proteins (e.g. viral proteins) to the TCR of CD8+ T cells (Belz, Shortman *et al.* 2005) while MHCII expressing APCs such as macrophages, B cells and DCs, are responsible for the internalisation, degradation, processing and presentation (of degraded proteins) of extracellular pathogens via the MHCII molecule to the TCR of CD4+ T cells (Irla, Kupfer *et al.* 2010).

1.5.2 Th1/Th2-Polarisation of the Adaptive Immune Response

Following encounter with antigen CD4+ (expressing MHCII) naïve T cells (also called T helper cells or Th0) differentiate to either Th1 or Th2 depending on their environment (Mosmann, Cherwinski *et al.* 1986). The secretion of Th1/Th2-polarising cytokines usually comes from APCs and other accessory cells.

Th0 cells differentiate into T helper 1 cells (Th1) upon exposure to IL-12 (Schmitt, Hoehn *et al.* 1994), resulting in activation of signalling pathways which induce sustained production of Th1 cytokines and block Th2 differentiation (Noble, Staynov *et al.* 1993). Cytokines which induce the Th1 immune-polarisation are IL-12, IL-27, IL-23 (Brombacher, Kastelein *et al.* 2003) and IFN- γ (Bradley, Dalton *et al.* 1996). Th1 cells produce and release IFN- γ and IL-2 (Cherwinski, Schumacher *et al.* 1987) and IL-3 (Mosmann, Cherwinski *et al.* 1986). Th1 cells are induced by and play a role in the defence against intracellular pathogens such as viruses, bacteria and protozoa .

Th0 cells differentiate into Th2 cells upon exposure to IL-4 (Noble, Staynov *et al.* 1993), resulting in activation of signalling which stimulates Th2 cytokine production and inhibits Th1 differentiation (Ria and Adorini 1998). IL-4 (Sornasse, Larenas *et al.* 1996), as well as IL-13 (Barner, Mohrs *et al.* 1998), induces the development of Th2 cells (Sornasse, Larenas *et al.* 1996). Th2 cells produce IL-3 (Mosmann, Cherwinski *et al.* 1986), IL-4 and IL-5 (Cherwinski, Schumacher *et al.* 1987), IL-6, IL-9, IL-10 and IL-13 (Seder and Paul 1994). Secreted IL-4 promotes the production of Th2 cytokines, reinforcing the Th2 profile in a positive feedback loop, while IL-10 prevents the generation of Th1 cytokines such as IFN- γ and IL-12 and the activation of the Th1 profile (Romagnani 1997; Romagnani 1999). Polarisation of Th0 cells to Th2 cells is induced by extracellular pathogens and antigens such as parasites (helminths), bacteria and allergens.

Th0 cells can differentiate into Th1 and Th2 cells under identical conditions (in the presence of IL-4 and IL-12) therefore indicating maturation is not always mutually exclusive (Palm, Germann *et al.* 1996-1997).

1.5.3 Normal B Cell Function

Under normal conditions the function of B cells, the sole cell type with the ability to generate antibodies, is to synthesise antibodies specific to foreign pathogen upon antigen presentation. B cells also generate IgE specific to invading allergens and therefore play a pivotal role in the development of allergy.

B cells arise from bone marrow pluripotent stem cells and mature in peripheral lymphoid tissue. B cells express B cell receptors (BCRs) which consist of membrane bound immunoglobulins (Ig) (Bonnerot, Lankar *et al.* 1995). BCRs recognise non-peptide antigens, small and large linear peptide chains, as well as secondary and tertiary peptide structures. B cells express either MHCI or MHCII. Upon BCR antigen binding B cells internalise, process and present antigen fragments to T cells via the MHC groove (Lanzavecchia 1985). BCR activation also results in B cell proliferation (Patterson, Kraus *et al.* 2006); when B cells become activated, they divide to form clones called plasma cells, whose role it is to produce and release thousands of antibodies specific to the initial antigen upon subsequent antigen exposure. This is enhanced by DCs (Fayette, Durand *et al.* 1998). A much smaller portion of activated B cells will become memory cells and these cells will "remember" this initial exposure to the antigen (Rajewsky 1996) and will result in a faster and more potent immune response upon subsequent antigen exposure. Plasma B cells can no longer act as APCs because they no longer sequester antigen or express MHC on their cell surface. T cell-derived cytokines such as IL-2, IL-5 and IL-6 enhance B

cell proliferation and differentiation into antibody secreting plasma cells (Splawski, McAnally *et al.* 1990; Emslie, D'Costa *et al.* 2008).

1.5.4 B Cell Antibody Class Switching

Naïve B cells express either IgM or IgD on their cell surface (Reynaud, Delpy *et al.* 2005). Upon stimulation mature B cells switch Ig classes and express either IgG, IgA or IgE. Only one antibody class type is expressed in a B cell at any time. This is discussed further in the following section.

1.5.5 Allergy; B Cell Generation of IgE

In the case of allergy and atopic disease, allergen binds to allergen-specific IgM on the B cell surface, where it is internalised, processed and presented to Th2 cells (TCR-CD4 complex), via MHCII (figure 1.01 below). Low affinity IgE receptor, FcεRII (CD23) is expressed on B cells and IgE bound to FcεRII facilitates B cell antigen processing (Getahun, Hjelm *et al.* 2005). Antigen presentation results in Th2 CD40 ligand expression (CD40L, CD154) (van Kooten, Gaillard *et al.* 1994). Interaction with B cell CD40 upregulates B cell CD80 (B7-1) and stimulates B cell proliferation (Yellin, Sinning *et al.* 1994). B cell CD80 binds to T cell CD28 which stimulates T cell IL-4 synthesis and secretion (Blotta, Marshall *et al.* 1996). IL-4 released from the T cell binds to B cell IL-4 receptor (IL-4R) which causes B cell proliferation and directs antibody class switching to IgE (Rothman, Lutzker *et al.* 1988). Specifically, the locus of the μ or the δ constant domains of IgM or IgD respectively, is deleted and class switching occurs resulting in the transcription of the ϵ constant domain of the IgE molecule (Stavnezer, Guikema *et al.* 2008). ϵ transcript synthesis and class switching is promoted by the transcription factors Stat-6 (through signalling at IL-4Ra) and NF- κ B (through signalling at CD40) and results directly in the production of IgE (Tinnell, Jacobs-Helber *et al.* 1998). The complex interactions which occur between B and Th2 cells and result in the generation of IgE is summarised in figure 1.01 below. Thus, in allergy, activated B cells produce and secrete thousands of identical allergen specific IgE molecules; IgE synthesis accounts for up to 50% of plasma cell protein synthesis. IL-13 and IL-4, which act through a common signalling pathway, both direct naïve B cells produce IgE and enhance MHCII antigen presentation pathway (Punnonen, Aversa *et al.* 1993).

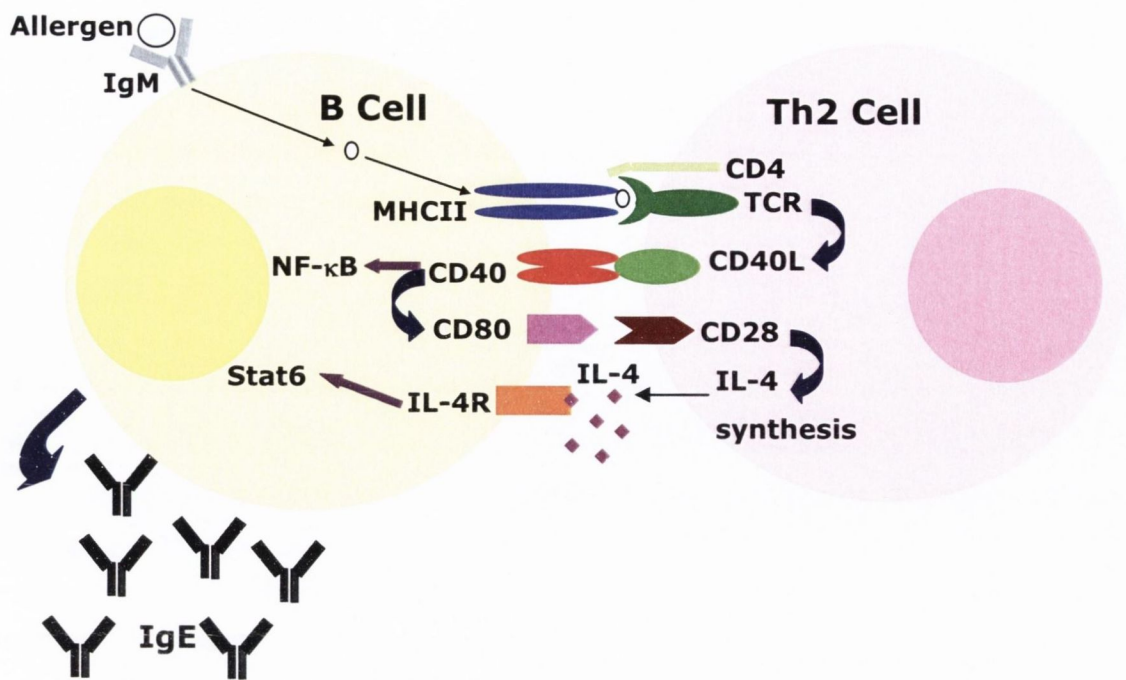


Figure 1.01: B cell-T cell interactions involved in IgE synthesis

1.5.6 IgE

IgE is structurally similar to other antibodies; it having identical heavy chains and two identical light chains, as shown in figure 1.02 below. IgE is a monomer of approximately 190 kd. As well as having a vital role in the onset of an allergic response (discussed in the following section), IgE prolongs mast cell survival (Sly, Kalesnikoff *et al.* 2008) and facilitates eosinophil-mediated antibody-dependent cell-mediated cytotoxicity against parasites (Gounni, Lamkhioed *et al.* 1994). IgE is the least abundant antibody class in the serum; it usually occurs at ~150 ng/ml in non-atopics, compared to 10mg/ml for IgG (Gould, Sutton *et al.* 2003).

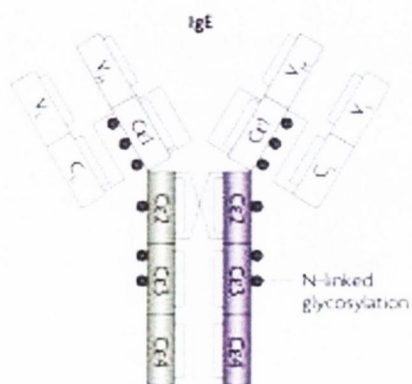


Figure 1.02: Structure of the IgE antibody (Gould and Sutton 2008)

1.5.7 FcεRI

The high affinity IgE receptor, FcεRI, is a member of the antigen receptor superfamily (which also includes BCRs, TCRs and some IgG receptor types). It is a tetrameric protein which consists of an α-chain, a β-chain and a γ-chain dimer (αβγ₂) (Blank, Ra *et al.* 1989). Mast cell FcεRI expression is regulated by serum IgE levels; IgE complexed to FcεRI leads to increased cell surface accumulation of FcεRI from within the cell which causes upregulation of FcεRI transcription leading to increased FcεRI at the cell surface (Hsu and MacGlashan 1996).

Multivalent IgE binds to the α- subunits of the FcεRI (FcεRIα) via its Cε3 region (the third constant (Fc) domain of the IgE molecule) region (Presta, Shields *et al.* 1994; Henry, Cook *et al.* 1997) in a 1:1 ratio (Keown, Ghirlando *et al.* 1998), an interaction which occurs with high affinity ($K_D = \sim 10^{-10}M$) (Presta, Shields *et al.* 1994; Henry, Cook *et al.* 1997). The intracellular cytoplasmic tails of the β- and γ- subunits contain conserved immunoreceptor tyrosine based activation motifs (ITAMs) which are responsible for initiating intracellular signalling events upon receptor activation. IgE does not undergo conformational change upon binding. The IgE-FcεRI complex has a half life of approximately 21 days (Miescher and Vogel 2002).

1.6 Mast Cell Sensitisation and Subsequent Activation; the Allergic Reaction

1.6.1 Allergen Sensitisation

The majority of IgE is bound to its specific high affinity receptor, FcεRI expressed on mast cells, where it remains. The initial exposure of the allergic individual to the allergen, the corresponding production of IgE by B cells and the binding of IgE to mast cell FcεRI is known as the sensitisation step of allergy. The individual is now "allergic" to that particular allergen.

1.6.2 Allergen Challenge; FcεRI Activation

After initial allergic sensitisation, subsequent allergen exposure will result in an allergic reaction. The activation of mast cell IgE-FcεRI complex upon allergen binding and the subsequent activation of downstream signalling transduction pathways which leads to mast cell degranulation are summarised below in figure 1.03. Multivalent allergen binds to two specific IgE molecules which are complexed to the FcεRIα subunits of two FcεRI on the mast cell surface (Keown, Ghirlando *et al.* 1998). Allergen-IgE binding results in the cross linking of Fc-bound- IgE which leads to FcεRIα subunit aggregation (Scholl, Kalkura *et al.* 2005). FcεRIα subunit

aggregation does not occur in the absence of antigen because of extensive glycosylation of the molecule (Letourneur, Sechi *et al.* 1995), however, the binding of a multivalent antigen to two Fc ϵ RI α bound IgE molecules overcomes the resistance of the α -chains to interaction .

The cytoplasmic tail of Fc ϵ RI α does not possess any enzymatic activity; instead intracellular signalling of activated Fc ϵ RI is mediated through the β - and γ - subunits (Repetto, Bandara *et al.* 1996). The Src family kinase Lyn is bound to Fc ϵ RI β under resting conditions and is activated upon receptor aggregation. Autophosphorylation of active Lyn is required for full kinase activity and active Lyn phosphorylates the β - and γ - immunoreceptor tyrosine based activation motif (ITAM) receptor subunits (Turner and Kinet 1999). P72Syk kinase (Syk) is recruited to the γ - receptor subunit where it binds, with higher affinity than to Fc ϵ RI β ; there it is phosphorylated and activated by Lyn. Lyn and Syk activation drives intracellular signalling cascades (Kopcec, Panaszek *et al.* 2006). Phosphorylation of both the β - and γ - ITAMs is required for complete receptor activation and subsequent downstream signalling to occur (Turner and Kinet 1999).

Fc ϵ RI α aggregation also activates the Src family kinase, Fyn, which phosphorylates the adaptor molecule Gab2 to activate the phosphatidylinositol-3-OH kinase (PI3K) pathway. Membrane phosphatidylinositol-4,5-bisphosphate (PIP2) is phosphorylated by PI3K to form phosphatidyl-3,4,5-triphosphate (PIP3) (Vanhaesebroeck, Leervers *et al.* 1997).

Another target of receptor activation is phospholipase (PL) C (PLC γ 1), which is recruited to the membrane where it is activated by tyrosine phosphorylation by Syk and Itk or Blk, both of which are Tec family non-receptor tyrosine kinases (Rivera, Fierro *et al.* 2008). PLC γ 1 hydrolyses PIP2 to form the second messengers diacylglycerol (DAG), which remains bound to the cell membrane and soluble inositol-1,4,5-triphosphate (IP3) (Vanhaesebroeck, Leervers *et al.* 1997). DAG activates protein kinase C (PKC) isoforms which activate further cytosolic signal transduction proteins.

Soluble IP3 migrates primarily to the smooth endoplasmic reticulum (ER), where it activates specific IP3 receptor calcium channels to induce the first stage of calcium (Ca²⁺) mobilisation; the transient release of Ca²⁺ from ER Ca²⁺ stores (Pacher, Csordas *et al.* 2000). STIM1, a sensor of ER Ca²⁺ concentration, couples the depletion of ER Ca²⁺ stores with the activation of store-operated calcium release-activated calcium (CRAC) channels in the plasma membrane via CRACM1, the pore forming unit of the CRAC channel. The result is the prolonged influx of exogenous Ca²⁺ into the cell (Di Capite and Parekh 2009). Calcium permeable, non-selective cation channels; transient receptor potential channels (TRPCs), are thought to

associate with STIM1 and CRACM1 to enhance Ca^{2+} entry while calcium-activated non-selective cation channels regulate the membrane potential and thus the influx of Ca^{2+} (Turner and Kinet 1999). Other effector molecules of receptor aggregation include GTPases which upon activation cause the activation of many complex downstream signalling pathways/networks, to act upon transcription factors (Turner and Cantrell 1997). Currently the complete mechanism of $\text{Fc}\epsilon\text{RI}$ receptor activation and downstream signalling is not understood.

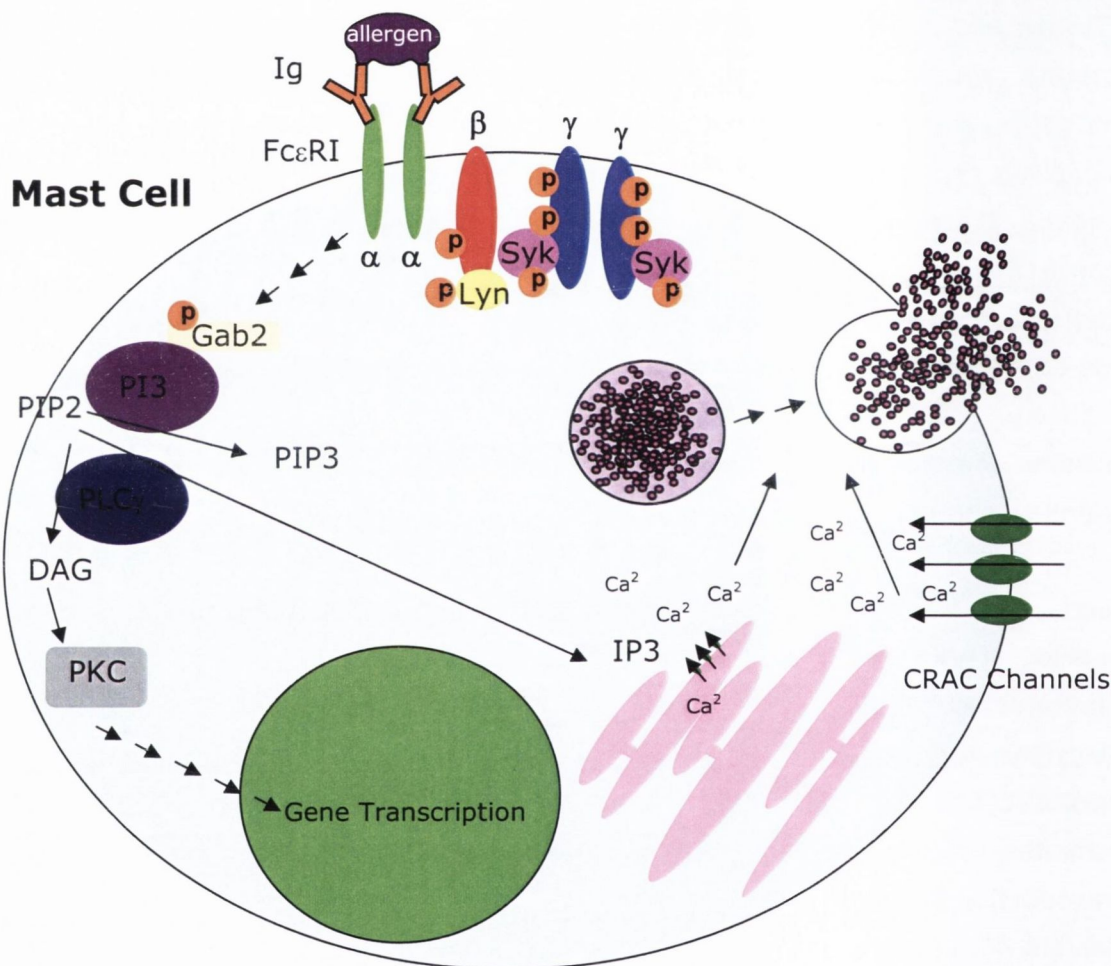


Figure 1.03: $\text{Fc}\epsilon\text{RI}$ activation and subsequent intracellular signalling

1.6.3 Termination of $\text{Fc}\epsilon\text{RI}$ Signalling

$\text{Fc}\epsilon\text{RI}$ activation persists only as long as the receptor is engaged and phosphorylated. Signalling is terminated when the antigen has disengaged from the IgE- $\text{Fc}\epsilon\text{RI}$ receptor complex and $\text{Fc}\epsilon\text{RI}$ is rapidly internalised following crosslinking (Santini

and Keen 1996). Termination of signal in part is Lyn dependent (Honda, Suzuki *et al.* 1997). It is thought the termination of signal transduction occurs when proteins which were phosphorylated upon receptor activation are dephosphorylated by protein tyrosine phosphatases which are bound to the Fc ϵ RI β intracellular ITAM domains, such the inositol phospholipid directed phosphatase SHIP (Leung and Bolland 2007).

1.6.4 Mast Cell Degranulation

Following signal transduction mediated by Fc ϵ RI activation, mast cell granules associate with the cell membrane and granule exocytosis occurs. Exocytosis is mediated by membrane fusion soluble N-ethyl-maleimide-sensitive factor-attachment protein receptor (SNARE) proteins. T-SNAREs such as syntaxins and soluble N-ethyl-maleimide-sensitive factor-attachment proteins (SNAPs) are located on the cell membrane and v-SNAREs, such as vesicle-associated membrane proteins (VAMPs) are located on the granule membrane. The t-SNARES SNAP-23 and syntaxin 4 regulate Fc ϵ RI-mediated mast cell exocytosis (Guo, Turner *et al.* 1998; Paumet, Le Mao *et al.* 2000). Rab GTPases also regulate exocytosis of mast cell granules. Synaptotagmin, the calcium sensor which plays a vital role in calcium-induced membrane fusion during exocytosis, is expressed in human mast cells and is involved in mast cell exocytosis (Kimura, Shiraishi *et al.* 2001). Cellular interactions which result in the degranulation of mast cells are shown below in figure 1.04.

Upon exocytosis mast cell mediators degranulation occurs in a sequential pattern (Turner and Kinet 1999). Mediator release begins after a lag period following degranulation of granules adjacent to the cell membrane the process moves towards more centrally located granules (Rohlich, Anderson *et al.* 1971) and maximum histamine release occurs from mast cells within 2-3 minutes of Fc ϵ RI activation (Ishizaka and Conrad 1983). Electron micrographs of a normal and a degranulating mast cell are shown in figures 1.05 and 1.06 below respectively.

Interaction of the released mediators with their respective mediators results in the familiar symptoms of allergy.

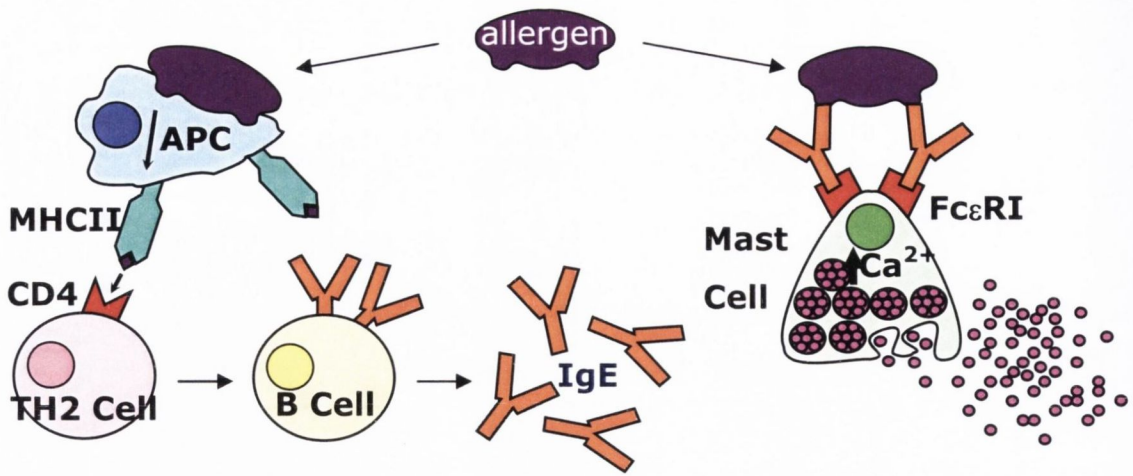


Figure 1.04: Generation of IgE, mast cell activation and subsequent degranulation

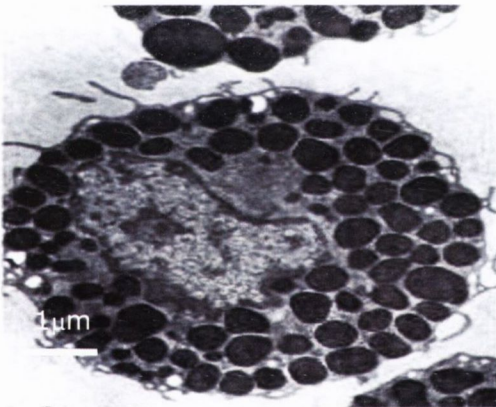


Figure 1.05: Low power electron micrograph of a normal mast cell. The cytoplasm of the cell is tightly packed with granules having a uniformly dense structure. X20000 (Rohlich, Anderson et al. 1971)

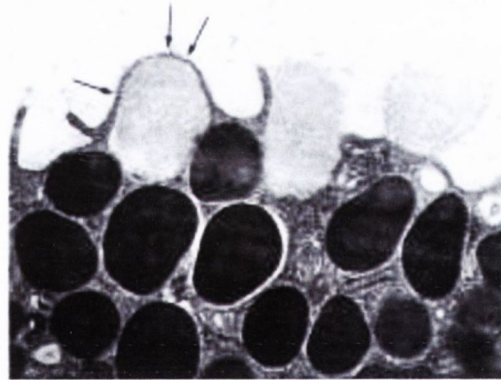


Figure 1.06: Electron micrograph of mast cell degranulation. Degranulation begins with peripheral granules. Arrows; Fusion between cell and granule membrane. Incubation with compound 48/80. X38000 (Rohlich, Anderson et al. 1971)

1.7 Mast Cell Mediators

Mast cells synthesise a variety of mediators which are released upon degranulation. These mediators are divided into three (non-exclusive) classes, namely preformed granule-associated mediators, newly synthesised lipid mediators and a variety of cytokines and chemokines.

1.7.1 Preformed Granule-Associated Mediators

Preformed mediators are synthesised in the mast cell and stored in mast cell granules from where they are released into the extracellular matrix via exocytosis upon mast cell activation. These mediators include the biogenic amines histamine and serotonin, the proteoglycans heparin and chondroitin sulphate E and the proteases tryptase, chymase (depending on MC_{TC} or MC_T mast cell type) and carboxypeptidase A (Dvorak, Galli *et al.* 1983).

1.7.1.1 Histamine

Histamine is implicated in the development of many of the symptoms of allergy (see H1R below). Mast cells are the primary source of tissue histamine in humans (Graham, Lowry *et al.* 1955) and histamine is the primary mediator-associated with mast cell degranulation in humans. Histamine is a biogenic amine which is formed when the amino acid histidine is decarboxylated by the enzyme L-histidine decarboxylase in the golgi apparatus of the mast cell. It is stored in mast cell granules under acidic conditions bound to carboxy groups on proteins and proteoglycans. Histamine remains in the mast cell granule until the mast cell becomes activated (Riley and West 1953; Rohlich, Anderson *et al.* 1971) when it dissociates from the proteoglycans and proteins by cation exchange with extracellular sodium at a neutral pH. Within minutes of release histamine is broken down to form methylhistamine, methylimidazole acetic acid or imidazole acetic acid, therefore histamine must act upon specific receptors which are located adjacent to the site of release. The synthesis and metabolism of histamine is outlined in figure 1.07 below. Rat peritoneal mast cells contain ten fold (10-30pg histamine/cell) the amount of histamine than that of their mucosal counterparts (1-3pg histamine/cell). Human mast cells contain similar amounts of histamine regardless of their location (lung, skin, lymphoid and intestinal mast cells contain 3-8pg histamine/cell) (Metcalf and Mekori 1997).

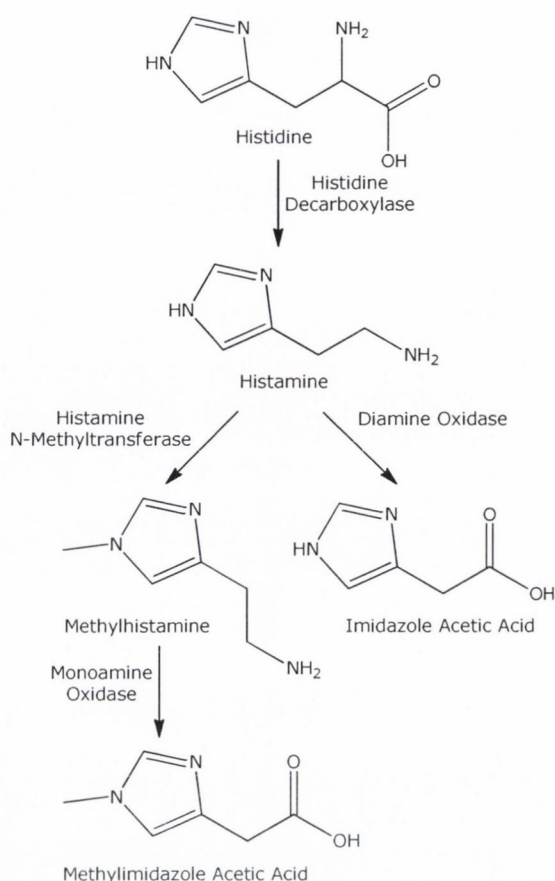


Figure 1.07: Synthesis and metabolism of histamine

1.7.1.2 Histamine Receptors

When histamine is released from mast cell granules upon mast cell activation, it exerts its effects via specific histamine receptors; namely histamine receptor 1 (H1R), histamine receptor 2 (H2R), histamine receptor 3 (H3R) and histamine receptor 4 (H4R). These are all G-protein coupled receptors (GPCRs) (Hill, Ganellin *et al.* 1997; Nguyen, Shapiro *et al.* 2001).

1.7.1.2.1 Histamine Receptor 1

Upon ligand binding histamine receptor 1 (H1R) activates PLC and the corresponding DAG IP3 signal transduction pathway via the G_q subunit of the GPCR (Jutel, Blaser *et al.* 2005). To a lesser extent it also activates adenylate cyclase to increase cyclic adenosine monophosphate (cAMP) concentrations (Moniri, Covington-Strachan *et al.* 2004).

The H1R is responsible for vasodilation and increased vascular permeability which occur during the non-specific inflammatory response as well as in allergy related inflammation (Long, Sprung *et al.* 1985). During the non-specific inflammation response histamine is released in response to tissue damage/infection by

connective tissue mast cells (as well as circulating basophils), resulting in the dilation and increased permeability of capillaries adjacent to the site of injury/infection. This allows for increased infiltration of phagocytes and other leukocytes as well as clotting agents to the area.

Histamine, through both H1R and histamine receptor 2 (H2R), modulates Th1/Th2-polarisation of the immune response and deletion of H1R in mice results in a dominant secretion of the Th2 cytokines IL-4 and IL-13 and increased levels of IgE and other antibodies when compared with mice lacking in the H2R (Jutel, Watanabe *et al.* 2001). H1R on T and B cells augments antigen/receptor-mediated immune responses (Banu and Watanabe 1999). The H1R is also located post synaptically in the brain hypothalamus and other limbic regions and is involved in the modulation of circadian rhythm (Long, Sprung *et al.* 1985). Histamine regulates excitability of sympathetic preganglionic neurons by acting on H1Rs expressed on these cells (Whyment, Blanks *et al.* 2006). It is also located in the gastrointestinal tract where it is involved in ileum contraction. In allergy the H1R is responsible for the characteristic symptoms of allergy such as urticaria (Hein 2002; Belsito 2010) due to wheal and flare response, runny nose (Hampel, Martin *et al.* 1999), sneezing (Kayasuga, Sugimoto *et al.* 2002), nasal blockage (Taylor-Clark, Sodha *et al.* 2005) and pruritis (Hossen, Fujii *et al.* 2005) including nasal pruritis (Tsumuro, Ogawa *et al.* 2005) and ocular pruritis (Fujishima, Fukagawa *et al.* 2006). The H1R is located on smooth muscle and is implicated in bronchoconstriction which occurs during an asthma attack (Albazzaz and Patel 1988).

1.7.1.2.2 Histamine Receptor 2

Upon ligand binding to the H2R, adenylate cyclase becomes activated via the $G\alpha_s$ subunit of the GPCR (Jutel, Blaser *et al.* 2005), thus increasing levels of the second messenger cAMP which results in an increase in intracellular Ca^{2+} concentrations (Florio, Flezar *et al.* 1992). The H2R is located in the heart where it is associated with the control of sinus rhythm. It is also expressed on oxyntic cells present in the epithelium of the stomach where it is involved in gastric acid secretion. The H2R is also located on smooth muscle where it is involved in smooth muscle relaxation. It is expressed on lymphocytes where, upon ligand binding, it prevents these cells from producing an antibody response (Eckert, Pfuller *et al.* 1985; Akdis and Blaser 2003). Histamine acts upon DC H2R causing an increase in IL-10 production and a concomitant reduction in IL-12 production. As a result DCs which mature in the presence of histamine become effector Th-2 promoting DC (Rottem, Hull *et al.* 1994; Mazzoni, Young *et al.* 2001), thus creating a positive feedback loop which could contribute to the severity of atopic diseases (DC are the most potent APC).

H2R is expressed on human mast cells (Lippert, Artuc *et al.* 2004) and therefore may be involved in allergy related immunomodulatory effects.

1.7.1.2.3 Histamine Receptor 3

Ligand binding at histamine receptor 3 (H3R) results in the inhibition of adenylate cyclase via the $G_{i/o}$ subunit of the GPCR (Jutel, Blaser *et al.* 2005). H3R is located on histaminergic neurons of the central nervous system (CNS) where it functions as an autoreceptor (Leurs, Bakker *et al.* 2005). It also negatively modulates the release of glutamine and noradrenaline. The H3R has been implicated to play roles in cognition, wakefulness, obesity, schizophrenia, memory/learning disorders and epilepsy.

1.7.1.2.3 Histamine Receptor 4

The histamine Receptor 4 (H4R) has only recently been discovered (Oda, Morikawa *et al.* 2000); (Nakamura, Itadani *et al.* 2000) using H3R homology studies and it has a high level of homology with the H3R (40%) (Nakamura, Itadani *et al.* 2000). The H4R is coupled to the $G_{i/o}$ subunit of the GPCR (Takeshita, Bacon *et al.* 2004) and ligand binding causes the inhibition of adenylate cyclase (Jutel, Blaser *et al.* 2005). The H4R is expressed on natural killer cells (NKs), monocytes, dendritic cells (DCs) (Gutzmer, Diestel *et al.* 2005), eosinophils (Ling, Ngo *et al.* 2004) and mast cells (Lippert, Artuc *et al.* 2004) and is responsible for mediating high affinity histamine-induced chemotaxis in these cells (Damaj, Becerra *et al.* 2007); (Ling, Ngo *et al.* 2004); (Hofstra, Desai *et al.* 2003) through histamine-induced actin polymerisation (Barnard, Barnard *et al.* 2008). The H4R suppresses IL-12 production in DCs (Gutzmer, Diestel *et al.* 2005). H4R, as well as H2R, control histamine-induced IL-16 release from CD8+ T cells (Gantner, Sakai *et al.* 2002). The H4R is implicated in pain and itch and has been shown to be expressed in the spinal cord, hippocampus, cortex, thalamus and amygdala, with highest expression in the spinal cord (Strakhova, Nikkel *et al.* 2009). The H4R has been implicated in colitis and is thought the H4R mediates recruitment of effector cells into inflammatory sites leading to chronic inflammation (Fogel, Jochem *et al.* 2007). The H4R is highly expressed in the bone marrow (Takeshita, Bacon *et al.* 2004).

1.7.1.3 Serotonin

It was thought that histamine was the sole biogenic amine to be released from human mast cells and although histamine is the primary human mast cell amine, occasional articles have been published indicating human mast cells secrete serotonin (5-hydroxytryptamine). It appears a sub population of human mast cells secrete serotonin and the release of serotonin in these cells, which is differential to

mediator release by degranulation, is mediated by polyamines (Kanerva, Lappalainen *et al.* 2009). Serotonin levels are also observed to be increased in patients with mastocytosis (Kushnir-Sukhov, Brittain *et al.* 2008). Serotonin is the primary mediator released upon mast cell degranulation in rodents. It is synthesised from the amino acid L-tryptophan by the enzyme tryptophan hydroxylase which hydroxylates L-tryptophan to form the intermediate 5-hydroxytryptophan. This intermediate is decarboxylated by the ubiquitous L-aromatic amino acid decarboxylase to form serotonin. Serotonin is degraded to 5-hydroxyindoleacetic acid by the enzymes monoamine oxidase and aldehyde dehydrogenase and to melatonin by the enzymes N-acetyl transferase and 5-hydroxyindole-O-methyltransferase. In allergy and atopic disease serotonin is required for the generation of the delayed phase of the hypersensitivity reaction of mice (Gershon, Askenase *et al.* 1975) and SCF has the ability to modulate the release of serotonin from rodent mast cells (Coleman, Buckley *et al.* 1995). Under some conditions serotonin is released differentially to histamine in the rat, indicating that serotonin release does not always indicate that mast cell degranulation has occurred (Theoharides, Bondy *et al.* 1982).

1.7.1.4 Proteoglycans; Heparin and Chondroitin Sulphate E

Proteoglycans are a family of structurally distinct polyanionic complex carbohydrates which are composed of repeating disaccharide units. Although all human mast cells produce chondroitin sulphate E and heparin sodium, heparin is found in much higher concentrations in MC_{TC} than MC_T mast cells. Chondroitin sulphate E and heparin sodium complex to and stabilise mast cell proteases and complex with a core of serglycin proteoglycan protein in the mast cell granule maintain granule homeostasis (Duelli, Ronnberg *et al.* 2009). Chondroitin sulphate E and Heparin sodium are released upon mast cell degranulation and increased levels of heparin are observed in the plasma of asthmatics compared to non-asthmatics (Lasser, Simon *et al.* 1987). Heparin can paradoxically inhibit allergen-induced eosinophil airway infiltration upon inhalation in animal models of asthma (Seeds and Page 2001).

1.7.1.5 Mast Cell Serine Proteases

As stated previously connective tissue type mast cells (MC_{TC}) contain both tryptase and chymase, while mucosal type mast cells (MC_T) contain only tryptase. Tryptase and chymase are stored in mast cell granules where they are bound to heparin. Upon mast cell degranulation they are released as a tryptase/chymase-heparin

complex. Carboxypeptidase A is stored in mast cell granules and is also complexed with proteoglycans.

1.7.1.5.1 Tryptase

Tryptase is an active tetramer of 134kDa, consisting of two subunits of 31-34kDa, each chain containing a single active site. Five homologous human tryptases have been cloned (1α , 4β) and α -tryptase is the predominant type in the blood of healthy individuals. Active tryptase is complexed to proteoglycans complexes which are smaller than those binding chymase and carboxypeptidase. Upon dissociation it undergoes conformational changes resulting in the formation of an inactive monomer (Welle 1997). Tryptase cleaves fibrinogen, activates collagenase, hydrolyses some neuropeptides and causes secretion of mucus. Tryptase accounts for 20-50% of the protein content in human mast cells. Mast cell tryptase is implicated in arthritis (Nakano, Mishiro *et al.* 2007; Shin, Nigrovic *et al.* 2009), blood tryptase can be measured to confirm anaphylaxis (Edston and van Hage-Hamsten 1998).

1.7.1.5.2 Chymase

Chymase is a monomer of 30kDa. It is bound to heparin and chondroitin sulphate in mast cell granules. Chymase converts angiotensin I (the precursor to angiotensin II; angiotensin I has no other function) to angiotensin II which increases blood pressure by acting on vascular smooth muscle cells (Welle 1997). Chymase also inactivates bronchoactive peptides such as bradykinin and kallidin as well as the neuropeptide substance P. Chymase stimulates mucus secretion and converts the IL-1 β cytokine precursor to bioactive IL-1 β (Mizutani, Schechter *et al.* 1991) and also a potent chemoattractant for monocytes and neutrophils (Tani, Ogushi *et al.* 2000). Chymase is a potent secretagogue for airway gland serous cells which are implicated in airway hypersecretion (Sommerhoff, Caughey *et al.* 1989). Increase chymase is expressed in congenital heart disease (Hamada, Terai *et al.* 1999).

1.7.1.5.3 Carboxypeptidase A

Carboxypeptidase A is a ~36kDa protease and in humans and rodents carboxypeptidase A is only associated with connective tissue type mast cells. Carboxypeptidase A is highly overexpressed in mastocytosis (D'ambrosio, Akin *et al.* 2003). Like chymase and tryptase, carboxypeptidase A is stored as an active form, however, granule pH (~pH5.5) is lower than that required for optimal activity (~pH7-9) (Pejler, Knight *et al.* 2009). Carboxypeptidase A is stored in association with heparin, which is complexed to a serglycin core (Schwartz, Riedel *et al.* 1982). The function of Carboxypeptidase is largely unknown. However, a recent

carboxypeptidase A knock out study demonstrated that it is required for mast cell staining, however, no ultrastructural granule defects were observed (Feyerabend, Hausser *et al.* 2005). The study did find that the degree of proteoglycan cross linking was reduced in the knockout mice and impaired storage of one variant of chymase, but not others or tryptase, was observed (Feyerabend, Hausser *et al.* 2005). Carboxypeptidase A seems to have an important role in the protection against certain snake venom toxins (Metz, Piliponsky *et al.* 2006).

1.7.2 Newly Synthesised Lipid Mediators

Newly synthesised lipid mediators are not preformed and are synthesised upon mast cell activation. These include leukotrienes (LT) such as LTB₄, LTC₄, LTD₄ and LTE₄, prostaglandins (PG) such as PGD₂ as well as platelet activating factor (PAF). These vary in the amount synthesised depending on mast cell location (Puxeddu, Piliponsky *et al.* 2003).

1.7.2.1 Leukotrienes and Prostaglandins

Upon mast cell activation phospholipase A₂ (PLA₂) is activated and generates arachidonic acid (ω 6) from membrane phosphatidylcholine. Arachidonic acid is metabolised by lipoxygenase to generate leukotrienes (LT) and cyclooxygenase to generate prostaglandins (PG) and Thromboxanes (TX).

1.7.2.1.1 Leukotrienes

Leukotrienes produced by mast cells include LTB₄ (Levi-Schaffer, Austen *et al.* 1987), LTC₄ (Levi-Schaffer, Austen *et al.* 1987), LTD₄ and LTE₄. LTC₄ is generated within the mast cell from LTA₄ by the enzyme LTC₄ synthase. LTC₄ is released into the extracellular fluid where it is converted to LTD₄ and LTE₄. Endothelial cells (Claesson and Haeggstrom 1988; Morelli, Norris *et al.* 1990) and can convert LTC₄ to LTD₄ and LTE₄. γ -glutamyl transpeptidase and aminopeptidase which produce LTD₄ and LTE₄ respectively are plasma membrane bound enzymes (Kuo, Lewis *et al.* 1984). Leukotrienes are involved in the cutaneous wheal and flare response, prolonged potent bronchoconstriction (10-1000 times more potent than histamine), increased vascular permeability (LTC₄), bronchial mucus secretion and constriction of vascular as well as intestinal smooth muscle. Leukotrienes are involved in the recruitment of immune cells such as eosinophils and neutrophils. LTC₄ is a potent bronchoconstrictor and as well as LTB₄ is a potent chemotactic agent. LTC₄ production is increased in the presence of IL-3 (Jakschik, Harrington *et al.* 1992) and can be modulated by SCF (Bischoff and Dahinden 1992).

1.7.2.1.2 Prostaglandins

PGD₂ is the major prostaglandin released by mast cells upon activation (Levi-Schaffer, Austen *et al.* 1987; Shichijo, Inagaki *et al.* 1998). PGD₂ is implicated in asthma where it is present in the bronchial airway lavage fluid of asthmatics (Wenzel, Westcott *et al.* 1989). *In vitro* PGD₂ causes the constriction of human bronchial smooth muscle (Norel, Labat *et al.* 1991). PGD₂ acts as a potent chemotactic agent for the recruitment of lymphocytes such as eosinophils (Powell 2003).

1.7.2.1.3 Platelet-Activating Factor

Platelet-activating factor (PAF) is synthesised from lysophosphatidylcholine and acetyl CoA by lysophosphatidylcholine transferase. PAF is a potent bronchoconstrictor (Spencer, Evans *et al.* 1991) and causes the aggregation and activation of platelets (Jamaluddin and Thomas 1992). PAF is a potent vasodilator and is implicated in the cutaneous wheal and flare allergic reaction (Petersen, Church *et al.* 1997).

1.7.2.2 Chemokines and Cytokines

Mast cells secrete a variety of chemokines and cytokines upon mast cell activation.

1.7.2.2.1 Chemokines

Chemokines produced include the CC chemokine ligand (CCL) 5 (CCL5), which is associated with Th2 type responses, as well as the CXC chemokine ligand 8 (CXCL8, IL-8) and CXCL10. Mast cells also produce CCL2, CCL3. Chemokines function as chemical signals to attract other immune cells such as macrophages and other mast cells etc. to the area during a non-specific inflammatory response (Kitaura, Kinoshita *et al.* 2005); (Puxeddu, Piliponsky *et al.* 2003).

1.7.2.2.2 Cytokines

Mast cells synthesise and release IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, GM-CSF, TNF- α , IFN- γ , IL-16, monocyte chemotactic protein-1 (MCP-1) and regulated on activation, normal T cell expressed and secreted (RANTES) upon mast cell activation (Bradding 1996; Metcalfe and Mekori 1997; Wedemeyer, Tsai *et al.* 2000).

Lorentz *et al.* demonstrated that mast cells produce the cytokines TNF- α , IL-1 β , IL-6, IL-8, IL-16 and IL-18 in the presence of SCF, but are capable of producing different cytokine profiles under various different conditions. The expression of TNF- α is enhanced upon activation with IgE-independent agonists such as bacteria as

well as with IgE-dependent agonists, where as activation of mast cells via FcεRI results in the expression of Th2 cytokines such as IL-3, IL-5, IL-9 and IL-13. IL-4 enhances the SCF-mediated production of IL-3, IL-5 and IL-13 while IL-4 also downregulates the expression of IL-6 (Lorentz, Schwengberg *et al.* 2000) while IL-4 alone enhances the release of certain cytokines such as IL-3, IL-4 and IL-8 from mast cells (Coleman, Buckley *et al.* 1995).

1.7.2.2.2.1 Tumor Necrosis Factor-α

Mast cells are the only tissue-resident cells which contain preformed TNF-α stored in granules, therefore, they are a principle source of this cytokine (Walsh, Trinchieri *et al.* 1991) in the early stages of inflammation (Gordon and Galli 1991). TNF-α released by mast cells activates dermal microvascular endothelium adjacent to activated mast cells (Walsh, Trinchieri *et al.* 1991) by rapidly inducing the glycoprotein endothelial-leukocyte adhesion molecule-1 (ELAM-1) on postcapillary dermal venules, which promotes adhesive interactions with circulating leukocytes (Klein, Lavker *et al.* 1989). This indicates that mast cell products other than histamine and other vasoactive amines have the ability to influence endothelium. Low levels of TNF-α are constitutively transcribed in mast cells.

1.7.2.2.2.2 Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) or VEGF-A, is a proinflammatory mediator and growth factor. It is a member of the larger VEGF family of proteins (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor) which regulate receptor tyrosine kinases to initiate signal transduction and downstream signalling. VEGF-A exists in nine different isoforms; four of these (VEGF121, VEGF165, VEGF189 and VEGF206) (Park, Keller *et al.* 1993) have identical function. VEGF stimulates vascular endothelial cell growth, survival (Gerber, McMurtrey *et al.* 1998) and proliferation and is a potent angiogenic factor (Leung, Cachianes *et al.* 1989). VEGF promotes the survival of existing vascular networks (Alon, Hemo *et al.* 1995) and enhances vascular permeability (Senger, Connolly *et al.* 1990) but is also essential for the growth of new vasculature both in embryonic development, after injury (Zhao, Egashira *et al.* 2004) and in normal adults. VEGF is implicated in tumour angiogenesis. VEGF is a potent inducer of endothelial cell and monocyte migration (Waltenberger, Claesson-Welsh *et al.* 1994; Barleon, Sozzani *et al.* 1996).

Mast cells are a source of VEGF (Vasiadi, Kalogeromitros *et al.* 2010). Endogenously produced corticotropin-releasing hormone, a polypeptide hormone and neurotransmitter which is secreted by the hypothalamus in response to stress, can induce the release of newly formed VEGF from mast cells via corticotropin releasing

hormone receptors, without the release of cytokines or preformed mediators (Cao, Papadopoulou *et al.* 2005) via the activation of the cAMP/PKA/p38 MAPK signalling pathway (Cao, Cetrulo *et al.* 2006). Mast cells release preformed VEGF as well as newly synthesising and releasing VEGF after FcεRI activation and also following treatment with calcium ionophore A23187 (Boesiger, Tsai *et al.* 1998).

1.8 Role of Mast Cells in Disease

Mast cells are central players in the development and maintenance of allergy and atopic disease, however, they have also been implicated in chronic inflammatory and non-inflammatory non-allergic disease.

1.8.1 Allergy and Atopic Disease

1.8.1.1 Asthma

Asthma is a disease of the upper respiratory tract characterised by bronchial hyperresponsiveness. During an asthmatic response, allergen-mediated release of mast cell mediators induces an immediate hypersensitive response characterised by bronchial mucosal oedema, mucus production and secretion and smooth muscle contraction resulting in airway obstruction and reduced airflow. In asthma serum IgE levels correlate with disease severity and bronchial hyperresponsiveness (Postma, Bleecker *et al.* 1995). A variety of inhaled allergens can provoke an asthma attack, among these are house dust mite protein (Sporik, Holgate *et al.* 1990), cockroach (Rosenstreich, Eggleston *et al.* 1997), cat dander (Bertelsen, Carlsen *et al.* 2010) and the common airborne fungus *Alternaria alternata* (Hedayati, Arabzadehmoghadam *et al.* 2009).

Numbers of circulating CD34+ mast cell progenitor cells are increased in asthma (Mwamtemi, Koike *et al.* 2001); these migrate to airways where they mature. Th2 cytokines such as IL-4, IL-5, IL-9 and IL-13 account for much of the pathophysiological manifestations of allergy and asthma and the Th2-polarised response mediates the recruitment of leukocytes, including Th2 cells, to the site of allergic response (Romagnani 2001). PGD₂ mediates bronchoconstriction (Hardy, Tattersfield *et al.* 1984) and PGD₂ levels in asthma patients are up to ten times higher than in non-asthmatic patients. Severity of atopic dermatitis, as well as hen's egg sensitisation and onset of rhinoconjunctivitis, in childhood is associated with a high frequency of the subsequent development of asthma (Ricci, Patrizi *et al.* 2006). LTE₄ has also been implicated in airway hyperresponsiveness in asthma (Paruchuri, Tashimo *et al.* 2009). Allergic sensitisation to allergen in asthma is either direct, where susceptible individuals develop asthma upon exposure to a specific allergen, or indirect, where individuals develop genetically determined

atopy and therefore become asthmatic as well as sensitised to ubiquitous aeroallergens (Duffy, Mitchell *et al.* 1998). Inhibition of mast cell activation prevents the onset of immediate as well as late phase allergic responses in asthma (Cockcroft and Murdock 1987).

1.8.1.2 Allergic Rhinitis

Allergic rhinitis or hay fever is a chronic allergic condition. Usually it occurs because of a hypersensitivity to pollen and therefore symptoms worsen during summer months. Symptoms include nasal pruritus, nasal congestion, sneezing, rhinorrhea and postnasal drainage. Patients also frequently have co-occurring non-nasal symptoms such as ocular pruritus, swelling, increased lacrimation and erythema as well as irritations of the throat with cough. Allergic rhinitis allergens include mites, cat, dog and *Alternaria* (a genus of fungi) but the prevalence of certain allergen hypersensitivities is dependent on geographical location (Moral, Roig *et al.* 2008). Acute perennial allergic rhinitis is associated with allergens such as dust mite protein and animal epithelium (Kocabeyoglu, Bozkurt *et al.* 2008).

All four histamine receptors are present in the nasal mucosa (Nakaya, Takeuchi *et al.* 2004) and histamine plays a crucial role in the development of nasal blockage, pruritus, sneezing and rhinorrhea (Shelton and Eiser 1994) and nasal lavage histamine levels correlate with nasal symptoms (Naclerio, Meier *et al.* 1983). In experimental models of allergic rhinitis nasal challenge with histamine results in a condition which mimics allergic rhinitis (Doyle, Boehm *et al.* 1990). However, although the H1R antagonist cetirizine reduces histamine-induced nasal blockage it does not abolish it (Taylor-Clark, Sodha *et al.* 2005). Other mast cell mediators are involved in the onset of symptoms; for example LTD₄ and TXA₂ are implicated in allergic rhinitis (Mizutani, Suda *et al.* 2008) while tryptase levels are increased in nasal fluids during pollen season (Di Lorenzo, Mansueto *et al.* 1997). Interestingly, it was recently shown that histamine induces Th2 activation and release of Th2 cytokines through the HR1 in house dust mite allergic rhinitis but not in asthmatics (Botturi, Lacoeyille *et al.* 2010).

1.8.1.3 Mastocytosis

Mastocytosis is a general term given to a spectrum of conditions caused by the presence of abnormally high amounts of systemic mast cells and CD34+ mast cell precursors. Mastocytosis variants include cutaneous mastocytosis of which urticaria pigmentosa is the most prevalent, systemic mastocytosis in which internal organs such as bone marrow and spleen as well as the skin are affected, mast cell leukaemia, aggressive systemic mastocytosis and mast cell sarcoma. Symptoms include pruritus, flushing, nausea, vomiting, diarrhoea, abdominal pain, lesions

(urticaria pigmentosa), vascular instability and anaphylaxis. Mastocytosis may occur at any age and prognosis depends on disease variant, severity and course. Serum tryptase levels are elevated in individuals with mastocytosis and measurement confirms the presence and indicates disease severity of urticaria pigmentosa (Heide, van Doorn *et al.* 2009). PGD2 metabolites are markedly increased in large amounts in patients with mastocytosis, these can also be quantified to confirm diagnosis of mastocytosis (Morrow, Guzzo *et al.* 1995). Mastocytosis is associated with increased risk of anaphylaxis (Brockow, Jofer *et al.* 2008).

1.8.1.4 Allergic Conjunctivitis

Allergic conjunctivitis is an allergic condition of the eye, specifically of the conjunctiva. The common form of acute allergic conjunctivitis is the seasonal form and is associated with seasonal allergens such as pollen (Kocabeyoglu, Bozkurt *et al.* 2008). Often seasonal allergic conjunctivitis is associated with allergic rhinitis, leading to the development of allergic rhinoconjunctivitis (Navarro, Colas *et al.* 2009). Symptoms of allergic conjunctivitis include oedema, erythema, pruritus and increased lacrimation. Mast cell-derived histamine, tryptase (Bacon, Irani *et al.* 2000), PGD2 (Sharif, Xu *et al.* 1996) and IL-4 (Macleod, Anderson *et al.* 1997) are increased following allergen exposure. Vernal keratoconjunctivitis, which is associated with increased IgE specific to dust mite protein and pollen (Kocabeyoglu, Bozkurt *et al.* 2008) and atopic keratoconjunctivitis are both chronic allergic conditions. Interestingly, cases of vernal keratoconjunctivitis and atopic keratoconjunctivitis have been recorded in non-atopic patients who received bone marrow transplant from atopic donors (Tabbara, Nassar *et al.* 2008).

1.8.1.5 Atopic Dermatitis

Atopic dermatitis is a chronic skin disease, characterised by the presence of erythema, oedema, vesiculation, crusting, excoriation (skin wearing), scaling, lichenification (skin thickening and hardening), pigmentation and pruritus. Atopic dermatitis has a strong co-incidence with other allergic diseases such as asthma and allergic rhinitis (Ricci, Patrizi *et al.* 2006). The course of atopic dermatitis, as well as healing time, is significantly related to egg sensitivity (Ricci, Patrizi *et al.* 2006).

1.8.1.6 Anaphylaxis

Anaphylaxis is an acute severe systemic hypersensitive allergic reaction which can occur upon exposure to allergen; it is caused by the immediate degranulation of mast cells either systemically or in one or more organ systems, has a rapid onset

and may cause death. Anaphylaxis is diagnosed in 95% of cases by the presence of some or all of the following symptoms: acute onset with involvement of the skin, mucosa or both; respiratory compromise; reduced blood pressure or associated symptoms of end organ dysfunction; persistent gastrointestinal symptoms; and low systolic blood pressure (Sampson, Munoz-Furlong *et al.* 2006). Although the incidence of anaphylaxis is unknown (Clark and Camargo 2007), it is thought that anywhere between 1% and 15% of the general population of the United States of America (Neugut, Ghatak *et al.* 2001) are affected. It is becoming more prevalent especially in the first 2 decades of life (Lin, Anderson *et al.* 2008). The most common allergens in anaphylaxis are: drugs such as NSAIDs (Conrnejo-Garcia, Blanca-Lopez *et al.* 2009) and β -lactams (Neugut, Ghatak *et al.* 2001); foods (Neugut, Ghatak *et al.* 2001) such as nuts, shellfish, milk, fish or eggs; insect stings (Neugut, Ghatak *et al.* 2001) such as wasp, bee or fire ant; insect bites from flies or mosquitoes; natural latex; monoclonal antibodies such as cetuximab, infliximab and omalizumab; animal dander such as horse or hamster; and grass pollen. Some non-immunologic triggers such as exercise, cold air or water, heat, radiation, ethanol and medications such as opioids can also cause anaphylaxis while some cases of anaphylaxis can be idiopathic, meaning they have no identifiable cause (Sampson, Munoz-Furlong *et al.* 2006).

The majority of anaphylaxis reactions are IgE-Fc ϵ RI-mediated. Increased levels of tryptase may indicate a risk for the development of anaphylaxis and serum tryptase levels can be measured to confirm mast cell burden during anaphylaxis (Schwartz 2006). Serum IL-2, IL-6, IL-10 and histamine levels are also significantly elevated in severe compared to moderate anaphylaxis while IL-4, IL-5, IL-13, IFN- γ and TNF- α levels are marginally elevated in severe anaphylaxis although this elevation does not correlate with reaction severity (Stone, Cotterell *et al.* 2009). Levels of IL-6, IL-10, tryptase and histamine correlate with the occurrence of hypotension in anaphylaxis (Stone, Cotterell *et al.* 2009).

Anaphylaxis is a complex condition and in some instances both IgE- and non-IgE-mediated anaphylaxis can occur in response to a single allergen; for example anaphylaxis from iodinated contrast media is largely IgE-mediated (73%), however, can also be non-IgE-mediated (26%) (Dewachter, Laroche *et al.* 2009).

Anaphylaxis following exposure to heroin (diamorphine, diacetylmorphine) is relatively common and it was found that following heroin injection plasma tryptase levels are increased not due to heroin exposure but because of the presence of morphine, a μ -opioid receptor agonist and metabolite of heroin (Rook, van Zanten *et al.* 2006). Increased serum tryptase levels after death from heroin overdose indicates anaphylaxis in these cases (Edston and van Hage-Hamsten 1998).

Anaesthetic heroin (diamorphine) has also been documented to cause anaphylaxis (Gooch and Gwinnutt 2006).

1.8.2 Mast Cells in Non-Allergic Diseases

Mast cells have been implicated in diseases which are not characterised by a Th2-polarised allergic response. These include autoimmune diseases, chronic non-inflammatory as well as inflammatory diseases.

1.8.2.1 Autoimmune Diseases

Autoimmune diseases are characterised by the presence of self reactive T and B cells which avoid destruction during negative selection in the early phases of their development. Instead they remain self reactive and when they encounter their target self antigens initiate a self-directed immune response.

1.8.2.1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic neurodegenerative disease which results in the demyelination of CNS neurons and a decline in viable neurotransmission. The disease is characterised by reduced mobility, fatigue, changes in sensation and concentration and memory problems.

Mast cells have been implicated in MS, they can be activated by myelin (Medic, Vita *et al.* 2008) and are found in increased numbers in and around MS lesions (Couturier, Zappulla *et al.* 2008). Studies using the experimental model of MS, experimental autoimmune encephalomyelitis (EAE), have found that mice lacking mast cells are resistant to myelin protein-induced EAE (Secor, Secor *et al.* 2000; Robbie-Ryan, Tanzola *et al.* 2003). However, more recently, Bennett *et al.* have found that although mast cells accumulate in the CNS in this experimental model they are not required for the development of disease (Bennett, Blanchet *et al.* 2009). Luteolin, a naturally occurring flavonoid and inhibitor of mast cell activation (Kempuraj, Tagen *et al.* 2008) and T cell activation, has been shown to inhibit EAE (Theoharides, Kempuraj *et al.* 2008). Despite the number of studies carried out in the area the role of mast cells in MS is still poorly understood.

1.8.2.1.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease of the joints. It is characterised by persistent inflammation of the synovium which leads to the erosion of cartilage and bone. Symptoms include pain and oedema which often result in reduced joint mobility as well as disability.

Histamine levels in synovial fluid of RA patients are reduced in comparison to controls (Adlesic, Verdrengh *et al.* 2007), indicating that despite increased mast cell

numbers in the synovium, histamine here is not responsible for RA-associated inflammation. Significant amounts of tryptase are present in the synovial fluid of rheumatoid arthritis while mice lacking the human ortholog show decreased arthritic responses (Shin, Nigrovic *et al.* 2009). Tryptase in the synovial fluid stimulates the proliferation of synovial fibroblast-like cells and the release of pro-inflammatory cytokines such as IL-8 (Nakano, Mishiro *et al.* 2007). Although mast cells degranulate via IgE-Fc ϵ RI-mediated mechanisms in the rheumatoid synovium (Schuerwegh, Ioan-Facsinay *et al.* 2010), the expression of CD153 (the ligand for CD30 which can activate mast cells to release chemokines in the absence of degranulation) on synovial mast cells indicates the possibility that mast cell mediators released in the absence of mast cell degranulation may be influential in RA (Carvalho, Ulfgren *et al.* 2009). Mice deficient in mast cells are resistant to the development of arthritis, an effect which is reversed upon mast cell graft (Lee, Friend *et al.* 2002).

1.8.2.2 Chronic Disease

Mast cells are implicated in chronic non-inflammatory disease such as diabetes mellitus and cystic fibrosis.

1.8.2.2.1 Diabetes Mellitus and Diet-Induced Obesity

Diabetes mellitus or diabetes is a disease characterised by high circulating glucose levels caused by a deficit in insulin levels, resulting in the inability to absorb glucose from the blood. Type I diabetes occurs in patients who are unable to produce insulin, while type II diabetes occurs in patients who are not producing enough insulin or who are insulin resistant. Type II diabetes is often associated with obesity which is usually diagnosed when body mass index (BMI; weight in kg/(height in meters)²) is greater than or equal to 30. Obesity is a major risk factor for cardiovascular disease and cancer as well as type II diabetes.

Mast cells have only recently been implicated in type II diabetes. An extensive study by Liu *et al.* has demonstrated the extent of mast cell involvement in diet-induced obesity and diabetes. They have shown that white adipose tissue from obese adults and mice contain increased mast cell numbers compared to controls, that mast cell stabilisation and genetic mast cell deficiency reduce weight gain in mice and that mast cells contribute to white adipose tissue angiogenesis and adipose apoptosis (Liu, Divoux *et al.* 2009). Insulin, in concert with antigen (human serum IgE), causes enhanced signal transduction upon Fc ϵ RI activation, altered cytoskeletal rearrangement and enhanced degranulation of mast cells sensitised to human serum IgE (Kettner, Di Matteo *et al.* 2010).

1.8.2.2.2 Cystic Fibrosis

Cystic Fibrosis (CF) is a diseases characterised by a dramatic decline in pulmonary function. It is an autosomal recessive condition which is caused by a deletion in the *cfr* gene which alters the expression of CF transmembrane conductance regulator (CFTR), a cAMP-dependent chloride channel which is expressed in epithelial cells; CFTR mutation leads to hyperviscous secretions (Konstan, Hilliard *et al.* 1994). The resulting airway obstruction and inefficient mucociliary clearance lead to a high incidence of chronic pulmonary infections (such as *Staphylococcus aureus* and *Haemophilus influenzae*) (Konstan, Hilliard *et al.* 1994) and associated inflammation in the lower respiratory tract which leads to progressive destruction of the bronchial wall. The incidence of CF in Ireland is the highest in the world (CFIreland 2010).

In CF, mast cell numbers increase slowly and continuously during fetal airway development; these numbers are significantly increased compared to non-CF developing foetuses (Hubeau, Puchelle *et al.* 2001), suggesting a role for mast cells in the early onset of inflammation in CF infants.

CF is often associated with poor nutrition due to the effect of the disease on the gastrointestinal tract (specifically dysmotility). There is a large influx of mast cells into the small intestine in experimental CF models (Canale-Zambrano, Poffenberger *et al.* 2007). Mast cells were recently found to also express functional CFTR (Kulka, Gilchrist *et al.* 2002) and IFN- γ upregulates its expression (Kulka, Dery *et al.* 2005).

1.8.2.3 Chronic Inflammatory Disease

Mast cells have also been implicated in chronic inflammatory diseases such as Celiac Disease and Cardiovascular Disease.

1.8.2.3.1 Coeliac Disease

Coeliac disease is a chronic inflammatory disease of the small intestine. Lesions are formed by the ingestion of proline and glutamine rich wheat gluten as well as related rye and barley proteins. Characteristic coeliac disease lesions show atrophy of villi, crypt hyperplasia, increased epithelial permeability, as well as infiltration of gluten protein-specific T cells. Coeliac disease is associated with increased mast cell numbers and increased histamine secretion in the intestinal mucosa (Strobel, Busuttil *et al.* 1983). Histamine, which is scavenged by gluten peptide, may contribute to the dominant Th1 response seen in coeliac disease (Qiao, Piper *et al.* 2005).

1.8.2.3.2 Cardiovascular Disease

Cardiovascular disease can refer to any disease of the heart and/or vascular system. Mast cells have been implicated in many of these.

As stated, chymase converts angiotensin I to angiotensin II, angiotensin II is found to be co-occurrent with chymase (Hamada, Terai *et al.* 1999) and mast cell chymase also plays a critical role in the formation of abdominal aortic aneurysm in experimental mouse models (Sun, Zhang *et al.* 2009).

Atrial fibrillation is a common arrhythmia which is associated with an increased risk of stroke and heart failure. Mast cell infiltration is induced by pressure overload in the heart in atrial fibrillation and mast cells have been found to play a critical role in atrial fibrillation through their interaction with cardiac fibroblasts (Liao, Akazawa *et al.* 2010). Cardiomyopathy, the deterioration of the myocardium, is associated with increased cardiac mast cell density as well as increased mast cell mediator release (Patella, de Crescenzo *et al.* 1997) while histamine and tryptase levels are increased in these hearts (Patella, Marino *et al.* 1998). Mast cells are implicated in atherosclerosis (Sun, Sukhova *et al.* 2007), or arterial disease and increased mast cell hyperplasia is seen in the raised lesions found in aortic and coronary artery segments (Atkinson, Harlan *et al.* 1994) indicating mast cells are involved in the development of atherosclerotic plaque. Bot *et al.* have shown that perivascular mast cell content correlates with the number of nerve fibres in the coronary atherosclerotic plaque and that neurotransmitters such as substance P can activate mast cells and thus promote mast cell-dependent plaque destabilisation (Bot, de Jager *et al.* 2010).

Mast cell numbers are increased in the hearts of patients with myocarditis (Turlington and Edwards 1988) while in myocardial infarction degranulation of mast cells is higher in segments of the left coronary artery where plaques have ruptured than in non-ruptured plaques (Laine, Kaartinen *et al.* 1999). Mast cells also contribute to the development of cerebral aneurysm by inducing chronic inflammation (Ishibashi, Aoki *et al.* 2010).

Mast cells and mast cell mediators such as TNF- α and histamine have been implicated in many other diseases such as psoriasis (Kirby and Griffiths 2001), migraine (Gazerani, Pourpak *et al.* 2003), irritable bowel syndrome (Buhner, Li *et al.* 2009), chronic inflammation (Oki, Izawa *et al.* 2009; Ishibashi, Aoki *et al.* 2010) and cancer (Coussens, Raymond *et al.* 1999; Esposito, Kleeff *et al.* 2002), indicating their role not only in allergy and atopic disease but in diseases with no obvious allergic component.

1.9 Non-Disease Mast Cell Functions

Mast cells are also functional in many normal, non-disease states such in the defence against parasitic infections and angiogenesis.

1.9.1 Parasite and Microbial Infections

It is thought that protective Th2-polarised IgE-mediated mast cell immune responses evolved to provide defence against parasitic infections (*Nippostrongylus brasiliensis*) and in countries where helminth infections are still prevalent mast cells provide a mechanism of defence against these parasites. The direct inhibition of FcεRI-mediated mast cell mediator release by ES-62, a glycoprotein secreted by filarial nematodes, which acts by selectively blocking several key signal transduction events upon receptor activation, may contribute to the lower incidence of allergic disorders in countries with a high incidence of nematode parasitic infections (Melendez, Harnett *et al.* 2007). This is discussed further in the causes of allergy sections below.

Mast cell-derived TNF-α is necessary for the defence against respiratory and gastrointestinal tract infections (Echtenacher, Mannel *et al.* 1996; Malaviya, Ikeda *et al.* 1996) and it is thought that early activation of mast cells by *Helicobacter pylori* (which causes gastritis) may be involved in the clearance of *H. pylori* from the body (Supajatura, Ushio *et al.* 2002).

1.9.2 Angiogenesis

Angiogenesis is the growth of new blood vessels from existing networks, the major angiogenic factor being VEGF. Increased mast cell numbers are seen in association with an increase in VEGF-mediated angiogenesis in lesions due to vitiligo (Aroni, Voudouris *et al.* 2010), a chronic condition characterised by cutaneous depigmentation. In asthmatics, the basement membrane of the bronchial mucosa is thicker than in non-asthmatics. Here VEGF+ cells and mast cells are increased in numbers in the bronchial mucosa and the numbers of VEGF+ cells are significantly related to the number of blood vessels as well as the number of mast cells present (Chetta, Zanini *et al.* 2005). Therefore, VEGF may play a role in angiogenesis in airway remodelling in asthma. While mast cells are themselves a source of VEGF, mast cell tryptase stimulates the release of VEGF from chondrocytes in arthritis, but not in non-arthritic patients (Masuko, Murata *et al.* 2007). IL-1β mast cell stimulation via LTB₄ receptor 2 (LTB₄R2) mediates secretion of mast cell IL-8, a potent angiogenic factor, secretion (Kim, Lee *et al.* 2010). A carcinoma is an invasive malignant tumor and in cervical lesions microvessel density is correlated with mast cell density, due to invasive carcinoma (Wilk, Lyszka *et al.* 2010).

1.10 Causes of Allergy

While there is no known single cause which determines the development of allergy and atopic disease it is accepted that genetics, epigenetics, helminth infection, hygienic environment and home environment all have a role to play.

1.10.1 Genetics

The contribution of genetic factors to the development of IgE sensitisation and to family history of IgE-mediated disease is between 70-80% (WAO 2010c); this high familial tendency indicates genetic influences in Th2 cell development and IgE production may exist. Several mutations have been implicated in allergy, namely those at the IL-10, IL-12, IL-4, IL-13, IL-4R α , IL-13R α , Fc ϵ RI β and filaggrin loci.

Many single nucleotide polymorphisms (SNPs) at the locus encoding *IL13* are implicated in the development of allergic conditions such as hay fever and asthma (Vladich, Brazille *et al.* 2005; Black, Teixeira *et al.* 2009). *IL13* SNPs are associated with increased IL-13 secretion from Th2-polarised cells (Cameron, Webster *et al.* 2006) as well as increased eosinophil count and increased serum total IgE and exacerbation of asthma when corticosteroids are used as treatment (Hunninghake, Soto-Quiros *et al.* 2007).

IL-4 is also implicated in allergy and SNPs in the IL-4 receptor gene, *IL4R*, are associated with a modest yet significant risk for asthma (Loza and Chang 2007; Zhang, Zhang *et al.* 2007) due to delayed termination of IL-4 signalling.

IL-12 plays a critical role in the development of Th1 cells thus antagonising Th2-polarisation. SNPs in *IL12A*, which is implicated in T-cell development, influence the development of cockroach allergy in children who have asthma, without affecting the development of asthma alone (Pistiner, Hunninghake *et al.* 2008). SNPs in the *IL12B* gene are also thought to be involved in the development of asthma (Randolph, Lange *et al.* 2004; Shimokawa, Nishiyama *et al.* 2009) in certain populations (Noguchi, Yokouchi *et al.* 2001).

SNPs in the gene encoding IL-18, which enhances IL-4 and IL-13 production thus stimulating IgE synthesis are a risk factor for the development of allergic dermatitis (Kim, Lee *et al.* 2007) and severity of adult asthma (Harada, Obara *et al.* 2009).

Specific SNPs in *IL10* promoter and *IL4R* genes are associated with immediate β -lactam allergies in females who have an atopic phenotype (Guglielmi, Fontaine *et al.* 2006) and a low IL-10 producing haplotype is more likely to be associated with severe asthma (Lim, Crawley *et al.* 1998). Variations in the *IL10* gene which promote high IL-10 production are associated with lower incidence of atopy in Gabonese schoolchildren (Van den Biggelaar, Thanh-Duc *et al.* 2007).

SNPs have been found in many more genes which are implicated in allergy such as CD14 (Zambelli-Weirner, Ehrlich *et al.* 2005) and filaggrin (Baurecht, Irvine *et al.* 2007; Weidinger, O' Sullivan *et al.* 2008). It is still unknown to what extent genetics influence the development of these specific conditions; however, it is currently understood that the onset of allergy and atopy is due to a complex interaction of both genetic and environmental factors.

1.10.2 Epigenetics

The concordance rate of allergies such as overall atopy and house dust mite sensitisation (Tovey, Sluyter *et al.* 1998), grass pollen and cat dander sensitisation (Strachan, Wong *et al.* 2001), peanut allergy (Sicherer, Furlong *et al.* 2000), atopic dermatitis (Schultz Larsen 1993), asthma (Skadhauge, Christensen *et al.* 1999) and allergic rhinitis (Rasanen, Laitinen *et al.* 1998) are high in monozygotic compared to dizygotic twins, but because a sizeable discordance between monozygotic twins exists in these diseases, other factors must influence the development of allergy. Epigenetics, changes in phenotype which occur because of stable and potentially heritable mechanisms other than changes in DNA sequences, have a sizeable influence on the development of allergies and may explain this difference (Jiang, Bressler *et al.* 2004). Epigenetic factors include DNA methylation, histone modifications (histone acetylation, methylation, phosphorylation etc.), other chromatin modifications and RNA-mediated modifications which ensure the appropriate expression or silencing of genes (Martino and Prescott 2010). It is believed that epigenetics provide the means for environmental factors to interact with genes and variations in patterns of epigenetic factors are thought to play a role in the development of disease. It is believed that up to 70% of this monozygotic twin variability can be explained by epigenetics (Petronis, Gottesman *et al.* 2003) while the 30% is believed to be due to environment. It is thought that smoking may play a role in causing epigenetic changes; *in utero* exposure to maternal smoking is associated with increased risk for asthma; however, interestingly, maternal exposure of grandmaternal smoking *in utero* is also associated with increased risk for asthma in grandchildren even if grandchildren had not been exposed to maternal smoking *in utero* indicating transmissible changes in epigenetics may be caused by smoking (Li, Langholz *et al.* 2005). Dietary folic acid intake correlates with promoter region methylation, a transmissible epigenetic modification.

1.10.3 Helminth Infections

While viral and bacterial infections prevent allergy by diverting the immune system to a Th1-polarised response thus preventing a Th2 immune response, helminth

infections stimulate potent Th2 immune responses. What is unusual about helminth infection is the paradoxical Th2-polarised immune response which occurs with the absence of a corresponding allergic response; although helminth infections do polarise the immune system to a Th2 response the infection is immunosuppressive explaining this seeming contradiction. Active helminth infections are protective against atopy (Cooper, Chico *et al.* 2003; Flohr, Nguyen-Tuyen *et al.* 2006). Van den Biggelaar *et al.* have shown that chronic helminth infection induces the synthesis and secretion of IL-10 which then suppresses the development of allergy and atopy (van den Biggelaar, van Ree *et al.* 2000). Interestingly the newly discovered nuocyte, which proliferates upon exposure to Th2 cytokines following infection with *Nippostrongylus brasiliensis*, is a source of IL-13, an important Th2 cytokine (Neill, Wong *et al.* 2010).

It is currently accepted that the sole beneficial effect of IgE is to protect against parasites and the development of allergy and atopic disease is in part the result of the continued presence of this ancient helminth defence mechanism which is in the most part redundant today because of the modern Western lifestyle. As discussed, the incidence of allergy is reduced where helminth infection is present and the increase in allergy observed in developing countries seems to be correlated to increased sanitation and hygiene in these countries, again reinforcing both the helminth hypothesis as well as the hygiene hypotheses discussed below.

1.10.4 The Hygiene Hypothesis

The hygiene hypothesis was first proposed by Strachan (Strachan 1989), who proposed that exposure to bacteria and viruses may play a protective role against the development of atopy and allergy and therefore increased incidence of allergy in westernised countries may be a consequence of living in a hygienic environment. Strachan proposed that this hygienic environment leads to the under stimulation of the immune system and a resulting disproportionate immune response to otherwise harmless antigens. In a similar manner no, or reduced, exposure to helminth infections which are otherwise allergy protective, may cause the development of allergic conditions.

Evidence provides a clear link between the increased incidence of allergy and improved hygiene; endotoxin levels in children's mattresses are inversely related to the occurrence of hay fever, asthma and atopic sensitisation and immune responses in these children are markedly reduced compared to controls (Braun-Fahrlander, Riedler *et al.* 2002). Large family size and position in the family (explained by exposure to older siblings) (Strachan 1989) as well as attendance at a crèche, poor sanitation (Flohr, Nguyen-Tuyen *et al.* 2006) and exposure to some viruses and

bacteria are all protective against the development of allergy; treatment of young children with antibiotics and the availability of clean water for both drinking and sewage (Flohr, Nguyen-Tuyen *et al.* 2006) are associated with an increased incidence of allergy.

Expanding on Strachan's original hygiene hypothesis is the currently proposed belief that when the child has been exposed to a hygienic environment, Th2 immune response polarisation occurs because no early life exposure to preventative substances such as microbial components occurs. However, when simultaneous exposure to antigen and preventative substance occurs, a Th1-polarisation of the immune system occurs in response to the antigen (Vogel, Blumer *et al.* 2008). After childhood there is a decrease in the numbers of Th0 cells which may become Th1 or Th2 cells and therefore the ratio of Th1:Th2 cells remain largely unaltered. Interestingly, it was found in an Irish study that the home environment does not differ between asthma sufferers and non-asthma sufferers, with homes in general being dust mite friendly and therefore a large percentage of the juvenile population are exposed to dust mite allergen (Leen, O' Connor *et al.* 1994), exposure which is not predictive of the incidence of allergy.

It is important to note that the majority of those people who have developed an allergic phenotype because of exposure to a hygienic environment in early childhood are asymptomatic or are only very mildly allergic. Although the reason for this is unknown, it may be related to a person's genetic predisposition.

1.10.5 Home Environment

The type of home location and environment is thought to have an influence on the development of allergy and disease. It has long been established that exposure to allergens such as house dust endotoxin (Zambelli-Weirner, Ehrlich *et al.* 2005) and domestic pet exposure (Williams, McPhee *et al.* 2006) in the home play a role in the development of allergic disease. However, more recently it has been demonstrated that farming and home location are important factors in the development of allergy and atopic disease.

1.10.5.1 Farming

It is well established that children who are raised in a farming environment have a lower incidence of allergy and atopy. Numerous studies have been conducted in this area and have found that exposure to stables and consumption of farm milk (Ege, Frei *et al.* 2007) is associated with a lower incidence of asthma, hay fever and atopic sensitisation and continuous long term exposure provides the most protection against the development of these allergic conditions (Riedler, Braun-

Fahrlander *et al.* 2001). Pig keeping, child's involvement in haying, use of silage and frequent stays in animal sheds were also all protective (Ege, Frei *et al.* 2007). It is thought that the protection given in a farming environment, while due to contact with animals, animal sheds and/or consumption of raw products like milk, is also due, at least in part, to the exposure of children to certain microorganisms which are present in this environment. These microorganisms shift the immune response to towards a Th1, and not Th2, profile and reduce allergic incidence (Debarry, Garn *et al.* 2007).

Interestingly maternal exposure to animal sheds during pregnancy exerts a protective effect on atopic sensitisation in children, while maternal contact with hay enhances the protective effect exposure to animal sheds has on grass pollen IgE levels in cord blood (Ege, Herzum *et al.* 2008).

Another study has revealed that farmers and their children who attend cowsheds during feeding are exposed to perennially high pollen concentrations which occurs in parallel with increased fungal and actinomycetal levels (no increase in bacterial levels observed), indicating that the combined effect of permanent exposure to livestock microbes and grass pollen may induce tolerance in children living on a farm (Sudre, Vacheyrou *et al.* 2009).

Taken in combination, the above findings indicate that growing up/living in a farming environment is protective against development of asthma, hay fever and atopic sensitisation.

1.10.5.2 Smoke, Exposure to Parental Smoking, Pollution and Weather

Baynam *et al.* have found that parental smoking interacts with genes associated with atopy (IL-4, IL-4Ra and IL-13 genes) to impair vaccine responses (Baynam, Khoo *et al.* 2007) and there is evidence for an interaction between smoking and a SNP in the *IL13* gene in causing allergy (Black, Teixeira *et al.* 2009).

Particulate air pollution such as wood smoke, road traffic particles and diesel exhaust particles all have the capacity to enhance allergic sensitisation (Samuelsen, Nygaard *et al.* 2008), while exposure to traffic-related air pollution such as particulate matter, soot and NO₂, is directly related to the development of asthma, but not eczema, allergic sensitisation and bronchial hyperresponsiveness, in children (Gehring, Wijga *et al.* 2010). Pollutant particle size seems to be important in this effect (Gent, Koutrakis *et al.* 2009).

A study carried out on behalf of the Israeli government found that childhood exposure to industrial pollution, composed of many organic and inorganic chemicals, results in the increased prevalence of asthma (Karakis, Kordysh *et al.*

2009). A recent study has shown that fluctuations in humidity and temperature also exacerbate asthma in children (Mireku, Wang *et al.* 2009).

Because the worldwide increase in the incidence of allergy is occurring too rapidly to be explained by changes in genetics it must be true that a complicated and as yet unknown interaction between environment, epigenetics and genetics results in the development of allergy and atopy.

1.11 Allergy Treatments and Therapies

Because no cure currently exists to prevent and eliminate allergic disease, various drug classes are used to try and control the symptoms of these diseases. These include mast cell stabilising compounds, traditional antihistamines and corticosteroids. If possible, the avoidance of allergen will prevent symptoms but this is not always possible. Immunotherapy is a relatively new treatment which aims to desensitise an individual to their allergen.

1.11.1 Mast Cell Stabilising Compounds

Mast cell stabilising compounds act to stabilise the mast cell by interacting with the IgE-Fc ϵ RI complex to prevent receptor crosslinking upon allergen exposure. Therefore, mast cells do not become activated to release their mediators and the allergic reaction which usually ensues is avoided. This drug class is composed of first-generation and second-generation mast cell stabilisers. First-generation mast cell stabilisers include disodium cromoglycate, nedocromil sodium, pemirolast and lodoxamide. These first-generation mast cell stabilising compounds require treatment for up to two weeks before their full mast cell stabilising potential is reached, therefore newer mast cell stabilising compounds, which also demonstrated H1R antagonist activity were developed. These newer dual acting compounds include ketotifen, olopatadine, azelastine and tiacrilast.

1.11.1.1 Disodium Cromoglycate (Cromolyn)

Disodium cromoglycate (DSCG) (figure 1.08), a chromone, was the first known mast cell stabilising compound. It was derived from khellin, a potent vasodilator which is derived from the seeds of *Ammi visnaga* (Meyer 2002). The development of DSCG meant that treatment of asthma was no longer restricted to the use of inhaled corticosteroids and bronchodilators. Experimentally, DSCG inhibits IgE-mediated histamine release (although mast cells eventually become resistant to DSCG *in vitro*) (Kusner, Dubnick *et al.* 1973) as well as calcium ionophore (Johnson and Bach 1975) and compound 48/80-mediated histamine release (Orr, Hall *et al.*

1971) from mast cells *in vitro*. *In vivo*, DSCG prevents the release of mast cell mediators and inhibits passive cutaneous anaphylaxis (Morse, Austen *et al.* 1969; Orr, Pollard *et al.* 1970). It is thought that chromones may act as specific anion channel blockers; DSCG blocks volume activated Cl⁻ currents, an action which occurs following its permeation across the cell membrane (Heinke, Szucs *et al.* 1995). DSCG is effective in the treatment of gastrointestinal manifestations of mastocytosis (Horan, Sheffer *et al.* 1990). DSCG is used in the treatment of asthma (Intal; Sanofi-Aventis) in conjunction with the use of bronchodilators, allergic conjunctivitis (Opticrom; Sanofi-Aventis) and allergic rhinitis (Rynacrom; Sanofi-Aventis).

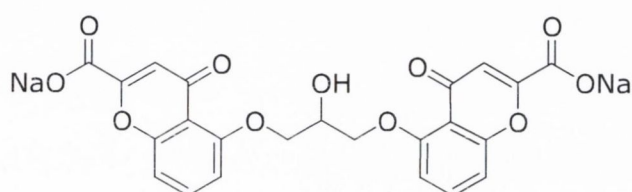


Figure 1.08: Disodium Cromoglycate

1.11.1.2 Nedocromil Sodium

Nedocromil sodium (figure 1.09), also a chromone, was the next mast cell stabilising compound to be synthesised. It exhibited equal and superior mast cell stabilising activity to that of DSCG both *in vitro* (Leung, Flint *et al.* 1986; Riley, Mather *et al.* 1987) and *in vivo* (Eady, Greenwood *et al.* 1985). However, subsequent human studies indicated nedocromil sodium possessed similar mast cell stabilising activity to DSCG (Schwartz, Blumenthal *et al.* 1996). Nedocromil sodium does, however, demonstrate significantly more effect than placebo in the treatment of mild to moderate asthma (Schwartz, Blumenthal *et al.* 1996) and therefore it is used to treat asthma that is not adequately controlled by bronchodilators alone (Tilade; Sanofi-Aventis).

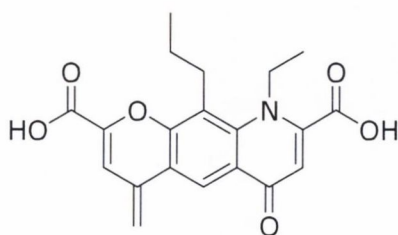


Figure 1.09: Nedocromil Sodium

1.11.1.3 Pemirolast

Pemirolast (figure 1.10) inhibits antigen, compound 48/80 (Fujimiya, Nakashima *et al.* 1991) and IgE, but not calcium ionophore (Yanagihara, Kasai *et al.* 1988), - induced mast cell degranulation *in vitro*. Pemirolast also inhibits passive cutaneous anaphylaxis *in vivo* (Yanagihara, Kasai *et al.* 1988). In phase III clinical trials pemirolast results in the reduction of conjunctival allergen challenge induced ocular pruritis and therefore may be beneficial to sufferers of allergic conjunctivitis (Abelson, Berdy *et al.* 2002). Pemirolast demonstrates similar activity to nedocromil sodium in the prevention of symptoms of allergic conjunctivitis such as redness, itching and eyelid swelling; however, pemirolast demonstrates a significant reduction in swelling at work/school in comparison to nedocromil sodium as well as increased comfort upon treatment (Shulman 2003). Pemirolast is not prescribed for treatment in Ireland.

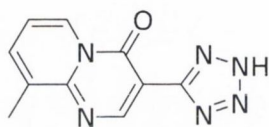


Figure 1.10: Pemirolast

1.11.1.4 Lodoxamide

Lodoxamide (figure 1.11) inhibits passive cutaneous anaphylaxis and symptoms in animal models of asthma (Johnson, Griffin *et al.* 1979) and plays a protective role in immediate asthmatic allergen response (Ollier, Graham *et al.* 1982). Lodoxamide is ineffective as a long term treatment for mild allergic asthma (Mann, Clement *et al.* 1985). However, it is effective in the treatment of allergic conjunctivitis (Denis, Bloch-Michel *et al.* 1998) where it shows superior activity and lower side effect incidence, than DSCG (Fahy, Easty *et al.* 1992) and nedocromil sodium (Yanni, Weimer *et al.* 1993). Lodoxamide reduces tryptase levels and eosinophil and neutrophil recruitment in tears following conjunctival antigen challenge in humans (Bonini, Schiavone *et al.* 1997) and inhibits eosinophil activation (Capron, Loiseau *et al.* 1998). Lodoxamide is used in the treatment of allergic conjunctivitis (Alomide; Alcon Laboratories).

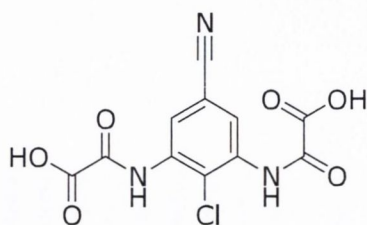


Figure 1.11: Lodoxamide

1.11.1.5 Ketotifen

Ketotifen (figure 1.12) stimulates histamine release at concentrations slightly higher than effective inhibitory concentrations in human *in vitro* conjunctival mast cell preparations (Yanni, Stephens *et al.* 1996). Ketotifen has been shown to be cytotoxic, resulting in the hemolysis of cells membranes *in vitro* (Brockman, Graff *et al.* 2000). Ketotifen inhibits passive cutaneous anaphylaxis *in vivo* (Awouters, Niemegeers *et al.* 1985) and is well tolerated and effective in the treatment of allergic conjunctivitis (Avunduk, Tekelioglu *et al.* 2005). Ketotifen is used in the treatment of allergic conjunctivitis, asthma and allergic rhinitis (Zaditen; Novartis).

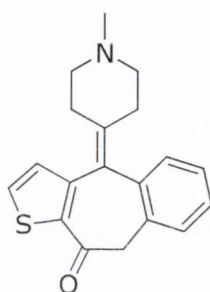


Figure 1.12: Ketotifen

1.11.1.6 Olopatadine

Olopatadine (figure 1.13) is an extremely effective mast cell stabiliser and antihistamine. It inhibits tryptase, PGD_2 (Sharif, Miller *et al.* 1996) and histamine release from human conjunctival mast cells *in vitro*, does not cause the release of histamine at high concentrations and also acts as a specific antihistamine *in vivo* (Yanni, Stephens *et al.* 1996). The H1R selectivity of olopatadine is superior to that of ketotifen and other antihistamines such as levocabastine, antazoline and pheniramine (Sharif, Miller *et al.* 1996). *In vitro* olopatadine exhibits superior conjunctival mast cell stabilising activity than DSCG, pemirolast and nedocromil sodium (Yanni, Miller *et al.* 1997; Abelson and Spitalny 1998; Butrus, Greiner *et al.* 2000). In comparison to ketotifen, olopatadine does not cause the lysis of cells *in vitro* (Brockman, Graff *et al.* 2000). Olopatadine is statistically and clinically superior to intranasal steroid spray (fluticasone furoate) for the relief of the symptoms of allergic conjunctivitis (Rosenwasser, Mahr *et al.* 2008) and it is effective in the treatment of allergen-induced nasal symptoms typical of allergic rhinitis (Pipkorn, Costantini *et al.* 2008). Olopatadine also inhibits scratching behaviour in the mouse and potentiates the inhibitory effect of prednisolone (a glucocorticoid) on the symptoms of atopic dermatitis *in vivo* (Kagawa, Izawa *et al.* 2010) indicating olopatadine may be effective in the treatment of atopic dermatitis.

Olopatadine is used for the treatment of allergic conjunctivitis (Opatanol, Alcon Laboratories) and although it is indicated for use twice daily it can be administered once daily at double dose and remain effective over 24 hours (Abelson, Spangler *et al.* 2007).

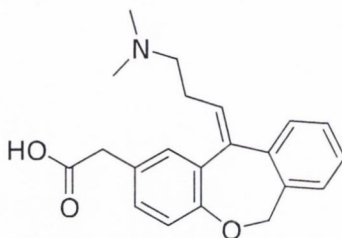


Figure 1.13: Olopatadine

1.11.1.7 Azelastine

Azelastine (figure 1.14) is a more potent inhibitor of mast cell activation both *in vitro* and *in vivo* than olopatadine (Lytinas, Kempuraj *et al.* 2002). However, azelastine, like ketotifen, demonstrates cytotoxic activity in conjunctival cells *in vitro* (Lee, Lee *et al.* 2008). Azelastine is effective in the treatment of allergic rhinitis (Shah, Nayak *et al.* 2009).

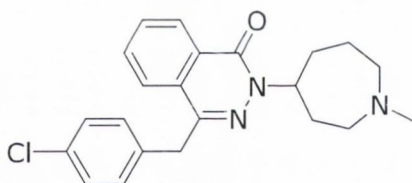


Figure 1.14: Azelastine

1.11.1.8 Tiacrilast

Tiacrilast (figure 1.15) inhibits mast cell degranulation and shows potent mast cell stabilising activity in several *in vivo* animal models (Welton, Hope *et al.* 1984). It is effective in the treatment of animal models of atopic dermatitis (Csato, Judak *et al.* 1990), Tiacrilast is not currently prescribed for treatment of allergy in Ireland.

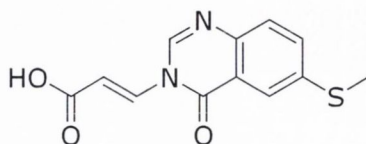


Figure 1.15: Tiacrilast

1.11.2 Histamine Receptor 1 Antagonists

H1R antagonists (antihistamines) act at the H1R to antagonise the activity of histamine which is released from activated mast cells. They are used in the treatment of allergy and result in the reduction of symptoms such as swelling, pruritis, urticaria, sneezing, sinus congestion and postnasal drip.

First-generation H1R antagonists can permeate the blood brain barrier and therefore affect histamine neurotransmission in the brain resulting in drowsiness and inducing sleep. These first-generation compounds include pyrilamine/mepyramine (figure 1.16), chlorpheniramine (chlorphenamine) (figure 1.17) and promethazine (figure 1.18). Mepyramine is used in the treatment of insect bites, stings and nettle rash (Anthisan; Sanofi-Aventis). Chlorpheniramine is used in the treatment of acute allergic reactions (Piriton; GlaxoSmithKline) and promethazine is used in the treatment of allergic conditions and reactions (Phenergan; Sanofi-Aventis).

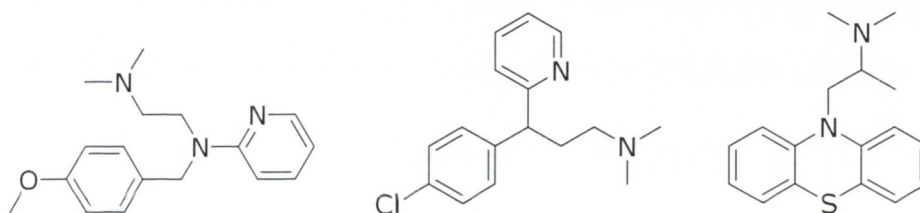


Figure 1.16: Mepyramine Figure 1.17: Chlorpheniramine Figure 1.18: Promethazine

Because of the undesirable sedating effects of first-generation antihistamine compounds, newer compounds which do not penetrate the blood brain barrier and therefore do not affect wakefulness were developed. These compounds include loratadine (figure 1.19), desloratadine (figure 1.20), cetirizine (figure 1.21), levocetirizine (figure 1.22), emedastine (figure 1.23) and fexofenadine (figure 1.24). Loratadine is used in the treatment of allergic rhinitis (Claritin; Schering-Plough and Histaclar; Gerard Laboratories), Desloratadine is used in the treatment of allergic rhinitis and urticaria (Neoclaritin; Schering-Plough). Cetirizine is used in the treatment of allergic rhinitis (Histek; Helsinn Birex Therapeutics, Zirtek; UBC Pharma and Zirtene; Gerard Laboratories), levocetirizine is used for the treatment of allergic rhinitis (Xytal; UCB Pharma, fexofenadine is used in the treatment of allergic rhinitis (Telfast; Sanofi-Aventis) and emedastine is used for the treatment of allergic conjunctivitis (Emadine; Alcon Laboratories).

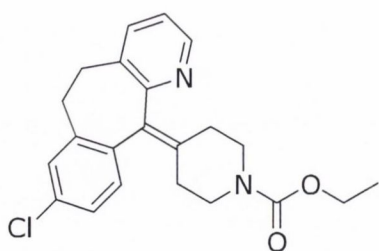


Figure 1.19: Loratadine

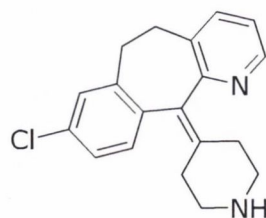


Figure 1.20: Desloratadine

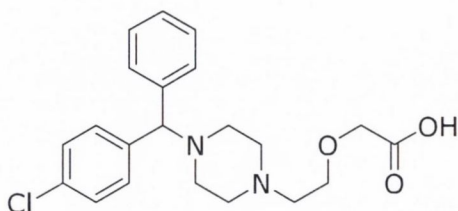


Figure 1.21: Cetirizine

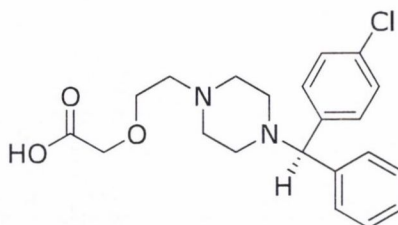


Figure 1.22: Levocetirizine

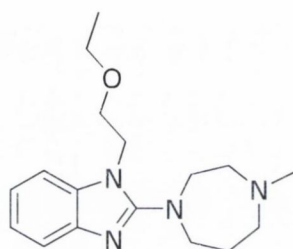


Figure 1.23: Emedastine

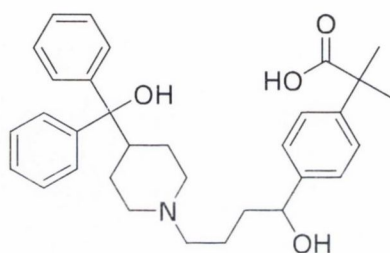


Figure 1.24: Fexofenadine

1.11.3 Immunotherapy

Immunotherapy is a relatively new approach in the treatment of allergy. Two of these approaches are allergen-specific immunotherapy and anti-IgE antibody therapy.

1.11.3.1 Allergen Immunotherapy/ Allergen-Specific Immunotherapy

Allergen-specific immunotherapy aims to induce immuno-tolerance in allergic individuals. Because allergic individuals can tolerate minute amounts of allergen, modulation of the immune response following gradual exposure (subcutaneous injection or sublingual) to increasingly larger doses of allergen leads to desensitisation/tolerance in the affected individual, thus reducing or eliminating the need for medication. It is thought that allergen-specific immunotherapy induces the expression of allergen-specific IgG, instead of IgE, via B cell antibody class switching, which therefore neutralises the allergen (Taudorf, Laursen *et al.* 1989). Because Th2 responses are induced in allergic individuals upon exposure to allergen and Th2 but not Th1 cells are detectable in the target organs of allergic individuals,

successful specific immunotherapy can therefore shift the allergen-specific response of allergic individuals from Th2 to Th1 (Shida, Kadoya *et al.* 2004). Accordingly, allergen-specific immunotherapy is associated with increased expression of IL-10 and IFN- γ and suppression of Th2 and induction of Th1 immune polarisation (Jutel, Pichler *et al.* 1995; Jutel, Akdis *et al.* 2003). Grazax tablets, which contain grass pollen (*Phleum Pratense*, ALK-Abello) and therefore may be used to induce tolerance, have recently been approved for use in the treatment of allergic rhinitis (ALK-Abello 2010). Allergen-specific immunotherapy is also used in the treatment of asthma, allergic rhinoconjunctivitis and is particularly effective in the treatment of cat dander, dust mite, tree pollen, grass pollen or weed pollen allergy. (Pollen shows strong cross reactivity and treatment using grass pollen often leads to desensitisation to all grass species (Norman 1985; Alexander, Kay *et al.* 2002)). Results of allergen-specific immunotherapy are long lasting (Passalacqua and Canonica 2002). In the treatment of IgE-mediated anaphylaxis (such as bee and wasp sting) allergen-specific immunotherapy can induce effective tolerance in 90% of cases. Rarely immunotherapy results in anaphylaxis (Frew 2010).

1.11.3.2 Anti-IgE Therapy

Because Fc ϵ RI expression levels correlate with serum IgE levels, targeting serum IgE with specific anti-IgE antibody results in the immobilisation of IgE and the subsequent reduction of free serum IgE which results in decreased expression of and load upon Fc ϵ RI (Belliveau 2005). Omalizumab (Xolair, Novartis) is a humanised monoclonal antibody manufactured by recombinant DNA technology in the Chinese hamster ovary (CHO) cell line that is used in the treatment of asthma. Omalizumab selectively binds to the C ϵ 3 domain of soluble IgE (Dodig, Richter *et al.* 2005) thus immobilising and preventing IgE-Fc ϵ RI binding. Clearance of the α -IgE-IgE complex is carried out by leukocytes and platelets. Mast cell Fc ϵ RI expression is downregulated after treatment and omalizumab shows effectiveness after at least 12-16 weeks (Novartis 2011). The decrease in serum IgE levels upon treatment with omalizumab occurs in a dose-dependent manner and treatment results in the inhibition of early and late phase allergic responses in asthma (Belliveau 2005). Anaphylaxis may occur upon administration of omalizumab.

1.12 Novel Mast Cell Stabilising Compounds

This project is concerned with the development of novel mast cell stabilising compounds. Ongoing research into the design, synthesis, *in vitro* and *in vivo* activity of novel compounds has resulted in the synthesis of several compounds which aim to stabilise the mast cell and prevent mast cell activation and

degranulation. Compounds synthesised in this lab fall into five groups. Groups I, II and III were synthesised by Finn (B.Sc., Ph.D. in preparation), while groups IV and V were synthesised by Zhang (Ph.D. 2010). The reasoning behind the synthesis of these compounds is discussed in the following chapters.

Group I consists of: DF1.01, DF1.02, DF1.03, DF1.04, DF1.05 and DF1.06,

Group II consists of: DF2.01, DF2.02, DF2.03, DF2.04 and DF2.05,

Group III is composed of: DF3.01, DF3.02, DF3.03 and DF3.04,

Group IV consists of: TZ1.01, TZ1.02, TZ1.03, TZ1.04, TZ1.05, TZ1.06 and TZ1.07,

Group V comprises; TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05, TZ2.06, TZ2.07, TZ2.08, TZ2.09, TZ2.10, TZ2.11, TZ2.12 and TZ2.13.

Group I, Group II, Group III, Group IV and Group V compounds are synthesised in two, three, eight or nine, from five to eleven and two or three steps respectively. Current drugs used to treat allergy, such as olopatadine, require up to seven synthesis steps (Ohshima, Otaki *et al.* 1992). Apart from the potential increased efficacy these compounds may have in comparison to current allergy drug treatments, it is clear that the majority of these novel compounds may be synthesised in a small number of steps. This may reduce not only the time taken but the cost of materials during the manufacturing process.

1.13 Aims

Following the synthesis of the novel compounds above it was necessary to evaluate their anti-allergy activity. Therefore, the aims of this project were:

1. To implement a method of rat peritoneal mast cell purification which would allow for further investigation into the mast cell stabilising activity by these compounds.
2. To establish mast cell stabilising activity *in vitro* in groups I to IV above, using both calcium ionophore and compound 48/80 to elicit histamine from a pure rat peritoneal mast cell population and to determine IC₅₀ values where appropriate.
3. To investigate whether a structure activity relationship exists for these compounds.

4. To assess compounds which demonstrate potent mast cell stabilising activity *in vitro* for *in vivo* mast cell stabilising activity using passive cutaneous anaphylaxis.
5. To evaluate systemic toxicity in representative compounds which demonstrate mast cell stabilising activity *in vivo*, using the SHIRPA behavioural observation protocol.

Results were obtained from the above studies, the project was evaluated and subsequent aims were then:

6. To develop a liquid chromatography mass spectrometry method to evaluate mast cell degranulation in rat peritoneal mast cells and to determine the mast cell stabilising activity of Group V compounds using this method.
7. To assess compounds which demonstrate suitable mast cell stabilising activity *in vivo* for H1R antagonist activity *ex vivo* using the guinea pig ileum protocol for H1R antagonist activity.
8. To evaluate compounds which demonstrate H1R antagonist activity *ex vivo* for *in vivo* anti-allergic pruritis activity using the scratching behaviour protocol.

Chapter 2

Investigations into the *In Vitro* Mast Cell Stabilising Activities of Novel Benzocycloalkanone Compounds

2.1 Introduction

Novel compounds with potent mast cell stabilising activity were previously developed in this lab by Barlow (Ph.D. 2002) (Barlow and Walsh 2008; Barlow and Walsh 2010), Byrne (Ph.D. 2006) and McHugh (M.Sc. 2006), the most potent of these being JWB4.61 and JWB5.12 which were synthesised by Barlow. However, because these compounds demonstrate poor solubility and poor stability over time it was necessary to develop newer compounds which addressed and attempted to rectify these issues. Novel compounds were therefore designed and synthesised by Finn and Zhang, using JWB4.61 and JWB5.12 as starting compounds. These newer compounds aim to improve solubility and overcome the stability problems of their parent compounds.

Compounds synthesised by Finn fall into three classes, namely;

- Group I; DF1.01, DF1.02, DF1.03, DF1.04, DF1.05, DF1.06
- Group II; DF2.01, DF2.02, DF2.03, DF2.04, DF2.05 and
- Group III; DF3.01, DF3.02, DF3.03, DF3.04.

Compounds synthesised by Zhang, known as Group IV are;

- TZ1.01, TZ1.02, TZ1.03, TZ1.04, TZ1.05, TZ1.06, TZ1.07.

The design of each of these groups of compounds is discussed below.

2.1.1 Group I Compounds

Group I compounds were synthesised, by Finn, as a continuation of work carried out by Barlow. JWB5.12 (figure 2.01 a) demonstrates promising mast cell stabilising activity *in vitro* (RPMC assay; $73 \pm 14\%$ inhibition of histamine release, $20 \mu\text{M}$), however, this activity is not maintained in an *in vivo* setting (PCA; 44% inhibition of PCA, 3 mg/kg). Because JWB5.12 contains two chiral centres, resulting in the formation of four stereoisomers, Finn synthesised several novel simplified versions of JWB5.12 with the aim of maintaining mast cell stabilising activity while reducing the number of stereoisomers formed in comparison with the lead compound. These novel compounds would only form two stereoisomers due to the presence of identical side chains in each compound, thus reducing the number of distinct chiral centres from two to one. The compounds synthesised in this group are: DF1.01, DF1.02, DF1.03, DF1.04, DF1.05 and DF1.06 (figure 2.01 b-g).

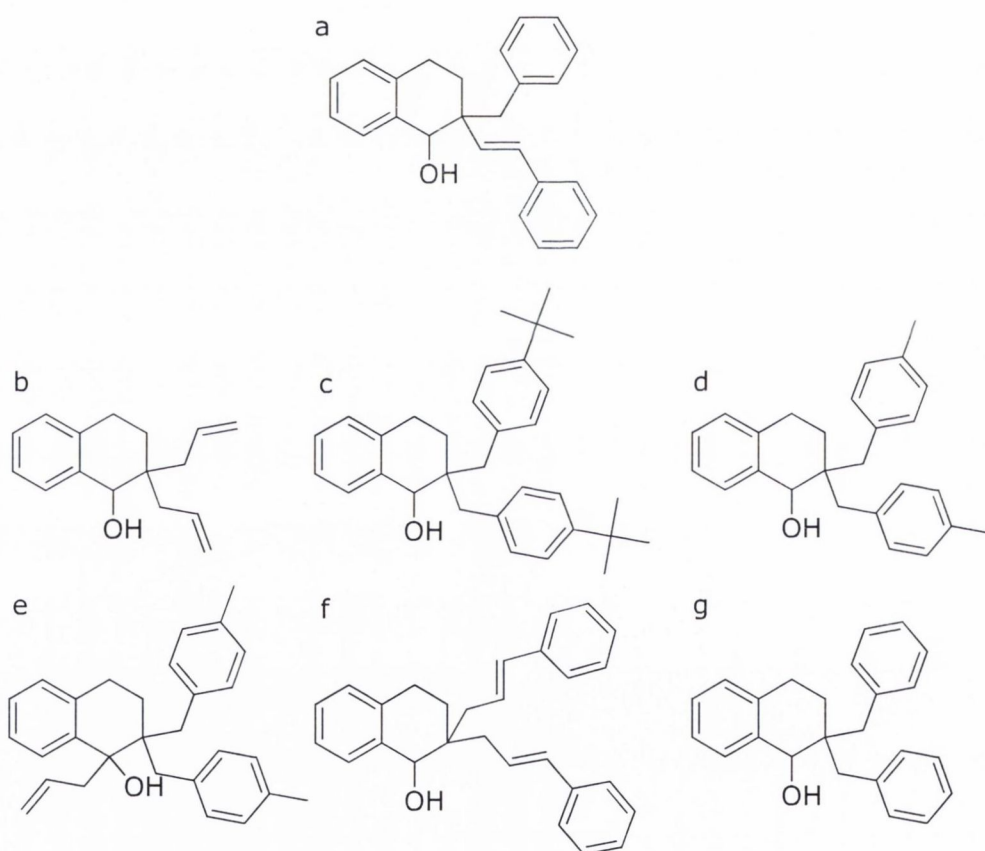


Figure 2.01: JWB5.12 and Group I compounds; a) JWB5.12, b) DF1.01, c) DF1.02, d) DF1.03, e) DF1.04, f) DF1.05 and g) DF1.06

2.1.2 Group II Compounds

Group II compounds were synthesised, again by Finn, with the aim of increasing the solubility of DF Group I compounds. Therefore, all of these Group II compounds contain additional functional groups which aim to improve their solubility. The resulting compounds are: DF2.01, DF2.02 and DF2.04 which are the succinate derivatives of DF1.06, DF1.02 and DF1.03 respectively; DF2.03 which is the glutarate derivative of DF1.06; and DF2.05 which utilises the basicity of an amine functionality to improve the solubility of DF1.06 (figure 2.02 a-e).

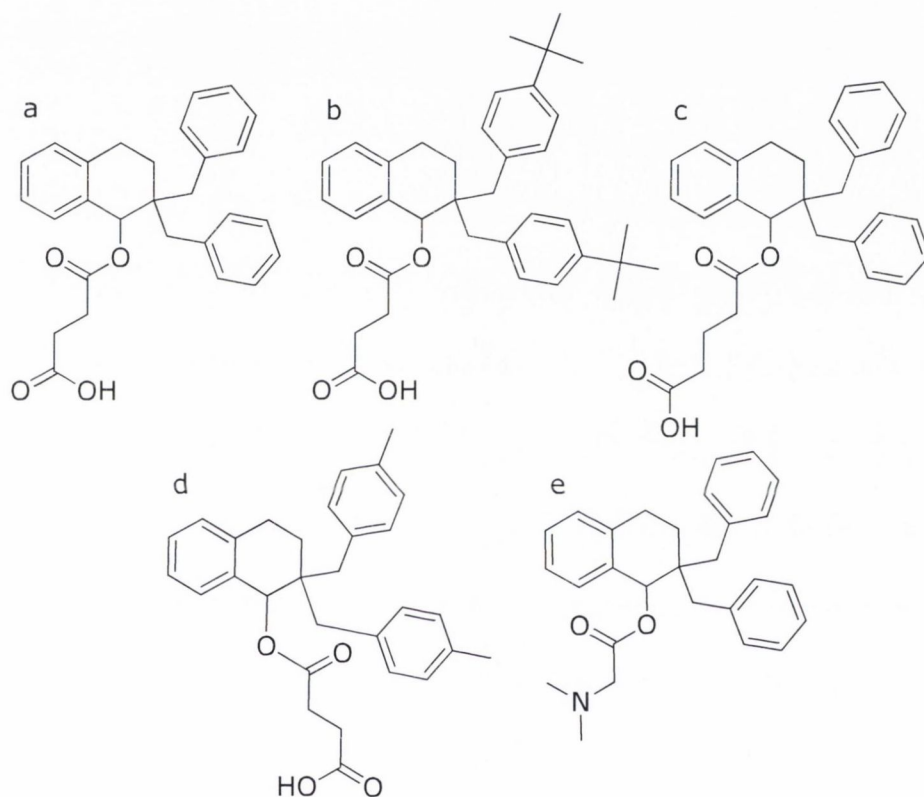


Figure 2.02: Group II compounds; a) DF2.01, b)DF2.02, c) DF2.03, d) DF2.04 and e) DF2.05

2.1.3 Group III Compounds

A second compound synthesised by Barlow which demonstrated potent mast cell stabilising activity *in vitro*, which was maintained *in vivo* was JWB4.61 (Barlow and Walsh 2008). However, JWB4.61 (figure 2.03 a) has a poor stability profile. Subsequent investigation by Finn demonstrates (unpublished data) that JWB4.61 fragments *in vitro* under physiological conditions and thus explains its poor stability. Because DF1.03 demonstrates potent mast cell stabilising activity a merged hybrid, a composite of JWB4.61 and DF1.03, was created. This would result in the formation of a more stable compound which would hopefully overcome the issues of stability inherent in JWB4.61 while hopefully maintaining the mast cell stabilising activity of both JWB4.61 and DF1.03. The compounds in this group are DF3.01, DF3.02 and DF3.03, as well as DF3.04, a merged hybrid which is a composite of JWB4.61 and DF1.05 (figures 2.03 b-e).

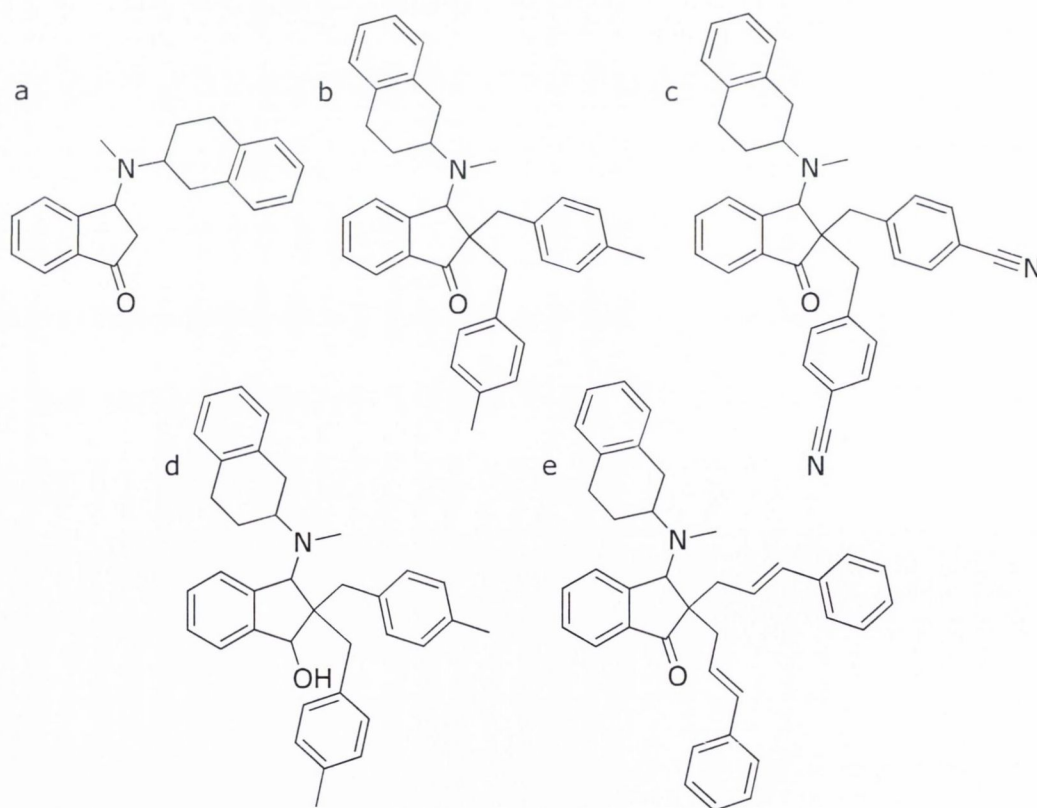


Figure 2.03: JWB3.61 and Group III compounds; a) JWB4.61, b) DF3.01, c) DF3.02, d) DF3.03 and e) DF3.04

2.1.4 Group IV Compounds

Because JWB4.61 fragments *in vitro* to form a conjugated compound, a fourth group of compounds (Group IV) were synthesised by Zhang. This series of compounds were designed to be simplified versions of JWB4.61 which could not fragment and therefore, would overcome the stability issues inherent in this compound (Barlow, Zhang, Woods and Walsh, paper in press, see Appendix II). The compounds which were synthesised in this group are: TZ1.01, TZ1.02, TZ1.03, TZ1.04, TZ1.05, TZ1.06 and TZ1.07 (figures 2.04 a-g).

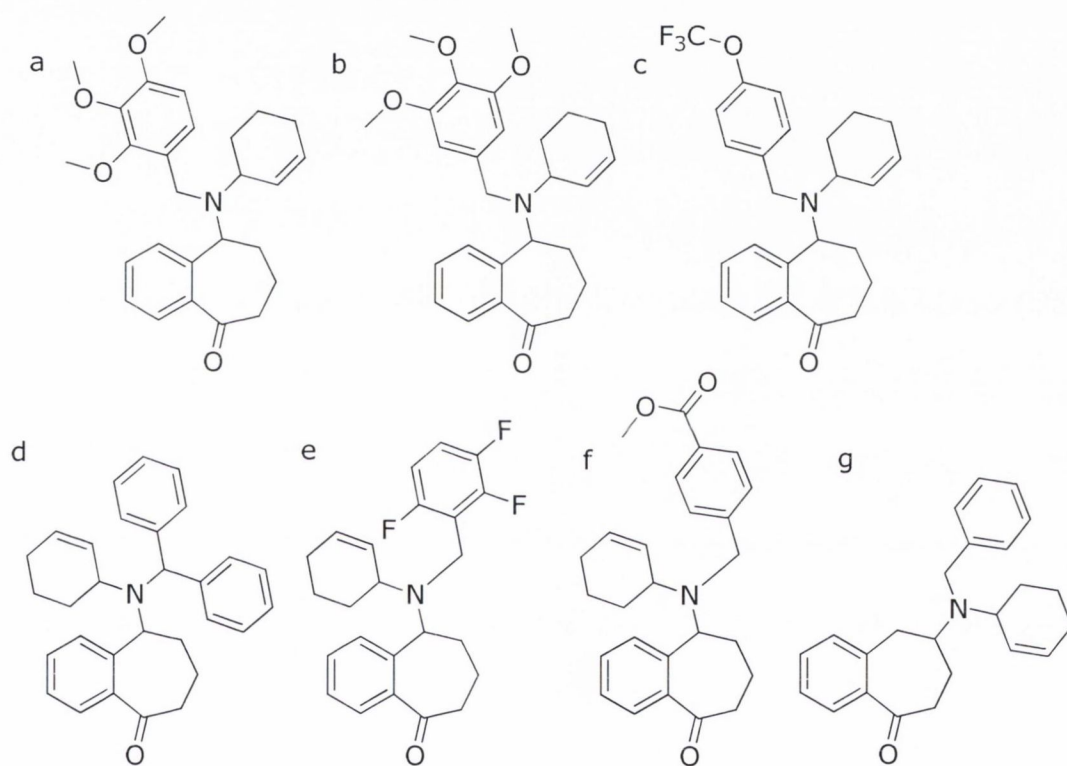


Figure 2.04: Group IV compounds; a) TZ1.01, b) TZ1.02, c) TZ1.03, d) TZ1.04, e) TZ1.05, f) TZ1.06 and g) TZ1.07

2.1.5 Aims:

Synthesis of these four groups of novel compounds meant it was necessary to evaluate their mast cell stabilising activity. Therefore, the primary aims of this project were:

- To select and implement a method for the purification of mast cells from the total rat peritoneal cell population,
- Following mast cell purification, to evaluate the novel compounds which were synthesised by Finn and Zhang above, for mast cell stabilising activity using the rat peritoneal mast cell (RPMC) assay. This follows the approach taken by Barlow, Byrne and McHugh to assess mast cell stabilising activity of their original compounds and
- To determine whether a theoretical structure activity relationship exists for these novel groups of compounds.

Decisions on what approach to take with respect to further compound synthesis and biological assays would be made depending on the results of these studies.

2.1.6 Rat Peritoneal Mast Cells

RPMS have been used to research the mechanisms and treatments of allergy for many years (O'Donnell, Ackerman *et al.* 1983; Heiman and Crews 1985; Kurosawa, Hisada *et al.* 1992; Choo, Park *et al.* 2003; Shin, Kim *et al.* 2005). Although, as already stated, rat mast cells primarily release serotonin and not histamine as in humans, they are readily accessible and therefore allow for the easy investigation of allergic processes. Although several cell lines, such as the *RBL-2H3*, exist to study mast cell mechanisms etc., isolated primary RPMS, which do not display altered phenotype in the manner of some cell lines, were chosen for this project.

2.1.7 Percoll

Percoll is composed of colloidal silica particles (15-30 nm diameter) coated with polyvinylpyrrolidone and is used to separate/purify cells, viruses and subcellular particles. It was first used in 1978 (Pertoft, Laurent *et al.* 1978) and separates cells on the basis of density gradient centrifugation. Essentially centrifugation of Percoll results in the formation of a density gradient due to the heterogeneity of particle sizes in the medium, therefore cells which are present band isopycnically as this gradient is formed (SigmaAldrich 2010). Working preparations of Percoll can be prepared to obtain desired density gradients. The method chosen for purification of peritoneal mast cells was developed by Rickard and Lagunoff (Rickard and Lagunoff 1994). Mast cells are separated from other peritoneal cells because they have a

higher density than that of other cells of the peritoneum, as well as the surrounding Percoll, which results in the formation of a highly purified mast cell pellet.

2.1.8 Rat Peritoneal Mast Cell Assay

The RPMC assay is frequently used to determine mast cell stabilising activity *in vitro* and is widely published in this use (Heiman and Crews 1985; Choo, Park *et al.* 2003; Shin, Kim *et al.* 2005). The assay comprises three main steps; firstly the test compound is incubated with RPMCs (purified or non-purified) to allow the stabilisation of mast cells by the compound, secondly an elicitor is added to the cells/compound matrix to cause the degranulation of mast cells and finally mast cell degranulation is quantified by measuring mast cell mediator release. A variety of elicitors can be used to induce mast cell degranulation; the calcium ionophore A23187 and compound 48/80 are used here.

2.1.9 Calcium Ionophore A23187

An ionophore is any molecule which facilitates the transport of ions across the cell membrane lipid bilayer. Calcium ionophore is a carboxylic acid antibiotic that is crystallised in broths of *Streptomyces chartreusensis* (Reed and Lardy 1972). It specifically binds divalent, but not monovalent, cations at physiological pH allowing for their transport across the cell membrane. The first studies on calcium ionophore A23187 found it uncouples oxidative phosphorylation while inhibiting ATP hydrolysis in rat liver mitochondria (Reed and Lardy 1972; Wong, Wilkinson *et al.* 1973). Calcium ionophore causes mast cell degranulation by facilitating transmembrane fluxes of calcium (Cochrane and Douglas 1974) and thus it is frequently used as a mast cell secretagogue.

2.1.10 Compound 48/80

Compound 48/80, a mixed polymer, is a condensation product of N-methyl-p-methoxyphenethylamine with formaldehyde and was first formed as a by product during attempts to synthesise a hypotensive tetrahydroisoquinoline (Lagunoff, Martin *et al.* 1983). Compound 48/80 activity is associated with tetrameric through to octameric derivatives, with the hexamer being the most active (Read and Lenney 1972). It was thought that compound 48/80 bypasses receptors to directly interact with and activate G proteins (Mousli, Bronner *et al.* 1990), causing dissociation of G proteins into α - and $\beta\gamma$ -subunits, increasing GDP-GTP exchange reaction and initiating downstream signal transduction pathways (Tomita, Inanobe *et al.* 1991) including the activation of PLD (Chahdi, Fraundorfer *et al.* 2000). However, Palomaki and Laitinen (Palomaki and Laitinen 2006) have demonstrated that compound 48/80 does not bypass receptors to directly interact with G proteins but

instead indirectly activates them by activating PLD which results in the generation of lysophosphatidic receptor activating phospholipids which activate receptor-dependent G proteins. Compound 48/80 mast cell degranulation requires the presence of calcium (Cochrane and Douglas 1974) and the influx of calcium into mast cells activated by compound 48/80 remains even after compound 48/80 has been washed out (Suzuki, Mori *et al.* 1982). Compound 48/80 is commonly used to cause mast cell degranulation in mast cell studies.

2.1.11 Quantification of Mast Cell Degranulation

Mast cell degranulation can be quantified by measuring a variety of preformed mast cell mediators which are released upon degranulation. These mediators include the enzymes β -hexosaminidase (Schwartz, Austen *et al.* 1979) and tryptase (Wenzel, Irani *et al.* 1986; Lavens, Proud *et al.* 1993) which may be quantified using colorimetric and ELISA methods, as well as histamine and serotonin (Ferjan and Erjavec 1998). Histamine is frequently measured following RPMC assay using the method developed by Shore *et al.* (Shore, Burkhalter *et al.* 1959) which was later enhanced (Shore 1971). In this method histamine is condensed under alkaline conditions with *o*-phthaldialdehyde (OPT) to form a fluorescent product (Yoshimura, Kaneuchi *et al.* 1987). This product is then acidified to enhance and stabilise histamine-OPT fluorescence. OPT may form condensation products with a number of biogenic amines apart from histamine, such as spermidine, however, the method has been optimised to ensure its specificity for histamine by optimising pH and incubation time (Hakanson, Ronnberg *et al.* 1972). Histamine release from mast cells can also be quantified using ELISA (Mori, Maru *et al.* 2000); however, the histamine-OPT reaction was chosen to ensure continuity between this work and the work of Barlow, Byrne and McHugh.

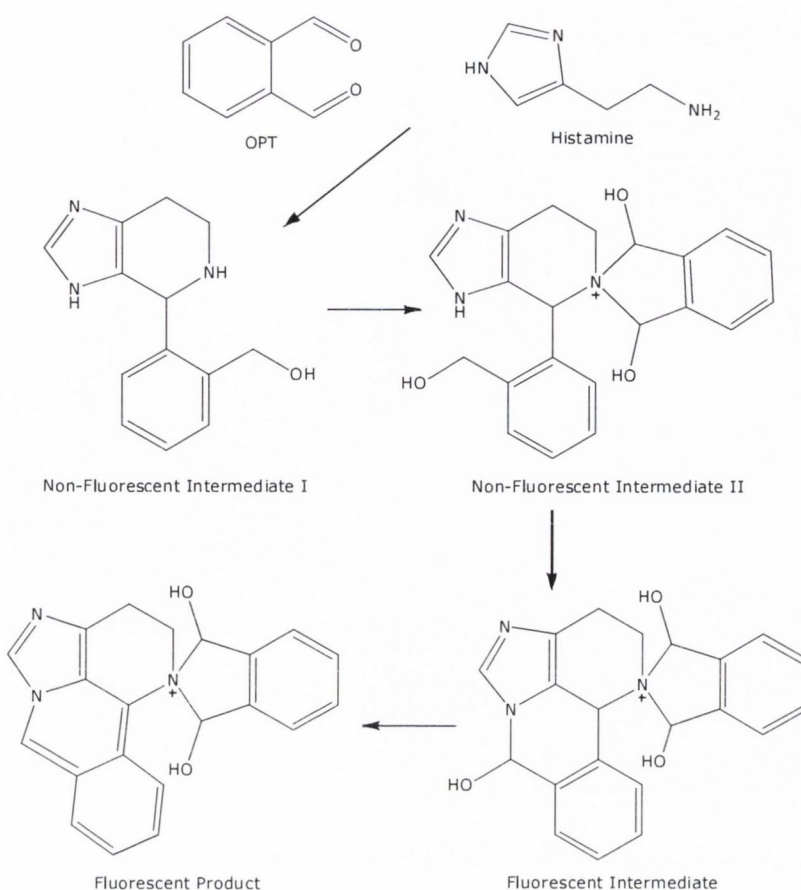


Figure 2.05: Derivatisation of Histamine with OPT to form a Fluorescent Product

2.1.12 Quantitative Structure Activity Relationship Analysis; Lipinski's Rule of Five

Quantitative structure activity relationship (QSAR) analysis is often carried out to determine if relationship between activity of a compound and its properties exist. Lipinski, working with Pfizer Pharmaceuticals, developed a set of rules known as Lipinski's Rule of Five into which the properties of compounds should ideally fit if they are desirable compounds for use in human treatments, when given orally (P.O.). (Lipinski, Lombardo *et al.* 2001). These rules state that poor absorption or permeability is more likely when there are more than 5 H-bond donors (sum of OH and NH), more than 10 H-bond acceptors (sum of N and O), molecular weight greater than 500 and the computed LogP (cLogP; referred to from this point as LogP) is greater than 5. If a compound obeys two or more of these rules it is possible it may have absorption problems. Of course if a certain compound ionises LogD_{7.4}, rather than its LogP, may be more relevant and therefore should be taken into account. Lipinski found that although the vast majority of orally active

compounds obey these rules, antibiotics, antifungals, vitamins and glycosides do not and therefore he included a *caveat*; compound classes which are substrates for biological transporters are exceptions to the rule.

2.2 Materials and Methods

2.2.1 Materials

Bovine serum albumin (BSA), calcium ionophore A23187, compound 48/80, dimethyl sulfoxide (DMSO), disodium cromoglycate (Cromolyn; DSCG), glucose, HEPES, histamine, KCl, KH_2PO_4 , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, NaCl, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, OPT, Percoll, toluidine blue and trypan blue were purchased from Sigma Aldrich, Arklow, Co. Wicklow, Ireland. $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ was purchased from Merck Laboratories, Nottingham, UK. Black, clear bottomed 96 well plates (Greiner 655096) were purchased from Cruinn, Dublin 12, Ireland.

2.2.2 Buffers

Purification of mast cells using Percoll; stock 1 (1.54 M NaCl, 27 mM KCl, 6.8 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$), stock 2 (0.1 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 M KH_2PO_4 , pH 7.3–7.4) and path-o-cyte 4 (35% BSA in BSSA minus BSA) were prepared weekly. BSSA (BSA wash buffer) (5 ml stock 1, 5 ml stock 2, 40 ml dH_2O , 0.7 ml path-o-cyte 4) and working Percoll solution (1.5 ml stock 1, 0.75 ml dH_2O , 0.21 ml path-o-cyte 4, 10.5 ml Percoll, adjust to pH 7.2 with 0.1 M KH_2PO_4) were prepared daily.

RPMC assay; mast cell medium (MCM; 150 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl_2 , 5.6 mM Glucose, 5 mM HEPES, pH 7.3–7.4) and hanks balanced salt solution (HBSS; 156 mM NaCl, 2.7 mM KCl, 0.4 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.5 mM CaCl_2 , 5.6 mM glucose, 5 mM HEPES, 2.7 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ pH 7.3–7.4) were prepared weekly.

2.2.3 Animals

Female Wistar rats (250–350 g) were sourced from the BioResources Unit, Trinity College Dublin. Animals were housed in groups of 8 or less, under controlled conditions at 22°C with 12 hour light and 12 hour dark cycles. All animals had access to food and water *ad libitum*. Animals were sacrificed using CO_2 .

2.2.4 Isolation of Rat Peritoneal Mast Cells

Following euthanasia the upper layer of skin was cut from the abdomen of female Wistar rats and 10 ml of buffer (MCM for calcium ionophore A23187 or HBSS for compound 48/80 assays) was injected into the peritoneal cavity, which was then massaged for three minutes to pool peritoneal cells. An incision was made rostrocaudal along the abdomen, buffer was removed using a Pasteur pipette and was centrifuged at 150 g (930 rpm) at 4 °C for five minutes. Supernatant was discarded and the cell pellet was resuspended in 4 ml of BSSA for use in the Percoll purification procedure.

2.2.5 Purification of Rat Peritoneal Mast Cells using Percoll

Mixed peritoneal cell pellet, resuspended in BSSA, was layered over 10 ml of working Percoll solution in 26.3 ml polycarbonate centrifuge tubes and centrifuged at 225 g (1140 rpm) at 4 °C for 20 minutes. Clear BSSA and the Percoll interface containing non-mast cells were removed using a Pasteur pipette, leaving a highly purified mast cell pellet. The cell pellet was resuspended in 10 ml BSSA and mast cells were separated from residual Percoll by centrifugation at 225 g (1140 rpm) at 4 °C for five minutes. Supernatant was again removed, leaving a mast cell pellet. Mast cells were resuspended in 21 ml of buffer (MCM for calcium ionophore A23187 or HBSS for compound 48/80) for RPMC assay.

2.2.6 Mast cell Purity following Purification with Percoll

Rat peritoneal mast cell purity was assessed after purification with Percoll using acidified, alcoholic toluidine blue (0.5% toluidine blue in dH₂O, diluted 1:10 in ethanol, adjusted to pH 3 with glacial acetic acid). One volume of mast cell suspension was added to equal volumes of toluidine blue and methanol. Cells were counted using a haemocytometer.

2.2.7 Mast cell Viability following Purification with Percoll

Rat peritoneal mast cell viability was assessed after purification with Percoll using the trypan blue exclusion method. Mast cell suspension was diluted (1:2) in trypan blue. Viability was demonstrated when cells remained clear, while non-viable cells turned blue due to the penetration of the dye into the cell. Cells were again counted using a haemocytometer.

2.2.8 Rat Peritoneal Mast Cell Assay

Tubes were numbered in duplicate; the first 10 tubes were always labelled as follows (table 2.01), while subsequent tubes were labelled according to test compound and test compound concentration being assayed.

Table 2.01: Tube layout for RPMC assay

Tube Number	Label
1+2	Basal
3+4	Max
5+6	Total
7+8	Blank
9+10	DSCG (40 µM)

4.5 ml of buffer (MCM for calcium A23187 experiments or HBSS for compound 48/80 experiments) was added to each tube. DSCG was chosen as a suitable mast cell stabiliser for use as a control compound. 25 µl of buffer was added to tubes 1-6, 25 µl of DMSO (vehicle) to tubes 7 and 8 and 25 µl of DSCG (40 µM in buffer) to tubes 9 and 10. A stock of test compound was prepared by dissolving compounds in 100% DMSO and subsequent concentrations were prepared by serial dilution, also in 100% DMSO. 25 µl of test compound at required concentrations was added, in duplicate, to the remaining tubes (0.5% DMSO final concentration in tubes). Tubes were vortexed and placed in a water bath at 37 °C. Peritoneal mast cell suspension was gently inverted to ensure a homogenous cell suspension and 500 µl of mast cell suspension was added to each test tube. All tubes were shaken for 10 minutes at 37 °C to allow compounds to stabilise mast cells. Shaking was stopped, 50 µl of buffer was added to tubes 1 and 2 while 50 µl of elicitor was added to remaining tubes and all tubes were left to stand at 37 °C according to the assay protocol below (table 2.02);

Table 2.02: RPMC assay elicitors and their corresponding assay conditions

Elicitor	Concentration	Buffer	Incubation time
Calcium Ionophore A23187	1 µg/ml	MCM	10 minutes
Compound 48/80	0.25 µg/ml	HBSS	5 minutes

Tubes were placed on ice for 10 minutes and 500 µl of ice cold buffer was added to each tube to stop the degranulation activity of the elicitor. Simultaneously tubes 5 and 6 were placed in boiling water to lyse the cells, resulting in the "Total" release of mast cell mediators. After boiling 500 µl of ice cold buffer was added to tubes 5 and 6 to ensure equal volume in all tubes. Tubes were then centrifuged at 150 g (930 rpm) at 4 °C for five minutes.

2.2.9 Derivatisation of Histamine with o-Phthaldialdehyde and Subsequent Detection using Fluorescence

A new set of tubes were labelled according to the assay layout and 600 µl of 1 M NaOH was added to each tube. Supernatant (3 ml) was removed from assay tubes and added to corresponding new tubes; all tubes were vortexed. 150 µl of OPT in methanol (0.01 g/ml) was added to each tube and tubes were again vortexed. Four minutes later 300 µl of 3 M HCl was added to each tube and again tubes were vortexed. The addition of OPT to basic histamine results in the formation of a highly

fluorescent histamine-OPT product, while acidification stabilises and enhances this product, as discussed above. 300 µl of supernatant was removed from each tube and added to the wells of a 96 well black clear bottom plate. Fluorescence was read at 350 nm (excitation) and 450 nm (emission) using a FLUOstar OPTIMA (BMG labtech) platereader. A histamine standard curve (0-400 ng/ml) was prepared and simultaneously derived with OPT to ensure linearity of measurement of histamine using this method; r^2 values obtained were > 0.98 .

2.2.10 Analysis of Data

Duplicate tubes were averaged and Basal values were subtracted from the average value of each duplicate pair. Percentage Max/Total values were calculated to determine that degranulation of mast cells had occurred. Drug activities were determined as percentage inhibition of histamine release, using the formula;

$$\% \text{ inhibition of histamine release} = 100 - [(drug - basal) / (blank - basal)] * 100$$

Except in the case of DSCG (which was prepared in assay buffer and not DMSO) where the following formula was used;

$$\% \text{ inhibition of histamine release} = 100 - [(drug - basal) / (max - basal)] * 100$$

2.2.11 Computation of Quantitative Structure Analysis Values

The theoretical properties of compounds were computed using MarvinSketch, ChemAxon (Chemaxon 2010). Properties determined were the partition coefficient (LogP or cLogP), the distribution coefficient (LogD_{7.4}), the acid dissociation constant (pKa), hydrogen (H+) bond donor and acceptor sites as well as 2D polar surface area (PSA).

2.2.12 Statistics

Statistical analysis of the data was carried out using GraphPad Prism, version 5. Percentage inhibition values of each test compound, at each concentration, are expressed as mean $\pm$ SEM. Values were tested for Gaussian distribution and where appropriate IC₅₀ values were determined.

To determine whether a structure activity may exist for these compounds LogP and/or LogD_{7.4} values were analysed against compound RPMC activity (expressed as inhibition of histamine release), using GraphPad Prism, version 5.

2.3 Results

2.3.1 Percoll Purification of Rat Peritoneal Mast Cells

2.3.1.1 Mast Cell Purity

Toluidine Blue staining after purification with Percoll indicated a percentage mast cell purity of $88.81 \pm 2\%$ (mean $\pm$ SEM, $n=3$).

2.3.1.2 Mast Cell Viability

Trypan Blue staining following purification with Percoll indicated a percentage mast cell viability of $91.55 \pm 2\%$ (mean $\pm$ SEM, $n=3$).

Mast cell purity and viability results above indicate Percoll purification of mast cells from the rat peritoneal total cell population results in a highly pure and viable mast cell population.

2.3.2 Rat Peritoneal Mast Cell Assay

2.3.2.1 Group I Compounds

Group I compounds were assessed for *in vitro* mast cell stabilising activity by fluorescence quantification of histamine released from mast cells following calcium ionophore A23187- and compound 48/80-induced mast cell degranulation. Most compounds in this group demonstrated mast cell stabilising activity superior to that of control compound DSCG (figures 2.06-2.11 below).

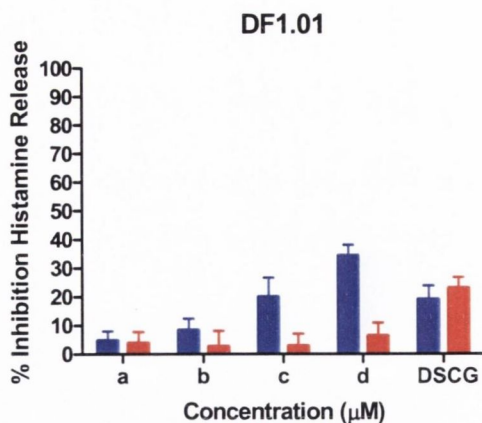


Figure 2.06: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF1.01 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 8

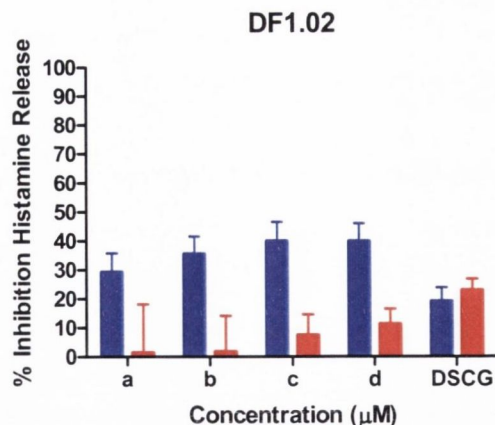


Figure 2.07: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF1.02 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 5 or 8

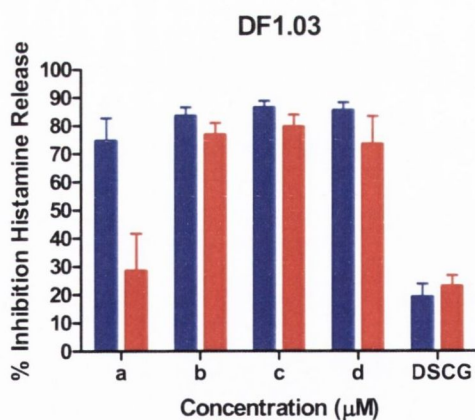


Figure 2.08: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF1.03 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 8.

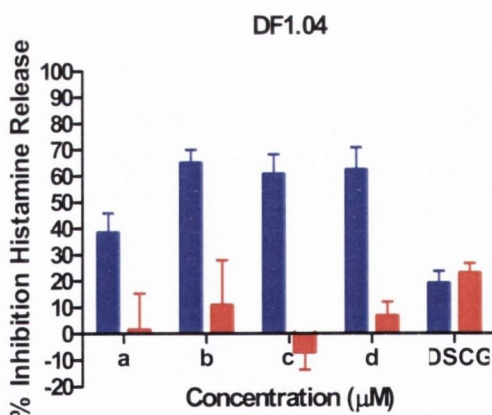


Figure 2.09: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF1.04 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 8

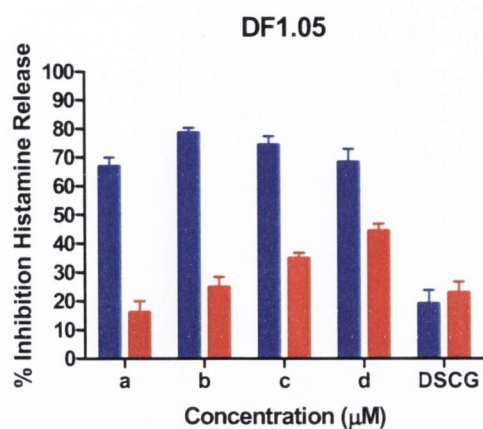


Figure 2.10: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF1.05 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=5 or 8.

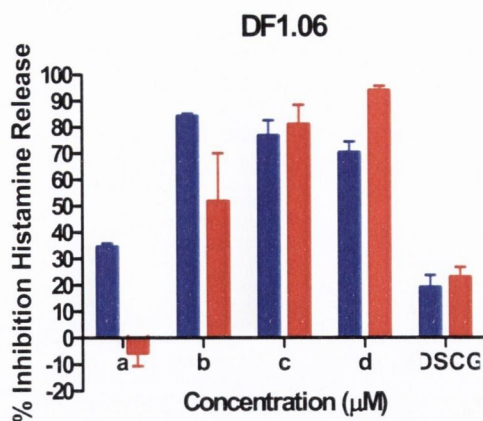


Figure 2.11: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF1.06 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 5 or 8.

2.3.2.2 Group II Compounds

Group II compounds were also assessed for mast cell stabilising activity using the RPMC assay. Encouragingly, all compounds in this group demonstrated mast cell stabilising activity (up to 20 μM) superior to that of DSCG (40 μM) in the presence of both calcium ionophore A23187 and compound 48/80 (figures 2.12-2.16 below).

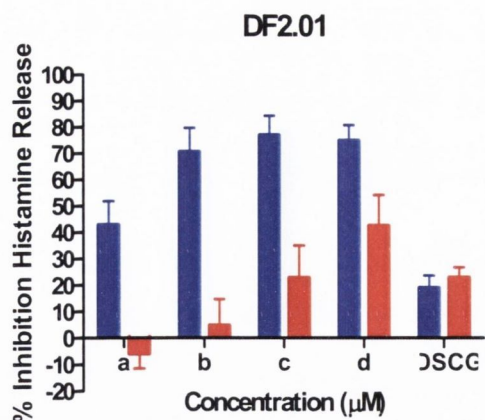


Figure 2.12: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF2.01 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 5 or 8

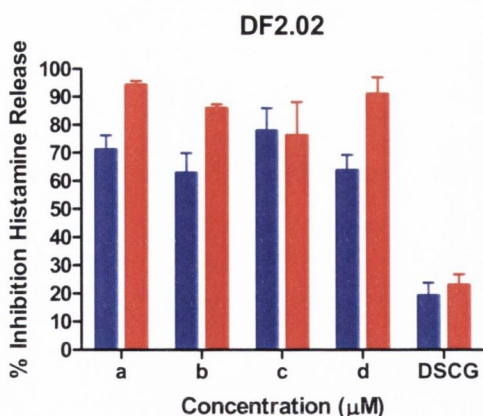


Figure 2.13: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM D2.02 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 8

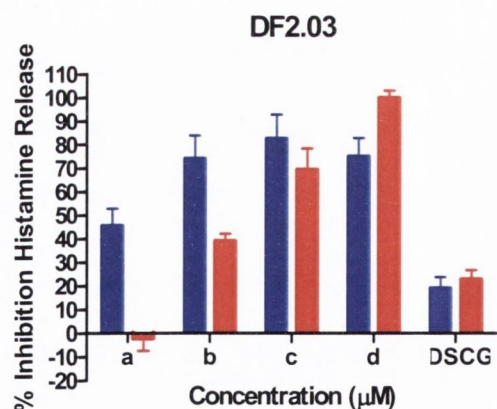


Figure 2.14: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF2.03 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 5 or 8

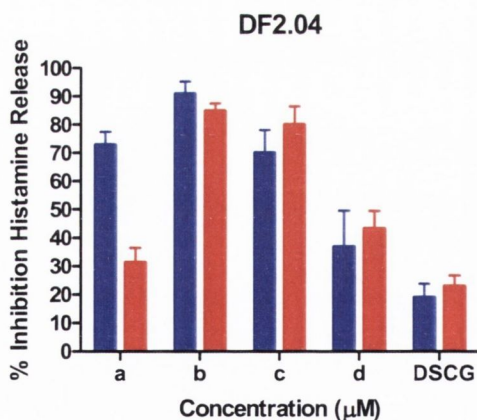


Figure 2.15: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF2.04 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 5 or 8

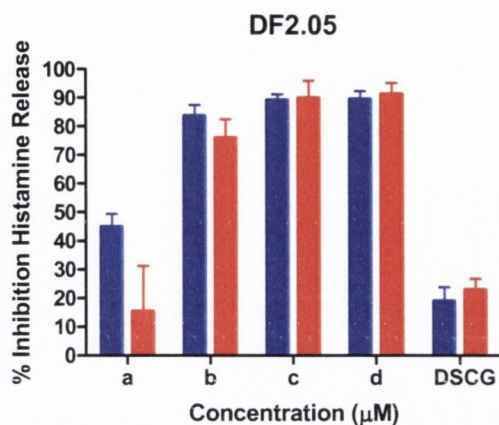


Figure 2.16: Effect of a) 1 μM, b) 5 μM, c) 10 μM and d) 20 μM DF2.05 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=3 or 4 or 8

2.3.2.3 Group III Compounds

Group III compounds were assessed for mast cell stabilising activity using the RPMC assay. All compounds in this group demonstrated mast cell stabilising activity (up to 20 μM) superior to that of DSCG (40 μM) in the presence of calcium ionophore A23187, however, this activity was generally not maintained in the presence of compound 48/80 (figures 2.17-2.20 below).

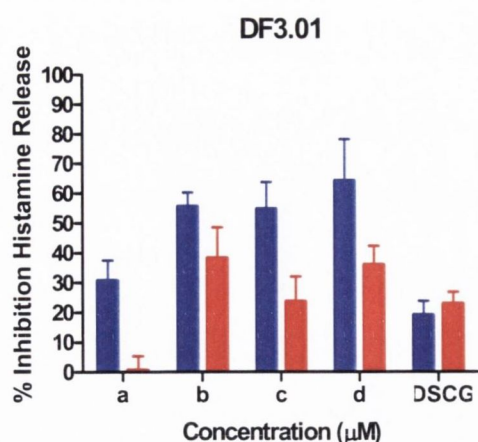


Figure 2.17: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF3.01 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 5 or 8.

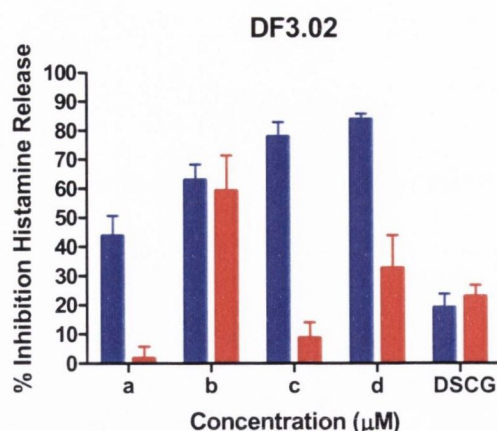


Figure 2.18: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF3.02 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 6 or 8

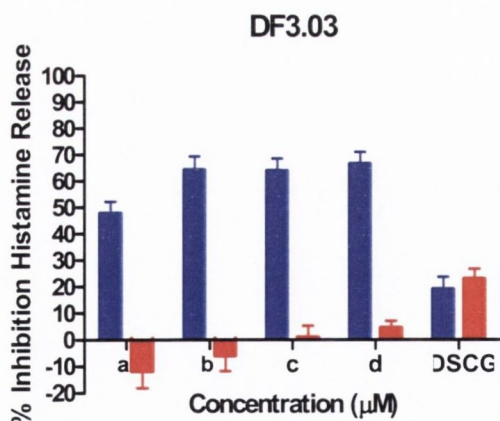


Figure 2.19: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF3.03 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 8

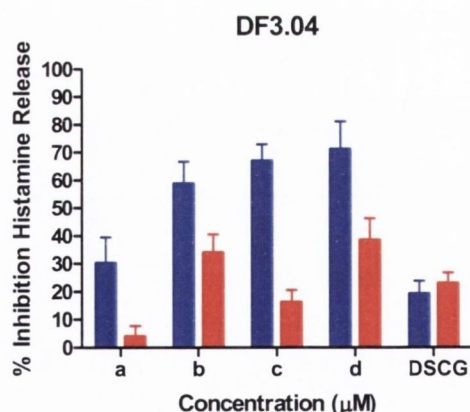


Figure 2.20: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM DF3.04 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 5 or 8

2.3.2.4 Group IV Compounds

Group IV compounds were assessed for mast cell stabilising activity using the RPMC assay. All compounds in this group demonstrated mast cell stabilising activity superior to that of DSCG (40 μ M) in the presence of both calcium ionophore A23187 and compound 48/80 (figures 2.21-2.27 below).

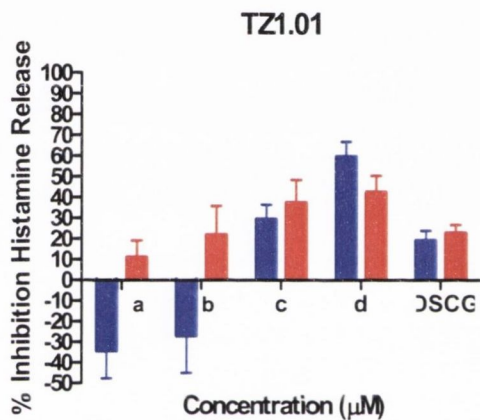


Figure 2.21: Effect of a) 1 μ M, b) 5 μ M, c) 10 μ M and d) 20 μ M TZ1.01 and control compound DSCG (40 μ M) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of n=4 or 5 or 8.

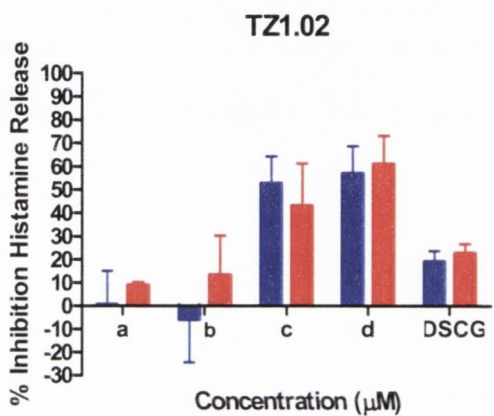


Figure 2.22: Effect of a) 1 μ M, b) 5 μ M, c) 10 μ M and d) 20 μ M TZ1.02 and control compound DSCG (40 μ M) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of n=4 or 8

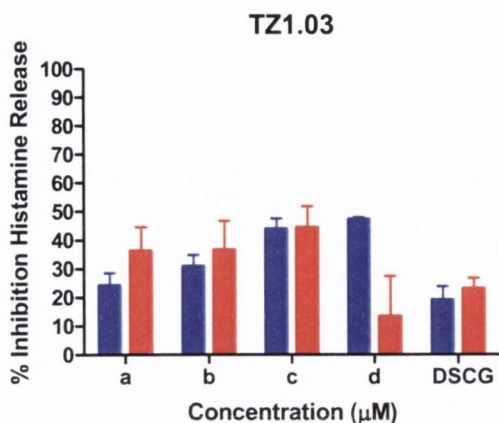


Figure 2.23: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM TZ1.03 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 8

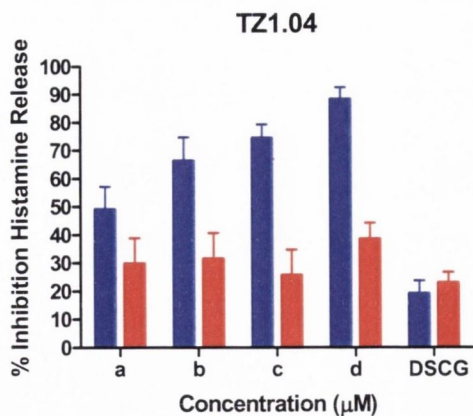


Figure 2.24: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM TZ1.04 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 5 or 6 or 8

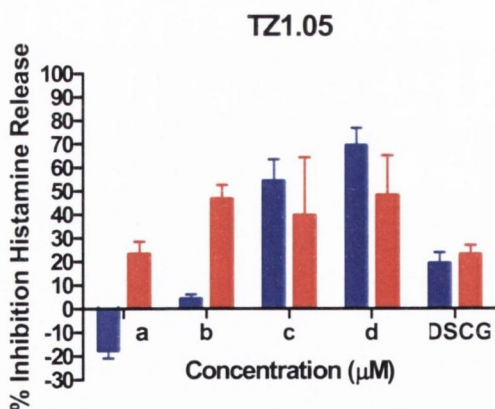


Figure 2.25: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM TZ1.05 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=3 or 4 or 5 or 8

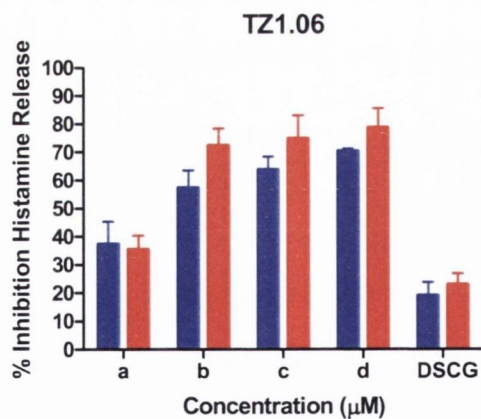


Figure 2.26: Effect of a) 1μM, b) 5μM, c) 10 μM and d) 20μM TZ1.06 and control compound DSCG (40 μM) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean ± SEM of n=4 or 8

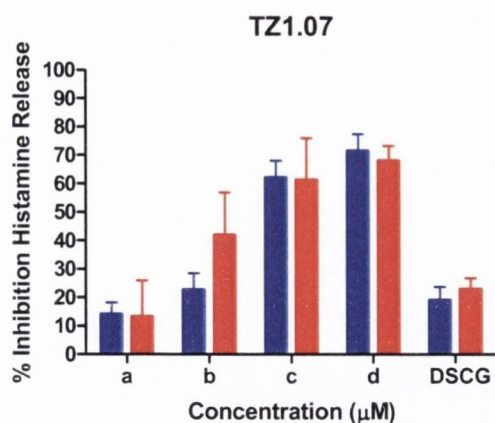


Figure 2.27: Effect of a) 1 μ M, b) 5 μ M, c) 10 μ M and d) 20 μ M TZ1.07 and control compound DSCG (40 μ M) on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of n=4 or 8

2.3.3 Rat Peritoneal Mast Cell Assay; Determination of IC₅₀ Values

Compounds from Groups I-IV which demonstrated potent mast cell stabilising activity, as well as favourable solubility profiles, were further evaluated in the RPMC assay. IC₅₀ values were determined for these compounds in the presence of calcium ionophore A23187 and/or compound 48/80 (figures 2.28-2.38).

2.3.3.1 Group I Compounds

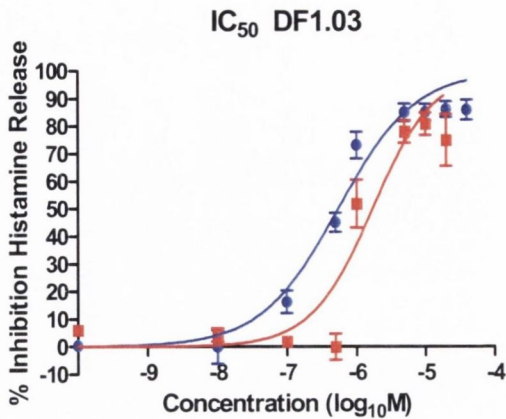


Figure 2.28: Dose response of DF1.03 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=4$ or 8.

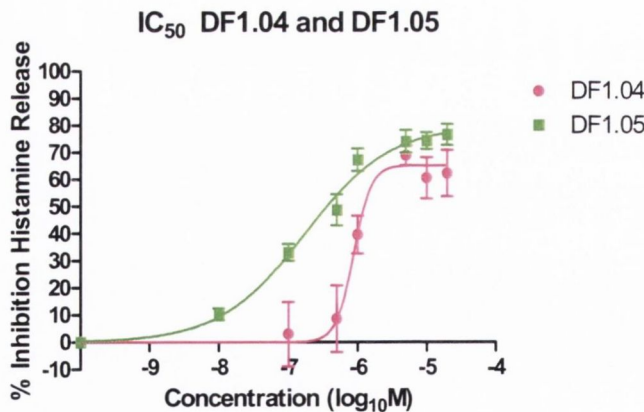


Figure 2.29: Dose response of DF1.04 (pink) and DF1.05 (green) on calcium ionophore A23187-induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=4$ or 8

2.3.3.2 Group II Compounds

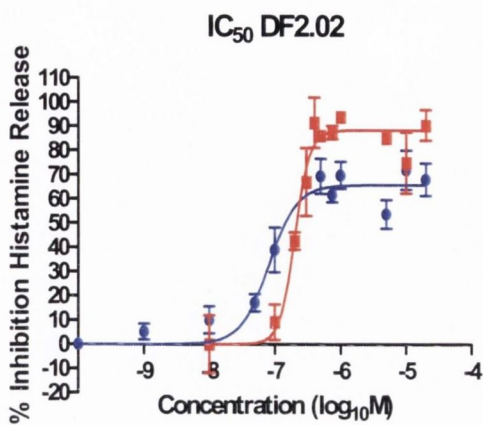


Figure 2.30: Dose response of DF2.02 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of n=4 or 5 or 8

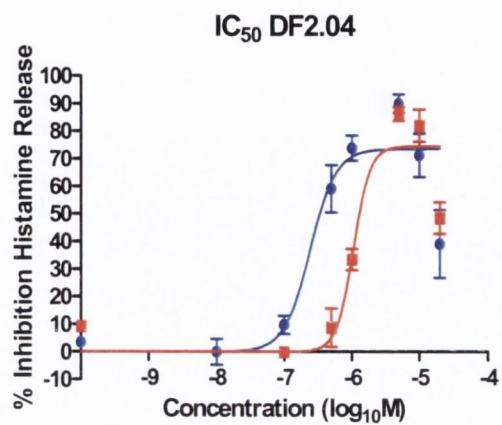


Figure 2.31: Dose response of DF2.04 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of n=4 or 5 or 8.

2.3.3.3 Group III Compounds

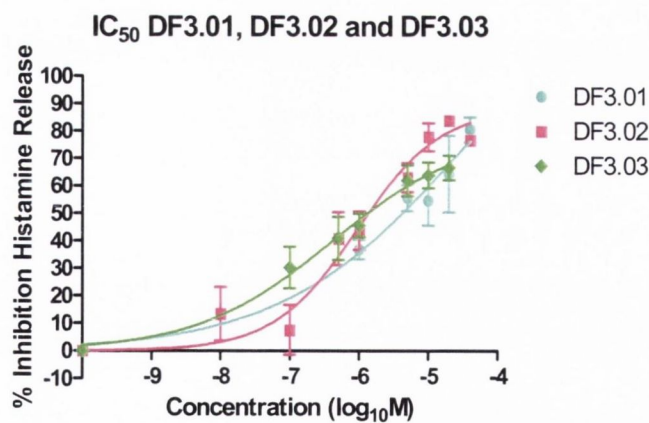


Figure 2.32: Dose response of DF3.01 (turquoise), DF3.02 (pink) and DF3.03 (green) on calcium ionophore A23187-induced histamine release from RPMC. Data represents mean $\pm$ SEM of n=3 or 4 or 5 or 8

2.3.3.4 Group IV Compounds

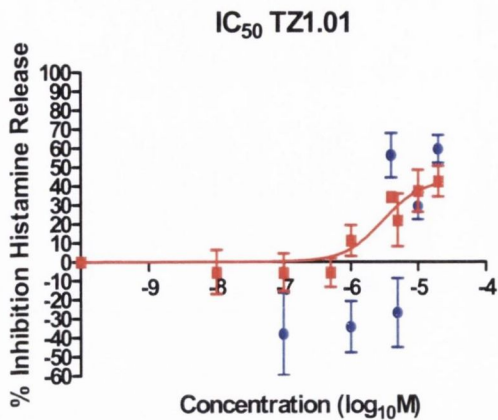


Figure 2.33: Dose response of TZ1.01 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=4$ or 5

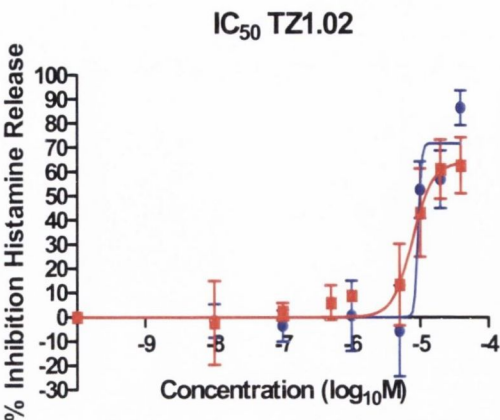


Figure 2.34: Dose response of TZ1.02 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=3$ or 4

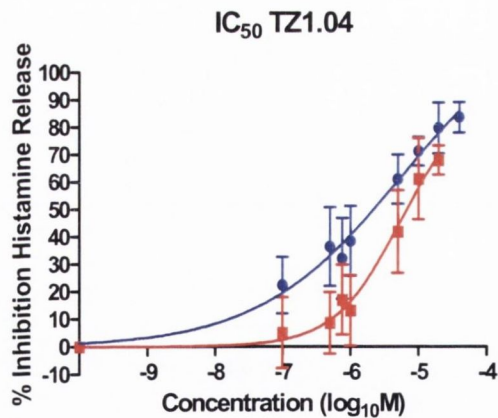


Figure 2.35: Dose response of TZ1.04 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=5$ or 7

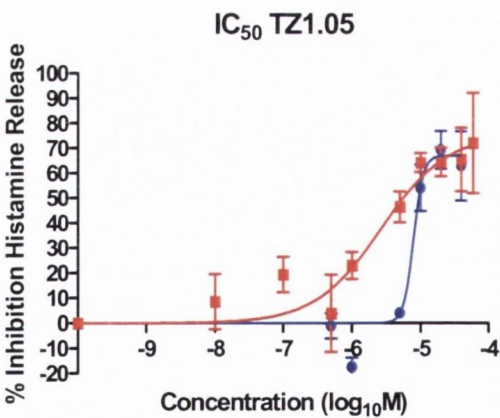


Figure 2.36: Dose response of TZ1.05 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=3$ or 4 or 5

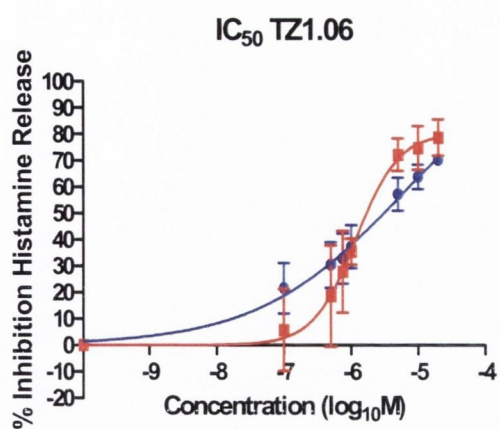


Figure 2.37: Dose response of TZ1.06 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=4$

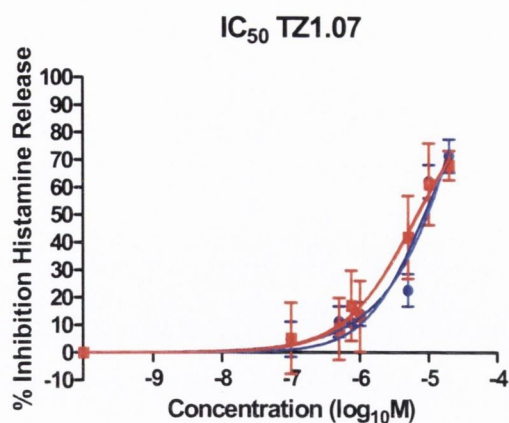


Figure 2.38: Dose response of TZ1.07 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=5$

2.3.3.5 Rat Peritoneal Mast Cell Assay IC₅₀ Values

Table 2.03: Best fit IC₅₀ values for test compounds following RPMC assay on Percoll purified mast cells using the elicitors Compound 48/80 and/or Calcium Ionophore A23187

Test Compound	Calcium Ionophore A23187	Compound 48/80
Group I		
DF1.03	0.55 µM	1.72 µM
DF1.04	0.88 µM	
DF1.05	0.17 µM	
Group II		
DF2.02	81.82 nM	0.20 µM
DF2.04	0.24 µM	1.05 µM
Group III		
DF3.01	31.59 µM	
DF3.02	0.95 µM	
DF3.03	0.41 µM	
Group IV		
TZ1.01	values do not converge	3.02 µM
TZ1.02	9.43 µM	7.78 µM
TZ1.04	5.73 µM	5.52 µM
TZ1.05	7.90 µM	2.67 µM
TZ1.06	5.49 µM	1.18 µM
TZ1.07	8.62 µM	7.02 µM

2.3.4. Quantitative Structure Activity Relationship Analysis

Theoretical computed structural properties were determined for all compounds in Groups I-IV using MarvinSketch. LogP, LogD_{7.4}, pKa, hydrogen (H⁺) bond donor and acceptor sites and 2D PSA were established to determine whether these compounds may have absorption issues (tables 2.04-2.07).

Following determination of these computed values, structure activity relationship analysis was carried out to determine if a relationship may exist between the structures of these compounds and their activities (figures 2.39-2.56). No structure activity relationship was observed for any compound group.

2.3.4.1 Group I Compounds

Table 2.04: Computed QSAR Properties of DF Group I Compounds, as calculated using MarvinSketch, ChemAxon

Compound	DF1.01	DF1.02	DF1.03	DF1.04	DF1.05	DF1.06
LogP	4.11	9.18	7.12	8.06	7.31	6.09
LogD _{7.4}	4.11	9.18	7.12	8.06	7.31	6.09
pKa	14	14	14	13.62	14	14
H ⁺ donor site	1	1	1	1	1	1
H ⁺ acceptor site	1	1	1	1	1	1
Polar surface area (2D)	20.23 Å ²	20.23 Å ²	20.23 Å ²	20.23 Å ²	20.23 Å ²	20.23 Å ²
Stereoisomers	2	2	2	2	2	2
Molecular Weight	228 g/mol	440 g/mol	355 g/mol	396 g/mol	379 g/mol	328 g/mol

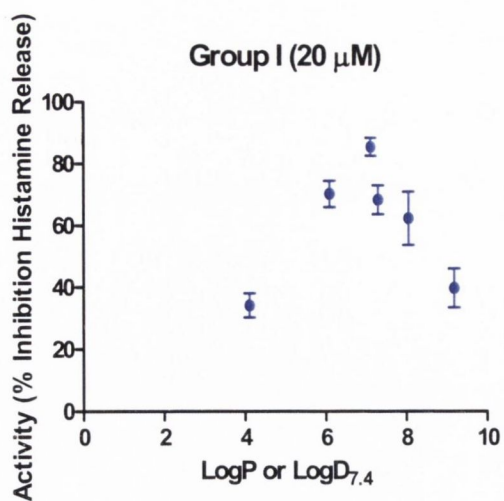


Figure 2.39: Relationship between LogP or LogD_{7.4} and RPMC activity of Group I compounds at 20 μ M, in the presence of calcium ionophore A23187. Data represents mean $\pm$ SEM of n=4 or 5 or 8 per compound

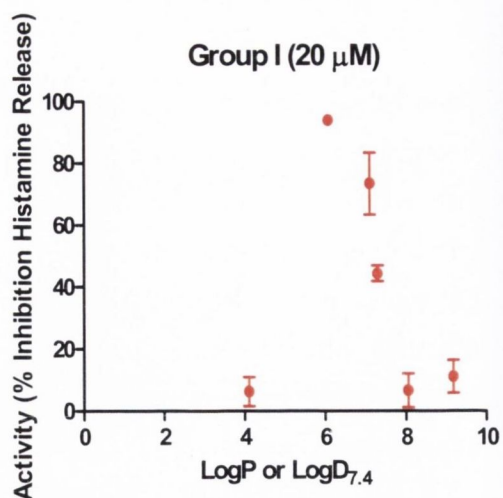


Figure 2.40: Relationship between LogP or LogD_{7.4} and RPMC activity of Group I compounds at 20 μ M, in the presence of compound 48/80. Data represents mean $\pm$ SEM of n=4 or 7 per compound

2.3.4.2 Group II Compounds

Table 2.05: Computed QSAR Properties of DF Group II Compounds, as calculated using MarvinSketch, ChemAxon

Compound	DF2.01	DF2.02	DF2.03	DF2.04	DF2.05
LogP	6.36	9.45	6.8	7.39	6.43
LogD _{7.4}	3.34	6.44	3.73	4.39	6.37
pKa	4.22	4.24	4.14	4.25	6.58
H+ donor site	1	1	1	1	0
H+ acceptor site	3	3	3	3	2
Polar surface area (2D)	66.43 Å ²	66.43 Å ²	66.43 Å ²	66.43 Å ²	29.54 Å ²
Stereoisomers	2	2	2	2	2
Molecular Weight	428 g/mol	562 g/mol	452 g/mol	456 g/mol	448 g/mol

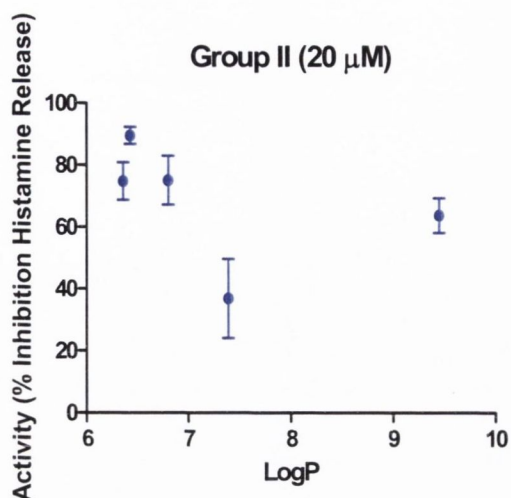


Figure 2.41: Relationship between LogP and RPMC activity of Group II compounds at 20 μ M, in the presence of calcium ionophore A23187. Data represents mean $\pm$ SEM of $n=4$ or 7 per compound

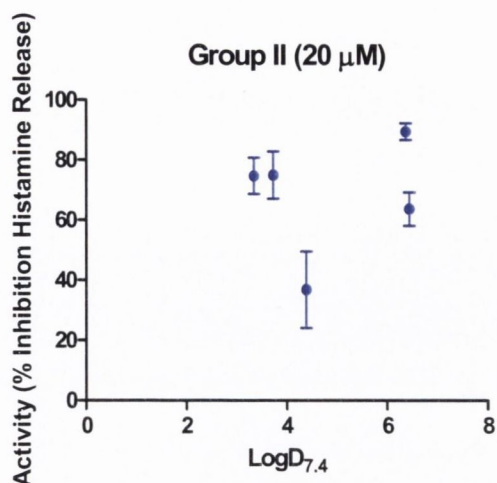


Figure 2.42: Relationship between LogD_{7.4} and RPMC activity of Group II compounds at 20 μ M, in the presence of calcium ionophore A23187. Data represents mean $\pm$ SEM of $n=4$ or 7 per compound

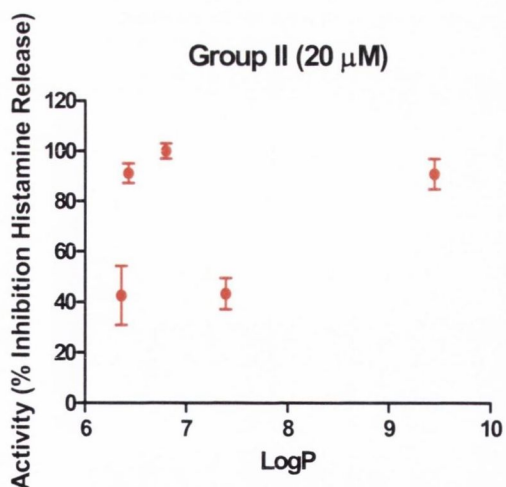


Figure 2.43: Relationship between LogP and RPMC activity of Group II compounds at 20 μ M, in the presence of compound 48/80. Data represents mean $\pm$ SEM of $n=4$ or 5 or 6 per compound

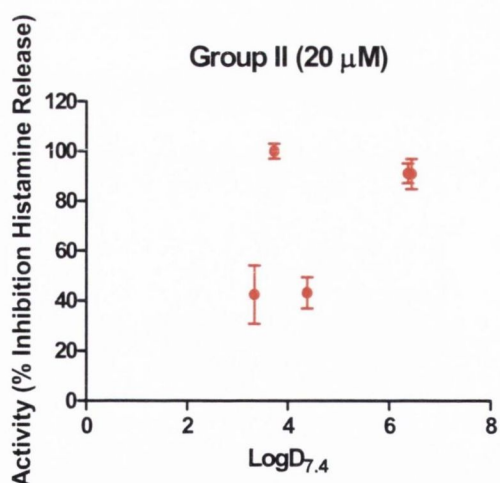


Figure 2.44: Relationship between LogD_{7.4} and RPMC activity of Group II compounds at 20 μ M, in the presence of compound 48/80. Data represents mean $\pm$ SEM of $n=4$ or 7 per compound

2.3.4.3 Group III Compounds

Table 2.06: Computed QSAR Properties of DF Group III Compounds, as calculated using MarvinSketch, ChemAxon

Compound	DF3.01	DF3.02	DF3.03	DF3.04
LogP	9.3	8.0	8.7	9.5
LogD _{7.4}	7.11	5.87	6.24	6.54
pKa	9.62	9.54	10.08, 13.88	9.62
H+ donor sites	0	0	1	0
H+ acceptor site	2	4	2	2
Polar surface area (2D)	21.51 Å ²	69.09 Å ²	24.67 Å ²	21.51 Å ²
Stereoisomers	4	4	4	4
Molecular Weight	499 g/mol	521 g/mol	487 g/mol	532 g/mol

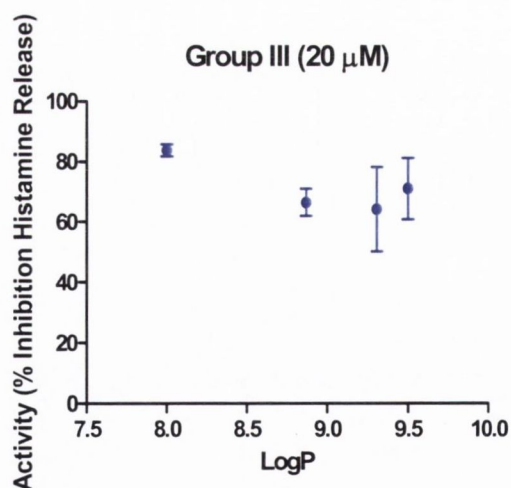


Figure 2.45: Relationship between LogP and RPMC activity of Group III compounds at 20 μ M, in the presence of calcium ionophore A23187. Data represents mean $\pm$ SEM of n=4 or 5 or 6 per compound

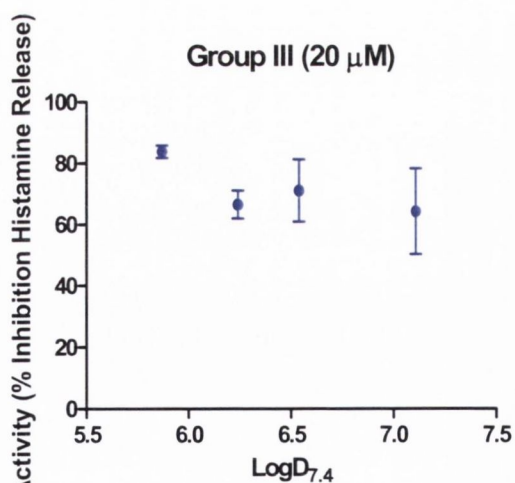


Figure 2.46: Relationship between LogD_{7.4} and RPMC activity of Group III compounds at 20 μ M, in the presence of calcium ionophore A23187. Data represents mean $\pm$ SEM of n=4 or 5 or 6 per compound

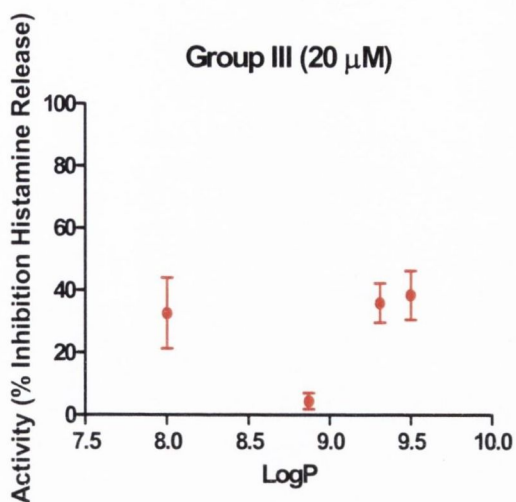


Figure 2.47: Relationship between LogP and RPMC activity of Group III compounds at 20 μ M, in the presence of compound 48/80. Data represents mean $\pm$ SEM of n=4 or 5 per compound

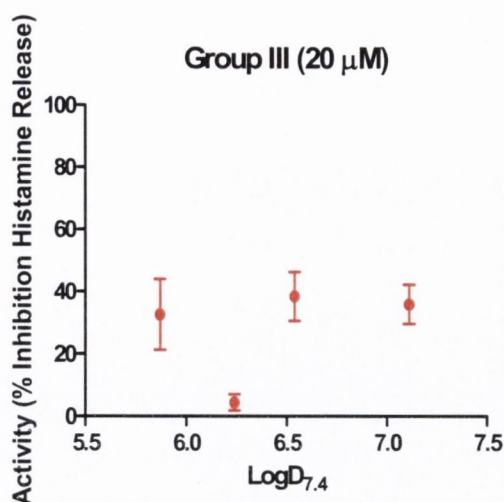


Figure 2.48: Relationship between LogD_{7.4} and RPMC activity of Group III compounds at 20 μ M, in the presence of compound 48/80. Data represents mean $\pm$ SEM of n=4 or 5 per compound

2.3.4.4 Group IV Compounds

Table 2.07: Computed QSAR Properties of DF Group IV Compounds, as calculated using MarvinSketch, ChemAxon

Compound	TZ1.01	TZ1.02	TZ1.03	TZ1.04	TZ1.05	TZ1.06	TZ1.07
LogP	5.05	5.05	6.95	7.31	5.95	5.52	5.37
LogD _{7.4}	4.70	4.39	6.03	6.28	5.92	4.77	3.42
pKa	7.49	7.95	8.27	8.39	6.23	8.07	9.35
H+ donor site	0	0	0	0	0	0	0
H+ acceptor site	5	5	3	2	2	3	2
Polar surface area (2D)	49.2 Å ²	49.2 Å ²	30.74 Å ²	21.51 Å ²	20.31 Å ²	47.81 Å ²	21.51 Å ²
Stereoisomers	4	4	4	4	4	4	4
Molecular Weight	472 g/mol	472 g/mol	466 g/mol	458 g/mol	434 g/mol	440 g/mol	381 g/mol

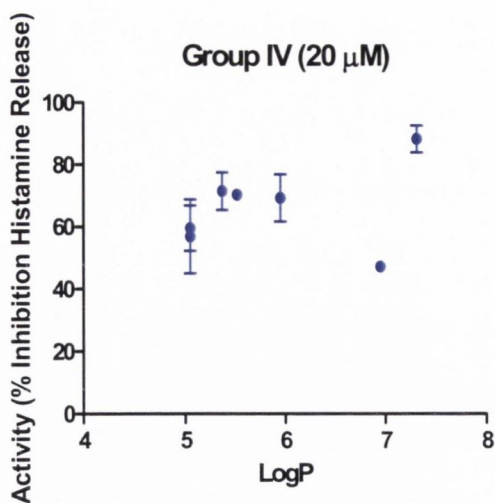


Figure 2.49: Relationship between LogP and RPMC activity of Group IV compounds at 20 μ M, in the presence of calcium ionophore A23187. Data represents mean $\pm$ SEM of $n=4$ or 5 per compound

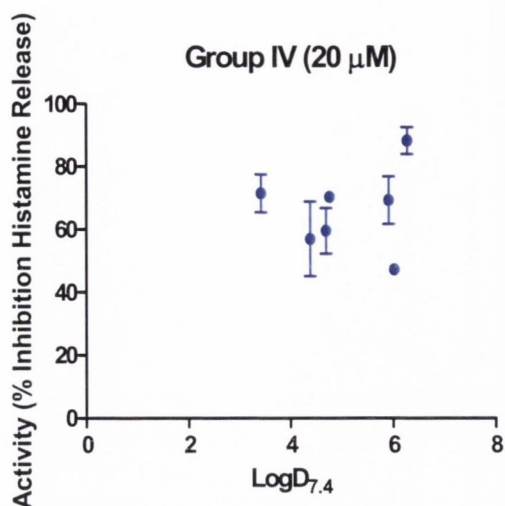


Figure 2.50: Relationship between LogD_{7.4} and RPMC activity of Group IV compounds at 20 μ M, in the presence of calcium ionophore A23187. Data represents mean $\pm$ SEM of $n=4$ or 5 per compound

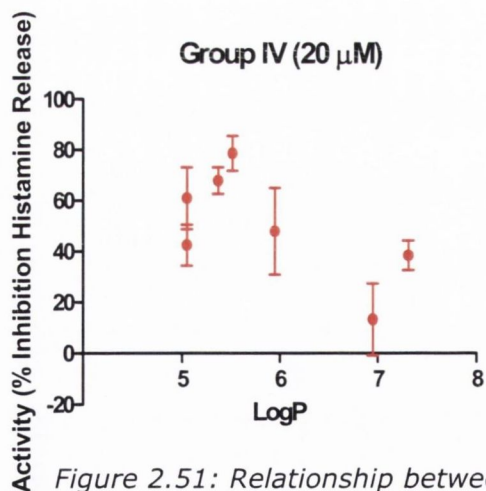


Figure 2.51: Relationship between LogP and RPMC activity of Group IV compounds at 20 μ M, in the presence of compound 48/80. Data represents mean $\pm$ SEM of $n=4$ or 5 per compound

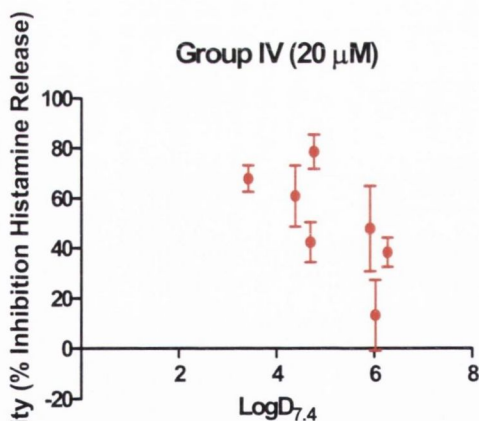


Figure 2.52: Relationship between LogD_{7.4} and RPMC activity of Group IV compounds at 20 μ M, in the presence of compound 48/80. Data represents mean $\pm$ SEM of $n=4$ or 5 per compound

2.3.4.5 Groups I - IV Combined

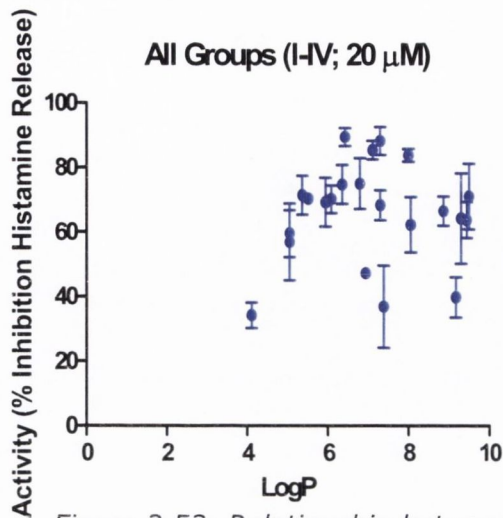


Figure 2.53: Relationship between LogP and RPMC activity of Groups I-IV compounds combined at 20 μM , in the presence of calcium ionophore A23187. Data represents mean $\pm$ SEM of $n=4$ or 5 or 6 or 7 or 8 per compound

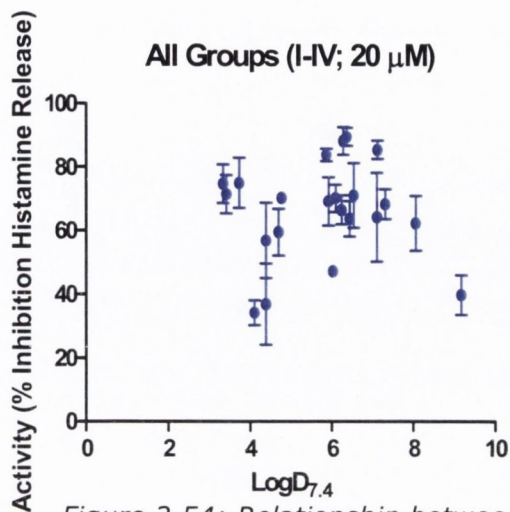


Figure 2.54: Relationship between $\text{LogD}_{7.4}$ and RPMC activity of Groups I-IV compounds at 20 μM , in the presence of calcium ionophore A23187. Data represents mean $\pm$ SEM of $n=4$ or 5 or 6 or 7 or 8 per compound

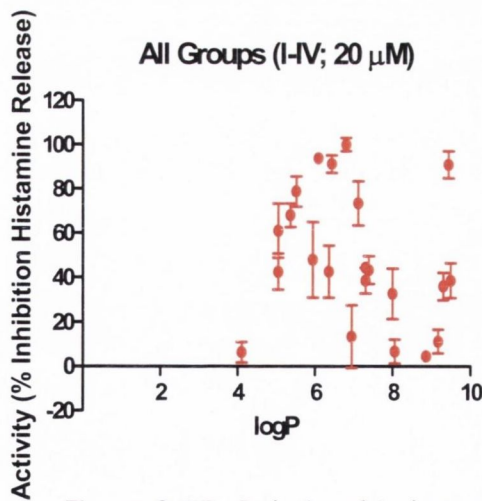


Figure 2.55: Relationship between LogP and RPMC activity of Groups I-IV compounds combined at 20 μM , in the presence of compound 48/80. Data represents mean $\pm$ SEM of $n=4$ or 5 or 6 or 8 per compound

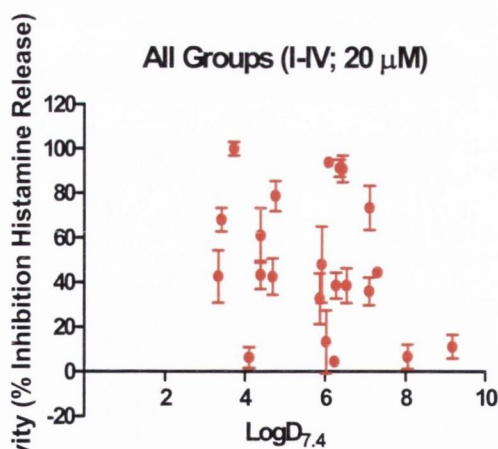


Figure 2.56: Relationship between $\text{LogD}_{7.4}$ and RPMC activity of Groups I-IV compounds combined at 20 μM , in the presence of compound 48/80. Data represents mean $\pm$ SEM of $n=4$ or 5 or 6 or 8 per compound

2.4 Discussion

2.4.1 Purification of Mast Cells using Percoll

The decision to purify mast cells was taken because mast cells only comprise approximately 1-5% of total pooled cells following isolation from the peritoneal cavity (Coleman, Holgate *et al.* 1981). Although work by Barlow, Byrne and McHugh was carried out using non-purified peritoneal mast cells, comprehensive conclusions can only be made from investigations where highly purified mast cell populations are used. For this reason these populations are widely used for investigations into the mast cell stabilising activity of novel compounds. A literature review was carried out to ascertain methods which would allow for the purification of mast cells from the total peritoneal cell population. Approaches which were considered were metrizamide (Coutts, Nehring *et al.* 1980) as well as ficoll (Sharma, Sharma *et al.* 2003). However, Percoll was chosen for use because of its favourable nontoxic properties. Purification using Percoll results in a highly pure ($88.81 \pm 2\%$, $n=3$) and viable ($91.55 \pm 2\%$, $n=3$) mast cell population in this instance, as assessed using toluidine blue (see section 2.3.1.1 above) and trypan blue (see section 2.3.1.2 above) staining respectively. Although RPMC purification using BSA, ficoll or metrizamide results in the inability of IgE-sensitised cells to release histamine following purification and somewhat diminishes calcium ionophore A23187 and compound 48/80-induced histamine release (Coutts, Nehring *et al.* 1980), purification using Percoll results in the release of histamine at similar levels to that of non-purified peritoneal cells when stimulated with calcium ionophore A23187 and compound 48/80 (Inagaki, Kawasaki *et al.* 1994). Percoll is also favourable because it does not cause mast cell aggregation (Nemeth and Rohlich 1980). Because of the highly pure and viable mast cell population obtained following purification with Percoll and because of its favourable properties, Percoll purified mast cell populations are appropriate for use in RPMC assays using calcium ionophore A23187 and compound 48/80 to induce histamine release.

2.4.2 Rat Peritoneal Mast Cell Assay

2.4.2.1 Group I Compounds

It is clear from RPMC results that Group I compounds demonstrate a mixed level of mast cell stabilising activity. DF1.01 ($34.22 \pm 4\%$, 20 μM , figure 2.06) and DF1.02 ($39.81 \pm 6\%$, 20 μM , figure 2.07) do not possess any remarkable activity when assayed with calcium ionophore, even at 20 μM . This activity is greatly reduced at lower concentrations for DF1.01 while activity is maintained by DF2.02 at lower

concentrations. DF1.01 and DF1.02 both demonstrate disappointing activity in the presence of compound 48/80 (DF1.01 $6.25 \pm 5\%$, 20 μM , figure 2.06; DF1.02 $11.13 \pm 5\%$, 20 μM , figure 2.07). DF1.03 displays potent mast cell stabilising activity in the presence of both calcium ionophore A23187 ($85.4 \pm 3\%$, 20 μM , figure 2.08) and compound 48/80 ($73.34 \pm 10\%$, 20 μM , figure 2.08), although this activity is reduced at 1 μM with compound 48/80 ($28.40 \pm 13\%$, 1 μM , figure 2.08). DF1.04 and DF1.05, although demonstrating significant mast cell stabilising activity with calcium ionophore A23187 (DF1.04 $62.3 \pm 9\%$, 20 μM , figure 2.09; DF1.05 $68.31 \pm 5\%$, 20 μM , figure 2.10) again show poor levels of activity in the presence of compound 48/80, although DF1.05 does perform better than DF1.04 (DF1.04 $6.60 \pm 5\%$, 20 μM , figure 2.09; DF1.05 $44.32 \pm 3\%$, 20 μM , figure 2.10). DF1.06 demonstrates good mast cell stabilising activity with both calcium ionophore A23187 ($70.18 \pm 4\%$, 20 μM , figure 2.11) and compound 48/80 ($93.86 \pm 2\%$, 20 μM , figure 2.11), although as with compound DF1.03, this activity is diminished at 1 μM ($-5.43 \pm 5\%$, 1 μM , figure 2.11) when assayed with compound 48/80. Although it is clear that mast cell stabilising activity is maintained in some of these compounds (DF1.03, DF1.05 and DF1.06) the group as a whole does not perform well.

2.4.2.2 Group II Compounds

Group II compounds demonstrate, on the whole, an improvement in mast cell stabilising activity when compared with Group I compounds. Each compound demonstrates good levels of activity in the presence of both calcium ionophore A23187 (DF2.02 $63.71 \pm 6\%$, 20 μM , figure 2.13; DF2.03 $74.97 \pm 8\%$, 20 μM , figure 2.14; DF2.04 $36.86 \pm 13\%$, 20 μM , rising to $90.92 \pm 4\%$, 5 μM , figure 2.15; DF2.05 $89.5 \pm 3\%$, 20 μM , figure 2.16) and compound 48/80 (DF2.02 $90.9 \pm 6\%$, 20 μM , figure 2.13; DF2.03 $100.00 \pm 3\%$, 20 μM , figure 2.14; DF2.04 $43.29 \pm 6\%$, 20 μM , rising to $84.84 \pm 3\%$, 5 μM , figure 2.15; DF2.05 $91.18 \pm 4\%$, 20 μM , figure 2.16), with the exception of DF2.01 which demonstrates better activity with calcium ionophore A23187 (DF2.01 $74.72 \pm 6\%$, 20 μM , figure 2.12) than with compound 48/80 (DF2.01 $42.55 \pm 12\%$, 20 μM , figure 2.12). Although this group was synthesised with the aim of increasing solubility of Group I compounds, there were in fact several issues with solubility noted in this group, namely with compounds DF2.01, DF2.03 and DF2.05, which ruled them out of further studies. DF2.02 and DF2.04 show promising activity at this stage.

2.4.2.3 Group III Compounds

Although Group III compounds demonstrate significant mast cell stabilising activity in the presence of calcium ionophore A23187 (DF3.01 $64.22 \pm 14\%$, 20 μM , figure 2.17; DF3.02 $83.79 \pm 2\%$, 20 μM , figure 2.18; DF3.03 $66.5 \pm 5\%$, 20 μM , figure 2.19; DF3.04 $71.06 \pm 10\%$, 20 μM , figure 2.20), like many compounds in Group I, they all demonstrate disappointingly poor activity in the presence of compound 48/80 (DF3.01 $35.9 \pm 6\%$, 20 μM , figure 2.17; DF3.02 $32.64 \pm 11\%$, 20 μM , figure 2.18; DF3.03 $4.47 \pm 3\%$, 20 μM , figure 2.19; DF3.04 $38.46 \pm 8\%$, 20 μM , figure 2.20).

2.4.2.4 Group IV Compounds

Again, Group IV compounds demonstrate a mixed mast cell stabilising profile. TZ1.06 and TZ1.07 were the most active compounds in this group in the presence of both calcium ionophore A23187 (TZ1.06 $70.26 \pm 1\%$, 20 μM , figure 2.26; TZ1.07 $71.45 \pm 6\%$, 20 μM , figure 2.27) and compound 48/80 (TZ1.06 $78.71 \pm 7\%$, 20 μM , figure 2.26; TZ1.07 $67.97 \pm 5\%$, 20 μM , figure 2.27), although TZ1.06 is more active. TZ1.02, although not as potent as TZ1.06 and TZ1.07, also demonstrates similar levels of mast cell stabilising activity when assayed with both calcium ionophore A23187 (TZ1.02 $56.9 \pm 12\%$, 20 μM , figure 2.22) and compound 48/80 (TZ1.02 $61.02 \pm 12\%$, 20 μM , figure 2.22). TZ1.01 and TZ1.04 demonstrated potent mast cell stabilising activity in the presence of calcium ionophore A23187 (TZ1.01 $59.51 \pm 7\%$, 20 μM , figure 2.21; TZ1.04 $88.19 \pm 4\%$, 20 μM , figure 2.24) but this was not repeated when these compounds were assayed in the presence of compound 48/80 (TZ1.01 $42.52 \pm 8\%$, 20 μM , figure 2.21; TZ1.04 $38.48 \pm 6\%$, 20 μM , figure 2.24). TZ1.03 demonstrates a low but seemingly constant level of activity with compound 48/80 (TZ1.03 $13.33 \pm 14\%$, 20 μM , figure 2.23), while its activity in the presence of calcium ionophore remains low (TZ1.03 $47.18 \pm 1\%$, 20 μM , figure 2.23), although higher than other compounds of this class at 1 μM (TZ1.03 $36.48 \pm 10\%$, 1 μM , figure 2.23). TZ1.05 showed mast cell stabilising activity in the presence of calcium ionophore A23187 (TZ1.05 $69.21 \pm 8\%$, 20 μM , figure 2.25), however, this activity was lost at 5 μM (TZ1.05 $4.03 \pm 2\%$, 5 μM , figure 2.25) in a similar manner to TZ1.01 (TZ1.01 $26.79 \pm 18\%$, 5 μM , figure 2.21), activity in the presence of compound 48/80 remains constant (TZ1.05 $47.93 \pm 17\%$, 20 μM , figure 2.25).

2.4.2.5 Rat Peritoneal Mast Cell IC₅₀

At this point activity profiles as well as solubility issues were considered and on this basis compounds were chosen for further investigation into their activity. DF1.01, DF1.02 and TZ1.03 were excluded from further studies because of their poor activities at 20, 10, 5 and 1 μM using both calcium ionophore A23187 and compound 48/80 as discussed above. DF1.06, DF2.01, DF2.03 and DF2.05 were also excluded from further studies because of poor solubility which was observed in RPMC assays above; frequently these compounds prepared in DMSO would crash out of solution upon introduction to the assay buffer. IC₅₀ values were established in compounds which show favourable solubility and mast cell stabilising activity.

IC₅₀ values are compiled in table 2.03 and were only obtained in the presence of calcium ionophore A23187 for compounds DF1.04 (IC₅₀ 0.87 μM), DF1.05 (IC₅₀ 0.17 μM), DF3.01 (IC₅₀ 31.59 μM), DF3.02 (IC₅₀ 0.94 μM) and DF3.03 (IC₅₀ 0.40 μM) because of their poor mast cell stabilising activities in the presence of compound 48/80, discussed above. IC₅₀ values using both calcium ionophore A23187 and compound 48/80 were obtained for DF1.03 (IC₅₀ 0.54 μM calcium ionophore A23187; IC₅₀ 1.72 μM compound 48/80), DF2.02 (IC₅₀ 81.82 nM calcium ionophore A23187; IC₅₀ 0.20 μM compound 48/80), DF2.04 (IC₅₀ 0.24 μM calcium ionophore A23187; IC₅₀ 1.04 μM compound 48/80), TZ1.01 (IC₅₀ could not be established using calcium ionophore A23187 as values do not converge, see figure 2.33; IC₅₀ 3.01 μM compound 48/80), TZ1.02 (IC₅₀ 9.43 μM calcium ionophore A23187; IC₅₀ 7.77 μM compound 48/80), TZ1.04 (IC₅₀ 5.72 μM calcium ionophore A23187; IC₅₀ 5.51 μM compound 48/80), TZ1.05 (IC₅₀ 7.90 μM calcium ionophore A23187; IC₅₀ 2.66 μM compound 48/80), TZ1.06 (IC₅₀ 5.49 μM calcium ionophore A23187; IC₅₀ 1.17 μM compound 48/80) and TZ1.07 (IC₅₀ 8.61 μM calcium ionophore A23187; IC₅₀ 7.02 μM compound 48/80). It is clear from table 2.03 that compound DF2.02 is the most potent mast cell stabilising compound of all of those assessed in this work, in the presence of both calcium ionophore A23187 and compound 48/80, of all of those assayed. Other compounds such as TZ1.02, TZ1.04 TZ1.06 and TZ1.07 possess a level of mast cell stabilising activity which warrants further investigation into their activities.

2.4.3 Structure Activity Relationship

To try and further understand the activity of these compounds, computational analysis was carried out into their properties using MarvinSketch 5.3.8 (Chemaxon 2010). The properties obtained using MarvinSketch were the cLogP (LogP), LogD_{7.4}, pKa, hydrogen bond donor and hydrogen bond acceptor sites and 2D PSA (tables

2.04-2.07). Investigations were carried out into the possible structure activity relationship for each group of compounds.

To determine if the computed LogP values determined using MarvinSketch are correct, laboratory investigations should be carried out. Computed values were not used to determine definitive values, but to gain a general indication of the properties of these compounds. Results below indicate that these compounds appear to be highly lipophilic in nature.

2.4.3.1 LogP

LogP affects the absorption of compounds through various lipid membranes, including the G.I. tract and cell membranes. For optimum absorption at different sites, compounds should possess certain absorption values; for CNS penetration, oral absorption, intestinal absorption, colonic absorption, sub-lingual absorption and percutaneous absorption compounds should have a LogP value of approximately 2, 1.8, 1.35, 1.32, 5.5 and 2.6 with low molecular weight, respectively. High LogP values indicate a compound is very lipophilic and therefore can easily traverse the lipid bilayer to enter the cell, however, if the lipophilicity is very high compounds can become "stuck" to the lipid bilayer. Very high LogP (4-7) can result in the toxic build up of the compound in adipose tissues.

Tables 2.04 to 2.07 above indicate computed LogP values for each compound group. Although the values calculated using MarvinSketch are suggested computed values, all have very high LogP values (greater than or equal to 5), except for DF1.01 (4.11), which although less than 5, is still relatively high suggesting they are highly lipophilic molecules.

"The Principle of Minimum Hydrophobicity in Drug Design" (Testa, Pliska *et al.* 1995) maintains that compounds should not enter the CNS unless desired. To ensure this does not occur, LogP value should be lower than 2.0. Group I-IV compounds all have a theoretical computed LogP value which are greater than 2.0 indicating they, in all probability, may permeate through the blood brain barrier and enter the CNS.

2.4.3.2 LogD_{7.4}

Because logP is the measure of the lipophilicity of a neutral compound at a certain pH and does not take into account the fact that they may ionise at different pH values LogD_{7.4}, the distribution coefficient at pH7.4, is used to express the lipophilicity of an ionisable species at blood serum pH. Because many of these compounds ionise (see pKa values), their computed LogD_{7.4} values were determined. In general LogD_{7.4} should be around 2.0 or less when it is considered in the place of LogP. High LogD_{7.4} (greater than 5) can result in low solubility, high

permeability and low metabolism resulting in potential toxic pooling in adipose tissue while $\text{LogD}_{7.4}$ of 3-5 also generally results in low solubility and high permeability with moderate to high metabolism. All compounds in Groups I-IV possess computed $\text{LogD}_{7.4}$ values greater than 3 (tables 2.04 - 2.07), with the majority of compounds having a $\text{LogD}_{7.4}$ greater than 5, DF1.02 ($\text{LogD}_{7.4}$ 9.18) and DF1.04 ($\text{LogD}_{7.4}$ 8.04) have the highest $\text{LogD}_{7.4}$ values. As with the LogP , $\text{LogD}_{7.4}$ values for all compounds are unfavourably high. Because $\text{LogD}_{7.4}$ less than 1 generally reflects the inability of compounds to penetrate the blood brain barrier and although $\text{LogD}_{7.4}$ values are theoretical computed values, results seem to reinforce computed LogP findings, which indicate these compounds may penetrate the CNS.

2.4.3.3 Structure Activity Relationship

In an attempt to determine SAR, LogP versus activity was plotted for each group of compounds at high (20 μM ; figures 2.39–2.52) and low (1 μM ; data not presented here because again like 20 μM , no relationship was found) concentrations in an attempt to establish a relationship between LogP and activity for each group, using both calcium ionophore A23187 and compound 48/80. The analysis was also carried out with the entire compound group (Groups I-IV; figures 2.53–2.56). Often a relationship exists between the activities and chemical properties of a compound class which allows the activity of these compounds to be predicted (Barfknecht and Nichols 1975; Grinius, Stanton *et al.* 2002). Linear regression analysis was therefore carried out to determine whether or not a simple linear relationship may exist between the computed LogP values and compound RPMC activities. In all cases no relationship was found to exist between LogP and activity within any group, or when all groups are analysed together (r^2 values are extremely low in all cases). Investigations were also carried out to determine whether a parabolic relationship may exist; no relationship was determined in any case using this analysis (data not shown) although Group I compounds appear visually to have a parabolic relationship when computed LogP values are plotted against RPMC assay activities (figure 2.39). $\text{LogD}_{7.4}$ versus activity were, in the same manner as LogP values, plotted for each group of compounds, as well as with the entire compound group, at high (20 μM) (and low; 1 μM) concentrations in an attempt to establish a relationship between $\text{LogD}_{7.4}$ and activity for each group, using both elicitors. In all cases linear regression and parabolic analysis was carried out and again no relationship was found to exist between $\text{LogD}_{7.4}$ and activity within any group (in all cases r^2 values again are extremely low), or within the group as a whole.

2.4.3.4 pKa

Most compounds studied here are ionised at blood serum pH, reflecting the difference in LogP and LogD_{7.4} values seen; Group I compounds are strong bases, Group II compounds are weak acids while Group III and Group IV are all weak bases (tables 2.04–2.07).

2.4.3.5 Hydrogen Bond Donor and Acceptor Sites

Excessive number of hydrogen bond donor groups impairs permeability across a membrane bilayer and Lipinsky determined that simply counting the amount of NH and OH bonds easily establishes H bond donor values for a compound (Lipinski, Lombardo *et al.* 2001). Smaller numbers of H bond donors correlate with better permeability. Too many hydrogen bond acceptor sites can also prevent permeability and in this case the sum of N and Os are used as a rough guide. Compounds in Groups I-IV all have 0 or 1 hydrogen bond donor sites and 1, 2, 3 4 or 5 hydrogen bond acceptor sites, these values are all acceptably low indicating hydrogen bond donor or acceptor status should not influence lipid bilayer permeability of these compounds (tables 2.04–2.07).

2.4.3.6 Lipinski's Rule of Five

Although all compounds in Groups I to IV obey the Rule of Five with respect to hydrogen bond donor and acceptor sites and their molecular weights are all below 500g/mol, all compounds in these groups, except DF1.01 (LogP 4.11), have a LogP value higher than 5 (although TZ1.01 and TZ1.02 have a LogP of 5.05). If LogD_{7.4} is considered instead 8 compounds have values less than 5; DF1.01 (LogD_{7.4} 4.11, table 2.04), DF2.01 (LogD_{7.4} 3.34, table 2.05), DF2.03 (LogD_{7.4} 3.73, table 2.05), DF2.04 (LogD_{7.4} 4.39, table 2.05), TZ1.01 (LogD_{7.4} 4.70, table 2.07), TZ1.02 (LogD_{7.4} 4.39, table 2.07), TZ1.06 (LogD_{7.4} 4.77, table 2.07) and TZ1.07 (LogD_{7.4} 3.42, table 2.07). All of these compounds possess computed LogP and LogD_{7.4} values which suggest they may traverse the blood brain barrier and may become tethered at lipid bilayers, as discussed above.

2.4.3.7 2D Polar Surface Area

PSA (Å²) is the measure of the amount of surface area of a compound which arises from polar atoms (nitrogen and oxygen, with their attached hydrogen; sulphur and phosphorous atoms can be included for calculations) (Ertl, Rohde *et al.* 2000). Although PSA was not one of the initial properties which was considered when trying to predict the permeability profile of compounds, as with Lipinski's Rule of Five, it is becoming more widely used because it often correlates with passive

diffusion of compounds through membranes including intestinal and blood brain barrier membranes. PSA can therefore help to predict the permeability properties of compounds. Obviously high PSA values, although indicating the solubility of compounds in aqueous media, lead to diminished transport of the compound across the lipid bilayer and across membranes. For most compounds which do not penetrate the CNS, PSA values are generally greater than $120 \text{ \AA}^2$ while for most CNS active compounds, PSA is below $70 \text{ \AA}^2$. PSA values greater than $140 \text{ \AA}^2$ can have intestinal absorption problems. As shown in the above tables, all of the compounds assessed in this chapter (Groups I-IV) have computed PSA values which are less than $70 \text{ \AA}^2$ (tables 2.04 – 2.07) which may support results obtained using LogP and LogD_{7.4} that these compounds probably cross the blood brain barrier and act centrally, as well as possibly become tethered at the lipid bilayer cell membrane.

Although QSAR analysis indicates that these compounds, while possessing favourable hydrogen bond acceptor and donor site numbers and low molecular weight, may traverse the blood brain barrier due to their LogP, LogD_{7.4} and PSA values, it is important to note that the prime reason that QSAR analysis was carried out was to determine whether a structure activity relationship may exist for these compounds. No relationship was found between LogP, LogD_{7.4} and mast cell stabilising activity. These results do not, however, diminish the findings that compounds assayed above possess potent mast cell stabilising activity in the presence of calcium ionophore A23187 and compound 48/80, the most potent of which being DF2.02. In RPMC assays DSCG was used as control compound and at concentrations comparable to those of novel compounds assayed DSCG activity is inferior to these compounds in the presence of both calcium ionophore A23187 (DSCG $40 \text{ }\mu\text{M}$ 23.19 ± 2.82 $n=53$, $20 \text{ }\mu\text{M}$ 6.05 ± 4.01 $n=6$) and compound 48/80 ($40 \text{ }\mu\text{M}$ 25.14 ± 3.46 $n=23$, $20 \text{ }\mu\text{M}$ 10.42 ± 2.08 $n=35$). Because the mast cell stabilising activity of some of these novel compounds is highly favourable, further studies should be carried out into the activities of these compounds despite what appears to be their unfavourable highly lipophilic nature. This would determine if this RPMC assay determined mast cell stabilising activity is maintained in a variety of other settings and thus indicate their potential for use as possible therapeutic agents in the future.

2.5 Conclusions

The studies undertaken in this chapter aimed to purify mast cells from the rat peritoneal cavity and to establish mast cell stabilising activity in a variety of novel compounds in this highly purified mast cell population. Some of these novel compounds which were designed to overcome a number of issues with JWB5.12 and JWB4.61, demonstrated potent mast cell stabilising activity in RPMCs, activity which was superior to that of DSCG. The most potent of these, DF2.02, as demonstrated by IC_{50} values using both calcium ionophore A23187 and compound 48/80, would require further investigation with respect to its mast cell stabilising activity. Other compounds, such as TZ1.02, TZ1.04 TZ1.06 and TZ1.07, which emerged as active compounds in the above studies would also require further evaluation.

Evaluation into the properties of these compounds was carried out with a view to establishing a SAR. SAR analysis using computed values revealed that it is likely that these compounds are all highly lipophilic compounds which may permeate the lipid bilayer. Computed values indicate high lipophilicity of these compounds which may suggest that they may remain tethered at the cell membrane and therefore may exert their effect there. Analysis of the lipophilicity of these compounds with respect to their activities did not reveal a relationship between their RPMC mast cell stabilising activity and their hydrophobicity using computed theoretical values.

Although many mechanisms of action may be discussed, because the aims of this project are to assist in the development of potent mast cell stabilising compounds, it was important to establish whether these active compounds maintain mast cell stabilising activity *in vivo*. Therefore, compounds which demonstrated potent mast cell stabilising activity in both calcium ionophore A23187 and compound 48/80 RPMC assays were assessed using the passive cutaneous anaphylaxis study for *in vivo* mast cell stabilising activity. This is discussed further in Chapter 3.

Chapter 3

***In Vivo* Passive Cutaneous Anaphylaxis Studies on *In Vitro* Mast Cell Stabilising Benzocycloalkanone Compounds**

3.1 Introduction

Rat peritoneal mast cell assay results obtained in Chapter 2 reveal that a number of novel compounds synthesised by Finn and Zhang possessed mast cell stabilising activity which is comparable, or superior, to that of DSCG *in vitro*, the most potent of these being DF2.02. Because these compounds demonstrated mast cell stabilising activity *in vitro*, it was necessary to determine whether this mast cell stabilising activity was maintained *in vivo*. This chapter therefore, presents the findings of the passive cutaneous anaphylaxis (PCA) assay, an *in vivo* allergy model by which a number of these compounds were assessed for mast cell stabilising activity.

Zhang synthesised a number of novel, Group V compounds which were also assessed for *in vivo* mast cell stabilising activity using the PCA model. These compounds are; TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05, TZ2.06, TZ2.07, TZ2.08, TZ2.09, TZ2.10, TZ2.11 and TZ2.12 (figure 3.01). The reasoning behind the synthesis of these compounds and the characteristics of this class will be discussed later in combination with the PCA results presented below (section 3.4.1.2).

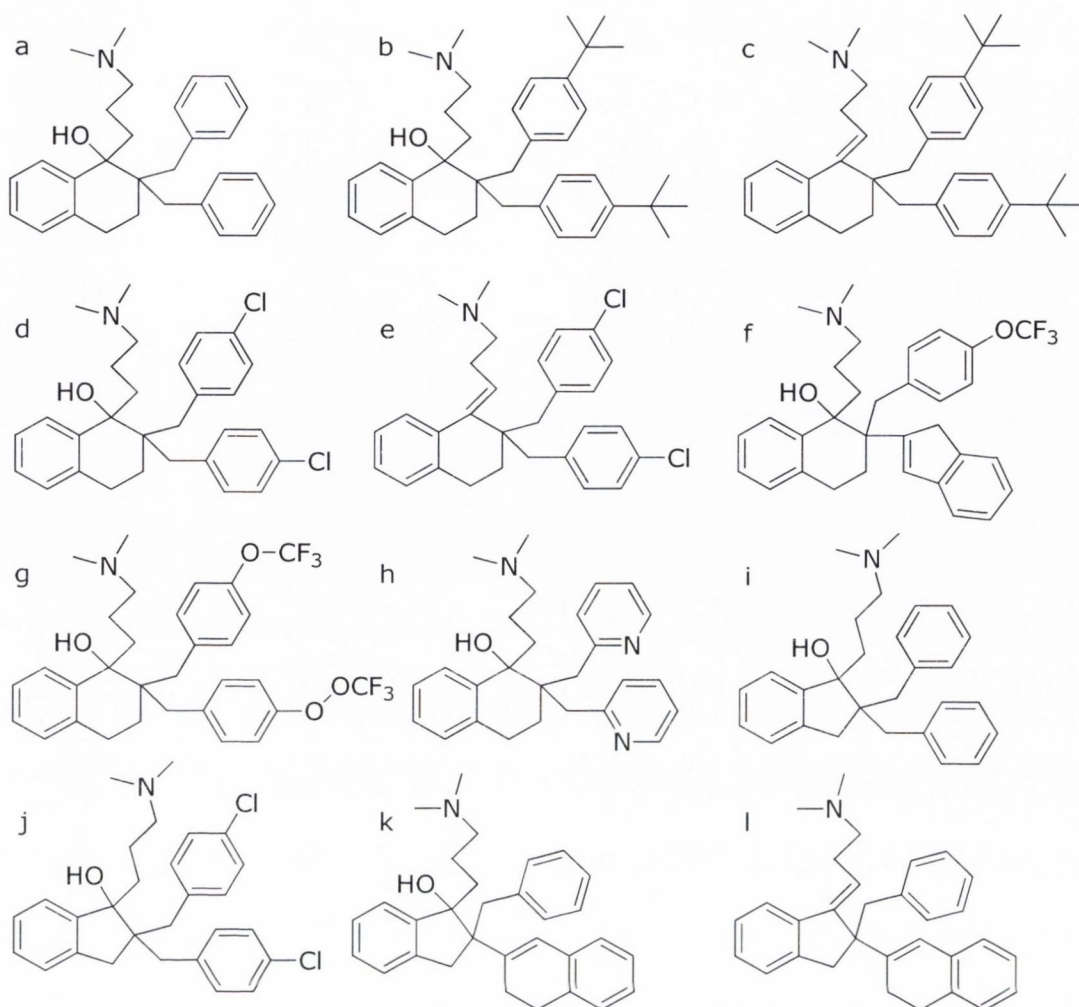


Figure 3.01: Group V Compounds: a) TZ2.01, b) TZ2.02, c) TZ2.03, d) TZ2.04, e) TZ2.05, f) TZ2.06, g) TZ2.07, h) TZ2.08, i) TZ2.09, j) TZ2.10, k) TZ2.11 and l) TZ2.12

3.1.1 Aims:

Because several compounds evaluated using the RPMC assay demonstrated potent *in vitro* mast cell stabilising activity, and because novel Group V compounds were synthesised by Zhang, the aims of this project were:

- To establish the *in vivo* mast cell stabilising activity of a selection of compounds which demonstrated *in vitro* mast cell stabilising activity in Chapter 2, using the PCA assay,
- To establish the *in vivo* mast cell stabilising activity of novel Group V compounds synthesised by Zhang, again using the PCA model,
- To determine the systemic toxicity of two representative compounds, using the SHIRPA behavioural observation protocol and
- To investigate whether a structure activity relationship exists within PCA active compounds.

3.1.2 Passive Cutaneous Anaphylaxis *In Vivo* Model of Allergy

The passive cutaneous anaphylaxis (PCA) model of allergy was first developed in the mouse by Ovary (Ovary 1958). Using this protocol, rats are passively sensitised by subcutaneous dermal injection of IgE containing serum raised against chicken egg albumin (CEA). These IgE molecules bind to FcεRI receptors on dermal mast cells and subsequent allergen (CEA) challenge results in the Prausnitz-Kustner reaction (Manwaring 1938); the characteristic wheal and flare response of allergy (Geha, Helm *et al.* 1985). Evans blue dye is administered along with allergen challenge and because mast cell activation, through the actions of histamine on its H1R, ultimately results in increased permeability of capillaries adjacent to activated mast cells, mast cell activation is quantified by measuring the amount of dye leakage into the dorsal skin inoculation sites. Test compounds are administered along with allergen and Evans blue dye, which therefore allows for the visualisation of the activity of compounds (Ovary 1958), in comparison with controls (Watanabe and Ovary 1977). Compounds which possess mast cell stabilising activity cause a reduction of Evans blue dye leakage into the skin.

3.1.3 Use of Adjuvant to Raise Serum

In general rats do not produce IgE; however, the use of an adjuvant to facilitate the induction of an immune response overcomes this issue. Several possible adjuvants exist for use, such as Freund's complete adjuvant, however, aluminium hydroxide gel and heat killed *Bordetella pertussis* are used here. Aluminium hydroxide is a widely used adjuvant in human vaccines (Clements and Griffiths 2002) while *B. pertussis* is a non-motile gram negative bacterium responsible for whooping cough. Aluminium hydroxide alone, or in combination with *B. pertussis* results in the production of serum rich in IgE, increasing the efficacy of administration with *B. pertussis* alone (Jarrett, Hall *et al.* 1980). Rats immunised with CEA and *B. pertussis* produce IgE which is sensitised to CEA (Jarrett, Hall *et al.* 1980) and immunisation with CEA in aluminium hydroxide increases specific IgE without significantly increasing total IgE levels (Salgado, Gilabert *et al.* 1989). The highest IgE levels are obtained with the combination of CEA with aluminium hydroxide with *B. pertussis* (Castell, Gisbert *et al.* 1987), therefore, this combination was used here. Aluminium hydroxide also acts to slow the release of antigen and attracts immune cells to the site of injection, facilitating antigen presentation (Salgado, Gilabert *et al.* 1989) while *B. pertussis* increases the rate of B cell division, resulting in increased amounts of antibodies being generated (Reed, Benner *et al.* 1972).

3.1.4 Disodium Cromoglycate

As previously discussed, DSCG inhibits both calcium ionophore A23187 and compound 48/80-mediated histamine release in the RPMC assay. DSCG inhibits PCA in rats which have been sensitised using serum raised against CEA (Goose and Blair 1969) and also inhibits PCA when administered orally (Shin, Kim *et al.* 2004). DSCG is therefore used frequently as control compound for the PCA *in vivo* assay, alongside novel test compounds (Kobayashi, Hiroi *et al.* 1993; Park, Choo *et al.* 2003; Kim and Shin 2005).

3.1.5 SHIRPA Behavioural Observation Profile

The SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; Imperial College School of Medicine at St. Marys'; Royal London Hospital, St. Bartholomew's and the Royal London School of Medicine; Phenotype Assessment (SHIRPA) behavioural observation protocol is based on the Irwin profile (Irwin 1968) and is similar to the functional observation battery (Moser 1995), both of which can be used to determine toxicity. The SHIRPA behavioural observation protocol was designed to allow for the characterisation of transgenic and inbred mouse phenotypes (Rogers, Fisher *et al.* 1997; Rogers, Jones *et al.* 1999) and has since been used to assess and quantify transgenic phenotype (Lalonde, Dumont *et al.* 2004), cognitive dysfunction and subsequent treatment (Reis, Comim *et al.* 2010), effects of parasite infection (Gulinello, Acquarone *et al.* 2010) and toxicity (Davies, Wang *et al.* 2005; Dadachova, Moadel *et al.* 2006).

The SHIRPA observation protocol consists of four subcategories of behavioural observation; undisturbed behaviour, active behaviour, restrained behaviour and manipulated behaviour, which together assess the general effects of a drug candidate on the CNS. It is based on observations of behaviour associated with neurotoxicity (convulsions, tremor), CNS stimulation (excitation, straub tail, jumping, stereotypies, hypersensitivity to external stimuli, aggressive behaviour), CNS depression (sedation, rolling gait, loss of balance, motor discoordination, hyposensitivity to external stimuli, decreased muscle tone) and autonomic function (respiration, defaecation, lacrimation, salivation). A simplified, shortened version of the SHIRPA protocol, in which the primary screen (behaviour) is carried out and the secondary (e.g. histology and biochemistry) and tertiary (e.g. learning and memory and magnetic resonance imaging) screens are omitted, is used here to quantify the behavioural and functional profile of animals upon administration of test compounds, with a view to establishing their safety.

3.2 Materials and methods

3.2.1 Materials

Aluminium hydroxide gel, chicken egg albumin (CEA), dimethyl sulfoxide (DMSO), disodium cromoglycate (cromolyn; DSCG), Evans blue, HPLC grade acetone, phosphoric acid, potassium hydroxide and Tween 80 were all purchased from Sigma Aldrich, Arklow, Co. Wicklow, Ireland. Na-Nu Vitality® maple syrup was purchased from Tesco Ireland, Greystones, Co. Wicklow.

3.2.2 Animals

Female Wistar rats (250-350 g) were sourced from the BioResources Unit, Trinity College Dublin. Animals were housed in groups of eight or less, under controlled conditions at 22 °C with 12 hour light and 12 hour dark cycles. All animals had access to food and water *ad libitum* throughout the experiment. Experimental procedures were subject to review by Trinity College Dublin Ethics Committee, were licensed by the Department of Health and were carried out in accordance with EC directive 86/609/EEC.

3.2.3 Raising of Antiserum

A stock of antiserum was previously raised by Byrne in this lab and was used for some PCA experiments; however, in time it was necessary to raise additional amounts of antiserum. *B. pertussis* which had been used by Byrne was used to raise the new batch of serum; 1 ml of heat killed *B. pertussis* suspension (10^{10} organisms/ml/animal), 0.5 ml of CEA (1.0 mg/animal, made in 0.9% saline) and aluminium hydroxide (2.5 mg/animal) were injected into the peritoneal cavity (intraperitoneal, I.P.) of two female Wistar rats. Both animals subsequently died within 24 hours and post mortem examination, carried out by Dr. Peter Nowlan (BioResources, Trinity College Dublin), revealed the animals had suffered septic shock, although it is likely that they may have suffered from anaphylactic shock due to the nature of the experiment.

A new stock of heat killed *B. pertussis* suspension (10^{10} organisms/ml) was received from Professor Kingston Mills' lab, Trinity College Dublin. This *B. pertussis* suspension was administered to six different animals at two separate doses (0.8 ml/animal and 1.0 ml/animal I.P.), with aluminium hydroxide and CEA in similar doses as before. This would determine the correct dose at which *B. pertussis* would maintain its adjuvant activity without harming the animals. All of these animals survived the 14 days required for the generation of adequate amounts of IgE rich antiserum. Original stock *B. pertussis* suspension was also given to two animals at a dose of 0.5 ml/animal with the standard amounts of CEA and aluminium

hydroxide and these animals also survived to day 14. On day 14 all animals were sacrificed by cardiac puncture under halothane or isoflurane (halothane was banned for use during this project due to its teratogenic properties, therefore a different choice of anaesthetic was required) anaesthetic and blood was collected and pooled from each *B. pertussis* dose group. The back of a test group of animals were shaved and animals were inoculated intradermally with various dilutions of serum (in 0.9% w/v saline; 100 µl/site). The titre of antiserum to be used in future was determined as being the lowest dilution capable of producing a wheal of 5mm in diameter after intravenous challenge with CEA (2.5 mg/animal) in 1% Evan's blue dye (in 0.9% w/v saline), 48 hours after inoculation.

For the original batch raised by Byrne a 1:16 dilution was the lowest effective dilution and so was used in PCA studies. The lowest effective dilution using the antiserum raised using 0.5 ml of the original *B. pertussis* was a 1:32 dilution, while the lowest effective dilutions using the antiserum raised from the *B. pertussis* sourced from Professor Kingston Mills' lab were a dilution of 1:1 (0.8 ml/animal *B. pertussis* used to raise the antiserum; results not optimal); and a dilution of 1:1 (1 ml/animal *B. pertussis* used to raise the antiserum), therefore, these dilutions were aliquoted and frozen for use in future PCA studies, apart from the batch raised using 0.8 ml/animal of *B. pertussis* from Professor Mills's lab which was discarded.

3.2.4 Passive Cutaneous Anaphylaxis *In Vivo* Assay

Female Wistar rats were anaesthetised and their backs shaved. To sensitise dermal mast cells three dorsal intradermal saline inoculations (0.9% w/v; 100 µl/site) and three dorsal intradermal serum (raised above) inoculations (100 µl/site) were performed on each animal and each site was marked with an insoluble red marker. Forty eight hours later animals were challenged intravenously, via their tail vein, with CEA (2.5 mg/animal) in 0.25 ml 2% Evans blue dye in saline (0.9% w/v) with 0.25 ml of test compound in vehicle or vehicle alone. Test compounds, and control compound DSCG, were administered at a dose of 3 mg/kg. Thirty minutes later animal were sacrificed by CO₂ asphyxiation, the dorsal skin was reflected and skin biopsies of the original inoculated areas were excised. Each tissue segment was added to 1 ml (1 M) KOH and digested overnight at 37 °C in a shaking water bath. The following day 0.625 ml (0.6 M) H₃PO₄ and 1.625 ml of HPLC grade acetone were added to 500 µl of each tissue digest in new labelled centrifuge tubes. Samples were shaken and centrifuged at 1560 g (3000 rpm) at 4 °C for 15 minutes. Supernatant was removed (300 µl) and added to the wells of a black 96 well plate. Red light absorbance, which quantified Evans Blue dye, was read at 620 nm (FLUOstar OPTIMA, BMG labtech).

3.2.5 Analysis of Data

Absorbance values were determined for each site of dermal serum or saline inoculation (figure 3.02).

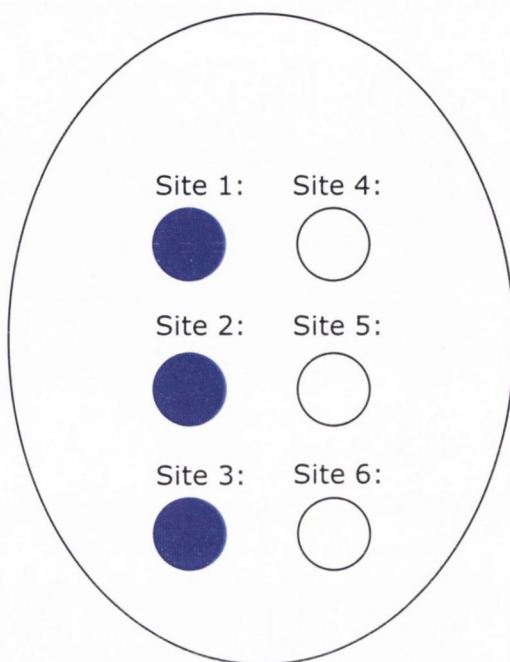


Figure 3.02: Sites of serum/saline inoculations, PCA assay

Because the amount of Evans blue dye leakage at each site corresponds to local vasculature leakage due to histamine release by adjacent, activated mast cells, calculations into the activity of novel mast cell stabilising compounds are carried out as follows:

$$\text{site 1 (serum) - site 4 (saline) = A}$$

$$\text{site 2 (serum) - site 5 (saline) = B}$$

$$\text{site 3 (serum) - site 6 (saline) = C}$$

During each PCA experimental run DSCG in vehicle, test compound in vehicle and vehicle alone were administered. Values (A, B and C) for each vehicle treated animal were obtained as described and values for the vehicle treated group were combined and averaged to give the true vehicle absorbance value. To quantify the activity of DSCG or test compounds, A, B and C were calculated for each drug treated animal and % inhibition of PCA was calculated;

$$\% \text{ Inhibition of PCA} = 100 - [(\text{any A, B or C value}) / (\text{true vehicle absorbance value})] * 100$$

3.2.6 SHIRPA following Acute Oral Exposure

Test compounds were evaluated as a suspension (10 mg/kg) in maple syrup. This was consumed voluntarily by animals when administered orally (P.O.) using a syringe. Behaviour was established in untreated animals (n=24; t=0), vehicle treated (n=8) and test compound treated (TZ2.04; n=8 or TZ2.01; n=7) female Wistar rats. Assessment consisted of four subcategories: undisturbed behaviour, active behaviour, restrained behaviour and manipulated behaviour. Compounds were evaluated over 24 hours, at 1, 2, 4, 8 and 24 hours post dose. The SHIRPA behavioural observation protocol was carried out as follows:

Table 3.01: SHIRPA behavioural observation protocol score sheet and appendices

Rat Drug SHIRPA Screening Behavioural Observation Profile

Date: Drug: Dose & Administration Route: Sex: Weight:

A Undisturbed Behaviour

#	Test	0	1	2	3	4	5
1	Body Position	Completely Flat	Lying on Side	Lying Prone	Standing or Sitting	Rearing on Hind Limbs	Repeated Vertical Leaping
2	Spontaneous Activity	None, Resting	Casual Scratching, Grooming, Slow Movement	Vigorous Scratching, Grooming, Moderate Movement	Vigorous, Rapid/ Dart Movement	Extremely Vigorous, Rapid/ Dart Movement	
3	Respiration Rate	Gasping, Irregular	Slow, Shallow	Normal	Hyperventilation		
4	Tremor	None	Mild	Marked			
5	Defaecation	Number of Pellets					
6	Urination	None	Present				

B. Active Behaviour

#	Test	0	1	2	3	4	5	6
7	Drop onto Arena Floor, Time Before Move	Time (Seconds)						
8	Transfer Arousal	Coma	Prolonged Freeze, then Slight Movement	Extended Freeze, then Moderate Movement	Brief Freeze (Few Seconds), then Active Movement	Momentary Freeze then Swift Movement	No Freeze, Immediate Movement	Extremely Excited, ("Manic")
9	Piloerection	Absent	Present					
10	Palpebral Closure	Wide Open	Half Closed	Closed				
11	Startle Response	None	Preyer Reflex	Jump $\leq$ 1 cm	Jump $\geq$ 1 cm			
12	Gait	Normal	Fluid but Abnormal	Limited Movement Only	Incapacity			
13	Pelvic Elevation	Markedly Flattened	Barley Touching	Normal	Elevated			
14	Tail Elevation	Dragging	Horizontally Extended	Elevated/ Straub Tail				

15	Touch Escape	No Response	Mild (Escape Response to Firm Stroke	Moderate (Rapid Escape Response to Light Stroke)	Vigorous (Escape Response to Approach)			
16	Positional Passivity	Struggles when Held by Tail	Struggles When Held By Neck (Finger Grip)	Struggles When Laid Supine	Struggles When Held by Hind Limbs	No Struggle		

C. Restrained Behaviour (On/Above Arena)

#	Test	0	1	2	3	4
17	Trunk Curl	Absent	Present			
18	Limb Grasping	Absent	Present			
19	Visual Placing	None	Upon Nose Contact	Upon Vibrissae Contact	Before Vibrissae Contact	Early Vigorous Extension
20	Grip Strength	None	Slight Grip, Semi Effective	Moderate Grip, Effective	Active Grip, Effective	Unusually Effective
21	Body Tone	Flaccid, No Return of Cavity to Normal	Slight Resistance	Extreme Resistance, Board Like		

22	Pinna Reflex	None	Active Retraction, Moderately Brisk Flick	Hyperactive, Repetitive Flick		
23	Corneal Reflex	None	Active Single Eye Blinking	Multiple Eye Blinking		

D. Manipulated Behaviour and Other Tests

#	Test	0	1	2	3	4
24	Body Length	(mm)				
25	Tail Length	(mm)				
26	Lacrimation	None	Present			
27	Provoked Biting	Absent	Present			
28	Salivation	None	Slight Margin of Sub-maxillary Area	Wet Zone Entire Sub-maxillary Area		
29	Abdominal Tone	Flaccid, No Return of Cavity to Normal	Slight Resistance	Extreme Resistance, Board Like		
30	Skin Colour	Blanched	Pink	Bright, Deep Red Flush	Dark Footpad or Pigmentation	
31	Limb Tone	No Resistance	Slight Resistance	Moderate Resistance	Marked Resistance	Extreme Resistance

32	Toe Pinch	Slight Withdrawal	Moderate Withdrawal, Not Brisk	Rapid, Brisk Withdrawal	Very Brisk Repeated Extension and Flexion	
33	Righting Reflex	No Impairment	Impaired	Fails to Right When Placed on Back		
34	Negative Geotaxis	Turns and Climbs the Grid	Turns but then Freezes	Moves, but Fails to Turn	Does Not Move Within 30 Seconds	Falls Off
35	Aggression	None	Provoked Biting or Attack			
36	Vocalisation	None	Provoked During Handling			
37	Bizarre Behaviour					
38	Convulsions					
39	Other Comments					

Bizarre Behaviour

HF Head Flicking or Head Shaking

HS Head Searching- repetitive

H Hallucinating- like animal appears to respond to objects not present, e.g. visual tracking or fear withdrawal

B Compulsive Biting

L Compulsive Licking- usually of glass

Jar

UW Upright Walking- hind limbs only

AW Aimless Wandering- slow plodding around with no apparent purpose

C Circling

W Waltzing- rapid turning in circles

R Retropulsion

HB Head Bobbing

SB Self Destructive Biting- usually of toes with Bleeding

P Prancing Forelimbs- shifting from one forelimb to another with Slight turn of body from side to side

D Spatial Disorientation- walking or stumbling into objects

Convulsions

Clonic Type: Alternative contraction & relaxation of the voluntary muscles

C Clonic- co-ordinated, unsymmetrical convulsion & natural, purposeful movements, e.g. running, sometimes preceded by (Rn)

Cs Clonic Symmetrical- Repetitive symmetrical jerks or twitches of the limbs

Rn Running Excitement- Often accompanied by mild colnus/leading to severe convulsion

Ch Champing- Clonus of the jaws only

P Popcorn- Animal repeatedly "pops" into the air

A Asphyxia- A terminal clonic or clonic-tonic convulsion resulting from respiratory failure

Tonic Type: Persistent contraction and spasm of a set of voluntary muscles

T Tonic- Sustained extension of hindlimbs, usually preceded by tonic flexion

Tf This is used if tonic flexion occurs without extension

Op Opisthotonus- Head, body and limbs are rigidly extended and arched backwards

Em Emprosthus- Opposite of Op i.e. extended forward

Miscellaneous Type

Rr Rock and Roll- Animal is prostrate on its back and rocks from side to side in a seeming effort to right itself, occasionally rolling over overshooting)

Su Sitting Up- Sits upright on hindlimbs during the seizure

Pr Praying-sitting up seizure in which forelimbs are held together or crossed in attitude resembling prayer

3.2.7 Statistics

PCA inhibition values for each treatment group were combined and percentage inhibition PCA values were expressed as mean $\pm$ SEM. A minimum of n=3 animals were evaluated at each treatment group. Inhibition of PCA activities of test compounds was compared to the activities of the control compound (DSCG) on GraphPad Prism, version 5, using a student t-test followed by the Mann-Whitney poc-hoc test, a non-parametric test which evaluates if two independent set of values have equally large values.

SHIRPA analysis was carried out using GraphPad Prism, Version 5. Where required column statistics (mean, SEM, SD etc.) were carried out. Comparisons were carried out using one-way ANOVA followed by Tukey's multiple comparison test which evaluates if the mean value of a group of data varies from another.

3.2.8 Computation of Quantitative Structure Analysis Values

The theoretical properties of Group V compounds were computed using MarvinSketch, ChemAxon (Chemaxon 2010), as carried out in Chapter 2 on compound Groups I-IV. Properties determined were the partition coefficient (LogP or cLogP), the distribution coefficient (LogD_{7.4}), the acid dissociation constant (pKa), hydrogen (H⁺) bond donor and acceptor sites as well as 2D polar surface area (PSA).

3.3 Results

3.3.1 Passive Cutaneous Anaphylaxis

Inhibition of passive cutaneous anaphylaxis was established initially for compounds which demonstrated mast cell stabilising activity in the *in vitro* RPMC assay (DF2.02, DF2.05, TZ1.04, TZ1.05, TZ1.06 and TZ1.07; figures 3.03-3.08 below). Although these compounds demonstrated disappointing activity in general, TZ1.04 demonstrated inhibition of PCA activity comparable to that of DSCG (figure 3.05 below).

Group V compounds were then assessed for inhibition of PCA activity (figures 3.09-3.20 below) and although several compounds demonstrated disappointing activity, TZ2.01, TZ2.03, TZ2.04 and TZ2.05 demonstrated activity which was comparable to that of DSCG.

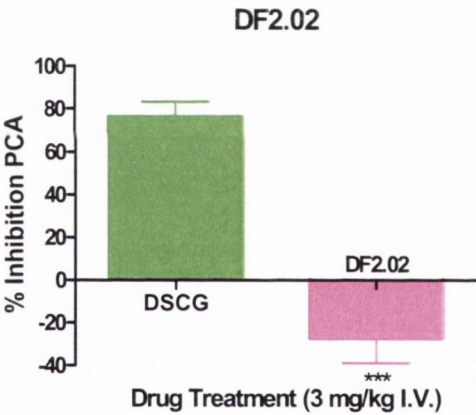


Figure 3.03: Effect of DF2.02 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=12$ or 38 sites.

*** $P<0.0001$

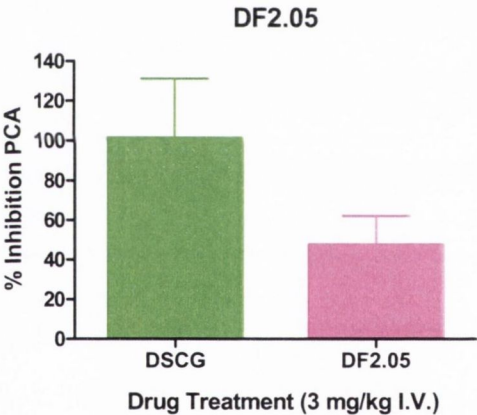


Figure 3.04: Effect of DF2.05 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=6$ or 8 sites

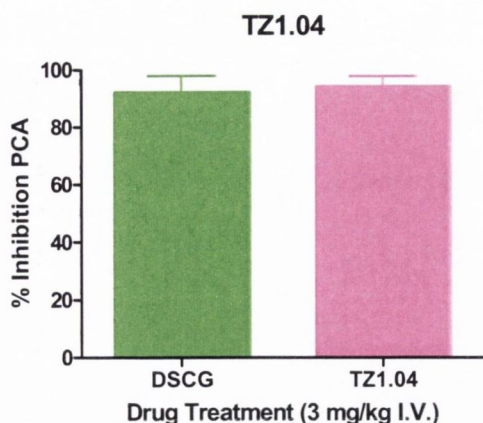


Figure 3.05: Effect of TZ1.04 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=6$ or 9 sites

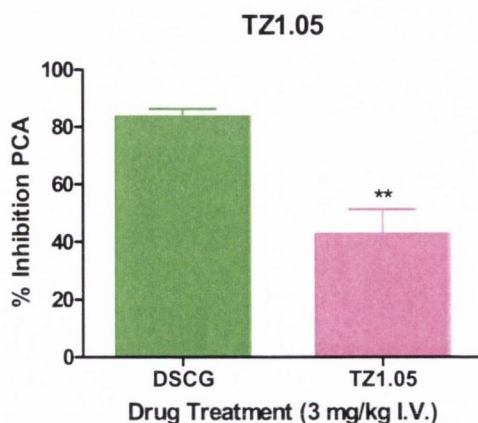


Figure 3.06: Effect of TZ1.05 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=8$ or 12 sites.

** $P<0.01$

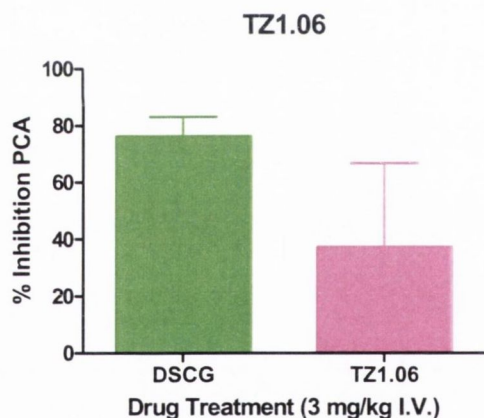


Figure 3.07: Effect of TZ1.06 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=9$ or 38 sites

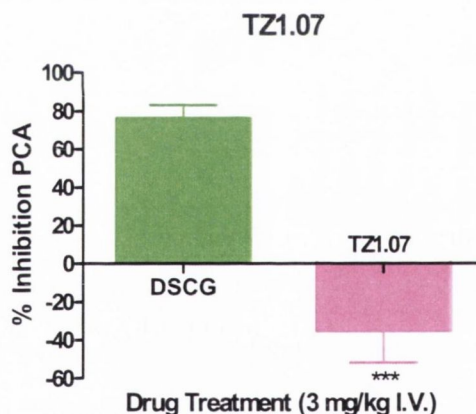


Figure 3.08: Effect of TZ1.07 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=15$ or 38 sites.

*** $P<0.001$

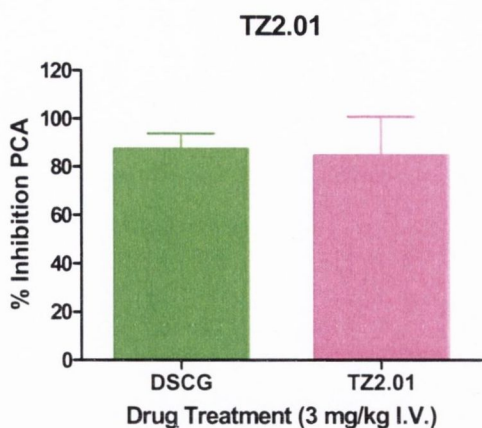


Figure 3.09: Effect of TZ2.01 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=8$ or 26 sites

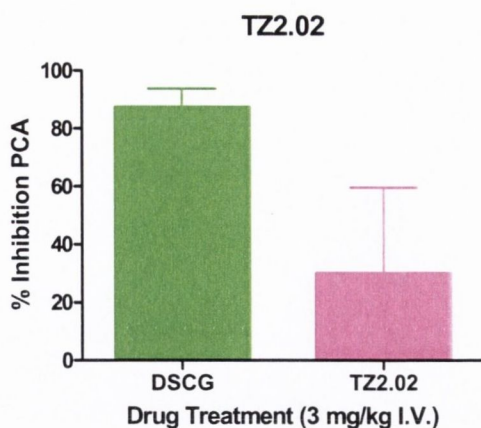


Figure 3.10: Effect of TZ2.02 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=12$ or 26 sites

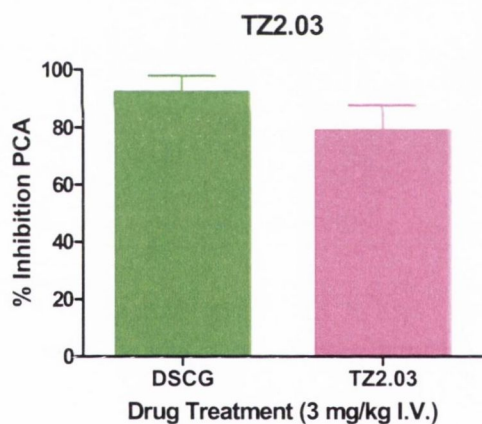


Figure 3.11: Effect of TZ2.03 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM $n=6$ or 9 sites

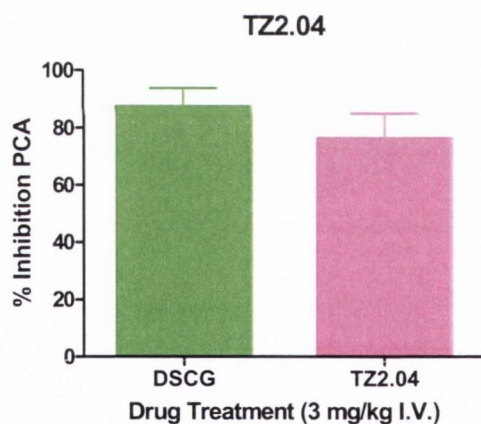


Figure 3.12: Effect of TZ2.04 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=12$ or 26 sites

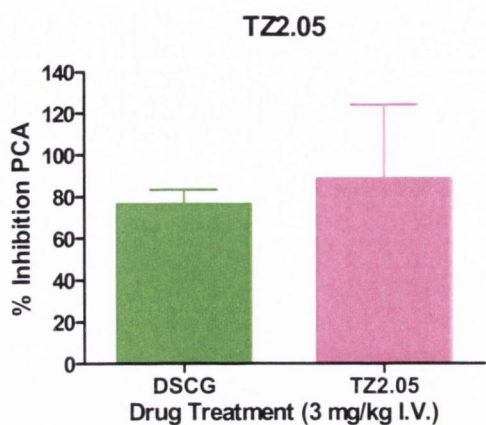


Figure 3.13: Effect of TZ2.05 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=6$ or 38 sites

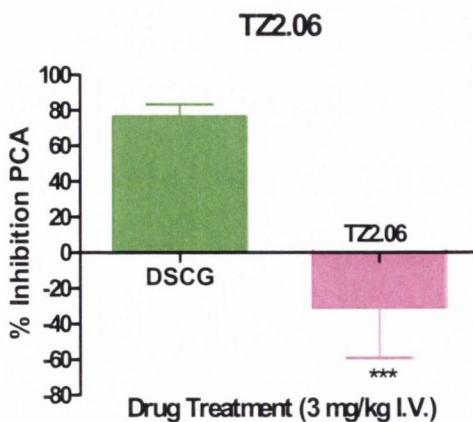


Figure 3.14: Effect of TZ2.06 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=9$ or 38 sites.

*** $P<0.001$

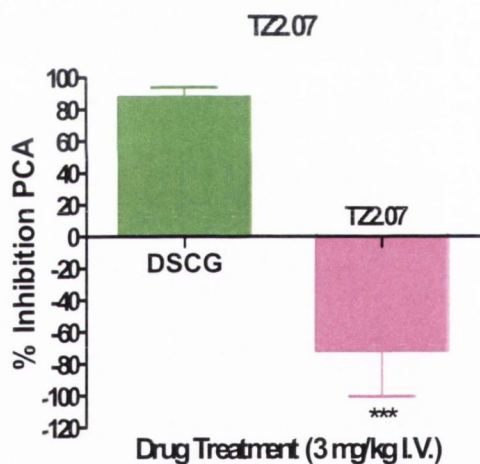


Figure 3.15: Effect of TZ2.07 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=9$ or 26 sites.

*** $P<0.001$

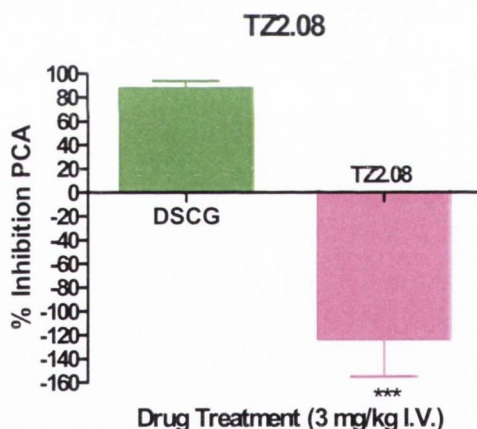


Figure 3.16: Effect of TZ2.08 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of $n=9$ or 26 sites.

*** $P<0.001$

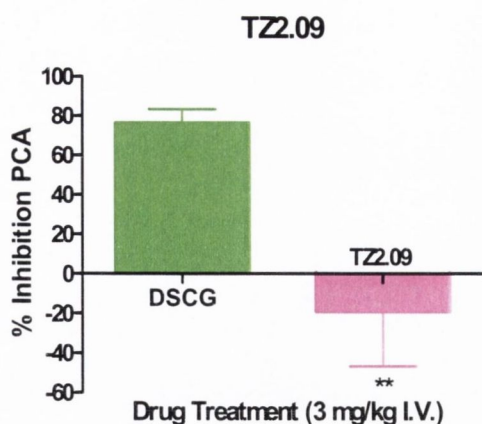


Figure 3.17: Effect of T22.09 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of n=10 or 38 sites.

**P<0.01

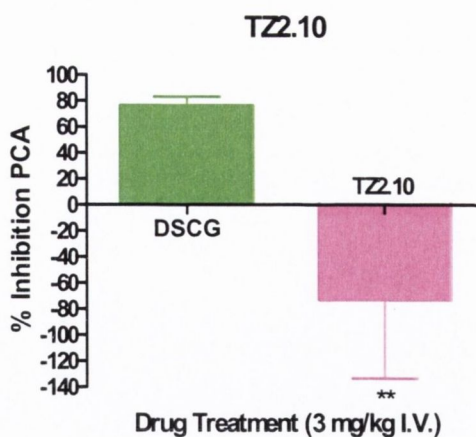


Figure 3.18: Effect of T22.10 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of n=9 or 38 sites.

**P<0.01

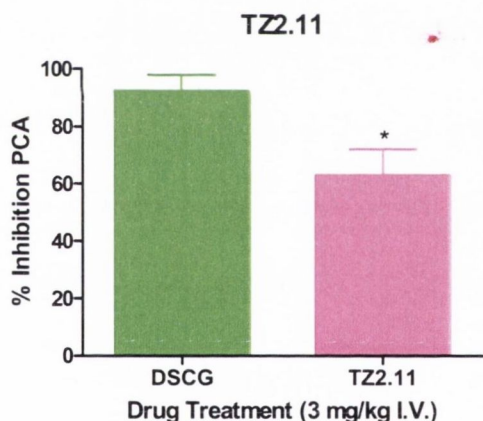


Figure 3.19: Effect of T22.11 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of n=6 or 9 sites.

*P<0.05

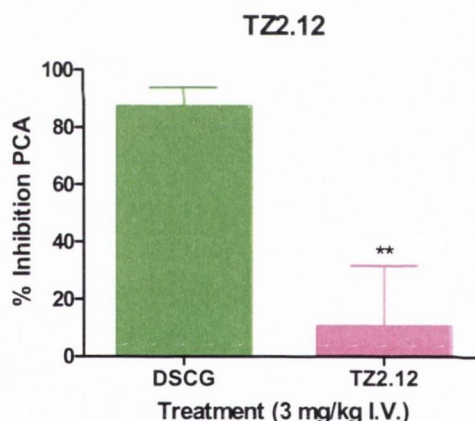


Figure 3.20: Effect of T22.12 and DSCG (both 3 mg/kg) on the PCA reaction. Each data represents the mean $\pm$ SEM of n=7 or 26 sites.

**P<0.01

3.3.2 SHIRPA Behavioural Observation Protocol

Table 3.02: Summary table showing SHIRPA behavioural observation results for untreated (n=24; t=0), vehicle treated (n=8; t= 1, 2, 4, 8 and 24 hours), TZ2.01 (n=7; t= 1, 2, 4, 8 and 24 hours) and TZ2.04 (n=8; t= 1, 2, 4, 8 and 24 hours)

	Test	Untreated (t=0)	Vehicle (t=1, 2, 4, 8, 24 hr)	TZ2.01 (t=1, 2, 4, 8, 24 hr)	TZ2.04 (t=1, 2, 4, 8, 24 hr)
1	Body Position	(2) Lying Prone or (3) Sitting or Standing	(2) Lying Prone or (3) Sitting or Standing	(2) Lying Prone or (3) Sitting or Standing	(2) Lying Prone or (3) Sitting or Standing
2	Spontaneous Activity	(1) Casual Scratching or Grooming	(0) None, Resting or (1) Casual Scratching or Grooming	(0) None, Resting or (1) Casual Scratching or Grooming	(0) None, Resting or (1) Casual Scratching or Grooming
3	Respiration Rate	(2) Normal	(2) Normal	(2) Normal	(2) Normal
4	Tremor	(0) None	(0) None	(0) None	(0) None
5	Defecation	4.3 ± 0.8	3.1 ± 0.3	3.3 ± 0.5	2.9 ± 0.4
6	Urination	(1) Present; Minimal	(1) Present; Minimal	(1) Present; Minimal	(1) Present; Minimal
7	Time Elapsed after Drop	0.18 ± 0.07 seconds	0.21 ± 0.05 seconds	0.22 ± 0.06 seconds	0.20 ± 0.05 seconds
8	Transfer Arousal	(4) Momentary Freeze, then swift movement or (5) No Freeze Immediate Movement	(4) Momentary Freeze, then swift movement or (5) No Freeze Immediate Movement	(4) Momentary Freeze, then swift movement or (5) No Freeze Immediate Movement	(4) Momentary Freeze, then swift movement or (5) No Freeze Immediate Movement
9	Piloerection	(1) Present; Slight	(1) Present; Slight	(1) Present; Slight	(1) Present; Slight

10	Palpebral Closure	(0) Wide Open	(0) Wide Open	(0) Wide Open	(0) Wide Open
11	Startle Response	(2) Jump ≤ 1 cm	(2) Jump ≤ 1 cm or (3) Jump ≥ 1 cm	(2) Jump ≤ 1 cm or (3) Jump ≥ 1 cm	(2) Jump ≤ 1 cm or (3) Jump ≥ 1 cm
12	Gait	(0) Normal	(0) Normal	(0) Normal	(0) Normal
13	Pelvic Elevation	(2) Normal	(2) Normal	(2) Normal	(2) Normal
14	Tail Elevation	(0) Dragging	(0) Dragging	(0) Dragging	(0) Dragging
15	Touch Escape	(1) Mild to (2) Moderate	(0) No response to (1) Mild	(0) No response to (1) Mild	(0) No response to (1) Mild
16	Positional Passivity	Generally Struggles in Each Situation	Generally Struggles in Each Situation	Generally Struggles in Each Situation	Generally Struggles in Each Situation
17	Trunk Curl	(1) Present	(1) Present	(1) Present	(1) Present
18	Limb Grasping	(1) Present	(1) Present	(1) Present	(1) Present
19	Visual Placing	Present	Present	Present	Present
20	Grip Strength	(2) Moderate Grip, Effective	(2) Moderate Grip, Effective to (3) Active Grip, Effective	(2) Moderate Grip, Effective to (3) Active Grip, Effective	(2) Moderate Grip, Effective to (3) Active Grip, Effective
21	Body Tone	(1) Slight Resistance	(1) Slight Resistance	(1) Slight Resistance	(1) Slight Resistance
22	Pinna Reflex	(1) Active Retraction, Moderately Brisk Flick	(1) Active Retraction, Moderately Brisk Flick	(1) Active Retraction, Moderately Brisk Flick	(1) Active Retraction, Moderately Brisk Flick
23	Corneal Reflex	(1) Active Single Eye Blinking	(1) Active Single Eye Blinking	(1) Active Single Eye Blinking	(1) Active Single Eye Blinking
24	Body Length	19.07 $\pm$ 0.1 cm	19.69 $\pm$ 0.2 cm	19.71 $\pm$ 0.4 cm	19.13 $\pm$ 0.2 cm
25	Tail Length	14.89 $\pm$ 0.1 cm	15.31 $\pm$ 0.2 cm	15.29 $\pm$ 0.2 cm	15.06 $\pm$ 0.1 cm
26	Lacrimation	(0) None	(0) None	(0) None	(0) None

27	Provoked Biting	(0) Absent to (1) Present	(0) Absent	(0) Absent	(0) Absent to (1) Present
28	Salivation	(0) None	(0) None	(0) None	(0) None
29	Abdominal Tone	(1) Slight Resistance	(1) Slight Resistance	(1) Slight Resistance	(1) Slight Resistance
30	Skin Colour	(1) Pink	(1) Pink	(1) Pink	(1) Pink
31	Limb Tone	(2) Moderate Resistance	(2) Moderate Resistance	(2) Moderate Resistance	(2) Moderate Resistance
32	Toe Pinch	(1) Moderate to (2) Brisk Withdrawal	(1) Moderate to (2) Brisk Withdrawal	(1) Moderate to (2) Brisk Withdrawal	(1) Moderate to (2) Brisk Withdrawal
33	Righting Reflex	(0) No Impairment	(0) No Impairment	(0) No Impairment	(0) No Impairment
34	Negative Geotaxis	(0) Turns and Climbs the Grid	(0) Turns and Climbs the Grid	(0) Turns and Climbs the Grid	(0) Turns and Climbs the Grid
35	Aggression	(0) None (1) in 2 of 24)	(0) None	(0) None	(0) None
36	Vocalisation	(1) Provoked During Handling	(0) None to (1) Provoked During Handling	(0) None to (1) Provoked During Handling	(0) None to (1) Provoked During Handling
37	Bizarre Behaviour	Absent	Absent	Absent	Absent
38	Convulsions	Absent	Absent	Absent	Absent
39	Other Comments	None	None	None	None

3.3.3 Quantitative Structure Activity Relationship Analysis

Theoretical computed structural properties were determined for all Group V compounds using MarvinSketch. LogP, LogD_{7.4}, pKa, hydrogen (H+) bond donor and acceptor sites and 2D PSA were established to determine whether these compounds may have absorption issues (tables 3.03 and 3.04 below).

Following determination of these computed values, structure activity relationship analysis was carried out to determine if a relationship may exist between the structures of these compounds and their activities (figures 3.21-3.27 below). No structure activity relationship was observed.

Table 3.03: Computed QSAR Properties of Group V Compounds, as calculated using MarvinSketch, ChemAxon. (Part 1)

Compound	TZ2.01	TZ2.02	TZ2.03	TZ2.04	TZ2.05	TZ2.06
LogP	6.61	9.7	10.44	7.82	8.56	8.06
LogD _{7.4}	4.54	7.73	8.11	5.75	6.23	5.99
pKa	9.49, 13.69	9.49, 13.69	9.76	9.49, 13.69	9.76	9.49, 13.63
H+ donor site	1	1	0	1	0	1
H+ acceptor site	2	2	1	2	1	3
Polar surface area (2D)	24.67 Å ²	24.67 Å ²	4.44 Å ²	24.67 Å ²	4.44 Å ²	33.9 Å ²
Stereoisomers	2	2	1	2	1	4
Molecular Weight	449.5 g/mol	516.5 g/mol	543.5 g/mol	517.5 g/mol	499.5 g/mol	557.5 g/mol

Table 3.04: Computed QSAR Properties of Group V Compounds, as calculated using MarvinSketch, ChemAxon. (Part 2)

Compound	TZ2.07	TZ2.08	TZ2.09	TZ2.10	TZ2.11	TZ2.12
LogP	9.63	3.93	6.17	7.38	6.63	7.36
LogD _{7.4}	7.55	1.84	4.1	5.3	4.56	5.03
pKa	9.49, 13.69	9.49, 13.68	9.49, 13.68	9.49, 13.62	9.49, 13.61	9.76
H+ donor site	1	1	1	1	1	0
H+ acceptor site	5	4	2	2	2	1
Polar surface area (2D)	52.36 Å ²	50.45 Å ²	24.67 Å ²	24.67 Å ²	24.67 Å ²	4.44 Å ²
Stereoisomers	4	2	2	2	4	2
Molecular Weight	617.5 g/mol	452 g/mol	435.5 g/mol	503.5 g/mol	473.5 g/mol	455.5 g/mol

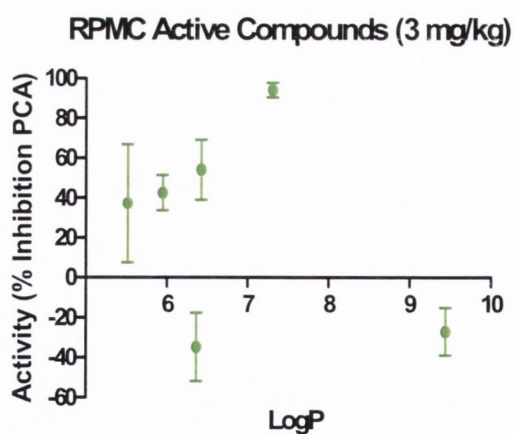


Figure 3.21: Relationship between LogP and PCA activity for RPMC active compounds. Data represents mean $\pm$ SEM of $n=7$, 9, 12 or 15 sites

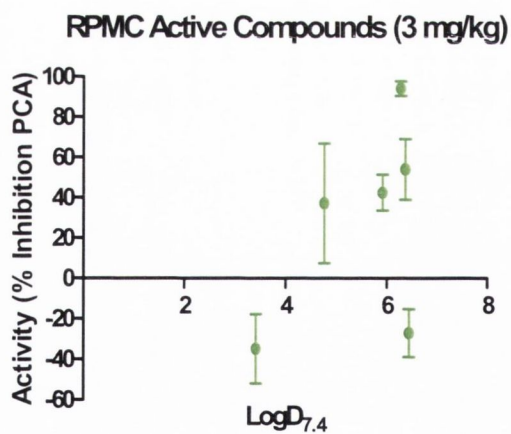


Figure 3.22: Relationship between LogD_{7.4} and PCA activity for RPMC active compounds. Data represents mean $\pm$ SEM of $n=7$, 9, 12 or 15 sites

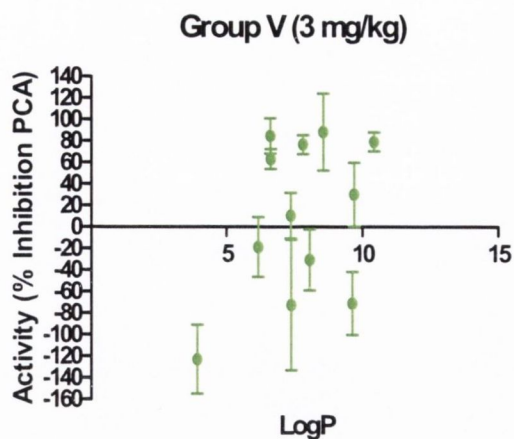


Figure 3.23: Relationship between LogP and PCA activity for Group V compounds. Data represents mean $\pm$ SEM of $n=6, 7, 8, 9, 10$ or 12 sites

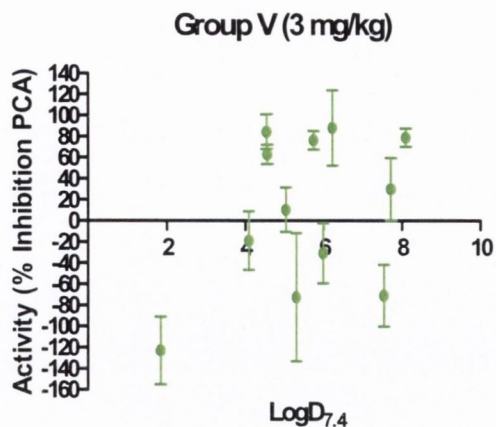


Figure 3.24: Relationship between LogD_{7.4} and PCA activity for Group V compounds. Data represents mean $\pm$ SEM of $n=6, 7, 8, 9, 10$ or 12 sites

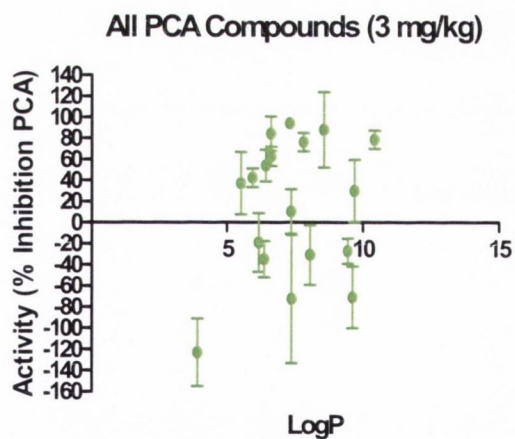


Figure 3.25: Relationship between LogP and PCA activity for all PCA assayed compounds. Data represents mean $\pm$ SEM of $n=6, 7, 8, 9, 10, 12$ or 15 sites

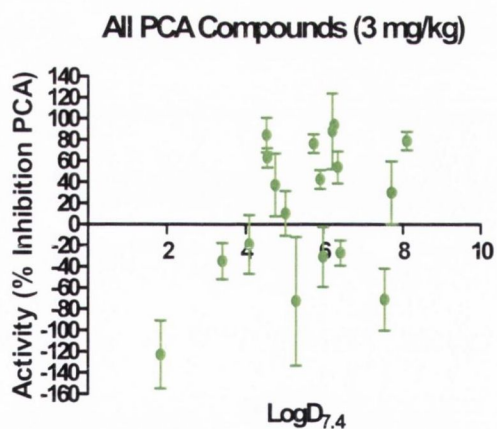


Figure 3.26: Relationship between LogD_{7.4} and PCA activity for all PCA assayed compounds. Data represents mean $\pm$ SEM of $n=6, 7, 8, 9, 10, 12$ or 15 sites

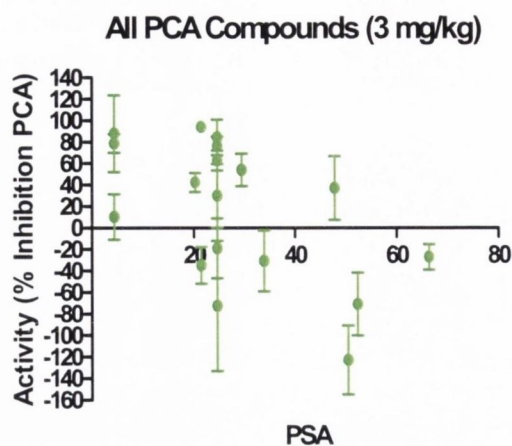


Figure 3.27: Relationship between PSA and PCA activity for all PCA assayed compounds. Data represents mean $\pm$ SEM of $n=6, 7, 8, 9, 10, 12$ or 15 sites

3.3.4 RPMC Assay Evaluation of Several Group V Compounds: TZ2.04, TZ2.06 and TZ2.08

Several representative Group V compounds which had not yet been evaluated for *in vitro* mast cell stabilising activity, TZ2.04, TZ2.06 and TZ2.08, were evaluated for *in vitro* mast cell stabilising activity. These compounds appeared to elicit the release of histamine from mast cells (figures 3.28 and 3.29 below).

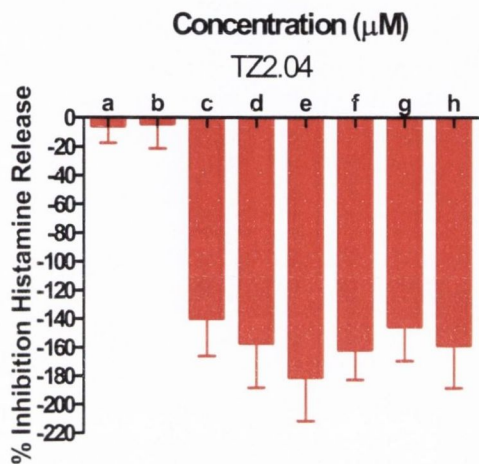


Figure 3.28: Effect of a) 0.1 μM, b) 0.5 μM, c) 0.75 μM, d) 1 μM, e) 5 μM, f) 10 μM, g) 20 μM and h) 40 μM TZ2.04 on compound 48/80-induced histamine release from RPMC. Data represents mean ± SEM of n=4

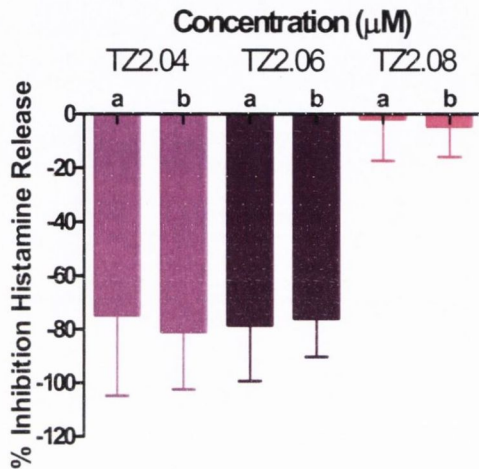


Figure 3.29: Effect of a) 10 μM and b) 20 μM TZ2.04, TZ2.06 and TZ2.08 on calcium ionophore A23187-induced histamine release from RPMC. Data represents mean ± SEM of n=3

3.4 Discussion

3.4 1 Passive Cutaneous Anaphylaxis

3.4.1.1 RPMC Active Compounds

In vivo mast cell stabilising activity was established for compounds which demonstrated mast cell stabilising activity in the *in vitro* RPMC assay (Chapter 2). The choice of vehicle was made on the basis of each compound's solubility. While some compounds readily dissolved in water for injection (WFI), others required the use of a solubilising agent such as DMSO or Tween 80, if required these were always used at the lowest concentration possible. DSCG values for each vehicle were averaged over a number of experiments in which that vehicle was used, to increase the number of control treated animals.

The first compound assessed *in vivo* was DF2.02 as it was the most potent mast cell stabilising compound in the RPMC assay in the presence of both calcium ionophore A23187 and compound 48/80. Because DF2.02 does not readily dissolve in WFI, 2% Tween 80 in WFI was used as vehicle. Unfortunately DF2.02 did not demonstrate mast cell stabilising activity *in vivo* (DF2.02 $-26.98 \pm 12\%$; DSCG $76.12 \pm 7\%$; figure 3.03), instead it appeared to cause the release of histamine, inducing an increase in Evans blue dye leakage in comparison to controls.

DF2.05 also demonstrated potent mast cell stabilising activity *in vitro*, in the presence of both calcium ionophore A23187 and compound 48/80. For the PCA assay DF2.05 was solubilised in 1% DMSO in physiological saline (0.9% w/v) and while it demonstrated some mast cell stabilising activity (DF2.05 $47.54 \pm 15\%$; DSCG $101.20 \pm 30\%$; figure 3.04), it was suspected that because of the presence of an ester, it may be susceptible to attack by esterases such as plasma esterase 1, which may explain its unexpectedly low *in vivo* activity.

TZ1.04, solubilised in 2.5% Tween 80 in WFI, demonstrated potent *in vivo* mast cell stabilising activity which was comparable to that of DSCG (TZ1.04 $94.15 \pm 3\%$; DSCG $92.15 \pm 6\%$; figure 3.05), while TZ1.05 solubilised in 5% Tween 80 in WFI demonstrated *in vivo* mast cell stabilising activity approximately half that of DSCG (TZ1.05 $42.50 \pm 9\%$; DSCG $83.36 \pm 3\%$; figure 3.06). TZ1.06 solubilised in 2% Tween 80 in WFI demonstrated mast cell stabilising activity (TZ1.06 $37.21 \pm 30\%$; DSCG $76.12 \pm 7\%$; figure 3.07) comparable to that of TZ1.05, although TZ1.06 demonstrated much more variability in its results. TZ1.07 solubilised in 2% Tween 80 in WFI, despite demonstrating potent mast cell stabilising activity in the *in vitro* RPMC assays, in a similar manner to DF2.02, caused the release of histamine *in*

vivo inducing an increase in Evans blue dye leakage in comparison with controls (TZ1.07 $-34.69 \pm 17\%$; DSCG $76.12 \pm 7\%$; figure 3.08).

3.4.1.2 Group V Compounds

Results from these merged compounds were disappointing overall because the mast cell stabilising activity observed in the most potent compounds *in vitro* did not translate to an *in vivo* setting, although DF2.05 showed some activity *in vivo*. Despite showing the most potent mast cell stabilising activity *in vitro*, in both the presence of calcium ionophore A23187 and compound 48/80, DF2.02 demonstrated extremely disappointing activity *in vivo*. Group V compounds were therefore synthesised by Zhang, with a view to rectifying the issues that were thought to occur with the above compounds. These Group V compounds were then assessed for mast cell stabilising activity *in vivo* using the PCA assay.

A stable linker unit was introduced into the first of these compounds, by using carbon-carbon linkers, to hopefully prevent possible cleavage by plasma esterases of DF2.02 which may occur when it is introduced into the blood. TZ2.01 is an extended relative of DF2.02 and DF1.06, while TZ2.02 and TZ2.03 were synthesised to be more robust versions of DF2.02 by removing the site of possible esterase cleavage. TZ2.01 in WFI (TZ2.01 $84.49 \pm 16\%$; DSCG $87.18 \pm 7\%$; figure 3.09), TZ2.02, also in WFI (TZ2.02 $30.00 \pm 30\%$; DSCG $87.18 \pm 7\%$; figure 3.10) and TZ2.03 which was solubilised in 2.5% Tween 80 in WFI (TZ2.03 $78.89 \pm 9\%$; DSCG $92.15 \pm 6\%$; figure 3.11) all demonstrated *in vivo* mast cell stabilising activity, although TZ2.02 is much less potent than TZ2.01 and TZ2.03, both of which demonstrated activity comparable to DSCG. These results suggest that cleavage of the linker unit in DF2.02 as predicted, may have been occurring, thus preventing its activity.

It was hypothesised that an issue of para-hydroxylation may have been associated with TZ2.01, therefore, TZ2.04 and TZ2.05 were synthesised to overcome the possibility of this occurring. TZ2.04 in WFI (TZ2.04 $76.31 \pm 9\%$; DSCG $87.18 \pm 7\%$; figure 3.12) and TZ2.05 solubilised in 2% Tween 80 in WFI (TZ2.05 $88.18 \pm 36\%$; DSCG $76.12 \pm 7\%$; figure 3.13) both demonstrated *in vivo* mast cell stabilising activity comparable to that of TZ2.01 and DSCG.

Impressed by the activity of these compounds, Zhang then synthesised TZ2.06, TZ2.07, TZ2.08, TZ2.09 and TZ2.10, all of which were assessed in the PCA *in vivo* assay. The compounds all demonstrated poor mast cell stabilising activity; TZ2.06 solubilised in 2% Tween 80 in WFI (TZ2.06 $-30.65 \pm 28\%$; DSCG $76.12 \pm 7\%$;

figure 3.14), TZ2.07 dissolved in water for injection (TZ2.07 $-70.98 \pm 29\%$; DSCG $87.18 \pm 7\%$; figure 3.15), TZ2.08 again dissolved in WFI (TZ2.08 $-122.80 \pm 32\%$; DSCG $87.18 \pm 7\%$; figure 3.16), TZ2.09 solubilised in 2% Tween 80 in WFI (TZ2.09 $-18.89 \pm 28\%$; DSCG $76.12 \pm 7\%$; figure 3.17) and TZ2.10 solubilised in 2% Tween 80 in WFI (TZ2.10 $-72.48 \pm 61\%$; DSCG $76.12 \pm 7\%$; figure 3.18) all caused the release of histamine resulting in increased Evans blue dye leakage when compared to controls. TZ2.09 is a ring contracted version of TZ2.01 and TZ2.10 is a ring contracted version of TZ2.04, therefore, the activities of these compounds (TZ2.06-TZ2.10) suggest a six ring structure is optimum for mast cell stabilising activity *in vivo*. TZ2.11 and TZ2.12 were synthesised based on a series of dimer compounds which were synthesised by Barlow and which were active in the *in vitro* RPMC assay but not in subsequent *in vivo* PCA investigations. Polar side chains were added to Barlow's compounds to improve their solubility, forming TZ2.11 and TZ2.12. TZ2.11 and TZ2.12 demonstrated some mast cell stabilising activity *in vivo* (TZ2.11 in 2.5% Tween 80 in WFI $67.97 \pm 9\%$; DSCG $92.15 \pm 6\%$; figure 3.19 and TZ2.12 dissolved in WFI $10.49 \pm 21\%$; DSCG $87.18 \pm 7\%$; figure 3.20), although TZ2.11 was far more potent than TZ2.12.

3.4.2 Structure Activity Relationship Analysis

3.4.2.1 LogP and LogD_{7.4}

Following QSAR analysis of compounds in the previous chapter using computed values, Group V compounds were analysed to determine their properties. Group V compounds appear to have high LogP values, all values being over 6 except for TZ2.08 which has a LogP value of 3.93 (tables 3.03 and 3.04). These values combined with high LogD_{7.4} values (tables 3.03 and 3.04), which all appear to be over 5 except TZ2.01 (LogD_{7.4} 4.54), TZ2.08 (LogD_{7.4} 1.84), TZ2.09 (LogD_{7.4} 4.10), TZ2.11 (LogD_{7.4} 4.56) and TZ2.12 (LogD_{7.4} 5.03), indicate that all Group V compounds appear to have extremely high lipophilic properties based on these computed values. This suggests these compounds, as with Groups I-IV, should easily penetrate the cell lipid bilayer but also the blood brain barrier and might act in the CNS. These compounds may also become tethered to the cell membrane, as discussed in Chapter 2. TZ2.08 is the only exception in Group V, having a LogD_{7.4} value of 1.84 suggesting it is sufficiently hydrophilic not to traverse the blood brain barrier.

Investigations into an activity/computed LogP or activity/computed LogD_{7.4} relationship were carried out again, as with Groups I-IV which were analysed using their RPMC assay activities, to determine if any relationship between the activities

and lipophilic properties of these compounds may exist.. No correlation was found between PCA activity and either LogP or LogD_{7.4} for Group V (figures 3.23 and 3.24). No correlation was found for the RPMC active compounds when analysed using their PCA values (figures 3.21 and 3.22) and no correlation was also found when all compounds assessed using the PCA assay were analysed together (figures 3.25 and 3.26).

Notably TZ2.08 which has poor *in vivo* mast cell stabilising activity, is the only compound with a LogD_{7.4} value suggesting it is sufficiently hydrophilic to be unable to traverse the blood brain barrier. It was considered that perhaps its poor PCA activity was due to its reduced ability to permeate the cell membrane in comparison with all other compounds evaluated in the PCA assay. However, because TZ2.08 is not the only compound which demonstrates poor PCA activity (DF2.02 figure 3.03, TZ1.07 figure 3.08, TZ2.06 figure 3.14, TZ2.07 figure 3.15, TZ2.08 figure 3.16, TZ2.09 figure 3.17 and TZ2.10 figure 3.18 all demonstrate negative mast cell stabilising activity suggesting they are eliciting mast cell mediator release) and because no relationship exists between *in vivo* mast cell stabilising activity and lipophilicity for these compounds (figures 3.21 to 3.26), this would suggest that the hydrophobicity of TZ2.08 is not the reason for its poor mast cell stabilising activity *in vivo*.

3.4.2.2 Polar Surface Area

Group V compounds all contain less than 5 hydrogen bond donor sites and less than 10 hydrogen bond acceptor sites indicating these compounds obey Lipinski's Rule of Five in this respect. The molecular weights of half of these Group V compounds (TZ2.01, TZ2.05, TZ2.08, TZ2.09, TZ2.11, TZ2.12) are below 500 g/mol, which is required to be optimal according to Lipinski's Rule of Five.

Polar surface area values for this group were varied. However, all PSA values are less than 70 Å² indicating they may penetrate the CNS. PCA evaluated compounds were assessed to establish if a relationship exists between PCA activity and PSA values. However, no relationship was observed when this comparison was made (figure 3.27).

3.4.3 SHIRPA Behavioural Observation Protocol

Because potent *in vivo* mast cell stabilising activity was observed in some of these Group V compounds, two representative compounds were chosen from this group for assessment into their systemic toxicity. TZ2.01 (inhibition of PCA activity 84%) and TZ2.04 (inhibition of PCA activity 76%) were chosen on the basis of their *in vivo* PCA mast cell stabilising activity. A modified SHIRPA behavioural observation

profile was used for this purpose. The SHIRPA protocol is based on the Irwin profile (Irwin 1968) which is a behavioural protocol routinely used to assess toxicity in novel drug candidates.

As summarised in table 3.01, no difference in behaviour was observed in any of the four subcategories; undisturbed behaviour, active behaviour, restrained behaviour and manipulated behaviour between the control (untreated, $t=0$) group and the vehicle (maple syrup) treated group at any of the time points ($t=1, 2, 4, 8$ and 24 hours). Neither TZ2.01 nor TZ2.04 treated animals demonstrated any differences in behaviour when compared with untreated and with maple syrup treated animals, at any time point ($t=1, 2, 4, 8$ and 24 hours). As well as the criteria outlined in the behavioural score sheet, no lethality, tiptoe, stereotypies such as repetitive chewing, or scratching, were observed in any treatment group at any time point.

Very slight piloerection was observed across all test groups during the course of the observation, however, this may be due to the slightly cooler temperature of the assessment room ($20-21\text{ }^{\circ}\text{C}$) in comparison to their housing room ($22\text{ }^{\circ}\text{C}$). Very slight differences in behaviour could be seen in some of the subcategories (e.g. test #2, spontaneous activity and test #15 touch escape, table 3.02). These observed behaviours were within the normal range, and may also be explained by increased handling of the animals as the experiment progressed, therefore, these observations may not be due to novel drug treatment. Because no adverse behaviour was observed in any of the subcategories it can be concluded that these compounds do not possess neurotoxic, CNS stimulating, CNS depressing or autonomic nervous system modulating activity and therefore can be deemed safe for administration at the administered dose (10 mg/kg , P.O.).

It would later emerge that the bioavailability of TZ2.04 when given P.O. is approximately 57% (Charles Rivers; see appendix) which implies the actual bioavailable dose assessed in the SHIRPA behavioural observation study is approximately 5.7 mg/kg . 10 mg/kg was originally chosen for assessment in the SHIRPA protocol because the bioavailabilities of TZ2.01 and TZ2.04 were unknown at the time and a sufficiently higher dose was chosen than that used in the PCA assay, to allow for the reduction in bioavailable compound which would occur upon P.O. dosing, when compared to I.V. administration. An actual dose of 5.7 mg/kg is higher than the PCA dose, which was the intended aim for the SHIRPA study. It is therefore encouraging to note that at this dose these compounds are safe for administration, at least at a systemic level.

3.4.4 Mechanism of Action of Group V Compounds

The *in vitro* RPMC-stabilising activity of several compounds did not transfer to the *in vivo* PCA assay, it may therefore be concluded that these compounds must not sufficiently prevent IgE-mediated mast cell mediator release *in vivo*. While this is disappointing the emergence of several new Group V *in vivo* mast cell stabilising compounds is encouraging. Results obtained here suggest that these compounds demonstrate sufficient mast cell stabilising activity to prevent IgE-mediated release, as occurs in the PCA assay. Because the *in vivo* activity of these compounds is now known, it is necessary to evaluate their mast cell stabilising activity profile *in vitro*. TZ2.04 was evaluated first, in the presence of Compound 48/80. However, it soon emerged that this compound was eliciting mast cell mediator release (figure 3.28). To investigate this further TZ2.04, TZ2.06 and TZ2.08 were evaluated at 20 μ M and 10 μ M using calcium ionophore A23187 to elicit histamine. Again the compounds appeared to cause the release of histamine from mast cells (figure 3.29).

At this stage it was decided to evaluate the RPMC mast cell stabilising activity of Group V compounds using liquid chromatography/mass spectrometry to quantify histamine released from mast cells. This would allow for the comparison of the *in vitro* activity of these Group V compounds to those which were previously assayed in Chapter 2.

It is interesting to note at this preliminary stage that the Group V compounds which are active in the PCA *in vivo* assay for mast cell stabilising activity, such as TZ2.04, seemed to be causing the release of histamine despite showing potent *in vivo* activity. Although the PCA assay is primarily and frequently used to determine *in vivo* mast cell stabilising activity it is interesting to note that mepyramine, a H1R antagonist with no mast cell stabilising activity, shows some activity in the PCA assay (Martin and Baggiolini 1981). Therefore, it is possible, if these compounds are shown to elicit histamine *in vitro* in proceeding studies, that their PCA activity may not be due to mast cell stabilising activity *in vivo*, but may instead be due to H1R antagonism, a property that to this point was not considered for these compounds.

3.5 Conclusions

Work presented in this chapter aimed to establish the *in vivo* PCA mast cell stabilising activity of compounds which demonstrated potent activity *in vitro* (Chapter 2). These compounds, specifically DF2.02 which was to date the lead compound of interest, unfortunately demonstrated disappointing mast cell stabilising activity when they were brought to an *in vivo* setting. This may be due to interactions with enzymes, such as plasma esterases, which may cleave these compounds when they are introduced into the blood during the PCA study.

Zhang subsequently synthesised novel compounds (Group V), which were also assessed for *in vivo* mast cell stabilising activity. Encouragingly several of these compounds possessed mast cell stabilising activity, specifically TZ2.01, TZ2.04 and TZ2.05, indicating their ability to prevent *in vivo* IgE-mediated mast cell mediator release.

Theoretical computed QSAR analysis was carried out on Group V compounds. Although compounds show favourable hydrogen bond acceptor and donor site numbers, they appear to be highly lipophilic compounds which may result in their absorption into the CNS. If these compounds have a highly lipophilic profile, as was indicated using MarvinSketch, it may be hypothesised that they may also become tethered at the cell membrane and not sufficiently enter the cytosol of the mast cell to interact with the processes of degranulation. TZ2.08 was the only Group V compound which demonstrated a more hydrophilic profile using MarvinSketch. Analysis of Group V compounds, as well as RPMC active compounds which were evaluated *in vivo*, indicated that no structure activity relationship appeared to exist for these compounds when their activities were analysed against their computed LogP, LogD_{7.4} and PSA values.

The theoretical properties which were determined using computational analysis here should be determined practically in the laboratory followed by repeated structure activity analysis investigation.

Two Group V compounds which were active in the PCA assay, TZ2.01 and TZ2.04, were assessed for systemic toxicity using the SHIRPA behavioural observation protocol which assessed undisturbed behaviour, active behaviour, restrained behaviour and manipulated behaviour and together assess the general effects of a drug candidate on the CNS. These compounds were found to be safe at the concentration evaluated (10 mg/kg P.O., actual bioavailable dose 5.7 mg/kg).

The *in vitro* mast cell stabilising activity of Group V compounds was unknown at this stage, therefore, it was necessary to establish this activity. It was decided to implement an alternative method to quantify mast cell mediator release instead of using histamine-OPT conjugate. *In vitro* mast cell stabilising activity would be evaluated by quantifying histamine release using liquid chromatography/mass spectrometry. This is discussed further in Chapter 4.

Chapter 4

Development of a Liquid Chromatography/Mass Spectrometry Method for Quantification of Mast Cell Histamine Release

4.1 Introduction

Passive cutaneous anaphylaxis results obtained in Chapter 3 indicate that TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05 and TZ2.11 prevented IgE-mediated activation of dermal connective tissue mast cells. Unfortunately, many other compounds from Groups I-IV which demonstrated promising activity *in vitro* (Chapter 2) did not possess mast cell stabilising activity *in vivo*. Because *in vivo*, but not *in vitro* results, were established for TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05 and TZ2.11, *in vitro* mast cell stabilising activity had to be established for these compounds.

As discussed in the previous chapter, TZ2.04, TZ2.06 and TZ2.06 were assessed as representative compounds from Group V in the RPMC assay and were found to cause the release of histamine from mast cells. Because these compounds show potent mast cell stabilising activity *in vivo*, it was decided a detailed liquid chromatography mass spectrometry (LCMS) study should be carried out to quantify Group V RPMC *in vitro* activity.

4.1.1 Aims

The aims of this work were therefore:

- To establish an LCMS method for the detection and quantification of histamine released from RPMCs,
- To assess TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05, TZ2.11 and TZ2.13 for *in vitro* mast cell stabilising activity using the RPMC assay followed by quantification of mast cell mediators using the above method and
- To determine if a LogP or LogD_{7.4}/RPMC activity relationship exists for these compounds.

4.1.2 Liquid Chromatography/Mass Spectrometry

The system used for this study is a Thermo Accela Liquid Chromatograph (HPLC) with a Thermo LTQ-XL-Orbitrap Discovery Mass Spectrometer (MS). Linear iontrap MS allows for the ionisation and subsequent detection of individual ions based on their mass while selected ion monitoring (MS/MS) allows for the detection of analyte fragment ions.

4.1.3 Detection of Histamine

Although histamine can be detected using several analytical methods without derivatisation, including thin layer chromatography (TLC) (Schwartzman and Halliwell 1975), HPLC/electrospray ionisation (ESI) quadrupole time of flight (TOF) MS (Moore, Gault *et al.* 2009) and normal phase HPLC/atmospheric pressure chemical ionisation MS/MS (Gianotti, Chiuminatto *et al.* 2008), due to its low

molecular weight it is appropriate to derivatise histamine before analysis using LCMS.

4.1.4 Agents used to Derivatise Histamine

Many derivatising agents exist for use in the detection of histamine, allowing for its quantification using a variety of methods. Histamine has been detected using HPLC following derivatisation with OPT (Huang, Mochizuki *et al.* 1998; Previati, Raspadori *et al.* 2002; Penissi, Rudolph *et al.* 2003), fluorescamine (Sattari-Ghandforoush and Mashayekhi 2008), dabsyl chloride (Pinho, Ferreira *et al.* 2004) and 4-chloro-3,5-dinitrobenzotrifluoride (Tang, Shi *et al.* 2009). Histamine has also been detected using ultra performance liquid chromatography and TOF MS following labelling with 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (Kawanishi, Toyo'oka *et al.* 2006), nano LCMS following derivatisation using 7-fluoro-4-nitrobenzoxadiazole (Song, Quan *et al.* 2004) and using capillary LCMS following derivatisation with 7-fluoro-4-nitrobenzoxadiazole (Song, Quan *et al.* 2004). Histamine has been detected using gas chromatography (GC) MS following conversion to an *N*- α -heptafluorobutyryl derivative (Mita, Yasueda *et al.* 1980) and using LCMS following derivatisation with 1,2-naphthoquinone-4-sulfonate (Garcia-Villar, Hernandez-Cassou *et al.* 2009). Histamine has also been detected following derivatisation with succinimidylferrocenyl (Bomke, Seiwert *et al.* 2009), dansyl chloride (DC) (Gosetti, Mazzucco *et al.* 2007) and 3,5-dinitrobenzoyl chloride (Kirschbaum, Rebscher *et al.* 2000). Although it is clear many derivatising agents may be used for the determination of histamine, DC was chosen for use in this study.

4.1.5 Dansyl Histamine

Histamine is routinely derivatised with DC to enable its quantification. Histamine has been detected in food such as in fish (Malle, Valle *et al.* 1996), olives (Hornero-Mendez and Garrido-Fernandez 1994) and dry sausages (Eerola, Hinkkanen *et al.* 1993) using HPLC following derivatisation with DC. DC has also been used to detect histamine in Korean soybean paste (Shukla, Park *et al.* 2010) and shrimp and tomato sauces (Saaid, Saad *et al.* 2009) using HPLC followed by UV spectrophotometry and in tomatoes (Bolygo, Cooper *et al.* 2000) and wine and beer (Loukou and Zotou 2003) using LCMS. Derivatisation with DC has also allowed for the quantification of histamine in alcoholic beverages (Loukou and Zotou 2003) and in donkey milk (La Torre, Saitta *et al.* 2010) using HPLC followed by atmospheric pressure chemical ionization MS, in wine using nano liquid chromatography (Hernandez-Borges, D'Orazio *et al.* 2007) and in wines using HPLC/diode array detector (mo Dugo, Vilasi *et al.* 2006). Dansyl histamine (DH) can be also

quantified using TLC (Hansen, Albeck *et al.* 1984) although it is difficult to obtain good precision when TLC is used. DC is reliable for use in the determination of histamine at the concentrations required here (Loukou and Zotou 2003), therefore it is a reliable derivatising agent for use in the detection of histamine.

4.1.6 Quantification of Mast Cell Degranulation

A variety of methods have been used to quantify histamine within or released from mast cells. Histamine has been quantified in single mast cells using electrochemical detection (Pihel, Hsieh *et al.* 1995) and in single primary cultured bone marrow-derived mast cells using matrix-assisted laser desorption/ionisation TOF MS (Shimizu, Ojima *et al.* 2003). Histamine release from RBL-2H3 cells has been measured using histamine microsensor with histamine oxidase (Iwaki, Ogasawara *et al.* 2002), while histamine release from rat mast cells was quantified following derivatisation with DC using high performance thin layer chromatography with densitometry (Singh, Periera *et al.* 1993). Mast cell degranulation has been quantified in human mast cell line (Igarashi, Kurosawa *et al.* 1996), in cultured human umbilical cord-derived mast cells (Shichijo, Inagaki *et al.* 1998) and in RPMCs using HPLC following derivatisation with OPT (Arakawa and Tachibana 1986; Florjanc and Erjavec 1991; Saito, Horie *et al.* 1992; Kanda, Akiyama *et al.* 1998) and also with fluorescamine (van Haaster, Engels *et al.* 1993). Nano-ESI with TOF MS was used to quantify histamine release from RPMCs (Sirikatitham, Yamamoto *et al.* 2007) while histamine release from RBL-2H3 has also been quantified using GCMS (Pittertschatscher, Hochreiter *et al.* 2002). Histamine has been measured in single RPMCs using MS coupled to flow cytometry, without histamine derivatisation (Fung and Yeung 1998) and HPLC followed by ESI MS/MS has been used to quantify basophilic cell line (KU812) degranulation (without deriving histamine) (Koyama, Takeuchi *et al.* 2009). However, to date no research group has published any paper using HPLC with ESI linear quadropole iontrap MS/MS to quantify histamine release from mast cells.

4.1.7 Internal Standard

Although many papers have been published using LCMS for analysis of DH without the presence of an internal standard (IS) during analysis (Dugo, Vilasi *et al.* 2006; mo Dugo, Vilasi *et al.* 2006; Gosetti, Mazzucco *et al.* 2007; Hernandez-Borges, D'Orazio *et al.* 2007; Saaid, Saad *et al.* 2009), it was decided an IS should be used in this study to improve reliability of data. The requirements of an IS are that it should mimic closely the properties of the analyte while differing slightly by chemically insignificant modification, possess similar chromatographic conditions to the analyte and have a close resemblance to the analyte in extraction and

derivatisation (Wieling 2002). An ideal internal standard would therefore be a stable isotope such as deuterated or ^{14}C -labelled histamine. However, cost prevented the use of an IS isotope in this study. A literature search was therefore carried out with the aim of finding a compound which complied with the above requirements for use in the analysis of histamine (molecular weight 111.15 g/mol) using HPLC or LCMS. Histamine is metabolised by methyltransferase to form methylhistamine, therefore, methylhistamine would be an ideal IS for use in this study. However, two separate methylhistamine species, 1- (tele-) or 3- (tau-) methylhistamine (198.09 g/mol), exist (figures 4.01 and 4.02).

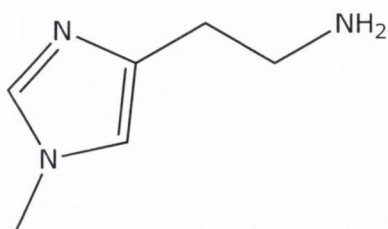


Figure 4.01: 1-methylhistamine

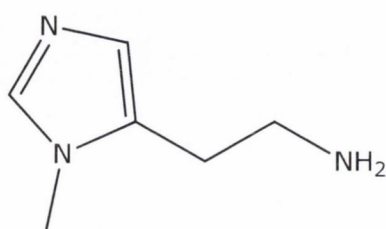


Figure 4.02: 3-methylhistamine

Further investigation into the use of methylhistamines revealed several contradictory papers; some groups state that 1-methylhistamine is endogenous (Hashimoto, Hashimoto *et al.* 1998; Kawanishi, Toyo'oka *et al.* 2006) and therefore 3-methylhistamine should be used as IS (Harsing, Nagashima *et al.* 1986), while others state that 3-methylhistamine is endogenous (Maslinski, Schippert *et al.* 1977; Erjavec and Irman-Florjanc 1986) and therefore 1-methylhistamine should be used as IS (van Haaster, Engels *et al.* 1993; Sattari-Ghandforoush and Mashayekhi 2008). Interestingly RPMCs have the ability to reuptake 1-methylhistamine (Florjanc and Erjavec 1991) which can be measured in mast cells (Goldschmidt, Khandelwal *et al.* 1984). Because of the contradictory nature of these papers, in addition to the fact mast cells play a role in the redistribution of 1-methylhistamine, it was decided that neither 1- nor 3-methylhistamine were suitable for use in this study.

Several groups have used 1,7-diaminoheptane (1,7-DAH) as IS for the detection of biogenic amines such as histamine using HPLC, following dansylation with DC (Hornero-Mendez and Garrido-Fernandez 1994; Loukou and Zotou 2003; Shukla, Park *et al.* 2010). Although retention time is determined by chromatographic conditions it was considered encouraging to note in these papers that the retention time of 1,7-DAH is within minutes of that of histamine.



Figure 4.03: 1,7-Diaminoheptane

1,7-DAH was therefore chosen for use as IS in this study because of its low molecular weight (130.23 g/mol) and because it would form a di-dansyl product.

4.1.8 Dansylation of Histamine and 1,7-DAH Using Dansyl Chloride

Histamine released in the RPMC assay was quantified following derivatisation with DC. Many protocols using DC to dansylate histamine require extraction steps prior to analysis (Hornero-Mendez and Garrido-Fernandez 1994; Loukou and Zotou 2003), however, the method used by Gosetti *et al.* (Gosetti, Mazzucco *et al.* 2007) is relatively quick, requires no extraction steps and was chosen because of the high number of samples that could be derived during each assay. Histamine is a biogenic amine which contains two amine groups; therefore, when it is derived using DH it forms a di-dansyl product (figure 4.04); the molecular weight of (dansyl)₂-histamine (DH) is 577.18 g/mol. Dansyl 1,7-DAH also forms a di-dansyl product (figure 4.05); the molecular weight of (dansyl)₂-1,7-DAH (D-1,7-DAH) is 596.8 g/mol (Loukou and Zotou 2003).

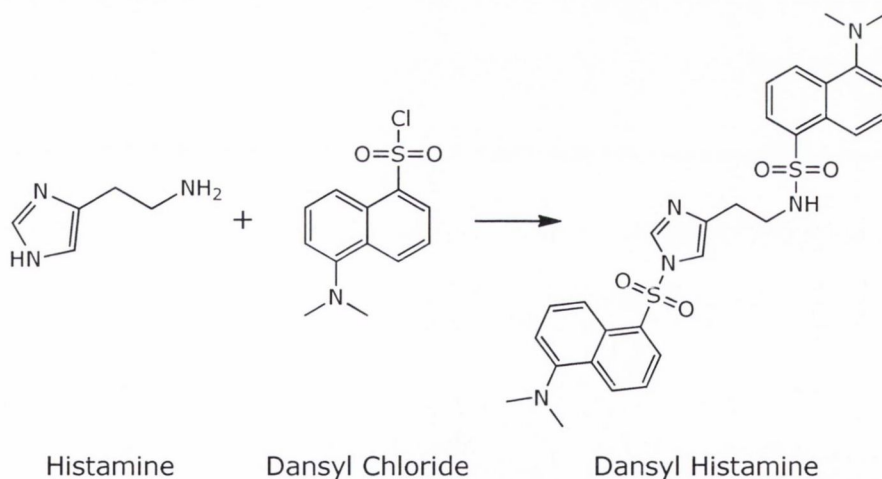


Figure 4.04: Derivatisation of histamine with dansyl chloride to form dansyl histamine

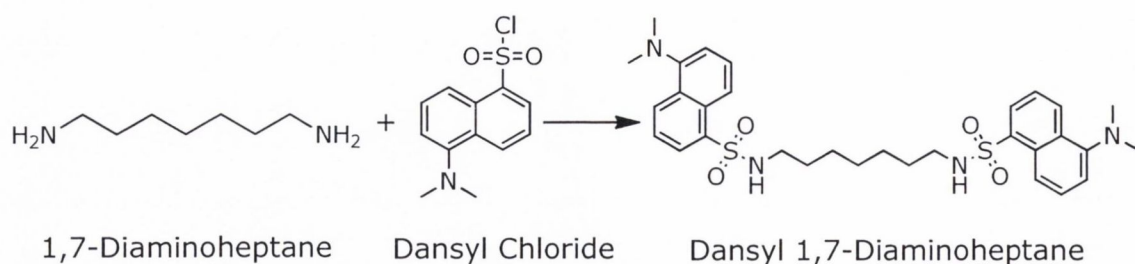


Figure 4.05: Derivatisation of IS 1,7-diaminoheptane with dansyl chloride to form dansyl 1,7-diaminoheptane

4.1.9 Method Validation

Although many different validation guidelines exist, such as those required by the U.S. Department of Health and Human Services, Food and Drug Administration (FDA 2001), dansylation of histamine and 1,7-DAH, and their subsequent detection and quantification using LCMS was validated here according to the European Medicines Agency (EMA), Committee for Medicinal Products for Human Use; Guidelines on Validation of Bioanalytical Methods (EMA 2009). Full validation was carried out for dansyl derivatives of both histamine and 1,7-DAH, allowing the use of 1,7-DAH as IS during subsequent analytical runs. EMA published guidelines state that the main objective of method validation is to demonstrate the reliability of a particular method for the determination of an analyte concentration in a specific biologic matrix. The main characteristics of a bioanalytical method that are essential to ensure acceptability of performance and reliability of analytical results are; lower limit of quantitation (LLOQ), the response function (calibration curve performance), accuracy, precision, selectivity, carry-over, dilution integrity, matrix effects, stability of the analyte and IS in the biological matrix and stock as well as working solutions under the entire period of storage and processing conditions. Therefore, these criteria were used to validate this analytical method.

4.1.9.1 Lower Limit of Quantitation

The LLOQ is the lowest amount of analyte which can be reliably quantified with acceptable accuracy and precision (see accuracy and precision below). The signal to noise ratio (response) of the LLOQ should ideally be at least 5 times the response of the blank sample (FDA 2001). The LLOQ can be adapted to the expected concentrations of the study.

4.1.9.2 Calibration Curve

A calibration curve is used to determine the response of the instrument with respect to the analyte of interest, accordingly a calibration curve is used to determine if a relationship exists between this response and the concentration of analyte analysed. The calibration curve is prepared using the matrix buffer. A calibration curve for each analyte studied was therefore used during method validation as well as during each analytical run. Calibration curves must contain a minimum of 6 calibration concentration levels (excluding the 0 sample) and a relationship should be determined for the calibration standards. Back-calculated concentrations of the calibration standards should be within $\pm 15\%$ of the nominal value, except for the LLOQ which should be within $\pm 20\%$. At least 75% of the calibration standards with a minimum of six must fulfil this criterion (excluding 0 samples). Calibration curves are prepared freshly during validation and RPMC analysis. At least 3 calibration curves should be analysed over 2 separate runs.

4.1.9.3 Accuracy

Accuracy is defined as the closeness of the determined value obtained by the method to the true concentration of the analyte. Accuracy is determined by replicate analysis of quality control (QC) samples using a minimum of 5 determinations at a minimum of 4 concentration levels which cover the calibration curve range; the LLOQ, within 3 times the LLOQ (low QC), around 50% of the calibration curve range (medium QC), and at about 75% of the upper calibration curve range (high QC). QC samples are analysed against the calibration curve and concentrations are compared with the nominal value. Accuracy is reported as a % of the nominal value and is evaluated within a single run (within run accuracy) and between runs (the between run accuracy) to support accuracy over time. For both within run and between run accuracy the mean accuracy value at each QC concentration point should be within 15% of the nominal values, except for the LLOQ which should be within 20% of the nominal value. Accuracy should be evaluated on three separate occasions, over 2 separate runs.

4.1.9.4 Precision

Precision is defined as the closeness of repeated individual measures of analyte and is expressed as the coefficient of variation (CV). The coefficient of variation is a normalised measure of dispersion of a probability distribution; it is defined as the ratio of the standard deviation to the mean. Precision is demonstrated for the LLOQ, low, medium and high QC samples within a run as well as between runs and therefore results generated for accuracy are also used to determine precision. The

CV for both within run and between run precision must not exceed 15% of the QC samples, except for the LLOQ which must not exceed 20%. Precision should be evaluated on three separate occasions, over 2 separate runs.

4.1.9.5 Selectivity

Selectivity is the specific evaluation of the analyte of interest in the matrix. It is essential that the analytical method chosen differentiates the analyte and the IS from other endogenous or non-endogenous components in the sample. Selectivity is shown using at least 6 sources of the appropriate blank matrix which are individually analysed and evaluated for interference. Absence of interfering components is accepted where the response is less than 20% of the LLOQ for the analyte.

4.1.9.6 Carry-Over

Carry-over occurs when analyte of interest from a certain sample carries-over to the next sample, therefore affecting the amount of analyte in the second sample. This occurs frequently when low concentration samples are run following high concentration samples. Carry-over may not affect accuracy and precision and therefore must be minimised during method development and study design. Carry-over may be minimised by inserting blank samples between samples which are expected to contain high and then low concentrations, or after the calibration standard curve. Randomisation of samples should be avoided as it may interfere with carry-over.

4.1.9.7 Dilution Integrity

Dilution integrity determines that dilutions of samples of the analyte do not interfere with accuracy or precision. Dilution integrity is demonstrated by preparing a sample above the upper limit of quantitation and diluting this sample with matrix (at least five determinations per dilution factor). Accuracy and precision should be within the criteria set above. Dilution integrity should cover the required dilutions of the study.

4.1.9.8 Matrix Effect

The matrix used is the components of a sample other than the analyte of interest, these components can interfere with the analysis of the analyte of interest, therefore, the effect of the matrix on the analysis of the analyte of interest should be determined. Matrix effect is investigated using at least 6 batches of matrix. Matrix factor should be calculated in each batch of matrix by calculating the ratio of the peak area in the presence of matrix (blank matrix spiked with analyte at a

maximum concentration of 3 times the LLOQ) to the peak area in the absence of any matrix (pure solution of the analyte). If this approach cannot be used the variability of the response from batch to batch should be assessed by analysing at least 6 batches of matrix in triplicate, spiked at a concentration of at least 3 times the LLOQ. The overall CV of the IS-normalised matrix factor calculated from the 6 batches should not be greater than 15%. The mean concentration should also be within 15% of the nominal concentration.

4.1.9.9 Stability

Stability should be evaluated to ensure that steps during sample preparation and analysis, as well as storage do not affect the concentration of the analyte. Stability should be ensured for every step in the analytical method and evaluated under similar conditions as those of an analytical run. Stability of the analyte and the IS in the matrix is evaluated using at least triplicate samples of the low and high QC samples which are analysed immediately after preparation and after the applied storage conditions that are to be evaluated. The QC samples are analysed against a freshly prepared calibration curve. The deviations of the concentrations obtained should be within 15% of nominal concentrations. Examples of stability tests which may be carried out are: stock solution and working solution stability, freeze thaw stability of the analyte in the matrix from freezer to room temperature and stability of the analyte in matrix stored in the refrigerator.

Because this project required the development of a new bioanalytical method, a complete validation was carried out on both DH and D-1,7-DAH following derivatisation with DC. If the method being used was an adaption of a previously validated method, a partial validation would have sufficed.

4.1.9.10 Ion Suppression/Enhancement

Ion suppression/enhancement is a form of matrix effect that particularly affects LCMS. It occurs because of the coelution of endogenous material present in biological samples, as well as the presence of exogenous material which is present due to contamination during sample preparation and can lead to adverse effects during quantitation such as poor detection, accuracy and precision by influencing the extent of analyte ionisation. Compounds which have a high concentration, mass and basicity and elute at the same retention time as the analyte of interest can often cause ion suppression. Using ESI, ion suppression often occurs at high concentrations of the analyte of interest. Although FDA guidelines indicate ion suppression should be determined, this is not a prerequisite for validation according to EMA guidelines.

4.1.10 TZ2.13

Upon evaluation of the results obtained below, Zhang synthesised a final compound, TZ2.13 (figure 4.06 a below), in an attempt to reduce the lipophilicity of this series of agents. TZ2.13 is an amino acid derivative of TZ2.01 (figure 4.06 b below) and was therefore evaluated along with TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05 and TZ2.11 to determine if this additional functional group would improve the activity of these compounds.

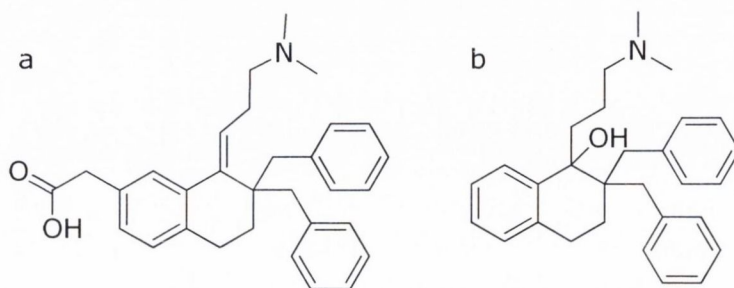


Figure 4.06: TZ2.13 (a) and its parent compound TZ2.01 (b)

4.2 Materials and Methods

4.2.1 Materials

Acetone for HPLC, dansyl chloride, histamine and 1,7-DAH (98% pure) were all purchased from Sigma Aldrich, Arklow, Co. Wicklow, Ireland. Formic acid, LCMS grade methanol, ammonium hydroxide (33%) and LCMS grade water were all purchased from Fisher Scientific, Ballycoolin, Dublin 15, Ireland.

4.2.2 Isolation, Purification and Assay of Rat Peritoneal Mast Cells

Isolation and purification using Percoll of RPMCs was carried out as described in Chapter 2. All compounds were assessed using both calcium ionophore A23187 and compound 48/80 to elicit histamine from mast cells. The RPMC assay was carried in an identical manner as previously described in Chapter 2, however, in this work supernatant is added to the dansylation mixture for derivatisation using DC instead of 1M NaOH for derivatisation with OPT.

4.2.3 Derivatisation of Histamine with Dansyl Chloride

Standard curves were prepared daily and were derived with DC along with assay samples. The method used was developed by Gosetti *et al* (Gosetti, Mazzucco *et al*. 2007); 1 ml of NaHCO₃ (0.25 M in dH₂O), 200 µl DC (0.02 M in HPLC grade acetone), 0.8 ml HPLC grade acetone and 500 µl of sample supernatant were added, without the removal of contaminants from samples, to each labelled 15 ml centrifuge tube. In the case of standard curve and QC samples, 100 µl was added to the dansylation mixture. Standard curves and QC samples were prepared using NaHCO₃ without spiked IS; however, assay samples were prepared using NaHCO₃ spiked with IS (final concentration 1080 ng/ml). Tubes were sealed and placed in a water bath at 60 °C for 20 minutes in the dark. Samples were allowed to come to room temperature before analysis.

4.2.4 Chromatographic Conditions

Reverse phase liquid chromatography was carried out using an XBridge C18 column (2.1 mm x 50 mm, 2.5 µm) (Waters Chromatography Ireland, Santry, Dublin 9). Mobile phase buffer was prepared (12.5 ml 33% NH₄OH in 250 ml LCMS grade H₂O, adjusted to pH 10 using concentrated formic acid to give 0.5% ammonium formate pH 10) and used to make mobile phases A (10:40:50; mobile phase buffer:LCMS grade H₂O:LCMS grade methanol) and B (10:90; mobile phase buffer:LCMS grade methanol). Mobile phases were eluted under gradient conditions as detailed in table 4.01 below. Mobile phase flow rate was 250 µl/min and partial loop sample injection volume was 5 µl. The column was perfused with mobile phase and allowed to

equilibrate over the course of 20 minutes before samples were run. Column temperature was set at 30 °C. HPLC was carried out at high pressure (minimum pressure 2 PSI; max pressure 10000 PSI). Each sample run time was ten minutes.

Table 4.01: Liquid Chromatography Mobile Phase Gradient Programme

Time (minutes)	% Mobile Phase A	% Mobile Phase B	µl/min
0	90	10	250
5	10	90	250
7.10	90	10	250
10	90	10	250

4.2.5 Mass Spectrometry Conditions

MS experiments were carried using Thermo LTQ-XL-Orbitrap Discovery Mass Spectrometer following HPLC using a Thermo Accela Liquid Chromatograph (Thermo Scientific). The LTQ-XL iontrap mass spectrometer was coupled to the Accela LC system via an ESI probe. A full scan was carried out using the Orbitrap while MS/MS was carried out on fragmented DH and D-1,7-DAH using the IonTrap; upon entry to the linear IonTrap DH and D-1,7-DAH were fragmented, at normalised collision energy of 35 arbitrary units, and their fragments 315 and 533 m/z respectively, were detected using MS/MS (table 4.02). The capillary temperature was maintained at 320 °C, sheath gas flow rate 60 arbitrary units, auxiliary gas flow rate 5 arbitrary units, sweep gas flow rate 0 arbitrary units; source voltage 4 kV, source current 100 µA, capillary voltage 20 V, tube lens 110 V. DH and D-1,7-DAH were detected in positive ion mode using selected ion monitoring (Table 4.02 below).

Table 4.02: Values of Characteristic Mass Fragments Obtained by HPLC/ESI MS

Peak	Dansyl-	M (m/z)	[M+H] ⁺ (m/z)	MS/MS (m/z)	Retention Time
Histamine	(Dansyl) ₂ -Histamine	577	578	315	6 minutes
1,7-DAH	(Dansyl) ₂ -1,7-DAH	596	597	533	6 minutes

4.2.6 LCMS Validation Protocol for Histamine and 1,7-DAH Following Dansylation

Stock solutions (3 µg/ml) of histamine and 1,7-DAH were prepared daily and serially diluted to generate calibration curves, as well as accuracy and precision QC samples. All calibration curve, accuracy and precision QC samples were then dansylated using DC as described above.

Calibration curve concentrations were prepared in the same concentration range as used in the RPMC assay (Chapter 2) for histamine. Internal standard 1,7-DAH was prepared in the same concentration range for ease. Concentrations used for histamine and 1,7-DAH were 0, 40, 90, 150, 300, 600, 900, 1200 and 1500 ng/ml; three freshly prepared calibration curves were analysed, over three separate runs.

For accuracy and precision studies four QC concentrations were chosen in accordance with guidelines; LLOQ (40 ng/ml), within three times the LLOQ (90 ng/ml), ~50% of the calibration curve range (800 ng/ml) and ~75% of the calibration curve range (1200 ng/ml) were prepared (n=5 at each concentration point for each run). Within run and between run accuracy and precision were determined using n=5 samples for each run. Three different runs were carried out on two different consecutive days.

Selectivity was determined over three runs, resulting in n=6 (max-basal)/(total-basal) RPMC assay values.

4.2.7 Analytical Run

Each analytical run began with a standard curve (histamine and IS) followed by QC samples (0, 90, 900, 1200 ng/ml of both histamine and IS). Analytical samples were then run and QC samples were again placed at the end of the analytical run to ensure method validity throughout the length of the run. As discussed, it was decided to evaluate the method using ng/ml histamine and not histamine:IS peak area ratios, therefore, IS was not used in test samples but only in QC and calibration curve samples. Blank samples (without histamine or 1,7-DAH) were placed strategically to prevent carry-over during the run).

4.2.8 Analysis of Data

All validation and assay samples were prepared in duplicate and values obtained were averaged. Area under the curve was integrated using Thermo Scientific Xcalibur software to determine ng/ml amounts of histamine and/or 1,7-DAH in each sample. Calculations of percentage inhibition of histamine release, for RPMC mast cell stabilising activity, was carried out as described in Chapter 2.

4.2.9 Statistics

Statistical analysis of the data was carried out using GraphPad Prism, version 5. For validation studies accuracy is expressed as mean $\pm$ SEM, while precision is expressed as CV. Calibration curve analysis is carried out using linear regression analysis.

RPMC assay results are expressed as mean $\pm$ SEM percentage inhibition values for each test compound, at each concentration.

4.2.10 Computation of Quantitative Structure Analysis Values

In a similar manner to Groups I-V discussed in Chapters 2 and 3, the theoretical properties of TZ2.13 were computed using MarvinSketch, ChemAxon (Chemaxon 2010). Properties determined were the partition coefficient (LogP or cLogP), the distribution coefficient (LogD_{7.4}), the acid dissociation constant (pKa), hydrogen (H⁺) bond donor and acceptor sites as well as 2D polar surface area (PSA).

4.3 Results

4.3.1 Liquid Chromatography Mass Spectrometry Validation

LCMS quantification of histamine release from mast cells was validated according to EMA validation criteria. Histamine was quantified after derivatisation with dansyl chloride. This was followed by quantification of 1,7-DAH, also after derivatisation with dansyl chloride.

4.3.1.1 Validation of Histamine following Dansylation using Dansyl Chloride

4.3.1.1.1 Histamine Calibration Curves

Table 4.03: Histamine Calibration Curve 1; r^2 0.9986, slope 200.70 ± 2.79 , Y intercept -1272 ± 2079 and X intercept 6.33

Nominal ng/ml	Peak Area	Actual ng/ml	% of Nominal
0	1428	0.00	
40	8192	34.57	86.43
90	17296	81.10	90.11
150	26723	129.28	86.19
300	57201	285.05	95.02
600	111958	564.91	94.15
900	187344	950.20	105.58
1200	239063	1214.53	101.21
1500	298899	1520.35	101.36

Table 4.04: Histamine Calibration Curve 2; r^2 0.9972, slope 146.60 ± 2.93 , Y intercept 4713 ± 2185 and X intercept -32.15

Nominal ng/ml	Peak Area	Actual ng/ml	% of Nominal
0	4176	0	
40	8389	28.55	71.36
90	16196	81.45	90.49
150	25966	147.63	98.42
300	51094	317.89	105.96
600	92906	601.17	100.19
900	146576	964.80	107.20
1200	175052	1157.74	96.48
1500	222733	1480.79	98.72

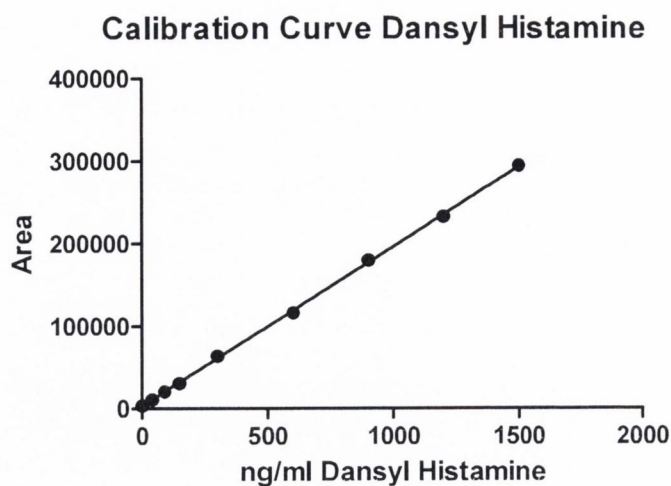


Figure 4.07: Histamine Calibration Curve 3; r^2 0.9996, slope 193.30 ± 1.37 , Y intercept 3146 ± 1021 and X intercept -16.28

Table 4.05: Histamine Calibration Curve 3; r^2 0.9996, slope 193.30 ± 1.37 , Y intercept 3146 ± 1021 and X intercept -16.28

Nominal ng/ml	Peak Area	Actual ng/ml	% of Nominal
0	3352	0.00	
40	10890	39.55	98.88
90	20386	88.31	98.13
150	30915	142.90	95.27
300	63759	313.18	104.39
600	116276	585.46	97.58
900	179870	915.16	101.68
1200	232469	1187.86	98.99
1500	294136	1507.57	100.50

4.3.1.1.2 Histamine Accuracy; Within Run and Between Run

Table 4.06: Within Run Accuracy Histamine, Run 1, $n=5$ per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean $\pm$ SEM)	43 $\pm$ 1	95 $\pm$ 1	834 $\pm$ 8	1247 $\pm$ 11
% of Nominal Value	107%	106%	104%	104%

Table 4.07: Within Run Accuracy Histamine, Run 2, n=5 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	44± 1	95± 2	750± 34	1078± 16
% of Nominal Value	110%	106%	94%	90%

Table 4.08: Within Run Accuracy Histamine, Run 3, n=5 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	39± 1	86± 1	765± 14	1118± 15
% of Nominal Value	97%	95%	96%	93%

Table 4.09: Between Run Accuracy Histamine, Runs 1, 2 and 3 combined, n=15 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	42± 1	92± 1	783± 59	1148± 21
% of Nominal Value	105%	102%	98%	96%

4.3.1.1.3 Histamine Precision; Within Run and Between Run

Table 4.10: Within Run Precision Histamine, Run 1, n=5 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	43± 1	95± 1	834± 8	1247± 11
CV	7%	2%	2%	2%

Table 4.11: Within Run Precision Histamine, Run 2, n=5 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	44± 1	95± 2	750± 34	1078± 16
CV	4%	5%	10%	3%

Table 4.12: Within Run Precision Histamine, Run 3, n=5 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	39± 1	86± 1	765± 14	1118± 15
CV	4%	2%	4%	3%

Table 4.13: Between Run Precision Histamine, Runs 1, 2 and 3 combined, n=15 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	42± 1	92± 1	783± 59	1148± 21
CV	7%	6%	8%	7%

4.3.1.1.4 Histamine Selectivity

Table 4.14: Selectivity of LCMS method for dansyl histamine

Blank Sample (0 ng/ml)	A	B	C	D	E	F
Actual ng/ml Histamine	3.43	2.89	0.68	-3.95	1.13	6.65
% of LLOQ (40 ng/ml Histamine)	9%	7%	2%	-10%	3%	17%

Table 4.15: Selectivity of LCMS method for dansyl histamine following RPMC assay

Average Values (ng/ml)	n=1	n=2	n=3	n=4	n=5	n=6
Basal	-5.43	0.22	-3.79	1.57	15.94	8.85
Max	159.21	292.94	194.31	246.28	85.35	77.73
Total	225.71	368.96	432.67	441.04	123.66	105.58
[(Max-Basal)/(Total/Basal)]%	71.23	79.38	45.39	55.68	64.43	71.21

4.3.1.1.5 Dansyl Histamine Representative Chromatogram

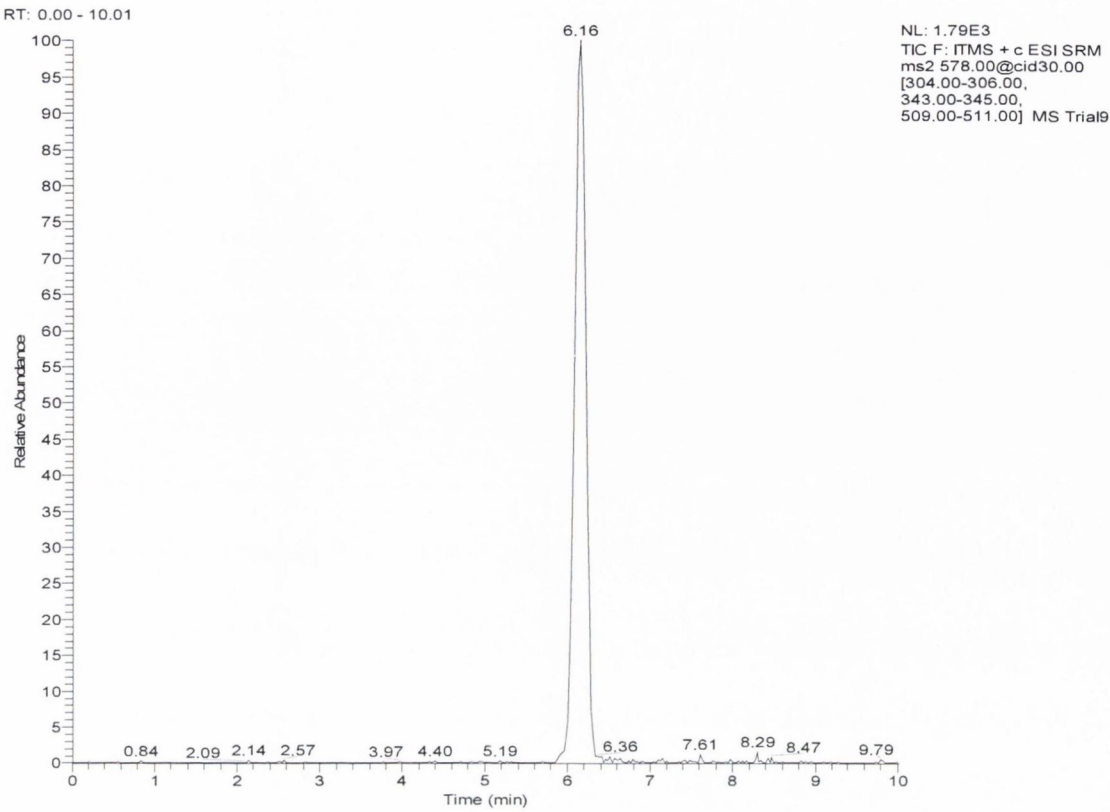


Figure 4.08: Representative chromatograph of dansyl histamine following MS/MS, 578 to 315 m/z transition

4.3.1.2 Validation of 1,7-Diaminoheptane following Dansylation using Dansyl Chloride

4.3.1.2.1 1,7-DAH Calibration Curves

Table 4.16: 1,7-DAH Calibration Curve 1; r^2 0.9971, slope 53.51 ± 1.09 , Y intercept -545.0 ± 812.70 and X intercept 10.19

Nominal ng/ml	Peak Area	Actual ng/ml	% of Nominal
0	96	0.00	
40	1690	30.48	76.19
90	4475	83.71	93.01
150	7008	132.13	88.09
300	14692	279.96	93.32
600	29965	571.04	95.17
900	51278	978.54	108.73
1200	61912	1181.83	98.49
1500	79767	1523.21	101.55

Table 4.17: 1,7-DAH Calibration Curve 2; r^2 0.9987, slope 52.72 ± 0.72 , Y intercept -64.89 ± 541.07 and X intercept 1.23

Nominal ng/ml	Peak Area	Actual ng/ml	% of Nominal
0	173	1.46	
40	2446	44.92	112.30
90	4554	85.22	94.69
150	8315	157.13	104.76
300	15494	294.39	98.13
600	31930	608.62	101.44
900	45664	871.20	96.80
1200	61886	1181.34	98.44
1500	80947	1545.77	103.05

Calibration Curve Dansyl 1,7-Diaminoheptane

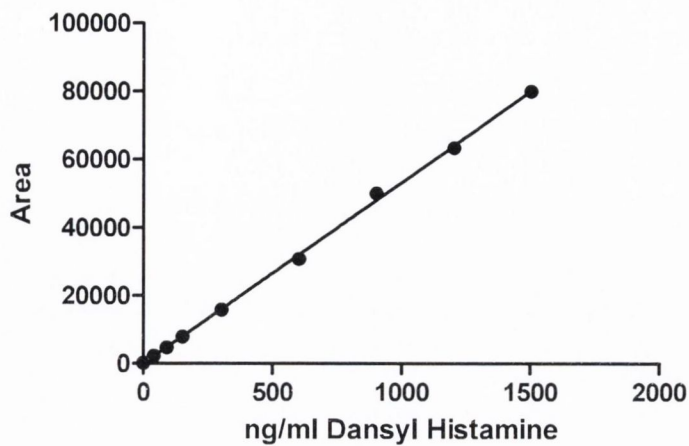


Figure 4.09: 1,7-DAH Calibration Curve 3; r^2 0.9991, slope 53.35 ± 0.60 , Y intercept 29.02 ± 449.10 and X intercept -0.54

Table 4.18: 1,7-DAH Calibration Curve 3; r^2 0.9991, slope 53.35 ± 0.60 , Y intercept 29.02 ± 449.10 and X intercept -0.54

Nominal ng/ml	Peak Area	Actual ng/ml	% of Nominal
0	206	0.00	
40	2274	38.51	96.27
90	4820	87.05	96.72
150	7951	146.11	97.41
300	15863	295.37	98.46
600	30801	577.17	96.20
900	50074	940.74	104.53
1200	63357	1191.32	99.28
1500	79917	1503.73	100.25

4.3.1.2.2. 1,7-DAH Accuracy; Within Run and Between Run

Table 4.19: Within Run Accuracy 1,7-DAH, Run 1, $n=5$ per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean $\pm$ SEM)	40 $\pm$ 2	93 $\pm$ 2	841 $\pm$ 7	1176 $\pm$ 20
% of Nominal	100%	104%	105%	98%

Table 4.20: Within Run Accuracy 1,7-DAH, Run 2, n=5 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	43± 2	97± 1	859± 12	1277± 22
% of Nominal	107%	107%	107%	106%

Table 4.21: Within Run Accuracy 1,7-DAH, Run 3, n=5 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	38± 1	85± 1	798± 9	1198± 16
% of Nominal	94%	94%	100%	100%

Table 4.22: Between Run Accuracy 1,7-DAH, Runs 1, 2 and 3 combined, n=15 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	40± 1	92± 2	833± 9	1217± 16
% of Nominal	100%	102%	104%	101%

4.3.1.2.3 1,7-DAH Precision; Within Run and Between Run

Table 4.23: Within Run Precision 1,7-DAH, Run 1, n=5 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	40± 2	93± 2	841± 7	1176± 20
CV	10%	4%	2%	4%

Table 4.24: Within Run Precision 1,7-DAH, Run 2, n=5 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean ± SEM)	43± 2	97± 1	859± 12	1277± 22
CV	10%	3%	3%	4%

Table 4.25: Within Run Precision 1,7-DAH, Run 3, n=5 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean $\pm$ SEM)	38 $\pm$ 1	85 $\pm$ 1	798 $\pm$ 9	1198 $\pm$ 16
CV	6%	2%	3%	3%

Table 4.26: Between Run Precision 1,7-DAH, Runs 1, 2 and 3 combined, n=15 per QC concentration point

Nominal ng/ml	40	90	800	1200
Actual ng/ml (Mean $\pm$ SEM)	40 $\pm$ 1	92 $\pm$ 2	833 $\pm$ 9	1217 $\pm$ 16
CV	10%	6%	4%	5%

4.3.1.2.4 1,7-DAH Selectivity

Table 4.27: Selectivity of LCMS method for dansyl 1,7-diaminoheptane

Blank Sample (0 ng/ml)	A	B	C	D	E	F
Actual ng/ml 1,7-DAH	2.15	6.06	1.46	-0.96	-3.07	0.36
% of LLOQ (40 ng/ml 1,7-DAH)	5%	15%	4%	-2%	-8%	1%

Table 4.28: 1,7-DAH was not detected in RPMC assay samples, indicating lack of interfering compounds in the assay matrix

Average Values (ng/ml)	n=1	n=2	n=3	n=4	n=5	n=6
Basal	2.92	2.16	4.63	2.02	2.57	2.87
Max	0.63	10.17	-0.27	1.69	2.56	1.96
Total	-0.27	2.98	6.03	3.05	3.35	2.23

4.3.1.2.5. Dansyl 1,7-Diaminoheptane Representative Chromatogram

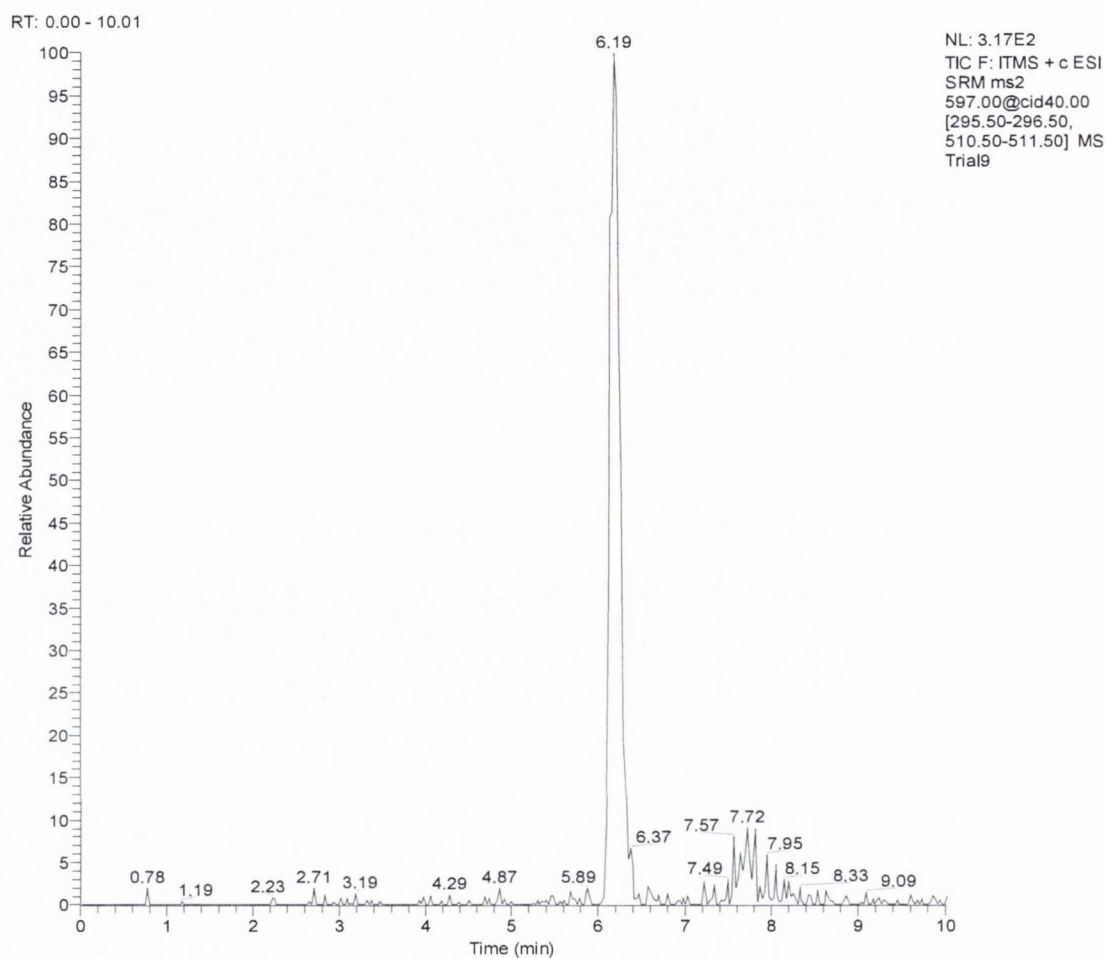


Figure 4.10: Representative chromatograph of dansyl 1,7-DAH following MS/MS, 597 to 533 m/z transition

4.3.2 Representative Total Ion Chromatogram and Spectra

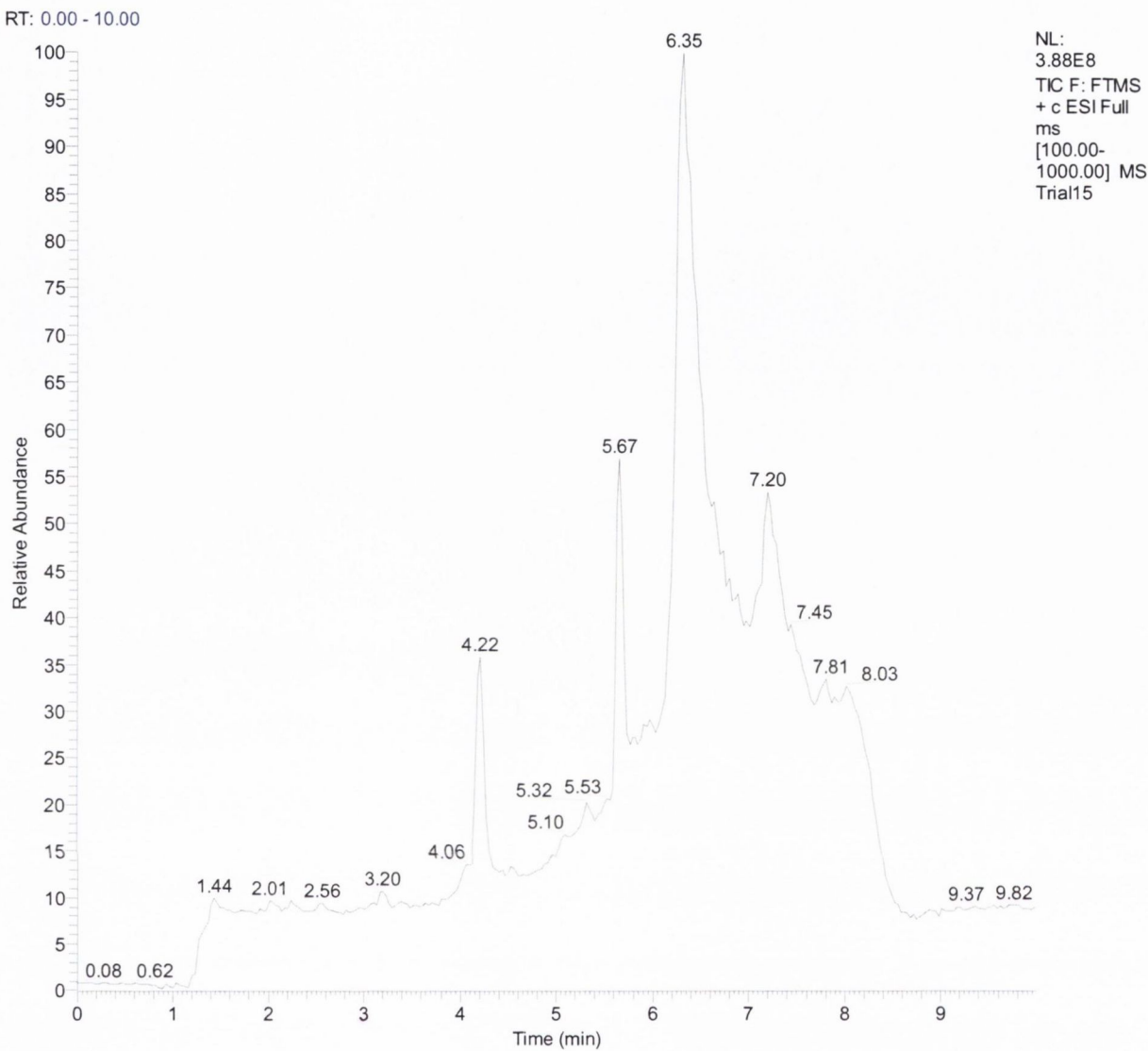


Figure 4.11: Representative total ion chromatogram from a "Total" assay sample in an analytical run

Trial15 #506 RT: 5.57 AV: 1 NL: 5.26E3
F: ITMS + c ESI Full ms2 578.00@cid35.00 [155.00-550.00]

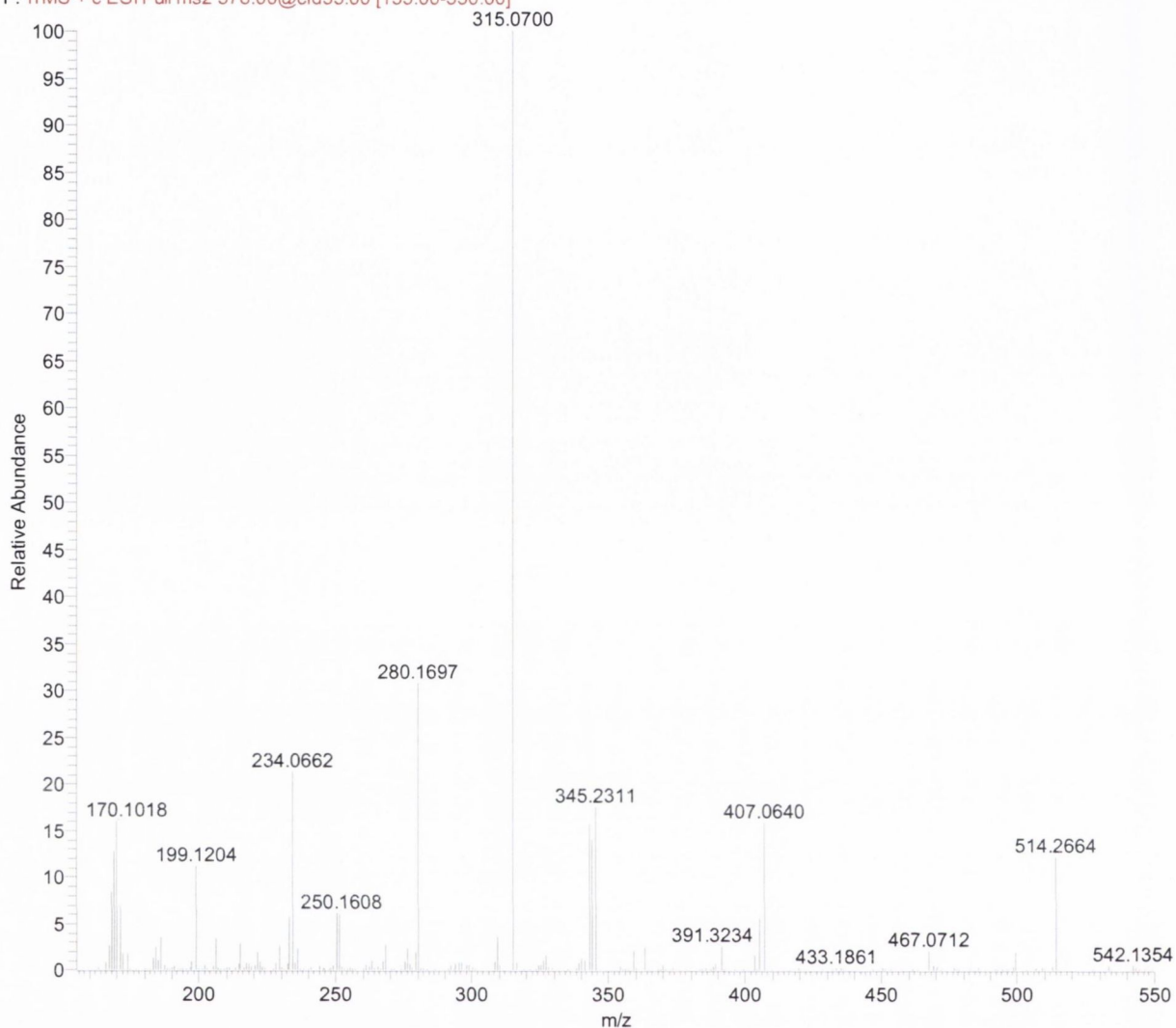


Figure 4.12: Representative dansyl histamine mass spectrum from a "Total" assay sample in an analytical run, without background clean-up. 578 to 315 m/z transition

Trial15 #516 RT: 5.66 AV: 1 NL: 2.40E5
F: ITMS + c ESI Full ms2 597.00@cid35.00 [160.00-550.00]

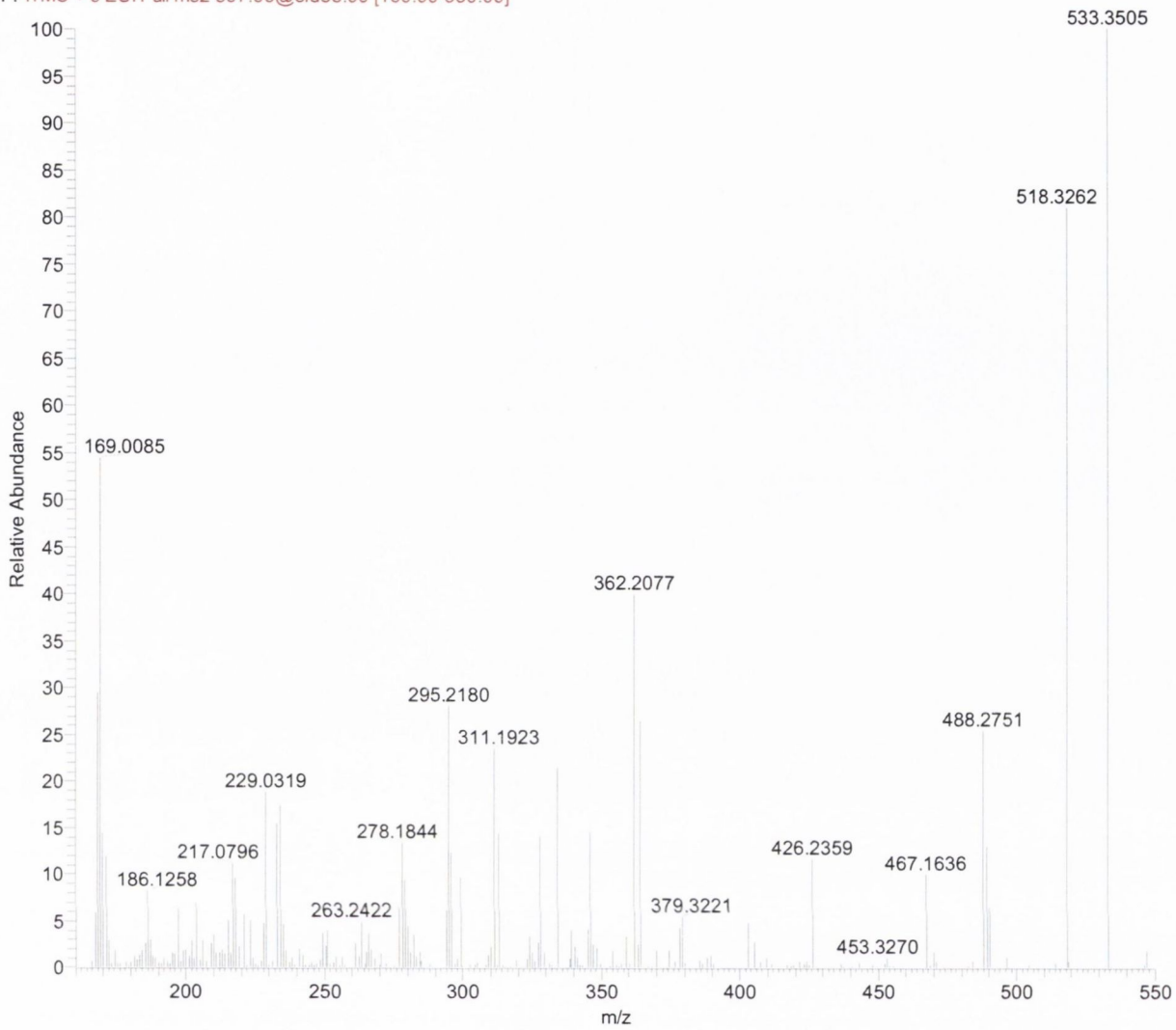


Figure 4.13: Representative dansyl 1,7-diaminoheptane mass spectrum, without background clean-up. 597 to 533 m/z transition

4.3.3 Rat Peritoneal Mast Cell Assay Results

TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05 and TZ2.11 were evaluated for mast cell stabilising activity, using the RPMC assay followed by the LCMS method developed and validated above. These compounds, despite demonstrating encouraging activity *in vivo* (Chapter 3), seemed to cause the release of histamine from mast cells (figure 4.11-4.16 below). TZ2.13, which was not evaluated *in vivo*, demonstrated mast cell stabilising activity (figure 4.17 below).

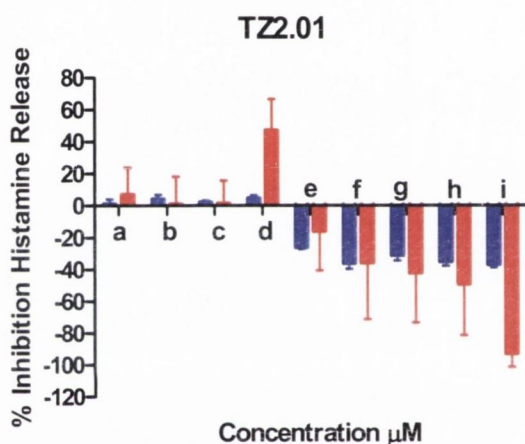


Figure 4.14: Effect of a) 0.01 μM , b) 0.1 μM , c) 0.5 μM , d) 1 μM , e) 5 μM , f) 10 μM , g) 20 μM , h) 40 μM and i) 60 μM TZ2.01 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=3$ or 4

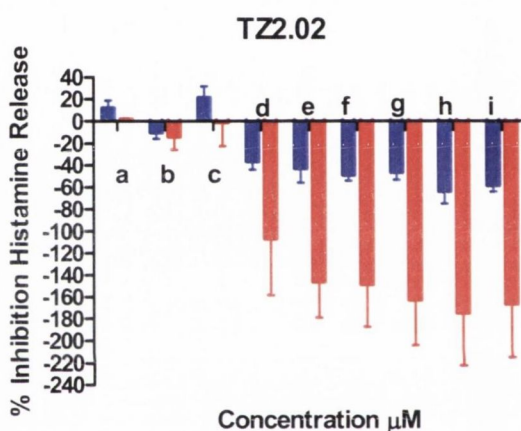


Figure 4.15: Effect of a) 0.01 μM , b) 0.1 μM , c) 0.5 μM , d) 1 μM , e) 5 μM , f) 10 μM , g) 20 μM , h) 40 μM and i) 60 μM TZ2.02 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=3$ or 4

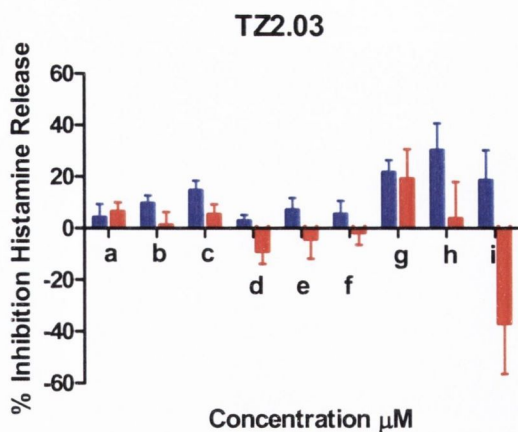


Figure 4.16: Effect of a) 0.01 μM , b) 0.1 μM , c) 0.5 μM , d) 1 μM , e) 5 μM , f) 10 μM , g) 20 μM , h) 40 μM and i) 60 μM TZ2.03 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=3$ or 4

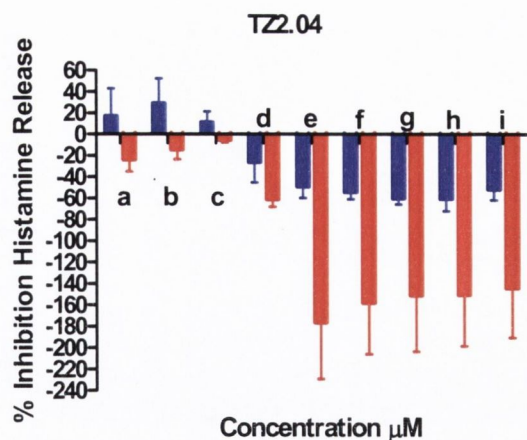


Figure 4.17: Effect of a) 0.01 μM , b) 0.1 μM , c) 0.5 μM , d) 1 μM , e) 5 μM , f) 10 μM , g) 20 μM , h) 40 μM and i) 60 μM TZ2.04 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=3$ or 4

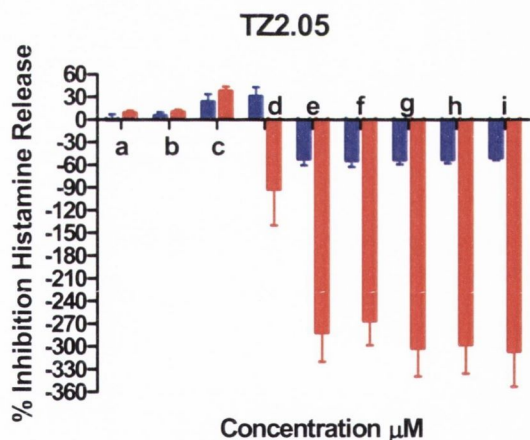


Figure 4.18: Effect of a) 0.01 μM , b) 0.1 μM , c) 0.5 μM , d) 1 μM , e) 5 μM , f) 10 μM , g) 20 μM , h) 40 μM and i) 60 μM TZ2.05 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=4$

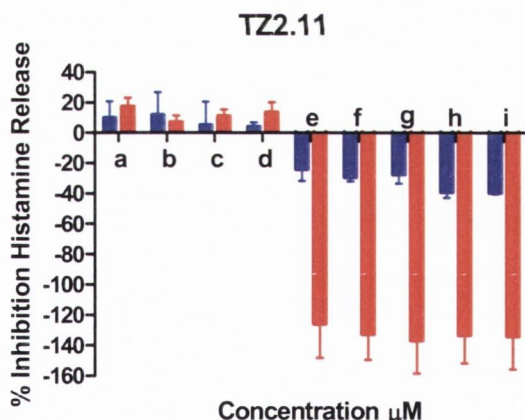


Figure 4.19: Effect of a) 0.01 μM , b) 0.1 μM , c) 0.5 μM , d) 1 μM , e) 5 μM , f) 10 μM , g) 20 μM , h) 40 μM and i) 60 μM TZ2.11 on calcium ionophore A23187- (blue) and compound 48/80- (red) induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=3$ or 4

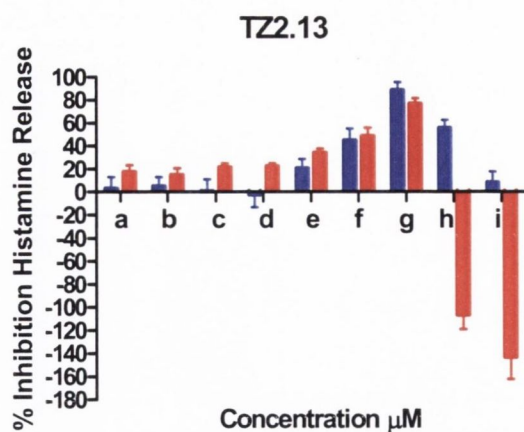


Figure 4.20: Effect of a) 0.01 μM , b) 0.1 μM , c) 0.5 μM , d) 1 μM , e) 5 μM , f) 10 μM , g) 20 μM , h) 40 μM and i) 60 μM TZ2.13 on calcium ionophore A23187 (blue) and compound 48/80 (red) -induced histamine release from RPMC. Data represents mean $\pm$ SEM of $n=3$ or 4

4.3.4 LogP and LogD_{7.4} Values of Assayed Compounds versus RPMC Activity

Theoretical computed structural properties were determined for TZ2.13 MarvinSketch (table 4.29 below). Computed LogP, LogD_{7.4}, pKa, hydrogen (H+) bond donor and acceptor sites and 2D PSA values were determined previously (Chapter 3) using MarvinSketch for TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05 and TZ2.11. structure activity relationship analysis was carried out to determine if a relationship may exist between the structures of these compounds and their activities (figures 4.18-4.21 below). No structure activity relationship was observed for any compound group.

Table 4.29: QSAR properties of TZ2.13 and TZ2.01, its parent compound

Compound	TZ2.13	TZ2.01
LogP	6.25	6.61
LogD _{7.4}	1.38	4.54
pKa	9.76	9.49, 13.69
H+ donor site	2	1
H+ acceptor site	4	2
Polar surface area (2D)	64.80 Å ²	24.67 Å ²
Stereoisomers	2	2
Molecular Weight	507.8 g/mol	449.5 g/mol

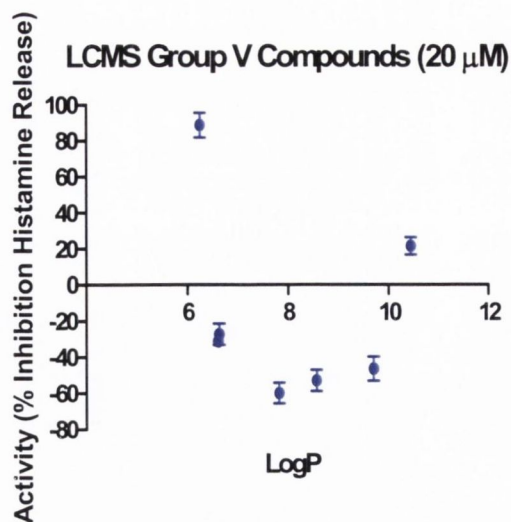


Figure 4.21: Relationship between LogP of group V compounds evaluated in the RPMC using LCMS to quantify histamine at 20 μ M, calcium ionophore A23187-induced histamine release. $n=3$ or 4 per compound

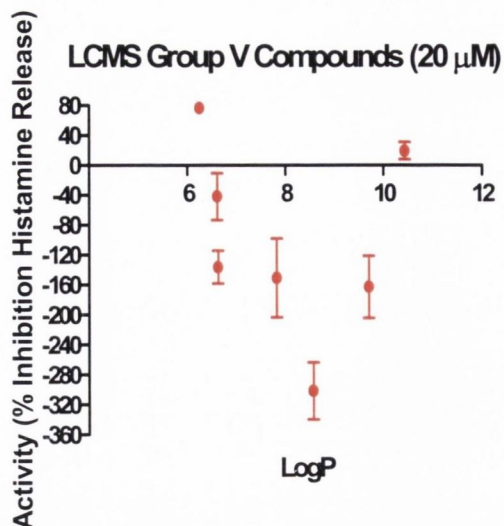


Figure 4.22: Relationship between LogP of group V compounds evaluated in the RPMC using LCMS to quantify histamine at 20 μ M, compound 48/80-induced histamine release. $n=3$ or 4 per compound

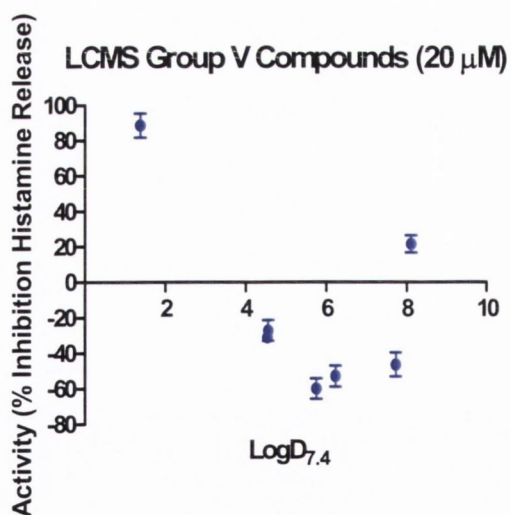


Figure 4.23: Relationship between LogD_{7.4} of group V compounds evaluated in the RPMC using LCMS to quantify histamine at 20 μ M, calcium ionophore A23187-induced histamine release. $n=3$ or 4 per compound

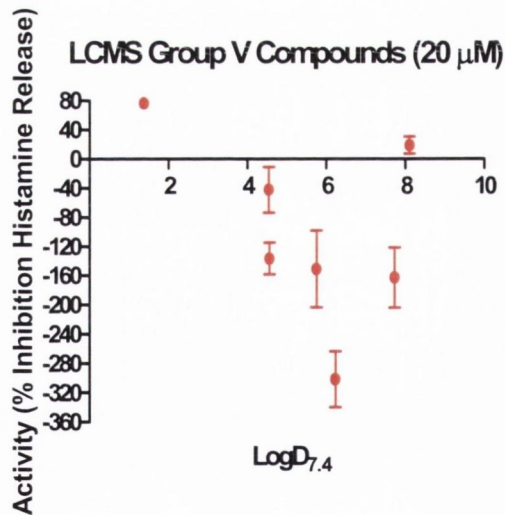


Figure 4.24: Relationship between LogD_{7.4} of group V compounds evaluated in the RPMC using LCMS to quantify histamine at 20 μ M, compound 48/80-induced histamine release. $n=3$ or 4 per compound

4.4 Discussion

4.4.1 LCMS Validation

Before RPMC assay samples could be analysed by LCMS it was necessary to fully validate both histamine and IS 1,7-DAH following dansylation with DC. Validation parameters used, according to EMA guidelines, were; LLOQ, calibration curve, accuracy, precision, selectivity, carry-over, dilution integrity, matrix effect and stability.

4.4.1.1 Lower Limit of Quantitation

The LLOQ was initially evaluated at 5 ng/ml histamine, however, values obtained were not acceptable at this level because peak area response was not at least five times the response of the blank samples (noise), as required. Therefore, the LLOQ was increased to 40 ng/ml histamine; at 40 ng/ml the mean peak area value for 40 ng/ml DH samples (10154 ± 450.40 , $n=12$) is at least five times higher than the mean peak area value of blank samples (1917 ± 123.20 , $n=7$), thus deeming this LLOQ acceptable for use. Because IS concentration can be chosen to suit the study and the LLOQ used for 1,7-DAH was also established at 40 ng/ml; mean peak area value for 40 ng/ml 1,7-DAH QC samples (2290 ± 86.07 , $n=12$) is also at least five times higher than the mean peak area value of blank samples (230.10 ± 36.68 , $n=7$), also validating 40 ng/ml as 1,7-DAH LLOQ.

4.4.1.2 Linearity/Calibration Curve

The histamine calibration curve initially fell within the same range as the one used in the RPMC assay using OPT (0, 5, 10, 20, 40, 60, 80, 100, 200, 300 and 400 ng/ml histamine). Although this calibration range exhibited consistent linearity using this method (r^2 values such as, 0.9925, 0.9661, 0.9974 for example) LLOQ of 5 ng/ml did not pass validation as discussed above. It was necessary to increase the range of the calibration curve to suit the higher LLOQ; therefore, the calibration curve range used was 0, 40, 90, 150, 300, 600, 900, 1200 and 1500 ng/ml histamine, to reflect this increase. For ease a similar calibration range was used to determine linearity of 1,7-DAH following dansylation. Histamine (tables 4.03 to 4.05, figure 4.07) and 1,7-DAH (tables 4.15 to 4.17, figure 4.09) both demonstrated linearity for this calibration range (r^2 values for histamine ≥ 0.991 and for 1,7-DAH ≥ 0.997) and at least 6 values determined in each calibration curve for both analytes lie within 15% of nominal values, apart from LLOQ which lies within 20%. This indicates that a correlation was found to exist between analyte concentration and the response. Therefore both histamine and 1,7-DAH comply with calibration curve validation criteria.

4.4.1.3 Accuracy

Accuracy was determined in 3 separate runs, over 2 separate days. Accuracy was first determined for histamine following dansylation. Within run and between run (within run values combined) accuracy results (tables 4.06 to 4.09) indicate that each QC concentration falls within the defined validation limits; $100 \pm 12\%$ of nominal values (as required to be $\pm 15\%$), except for LLOQ which falls within $100 \pm 10\%$ of nominal values (as required to be $\pm 20\%$). This indicates that our assay is quite accurate for the detection of dansylated histamine and therefore, this histamine assay conforms to accuracy validation criteria. Validation of accuracy of 1,7-DAH was also carried out; results obtained indicate that within run and between run accuracy (tables 4.19 to 4.22) for 1,7-DAH again fall within the validation limits of $100 \pm 8\%$ of nominal values (as required to be $\pm 15\%$), except for LLOQ which falls within $100 \pm 7\%$ of nominal values (as required to be $\pm 20\%$). This indicates that our assay is also quite accurate for the detection of 1,7-DAH and therefore, 1,7-DAH also complies with accuracy validation criteria. Because the RPMC assay is self-controlled and raw data from day to day was never compared, it was not necessary to evaluate inter-day accuracy. Instead mast cell stabilising activities were determined and compared from day to day.

4.4.1.4 Precision

Precision values were also determined in 3 runs over 2 separate days. Precision validation criteria dictate that the CV must not fall outside 15% of nominal values for QC samples, except for LLOQ of which the CV which must lie within 20% of nominal values. Within run and between run (within run values combined) histamine precision results (tables 4.10-4.13) indicate that each QC CV value does not exceed of the QC samples; $100 \pm 11\%$ (as required to be $\pm 15\%$), except for LLOQ in which the CV does not exceed $100 \pm 7\%$ of QC samples (as required to be $\pm 20\%$). This indicates that our assay is quite precise for the detection of dansylated histamine and therefore, histamine conforms to precision validation criteria. Validation of precision of 1,7-DAH was also carried out; results obtained indicate that within run and between run precision CV values (tables 4.23-4.26) for 1,7-DAH again do not exceed the validation limits of $100 \pm 7\%$ CV (as required to be $\pm 15\%$), except for LLOQ which does not exceed $100 \pm 11\%$ of QC samples (as required to be $\pm 20\%$). This indicates that our assay is also quite precise for the detection of 1,7-DAH and therefore, determined 1,7-DAH also complies with precision validation criteria and is suitable for use as an internal standard. Because the RPMC assay is self-controlled and raw data from day to day was never compared, it was not necessary to evaluate inter-day precision.

4.4.1.5 Selectivity

Selectivity of the analyte of interest was determined by analysing the analyte of interest in blank samples of the matrix. Results above indicate that blank samples (0 ng/ml) analysed for both histamine (table 4.14) and 1,7-DAH (table 4.27) both contain less than 20% of the LLOQ amount of the analyte of interest. This indicates that the method developed here falls within allowed validation limits (0 ng/ml blank response must be less than 20% of the LLOQ for the analyte) and therefore is selective for both dansyl histamine and dansyl 1,7-DAH.

Selectivity of the method for the RPMC assay, although not required, was also determined because histamine is endogenous. Therefore, selectivity was demonstrated following 6 RPMC assays. Basal, max and total values were determined as described in Chapter 2. These were dansylated according to the protocol above and then were quantified using LCMS. When the percentage $(\text{max-basal})/(\text{total-basal})$ values were calculated for each assay it was found that values obtained were similar to assay values obtained using the OPT fluorescence method for detection of histamine (table 4.15). Basal, max and total samples were also checked for the presence of 1,7-DAH which was confirmed to be absent as expected (table 4.28) indicating a lack of interference between other compounds in the assay matrix. Selectivity for histamine was also demonstrated by the accuracy and precision results discussed above.

The mean value when basal, max and total assay values were combined (2.84 ng/ml), although extremely low, did not equal 0.00 ng/ml. Using the definition of selectivity from validation criteria, this value is 7.1% of the LLOQ (40 ng/ml 1,7-DAH; values must be no more than 20% of the value of the LLOQ of the analyte of interest) and therefore it can be inferred that there is no compound present following PRMC assay which interferes with the detection of 1,7-DAH in the dansylation matrix.

4.4.1.6 Carry-Over

Carry-over was minimised by inserting a blank sample between samples containing high and low concentrations. A wash step was also included in the LCMS protocol following the sampling of each sample by the LCMS autosampler to ensure minimal sample carry-over.

4.4.1.7 Dilution Integrity

Serial dilution of freshly prepared stock solutions of both histamine and 1,7-DAH for each validation and analytical run were integral parts of the study design. Therefore, because validation of the method includes serial dilutions it was deemed

unnecessary to perform dilution integrity validation in this instance. As the method passes validation it follows that dilution did not affect the analytical method.

4.4.1.8 Matrix Effect

Histamine is present in the biological matrix and because it is impossible to obtain biological matrix without histamine, it is not possible to evaluate biological matrix effects for this experiment. For this reason all validation was carried out beginning starting at the dansylation procedure. Validation procedures were carried out using QC samples prepared in assay buffer. Selectivity in RPMC samples ensures the validated procedure is effective for use in the RPMC assay.

4.4.1.9 Stability

It was decided to prepare working solutions daily to avoid the issue of stability, therefore all stock solutions of histamine and 1,7-DAH were prepared daily and all dilutions were made using these preparations. MCM/HBSS buffer for RPMC assay was made as needed, generally every couple of days and at least once weekly, therefore validation was carried out using MCM/HBSS which had been stored at 4 °C for one week. For the dansylation procedure DC as well as NaHCO₃ preparations would also be made daily, avoiding storage stability issues.

The validation protocol carried out demonstrates that this analytical method is valid for LLOQ, calibration curve (linearity), precision, accuracy and selectivity. Because the study was designed to overcome any possible storage or carry-over issues and because it is not possible to evaluate matrix effect because of the nature of this study, it can be concluded that this analytical method was validated and fit for use. It was therefore used to quantify mast cell stabilising activity of compounds in the *in vitro* RPMC assay, samples were first dansylated and subsequently histamine released was quantified by measuring fragmented DH and D-1,7-DAH using MS/MS.

4.4.1.10 Ion Suppression/Enhancement

Because determination of ion suppression/enhancement was not required for validation of the method according to EMA guidelines, it was not determined in this instance. However, the importance of this variable is noted therefore its determination would be included in the design, development and validation of subsequent LCMS methods.

4.4.2 Representative Total Ion Chromatograph and Spectra

A total ion chromatogram (TIC) (figure 4.11) and spectra showing dansyl histamine (figure 4.12) and dansyl 1,7-DAH (figure 4.13) are indicated above. The TIC chromatogram and dansyl histamine spectrum are representative of an analytical run "Total" assay sample, where clean-up was not carried out.

4.4.3 Rat Peritoneal Mast Cell Assay

Six compounds were analysed for their *in vitro* mast cell stabilising activity following RPMC assay, using the above LCMS method to separate, ionise and detect histamine which had been derived using DC. It is important to note that the sole reason for establishing this method was to quantify mast cell mediator release in this mast cell population. Therefore, no attempt was made to validate the assay at a lower LLOQ, as the LLOQ used was suitable for the aim of the study. It may be desirable in the future to validate this method using a lower LLOQ. This study is fully validated and therefore is fit for its intended purpose.

The compounds which were evaluated in the RPMC assay were chosen because of their potent *in vivo* mast cell stabilising activity (Chapter 3); these were TZ2.01 (PCA 84.49 ± 16.37), TZ2.02 (PCA 30.00 ± 29.64), TZ2.03 (PCA 78.89 ± 8.766), TZ2.04 (PCA 76.31 ± 8.689), TZ2.05 (PCA 88.18 ± 35.90) and TZ2.11 (PCA 67.97 ± 9.234). TZ2.12 was excluded from this study because its PCA activity was minimal (PCA 10.49 ± 21.18). Because these compounds demonstrate mast cell stabilising activity *in vivo* it was necessary to determine their activities *in vitro* using the RPMC assay. Although LCMS results can be expressed as analyte to IS peak area ratio and 1,7-DAH was fully validated for use as IS, because the method was validated using ng/ml of histamine, results were calculated using ng/ml histamine values and not using peak area ratio. This means however, that in future studies if an IS was required 1,7-DAH, has been fully validated and therefore, would be suitable for use.

Results obtained in the RPMC assay, using LCMS, indicate that compounds TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05, TZ2.05 and TZ2.11 are inducing histamine release from mast cells (figures 4.14-4.19). Although these compounds possess potent mast cell stabilising activity *in vivo* (Chapter 3) it appears that because of their high level of lipophilicity they cause the release of histamine. This may occur because these compounds may permeate the cell membrane. TZ2.03 demonstrates some level of mast cell stabilising activity, although only at high concentrations (20 μ M and 40 μ M; figure 4.16).

It is clear that validation of the above method of detection and quantification of dansylated histamine has provided a novel use for the Thermo Accela Liquid Chromatograph with Thermo LTQ-XL-Orbitrap Discovery Mass Spectrometer. As discussed previously, this system has never been used in this capacity. Validation of the above method within required guidelines indicates that although no effort was made specifically to estimate the efficacy of the derivatisation process, the method of derivatisation is extremely efficient and reduction of histamine/1,7-DAH concentration present in the sample does not occur following dansylation. The use of this system to quantify histamine released from mast cells is clearly demonstrated in the determination of mast cell stabilising activity of novel compounds using the RPMC assay results, as discussed above (figures 4.14-4.20).

4.4.4 Relationship between LogP/LogD_{7.4} and RPMC Assay Activity

Group V compounds assessed here are highly lipophilic and investigations reveal that no relationship between PCA activity and computed theoretical LogP or LogD_{7.4} exists (Chapter 3). Investigations were therefore made to establish if a relationship exists between the RPMC activity and the computed LogP or LogD_{7.4} values of these Group V compounds. It is clear (figures 4.21–4.24) that although it appears a parabolic relationship may exist in certain instances, no relationship exists between the lipophilicity of these compounds and their RPMC activity at 20 μ M; all r^2 values fall below 0.39, for both LogP and LogD_{7.4}, in the presence of both elicitors. As with compounds in Chapter 2, these compounds were also evaluated for the existence of LogP or LogD_{7.4}/activity relationships at 1 μ M, however, as with compounds in Chapter 2, these compounds demonstrated extremely low r^2 values; even lower than those seen at 20 μ M (data not shown), reinforcing the fact that no relationship exists.

4.4.5 TZ2.13

The Group V compounds assayed here (TZ2.01-TZ2.05 and TZ2.11) appear to induce histamine release from mast cells, an effect which may be due to their ability to traverse the lipid bilayer, enter the cell and disrupt normal cell function. Therefore, TZ2.13 was synthesised with the aim of reducing the lipophilicity of these compounds. An acetic acid side chain was added to TZ2.01 (figure 4.06) to create a zwitterionic compound which would be unable to cross the cell membrane, thereby hopefully preventing the induction of histamine release from mast cells. RPMC results indicate that the addition of this charged species resulted in an increase in mast cell stabilising activity (TZ2.13 figure 4.20 compared to TZ2.01 figure 4.14), suggesting that the ability of Group V compounds to enter the cell is

the reason for their histamine eliciting activity. The activity of TZ2.13 in the presence of both calcium ionophore A23187 and compound 48/80 was at its most potent at 20 μ M ($88.62 \pm 7\%$ calcium ionophore A23187 and $76.52 \pm 5\%$ compound 48/80). At 40 μ M and 60 μ M in the presence of calcium ionophore, the mast cell stabilising activity of TZ2.13 was reduced ($8.40 \pm 9\%$) while in the presence of compound 48/80 TZ2.13 elicited the histamine release from mast cells ($143.20 \pm 19\%$). However, it is very encouraging to note that at concentrations of 20 μ M and lower, in the presence of both elicitors, the addition of a charged side chain augmented mast cell stabilising activity in Group V compounds.

The introduction of a carboxylic acid functional group side chain to TZ2.01 was carried out to create a zwitterionic compound which would reduce the lipophilicity of these Group V compounds. Virtual QSAR properties were calculated for TZ2.13 and were compared to those of TZ2.01. TZ2.13 demonstrates a slightly lower LogP value (6.25) than that of TZ2.01 (6.61). However, because these compounds ionise at pH 7.4, LogD_{7.4} is more indicative of their lipophilicity at physiological pH. TZ2.13 has a greatly reduced LogD_{7.4} value (1.38) than TZ2.01 (4.54), suggesting this compound is unable to traverse the lipid bilayer or penetrate the blood brain barrier. These results taken in concert with the increased PSA values of TZ2.13 ($64.80 \text{ \AA}^2$ TZ2.13 compared to $24.67 \text{ \AA}^2$ TZ2.01) indicate the aim of this synthesis was achieved and reinforce the improved RPMC assay results seen with TZ2.13 when compared to TZ2.01.

4.4.6 Do these Group V Compounds have an Alternate Mechanism of Action?

Group V compounds assayed here, with the exception of TZ2.13, elicited the release of histamine from mast cells; this was despite their potent inhibition of PCA (Chapter 3). The RPMC assay, while ideal for the *in vitro* study of mast cell stabilising activity of compounds, is a highly simplified system in comparison to the *in vivo* situation. While *in vitro* results indicate that mast cell degranulation should occur *in vivo* upon administration of test compound, PCA results indicate that these compounds do not demonstrate an overall mast cell degranulating response. These paradoxical findings lead to the question: if these compounds are causing the release of histamine from mast cells *in vitro*, by what means are they demonstrating such potent inhibition of PCA *in vivo*?

It is possible that these compounds possess a more potent alternate mechanism of action that accounts for their activity *in vivo*. As discussed in Chapter 1, mediators

released from activated mast cells interact with their specific receptors to cause the symptoms of allergy. Following PCA results it was believed that the activity of these compounds was due to their prevention of mast cell activation. It may be the case that these compounds do not influence mast cell activation but instead antagonise receptors to prevent mediator-receptor interaction from occurring. Because these compounds are potent in the PCA *in vivo* assay and because vascular permeability is mediated by the H1R, it is reasonable to hypothesise that these compounds may actually be H1R antagonists and may not be mast cell stabilisers at all.

Many studies indicate that a variety of compounds/agents demonstrate activity in experiments which aim to investigate inhibition of mast cell activation as well as those which investigate H1R antagonist activity. White rose petal extract (hexane fraction) demonstrates potent PCA activity, inhibits RBL-2H3 cell mediator release and also inhibits histamine-induced scratching behaviour in mice (Jeon, Kwon *et al.* 2009). Luteolin, a flavone found in plants such as celery, inhibits histamine- as well as compound 48/80- and PCA-induced scratching behaviour in mice (Baolin, Weiwei *et al.* 2005). VUF-K-8788, a novel anti-allergy compound, inhibits PCA in guinea pigs and rats, as well as histamine-induced contraction of guinea pig ileum (Takizawa, Matsumoto *et al.* 2001). *Ixeris dentate*, a herbal medicine used in Korea and China, fermented with lactic acid bacteria such as *Lactobacillus acidophilus*, inhibits PCA IgE-mediated degranulation of RBL-2H3 cells, as well as compound 48/80-induced scratching behaviour (Park, Sung *et al.* 2008). Similarly, curcumin inhibits PCA *in vivo* and RBL-2H3 degranulation *in vitro*, but also inhibits compound 48/80-induced scratching behaviour (Trinh, Bae *et al.* 2009). Persimmon leaf extract and astragalin inhibit histamine release from KU812 cells as well as PCA ear swelling and scratching behaviour in mice (Kotani, Matsumoto *et al.* 2000). These are all novel anti-allergy agents which demonstrate both mast cell stabilising and H1R antagonist activity in a variety of settings, therefore, it is reasonable to hypothesise that our compounds which appear to demonstrate mast cell stabilising activity *in vivo*, may possess H1R antagonist activity.

It is also interesting to note that several established H1R antagonists demonstrate activity in mast cell-mediated experiments. Compounds discussed here are not dual mast cell stabilising/H1R antagonising compounds such as ketotifen, but are considered pure H1R antagonists. Ebastine and cetirizine, non-sedating H1R antagonists, both demonstrate activity in the PCA assay (Yakuo, Yabuuchi *et al.* 2001). Chronic administration of chlorphenamine, a first-generation H1R antagonist, effectively inhibits CEA IgE-mediated nasal scratching following topical

administration of antigen (CEA) (Tsumuro, Ogawa *et al.* 2005). Azelastine and astemizole, second-generation H1R antagonists, both inhibit IgE-mediated peritoneal mast cell mediator release *in vitro* (Chand, Pillar *et al.* 1985), azelastine demonstrates potent PCA activity (Chand, Harrison *et al.* 1985) and also inhibits compound 48/80-induced scratching behaviour in mice as well as RPMC degranulation induced by compound 48/80 (Shin, Bae *et al.* 2007). Chlorphenamine and oxatamide, both first-generation H1R antagonists, and terfenadine and astemizole, both second-generation H1R antagonists all prevent compound 48/80-induced scratching behaviour in the mouse (Sugimoto, Umakoshi *et al.* 1998) and terfenadine and cetirizine inhibit PCA as well as compound 48/80 and histamine-induced scratching and vascular permeability in mice (Inagaki, Nakamura *et al.* 1999). Mepyramine, a classic first-generation H1R antagonist, demonstrates activity in PCA *in vivo* assays (Braga and Mota 1976) Bepotastine, a second-generation H1R antagonist, has mast cell stabilising activity *in vivo* (Williams, Gow *et al.* 2010).

Many novel anti-allergy agents demonstrate mast cell stabilising as well as H1R antagonising activity in a variety of experimental settings. Also a number of H1R antagonists demonstrate activity in protocols investigating mast cell stabilising activity. Therefore, it was necessary to evaluate these Group V compounds which demonstrate potent PCA activity, while eliciting mast cell mediators *in vitro*, to determine if they possess H1R antagonist activity. This was carried out with a view to establishing a possible alternative mechanism.

4.5 Conclusions

The primary aim of this work was to establish a method for the quantification of histamine using LCMS. Derivatisation of histamine was carried out using DC which allowed for robust measurement of this analyte. MS/MS allowed for the detection of DH and D-1,7-DAH fragments. Full validation of this method was carried out according to EMA guidelines. This method complies with validation criteria at the concentrations assessed above. As discussed previously, this method is a novel method which allows for the high throughput screening of mast cell stabilising compounds. Similar methods using large numbers of mast cell derived samples have not been carried out. While other methods, such as OPT, exist for the quantification of histamine release from mast cells, the sensitivity and accuracy that LCMS provides means that this is a highly favourable method. Therefore, this novel and useful method for the determination of mast cell stabilising activity of novel compounds may be a highly useful tool in this field of research.

While it may be considered that 40 ng/ml is a relatively high LLOQ, it is important to note that this method was validated for the application of quantifying mast cell histamine release in the RPMC assay, as carried out here. Future studies may include investigation into using this method at lower concentrations.

Upon validation of the LCMS method, seven compounds (TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05, TZ2.11 and TZ2.13) were assessed to determine their *in vitro* mast cell stabilising activity. Overall, results obtained were disappointing; these compounds despite their apparent mast cell stabilising activity *in vivo* did not stabilise mast cells in the RPMC assay, in the presence of calcium ionophore A23187 and compound 48/80, except at very low concentrations. TZ2.13, a zwitterionic compound which is based on TZ2.01, demonstrated improved mast cell stabilising activity when compared with other Group V compounds assayed here.

Because many substances which demonstrate PCA inhibitory activity also demonstrate activity in H1R experiments, as discussed above, it is possible that these *in vivo* active compounds are eliciting their *in vivo* PCA effect not by stabilising mast cells, but instead by antagonising histamine at the H1R. Investigations into the H1R antagonising activity of these compounds will be discussed in the next chapter, with a view to explaining the PCA *in vivo* activity of these compounds.

Chapter 5

Studies on the H₁R Antagonist and Anti Pruritic Activities of Novel Compounds

5.1 Introduction

Although TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05 and TZ2.11 demonstrated potent inhibition of PCA, results obtained using LCMS to determine their *in vitro* activity indicate that these compounds elicit histamine release from mast cells. TZ2.13, a zwitterionic compound which was synthesised to reduce the lipophilicity of these compounds, demonstrates improved mast cell stabilising activity in the RPMC assay.

Because many classical H1R antagonists demonstrate some level of activity in mast cell-mediated experiments as discussed in the previous chapter and because several novel agents demonstrate activity in both mast cell and H1R-mediated experiments, the above compounds must be evaluated to determine if they possess H1R antagonist activity.

TZ1.04 demonstrates potent mast cell stabilising activity in the presence of calcium ionophore A23187 and compound 48/80 in the RPMC assay (Chapter 2) and also demonstrates *in vivo* inhibition of PCA equivalent to the activity of DSCG (Chapter 3). It was therefore decided to evaluate this compound (in the place of TZ2.11) to determine if it contains H1R antagonist activity and therefore, a dual activity, or if its PCA activity is due to its mast cell stabilising activity alone.

5.1.1 Aims:

Because it was necessary to determine the H1R antagonist activity of TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05 and TZ1.04 the aims of this work were;

- To carry out a screen on these compounds using the *ex vivo* guinea pig (GP) ileum protocol for H1R antagonist activity.
- To evaluate compound TZ2.05 for its *in vivo* anti-atopic pruritic activity using the scratching behaviour protocol.

5.1.2 Histamine Receptor 1 Antagonist Activity

As discussed in Chapter 1 histamine exerts its effect on its specific receptors (H1R, H2R, H3R and H4R) upon mast cell degranulation. The H1R is implicated in the onset of familiar symptoms of allergy such as urticaria (Hein 2002; Belsito 2010), runny nose (Hampel, Martin *et al.* 1999), sneezing (Kayasuga, Sugimoto *et al.* 2002), nasal blockage (Taylor-Clark, Sodha *et al.* 2005), pruritis (Hossen, Fujii *et al.* 2005) including nasal pruritis (Tsumuro, Ogawa *et al.* 2005) and ocular pruritis (Fujishima, Fukagawa *et al.* 2006) and bronchoconstriction which occurs during an asthma attack (Albazzaz and Patel 1988). As discussed, blockage of the H1R by

H1R antagonists results in a decrease in these symptoms, an approach which is frequently used in the treatment of allergy.

Because the H1R is also located post synaptically in the brain hypothalamus and other limbic regions where it is involved in the modulation of circadian rhythm (Long, Sprung *et al.* 1985), first-generation H1R antagonists which can permeate the blood brain barrier interfere with sleep/wake cycles to cause drowsiness. This led to the development of newer H1R antagonists which do not penetrate the CNS and therefore do not have this effect.

5.1.3 Guinea Pig Ileum *Ex Vivo* Protocol for H1R Antagonist Activity

The GP ileum *ex vivo* protocol has long been used to determine receptor antagonist activity for a variety of biological molecules including serotonin and acetylcholine (Adebiyi, Adiakan *et al.* 2004). Although differences exist between the human and GP H1R (Seifert, Wenzel- Seifert *et al.* 2003), the GP ileum protocol has also long been used for the determination of histamine H1R antagonist activity (Takizawa, Matsumoto *et al.* 2001; Shah and Parmar 2003; Pertz, Gornemann *et al.* 2006). The GP ileum does not contain H2Rs which cause smooth muscle contraction (Leurs, Brozius *et al.* 1991), therefore, administration of histamine results in H1R-mediated ileum contraction (Corcostegui, Labeaga *et al.* 2005; Pertz, Gornemann *et al.* 2006).

Firstly, a response to histamine is recorded in the absence of test compound for each ileum segment and following equilibration time this is repeated in the presence of a test compound. Any reduction in contractile response upon administration of histamine which occurs in the presence of test compound is therefore due to the H1R antagonising actions of the compound. Mepyramine was chosen for use as control compound in the GP ileum H1R antagonist *ex vivo* experiment because it is a H1R antagonist which demonstrates potent H1R antagonist activity in this protocol (Elz, Kramer *et al.* 2000; Adebiyi, Adiakan *et al.* 2004). Ileum segments are also incubated in the presence of vehicle alone, in the place of test compound and any response observed will be due not only to vehicle treatment, but will also be due to a reduction in responsiveness of the tissue which occurs over time due to fatigue and time spent *ex vivo*. It is not necessary to separate out the vehicle and fatigue responses because all test compounds are evaluated in the vehicle. Mean values obtained for vehicle treated segments are subtracted from compound treated values during analysis to remove the vehicle and fatigue responses to allow for evaluations on compound treated responses alone to be carried out.

5.1.4 Scratching Behaviour

In vivo scratching behaviour can be induced using serotonin, histamine (Jeon, Kwon *et al.* 2009), compound 48/80 (Trinh, Bae *et al.* 2010), passive cutaneous anaphylaxis (Inagaki, Nakamura *et al.* 1999) and substance P (Inagaki, Nagao *et al.* 2000) and accordingly is used to determine the receptor antagonist as well as the anti-pruritic activity of test compounds, although mouse strain seems to be important when deciding which stimulus to use (Inagaki, Nagao *et al.* 2001).

Interestingly, H1R antagonists are not only effective at reducing histamine-induced scratching behaviour, but also compound 48/80 and PCA-induced (Inagaki, Nakamura *et al.* 1999), as well as substance P-induced scratching behaviour (Inagaki, Nagao *et al.* 2000).

Studies carried out into the mechanisms of scratching/pruritis have shown it occurs because of a complex variety of interactions between many mast cell mediators and their respective receptors. H1R-deficient mice demonstrate reduced, but not abolished, scratching behaviour response upon induction of scratching using PCA while compound 48/80 also induces scratching in these mice (Sugimoto, Nakamura *et al.* 2003), indicating histamine is not the sole mediator responsible for the induction of pruritis. Substance P (Andoh, Nagasawa *et al.* 1998) and LTB₄ (Andoh and Kuraishi 1998; Tsuji, Aono *et al.* 2010) can induce scratching behaviour, while mast cell tryptase via activation of proteinase-activated receptors 2 is also implicated in scratching behaviour (Yoshizaki and Sato 2009).

Although histamine-induced scratching behaviour is routinely used to determine H1R antagonist activity, the implication of H2Rs in histamine-induced scratching behaviour (Inagaki, Nakamura *et al.* 1999) and the complex mechanism of pruritis discussed above means the use of histamine to induce scratching behaviour would not conclusively determine H1R antagonist activity. It was therefore decided to evaluate test compounds for their anti-atopic pruritic activity using compound 48/80-induced scratching behaviour.

5.1.4.1 Compound 48/80-Induced Scratching Behaviour

Subcutaneous administration of compound 48/80 induces mast cell degranulation and results in pruritis. Therefore, compound 48/80-induced scratching behaviour is routinely used to determine the anti-allergy/anti-pruritic activities of novel compounds (Sugimoto, Umakoshi *et al.* 1998; Shin, Bae *et al.* 2001; Baolin, Weiwei *et al.* 2005; Lee, Shin *et al.* 2008; Tanizaki, Kambe *et al.* 2008; Trinh, Bae *et al.* 2009). Terfenadine, a second-generation antihistamine, was chosen for use as

control compound because it inhibits compound 48/80-induced scratching behaviour (Sugimoto, Umakoshi *et al.* 1998).

5.2 Materials and Methods

5.2.1 Materials

Atropine, CaCl_2 , Hp- β -cyclodextrin, *N-N*-dimethylacetamide, glucose, histamine, KCl, mepyramine, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, NaCl, NaHCO_3 and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ were purchased from Sigma Aldrich, Arklow, Co. Wicklow, Ireland. Na-Nu Vitality[®] maple syrup was purchased from Tesco Ireland, Greystones, Co. Wicklow, Ireland.

5.2.2 Animals

Female outbred guinea pigs (400 g) and female CD1 mice (20 g) were provided by the BioResources Unit, Trinity College Dublin. Animals were housed at environmentally controlled conditions at 22°C with 12 hour light and 12 hour dark cycles. Animals had *ad libitum* access to food and water. Guinea pigs were sacrificed using CO_2 asphyxiation. Upon completion of the experiment CD1 mice were donated to the BioResources Unit, Trinity College Dublin.

5.2.3 Buffers

Tyrode's Buffer for guinea pig ileum experiment; NaCl 136.9 mM, KCl 2.68 mM, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.42 mM, NaHCO_3 11.9 mM, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1.05 mM, CaCl_2 1.8 mM, Glucose 5.5 mM.

5.2.4 Guinea Pig Ileum *Ex Vivo* Protocol for Histamine Receptor Antagonist Activity

Ileum was excised and washed gently with Tyrode's buffer, continuously gassed with 95% O_2 and 5% CO_2 , to remove any contents and were then cut into 1.5 cm segments. Tissue preparations were mounted under resting tension of 1 g in 10 ml jacketed organ baths containing Tyrode's buffer, to which atropine (0.01 mM) had been added to prevent spontaneous muscarinic acetylcholine receptor activity. Organ bath preparations were continuously gassed with 95% O_2 and 5% CO_2 and maintained at 37 °C.

Tissues were allowed to equilibrate for 45 minutes, during which time buffer was changed regularly. Following equilibration, a cumulative concentration (the addition of consecutive doses without washout of the previous doses) response to histamine, ranging in concentration from 10 nM to 1.3 mM, was administered every 30 seconds. Preparations were then washed three times with Tyrode's buffer containing atropine and again allowed to equilibrate for a further 30 minutes, while

changing buffer regularly. Following this second equilibration test compounds (10 μ M or 1 μ M in 1% DMSO in Tyrode's buffer) were added to the organ bath and were left for 30 minutes, without washing, to allow interaction with the H1R. Following drug incubation, addition of the cumulative concentration response curve to histamine was repeated, in the presence of test compounds. Mepyramine, a first-generation H1R antagonist, was used as control compound. Only one concentration of test compound was tested on each tissue segment.

Compounds were first assessed at 10 μ M (TZ2.01 (n=5), TZ2.02 (n=4), TZ2.03 (n=5), TZ2.04 (n=5), TZ2.05 (n=5), TZ1.04 (n=5) and mepyramine (n=5)). Those compounds which demonstrate activity at 10 μ M were evaluated at 1 μ M (TZ2.01 (n=5), TZ2.04 (n=5) and Mepyramine (n=5)). To ensure viability of segments over the duration of the experiment, ileum segments were also assayed for their response to histamine in the presence of vehicle (n=8) alone.

All ileum contraction activity was recorded using an isometric transducer connected to a pen, traces were later counted and analysed.

5.2.5 Compound 48/80-Induced Scratching Behaviour

One female CD1 mouse was placed in an observation cage and allowed to acclimatise for ten minutes. Eight animals were used for each treatment group; a) compound 48/80 injected, b) saline injected, c) vehicle (2:1:1; maple syrup:20% Hp- β -cyclodextrin in 0.9% saline (added drop by drop):*N-N*-dimethylacetamide) treated, d) terfenadine (30 mg/kg) treated and TZ2.05 ((e) 15, (f) 30 and (g) 60 mg/kg) treated.

Animals were scruffed and compound 48/80 (50 μ g/50 μ l in 0.9% w/v saline) or saline (for saline-treated control animals) was injected subcutaneously into the rostral part of the back. Test compound TZ2.05 was prepared in vehicle and administered orally at 15, 30 or 60 mg/kg 4 hours before compound 48/80 injection. Control compound, terfenadine, was also prepared in vehicle and administered orally (30 mg/kg) 1 hour prior to compound 48/80 injection, as carried out by Sugimoto *et al.* (Sugimoto, Umakoshi *et al.* 1998). For vehicle treated control animals vehicle was administered 4 hours before compound 48/80 injection. Scratching incidences were recorded using a Mini DV Canon video camera and animals were observed for 60 minutes. Scratching incidences at the injection site with hind legs were counted for each animal.

5.2.6 Analysis of Data

Traces obtained following the GP ileum protocol for H1R antagonist activity were counted. All data was normalised to 100% of the maximal response. Each experiment has two results; a) the histamine cumulative concentration response

curve in the absence of test compound and b) histamine cumulative concentration response curve in the presence of test compound. Vehicle treated value was subtracted from each drug treated segment to establish the true drug effect. For each treatment group curves which were obtained in the absence of test compound (no antagonist) were combined and averaged, curves obtained in the presence of test compound were also combined and averaged. Antagonist activity is determined when a significant reduction in the histamine cumulative concentration response occurs in the presence of test compound.

Scratching behaviour incidences were counted and combined for each treatment group.

5.2.7 Statistics

Statistical analysis of the data was carried out using GraphPad Prism, version 5.

The difference between histamine cumulative concentration response curves in the absence and in the presence of test compound were examined following GP ileum H1R antagonist studies. Data is expressed as % of maximum response (mean $\pm$ SEM) at each concentration of agonist (histamine) following transformation and normalisation of data. Results were analysed for Gaussian distribution using the D'Agostino and Pearson omnibus test for normality and because all data did not display Gaussian distribution and therefore did not vary normally, results were analysed for significance using the Friedman test. The Friedman test is a non-parametric, repeated measures statistical test which compares the means of three or more matched groups and is similar to the one way, repeated measures ANOVA parametric test. Following the Friedman test the data was analysed to compare columns of data using the Bonferroni-Dunn Multiple Comparison post hoc test. The Bonferroni-Dunn Multiple Comparison test is used to compare the difference in the sum of ranks between two columns with the expected average difference.

Scratching incidences for each treatment group are expressed as total scratching incidences (mean $\pm$ SEM). Results were analysed for Gaussian distribution using the D'Agostino and Pearson omnibus test for normality and because some treatment groups did not display Gaussian distribution and therefore did not vary normally, results were analysed for significance the Mann Whitney U test for non-parametric distribution. The Mann Whitney U test compares the distributions of two unmatched groups.

5.3 Results

5.3.1 Guinea Pig Ileum *Ex Vivo* H1R Antagonist Study

The H1R antagonising activity of TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05 and TZ1.04 were determined using the guinea pig ileum protocol for H1R antagonist activity. Mepyramine was used as a control substance. Several of these novel compounds, TZ2.01 (figure 5.02), TZ2.04 (figure 5.04) and TZ2.05 (figure 5.05) demonstrated H1R antagonist activity at 10 μ M, while TZ2.01 maintained activity at 1 μ M.

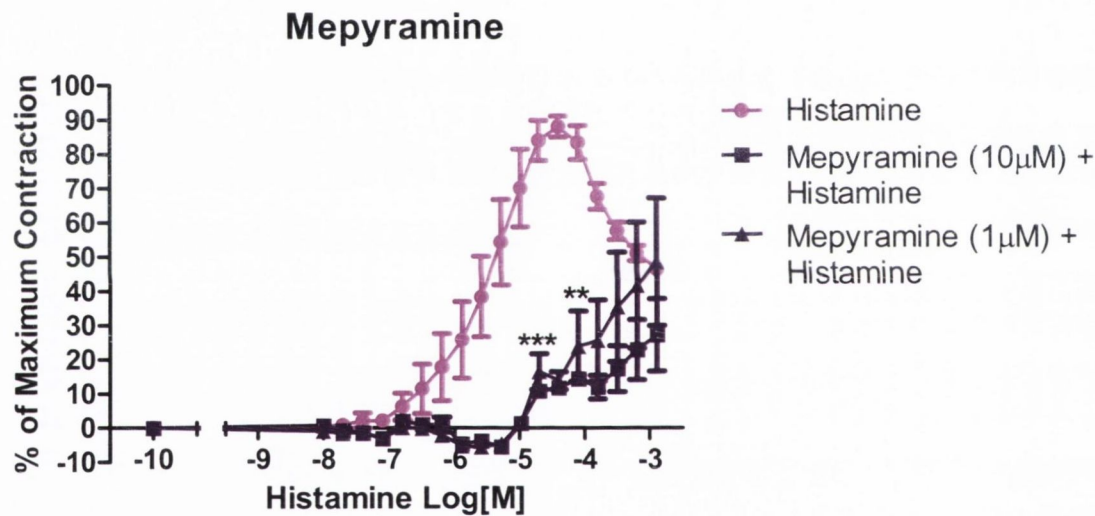


Figure 5.01: H1R antagonist activity of mepyramine, measured by inhibition of histamine-induced ileum contraction. *** $P < 0.001$, 10 μ M and *** $P < 0.01$, 1 μ M. Friedman test followed by Bonferroni-Dunn Multiple Comparison post hoc test

TZ2.01

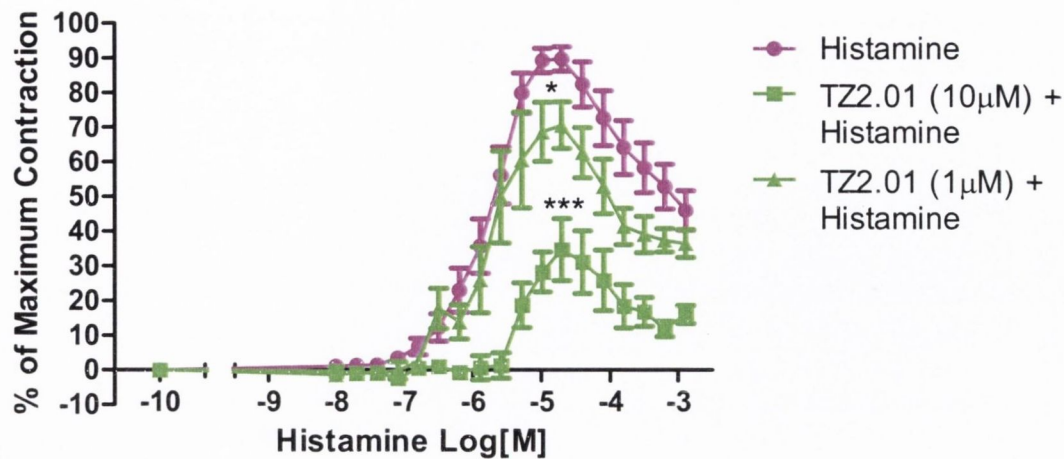


Figure 5.02: H1R antagonist activity of TZ2.01 (10 μ M and 1 μ M), measured by inhibition of histamine-induced ileum contraction. *** $P < 0.001$, 10 μ M and * $P < 0.05$, 1 μ M. Friedman test followed by Bonferroni-Dunn Multiple Comparison post hoc test

TZ2.02 and TZ2.03

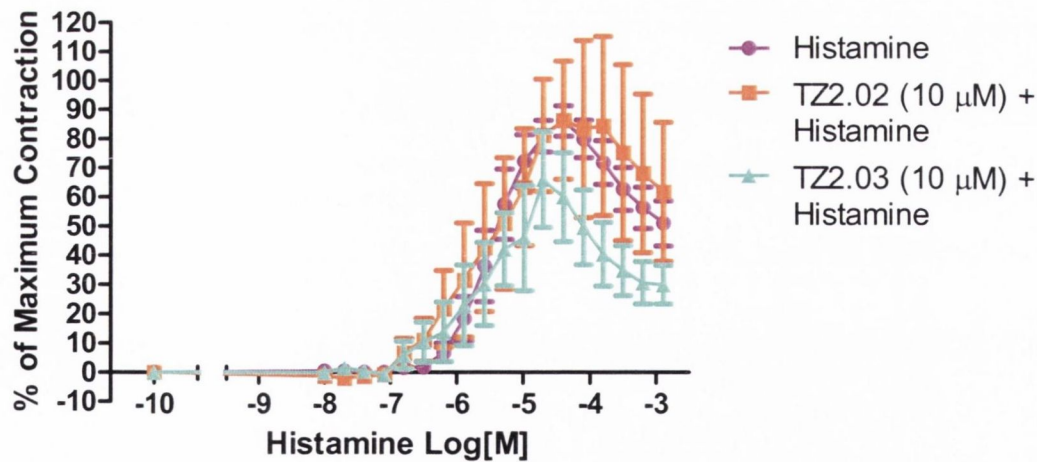


Figure 5.03: H1R antagonist activity of TZ2.02 and TZ2.03 (both at 10 μ M), measured by inhibition of histamine-induced ileum contraction. No Significance TZ2.02 or TZ2.03. Friedman test followed by Bonferroni-Dunn Multiple Comparison post hoc test

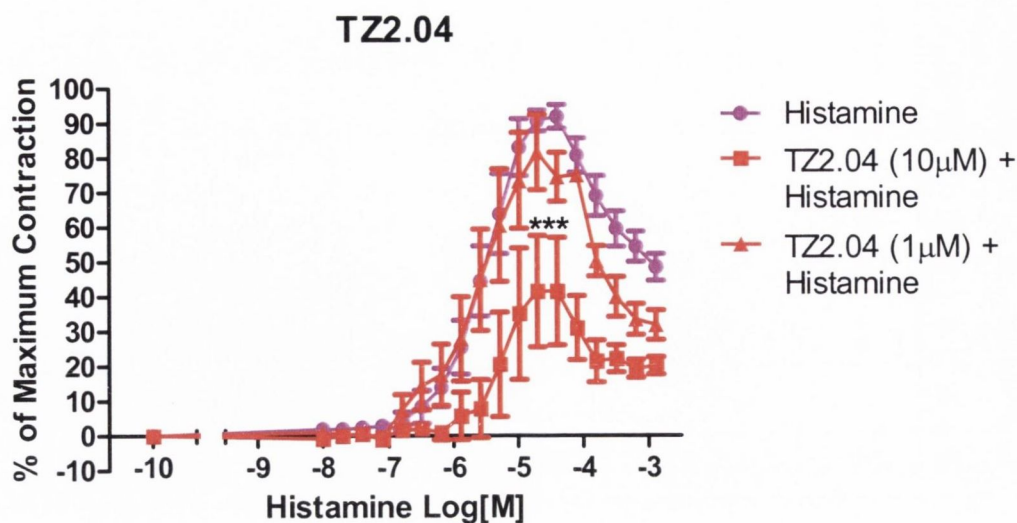


Figure 5.04: H1R antagonist activity of TZ2.04 (10 μ M and 1 μ M), measured by inhibition of histamine-induced ileum contraction. *** $P < 0.001$, 10 μ M. Friedman test followed by Bonferroni-Dunn Multiple Comparison post hoc test

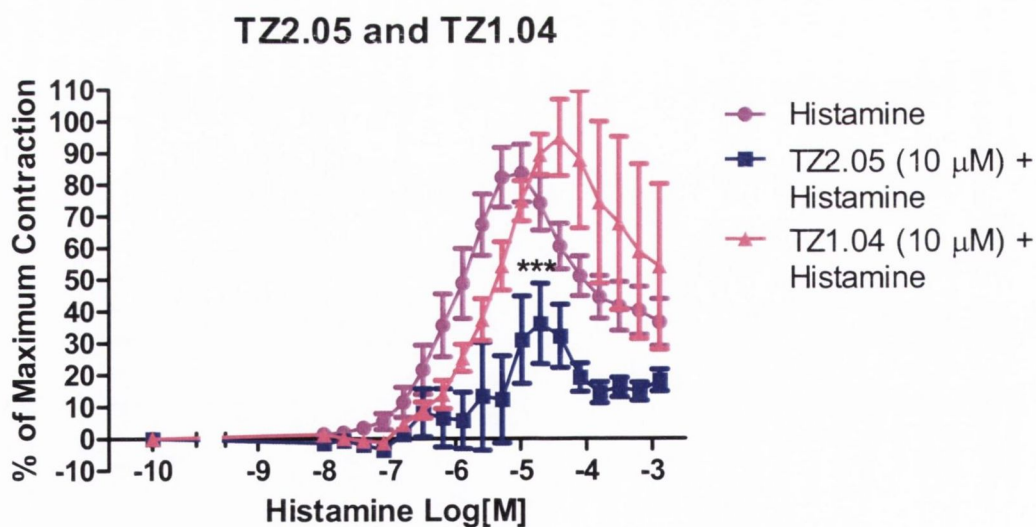


Figure 5.05: H1R antagonist activity of TZ1.04 and TZ2.05 (both at 10 μ M), measured by inhibition of histamine-induced ileum contraction. *** $P < 0.001$ TZ2.05, 10 μ M. Friedman test followed by Bonferroni-Dunn Multiple Comparison post hoc test

5.3.2 Compound 48/80-Induced Scratching Behaviour

Following the determination that TZ2.05 possessed H1R antagonist activity, this compound was evaluated for anti-pruritic activity using compound 48/80-induced scratching behaviour. Although TZ2.05 did not demonstrate anti-pruritic activity at 15 or 30 mg/kg, it did significantly prevent the number of compound 48/80-induced scratching events when administered (P.O.) at 60 mg/kg (figure 5.06).

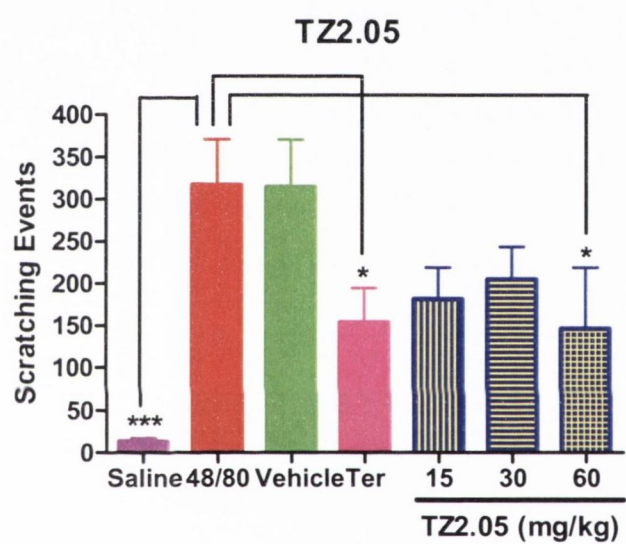


Figure 5.06: Effect of TZ2.05 (15, 30 and 60 mg/kg) on dorsal scratching by histamine in CD1 mice. Ter; terfenadine 30 mg/kg. Each bar represents mean ± SEM (n=8). * $P < 0.5$, *** $P < 0.01$ Mann Whitney U test

5.4 Discussion

5.4.1 Guinea Pig Ileum *Ex Vivo* Protocol for Histamine Receptor 1 Antagonist Activity

Results obtained in the GP ileum *ex vivo* protocol for H1R antagonist activity indicate that TZ1.04 possesses no H1R antagonist activity (figure 5.05) when evaluated at 10 μ M. This suggests that the potent inhibition of PCA demonstrated by TZ1.04 (Chapter 3) is due to mast cell stabilising activity (Chapter 2) alone and is not due to dual H1R antagonist/mast cell stabilising activity. Therefore, TZ1.04 seems to be a potent *in vivo* mast cell stabilising compound.

TZ2.02 and TZ2.03 (figure 5.03) also have no H1R antagonist activity when assayed at 10 μ M. Both of these compounds demonstrate inhibition of PCA (Chapter 3), although they are ineffective at inhibiting the release of histamine from rat peritoneal mast cells (Chapter 4). It seems that both of these compounds are neither mast cell stabilising nor H1R antagonising at the concentrations assessed in this study.

TZ2.01 (figure 5.02), TZ2.04 (figure 5.04) and TZ2.05 (figure 5.05) all demonstrate H1R antagonist activity at 10 μ M, although this significance is lost at 1 μ M for both TZ2.01 and TZ2.04. It is clear from the results in the previous chapter (Chapter 4) that these compounds are not mast cell stabilising compounds therefore their potent *in vivo* inhibition of PCA (Chapter 3) may be due, at least in part, to their H1R antagonist activity observed here. Because these compounds cause a downward shift in response compared to agonist alone, while not reaching maximal response, it may be concluded these compounds seem to be non-competitive H1R antagonists.

Although several compounds assessed here demonstrate H1R antagonist activity (TZ2.01, TZ2.04 and TZ2.05), their activity is not as potent as that of mepyramine which maintains significant H1R antagonist at 1 μ M (figure 5.01). Although mepyramine, a competitive H1R antagonist (Martinez, Novella *et al.* 1997), causes a shift in response to agonist to the right, it does not reach maximal concentrations. This indicates that perhaps increased concentrations of both mepyramine and histamine are needed to allow histamine exert its effect and return the response of mepyramine to 100% the response of histamine, as seen with competitive antagonists.

The results obtained in the GP ileum *ex vivo* protocol for H1R antagonist activity indicate that 2 classes of lead compounds have emerged;

- 1) TZ2.01, TZ2.04 and TZ2.05 which are H1R antagonists, thus explaining their inhibition of PCA. These compounds are not mast cell stabilisers and
- 2) TZ1.04, which has no H1R antagonising activity, indicating inhibition of PCA is due to its mast cell stabilising activity alone.

5.4.2 Outsourced Studies; Histamine Receptor 1 Binding and Histamine Receptor 1 GTP γ S Binding Studies

Although TZ2.04 was evaluated at 10 μ M and 1 μ M and TZ2.05 was only evaluated at 1 μ M, consensus agreed at this stage that TZ2.04 most likely degrades to form TZ2.05, it is thought that the alcohol group present on TZ2.04 naturally degrades to the more stable carbon-carbon double bond of TZ2.05; therefore, any TZ2.04 sample assessed would possibly contain two closely related, but distinct compounds. To resolve this issue it was decided to evaluate TZ2.05 in any future work to ensure that at least only one compound would be present during each experiment.

GP ileum *ex vivo* evaluation for H1R antagonist activity revealed that a number of these novel compounds (TZ2.01, TZ2.04 and TZ2.05) possess H1R antagonist activity. TZ2.05 and TZ2.14 (structurally related to TZ2.01, as TZ2.04 is related to TZ2.05) were sent for H1R binding studies to MDS Pharma Services (Now Ricerca Biosciences), Taiwan (see Appendix for results). Compounds were evaluated in a low number of experiments (n=2) with a view to establishing if activity exists rather than obtaining statistically significant results.

Compounds were first tested in Chinese Hamster Ovary (CHO-K1) cells which were transfected with the human H1R gene. Mepyramine binds to the H1R with high affinity (Ison, Franks *et al.* 1973) and TZ2.05 demonstrated 94% inhibition of specific [3 H]mepyramine binding while TZ2.14 demonstrated 92% inhibition of specific [3 H]mepyramine binding when both compounds were tested at 10 μ M, indicating an affinity for and binding at the H1R by these compounds. This does not indicate whether the compound is an agonist or an antagonist only that it binds to the receptor protein. Although GP ileum results indicate that TZ2.01, TZ2.04 and TZ2.05 are non-competitive antagonists, these results suggest their antagonism may be competitive in nature due to the fact they inhibit specific mepyramine binding at the histamine binding site. It is also possible of that these compounds

are non-competitive and allosterically alter the conformation of the receptor to prevent mepyramine binding at the histamine binding site therefore resulting in its displacement.

TZ2.05 and TZ2.14 were also assessed for inhibition of histamine-induced GTP γ S receptor binding using CHO-K1 cells transfected with the human H1R gene. Activation of the H1R by histamine induces intracellular GTP γ S binding and subsequent intracellular signaling. Inhibition of histamine-induced binding of radiolabelled GTP γ S to the receptor indicates that interaction of test compound with H1R has occurred to antagonise receptor activation and prevent histamine-induced GTP γ S binding. Results were considered significant when $\geq 50\%$ inhibition of histamine-induced bound [35 S]GTP γ S was found. TZ2.05 showed 85% inhibition while TZ2.14 showed 67% inhibition of histamine-induced bound [35 S]GTP γ S when they were tested at 10 μ M (n=2).

Although a low number of experiments were carried out in these studies, results suggest a reaffirmation of the H1R antagonist activity observed in the GP ileum protocol discussed above.

5.4.3 Compound 48/80-Induced Scratching Behaviour

GP ileum *ex vivo* results, preliminary data obtained from MDS Pharma Services and the fact that H1R and H2R receptors are involved in histamine-induced scratching behaviour informed the decision to evaluate TZ2.05 for *in vivo* anti-pruritic activity, using compound 48/80 to stimulate scratching due to the induction of localised mast cell degranulation. This was instead of assessing *in vivo* H1R antagonist activity using scratching behaviour.

Results obtained for compound 48/80-induced scratching behaviour in mice indicate that TZ2.05 (administered P.O.) demonstrates no anti-pruritic activity at 15 or 30 mg/kg. However, anti-pruritic activity was achieved at 60 mg/kg (figure 5.06) and this activity is comparable to that of terfenadine administered at 30 mg/kg (P.O.). This suggests that TZ2.05 displays anti-pruritic activity comparable to terfenadine in this experimental setting.

Terfenadine (0.1-10 mg/kg) significantly inhibits scratching behaviour in ICR mice, however this activity was observed when a very low dose of compound 48/80 (only 3 μ g) was administered to induce scratching, cetirizine (0.1-10 mg/kg) is also effective at this dose (Inagaki, Nakamura *et al.* 1999). Later, this group found that terfenadine and also ketotifen at doses of 0.1-10 mg/kg do not significantly inhibit

scratching behaviour in BALB/c mice when compound 48/80 was given at a dose of 100 µg (Inagaki, Igeta *et al.* 2002). Sugimoto *et al.* found that terfenadine (1, 3 and 10 mg/kg), astemizole (3, 10 and 30 mg/kg) and azelastine (1, 3 and 10 mg/kg) as well as chlorpheniramine (3 and 10 mg/kg) all inhibit compound 48/80-induced scratching behaviour in BALB/c mice while DSCG (30-300 mg/kg) is ineffective (Sugimoto, Umakoshi *et al.* 1998). Terfenadine also does not significantly inhibit PCA-induced scratching behaviour at 100 mg/kg (Takizawa, Matsumoto *et al.* 2001). It must be noted that dose and strain can influence results obtained using scratching behaviour (Green, Young *et al.* 2006).

Historically, terfenadine was prescribed for the treatment of allergic rhinitis and allergic skin conditions. However, because it did not display a favourable side effect profile the Irish Medicines Board (IMB) changed the status of terfenadine from over the counter to prescription only because of its potential to cause cardiac arrhythmia when used in combination with other drugs such as erythromycin and ketoconazole (IMB 1997). It was recommended that patients with cardiac or hepatic disease should therefore not use this product. At the end of 1998, following a review carried out by the IMB, along with the EU Committee for Proprietary Medicinal Products, terfenadine was withdrawn from all EU markets (IMB 1999).

As stated above, TZ2.04 degrades to form TZ2.05, a compound with an identical structure to that of TZ2.04, with the exception of the presence of a carbon-carbon double bond in the place of the alcohol functional group in TZ2.04. It is therefore encouraging that TZ2.05 demonstrates potent anti-pruritic activity, comparable to terfenadine in this setting, while TZ2.04 demonstrates a favourable side effect profile when evaluated using the SHIRPA behavioural observation protocol. This would suggest that if evaluated TZ2.05 would perhaps display the same profile as TZ2.04. The side effects observed upon evaluation of TZ2.05 may be favourable in comparison to terfenadine and therefore this compound may be viable for future use for the treatment of allergic pruritis in humans. It would be desirable to evaluate TZ2.05 in the SHIRPA behavioural observation protocol as well as in other toxicity and cytotoxicity studies.

Interestingly, although terfenadine causes severe cardiac arrhythmias, fexofenadine, the pharmacologically active metabolite of terfenadine, displays a much more favourable side effect profile and these cardiac effects are not observed upon its treatment (DuBuske 1999). Fexofenadine (Telfast; Sanofi-Aventis) is therefore currently used to treat allergic rhinitis and urticaria. Fexofenadine (2, 5 and 10 mg/kg) however does not inhibit histamine- or PCA-induced scratching behaviour in

ICR mice when given 2 hours pre-challenge (Hossen, Fujii *et al.* 2005) and fexofenadine (3, 10 and 30 mg/kg) also does not inhibit compound 48/80-induced scratching behaviour (20 µg compound 48/80/site) in ddY mice (Kobayashi, Takahashi *et al.* 2005). This would suggest that TZ2.05, having anti-pruritic activity, may be desirable for use as an alternative to fexofenadine, as well as terfenadine.

As discussed in Chapter 3, studies carried out by Charles Rivers (see appendix) indicate the bioavailability of TZ2.05 is approximately 57% P.O.; therefore, the bioavailable dose which is effective here, upon administration of 60 mg/kg P.O., is 34.2 mg/kg. The activity of TZ2.05 in the compound 48/80 induced scratching behaviour assay, when compared with that of terfenadine and its active metabolite fexofenadine, as well as with the unfavourable side effects of terfenadine indicate strongly that TZ2.05 should be further evaluated with respect to its anti-pruritic anti-allergic activity. At this stage this compound is a strong candidate for future use in the treatment of human allergic conditions.

5.5 Conclusions

The aims of this chapter were to evaluate TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05 and TZ1.04 for H1R antagonist activity and also to evaluate TZ2.05 for anti-pruritic activity. These compounds have been shown to possess *in vivo* PCA (TZ2.01, TZ2.02, TZ2.03, TZ2.04 and TZ2.05) or *in vitro* mast cell stabilising activity (TZ1.04). Results from the GP ileum H1R antagonist study reveal that TZ2.01, TZ2.04 and TZ2.05 all possess some degree of non-competitive H1R antagonist activity at concentrations evaluated.

Results obtained from MDS Pharma Services confirm that TZ2.05 has affinity for and antagonises the human H1R in a transfected cell line. TZ2.14, which is structurally related to TZ2.01, was also evaluated and was shown to possess similar activity. These results, although carried out in low numbers, suggest a reinforcement of *ex vivo* GP ileum protocol findings.

Investigation was carried out into the *in vivo* anti-pruritic activity of TZ2.05, a compound which to this point demonstrates potent *in vivo* PCA activity, *ex vivo* H1R non-competitive antagonism, as well as human H1R affinity and antagonism in cell culture. Although this compound does not demonstrate any anti-atopic pruritis activity at lower doses, at a higher dose (60 mg/kg, P.O.) TZ2.05 demonstrates anti-pruritic activity; this corresponds to a bioavailable dose of 34.2 mg/kg. TZ2.05 may be effective in the relief of symptoms of allergic atopic dermatitis and other allergic diseases.

The work carried out in this chapter indicates that two distinct lead compound classes have emerged.

- 1) H1R antagonists which have no mast cell stabilising activity; TZ2.01, TZ2.04 and TZ2.05. These compounds are active in the PCA assay and this activity may be explained by their H1R antagonist activity and
- 2) Mast cell stabilisers which have no H1R antagonist activity; TZ1.04. This compound demonstrates PCA activity which may be due to the mast cell stabilising activity it demonstrated *in vitro*.

Further investigation into the mechanism of action of these compounds should ideally be carried out to determine the exact use for these compounds and suggested further studies are discussed in Chapter 6. At this point, findings are encouraging; work carried out and presented above has determined that two

classes of anti-allergy compound which warrant further investigation were synthesised in this lab.

Chapter 6

Conclusions and Future Studies

6.1 Conclusions

The primary aim of this project was to evaluate the anti-allergy activity of several groups of novel benzocycloalkanone based compounds. Test compounds under investigation were evaluated in a variety of *in vitro* and *in vivo* assays.

Initial studies investigating the *in vitro* mast cell stabilising activity of these novel compounds were carried out using Percoll purified RPMCs. Histamine released from mast cells was quantified by fluorescence following its derivatisation with OPT. Results obtained were very encouraging: several Group I (DF1.03, DF1.05 and DF1.06), Group II (DF2.01, DF2.02, DF2.03 and DF2.04, DF2.05), Group III (DF3.01, DF3.02 and DF3.04) and Group IV (TZ1.01, TZ1.02, TZ1.03, TZ1.04, TZ1.05, TZ1.06 and TZ1.07) compounds all demonstrated mast cell stabilising activity superior to that of the control compound, DSCG, in the presence of both calcium ionophore A23187 and compound 48/80 while DF1.01, DF1.02, DF1.04, DF3.03, demonstrated mast cell stabilising activity superior to that of DSCG in the presence of calcium ionophore A23187, but not in the presence of compound 48/80.

These findings compare favourably with published work carried out on a variety of mast cell stabilising and anti-allergy agents. As demonstrated in Chapter 2, DSCG has low levels of activity when evaluated at similar concentrations to the test compounds and the IC_{50} of DSCG in the presence of calcium ionophore A23187 was shown to be 78 $\mu\text{g/ml}$ (approximately 140 μM) and 54 $\mu\text{g/ml}$ (approximately 105 μM) in the presence of compound 48/80 (Sharma, Sharma *et al.* 2003). Most compounds evaluated in Chapter 2 demonstrated IC_{50} values below 32 μM , mostly below 10 μM , in the presence of both calcium ionophore A23187 and compound 48/80. DF2.02, in particular, demonstrated extremely potent activity in comparison to DSCG (DF2.02: IC_{50} 81.82 nM in the presence of calcium ionophore and IC_{50} 0.20 μM in the presence of compound 48/80). Other anti-allergy agents such as ketotifen, a dual acting mast cell stabiliser and H1R antagonist and azelastine, a second generation H1R antagonist with mast cell stabilising activity demonstrate activities which are less favourable than novel compounds evaluated here. Ketotifen required a very high concentration of 100 $\mu\text{g/ml}$ (approximately 235 μM) to significantly inhibit (74%) compound 48/80-induced histamine release from RPMCs (Kanda, Akiyama *et al.* 1998) while the IC_{50} s of calcium ionophore A23187-induced histamine release from RPMCs of azelastine, diphenhydramine (a first-generation H1R antagonist) and ketotifen were 5, 52 and 200 μM respectively (Chand, Pillar *et al.* 1983). Although azelastine is obviously a very potent anti-allergy agent, many compounds, including DF1.03, DF1.04, DF1.05, DF2.02, DF2.04, DF3.02, DF3.03,

TZ1.01, TZ1.04, TZ1.05 and TZ1.06 display IC₅₀ values of approximately 5 µM or lower, in the presence of calcium ionophore A23187 or compound 48/80 or both. These results indicate that several very promising novel compounds which show potential for use in the treatment of allergic conditions have emerged from these studies.

Selected aspects of the work carried out in Chapter 2 of this thesis have been accepted for publication in the journal "Medicinal Chemistry". Similar work carried out by the author, to evaluate the *in vitro* mast cell stabilising activity of novel compounds (synthesised by Barlow) which are not documented in this thesis, was recently published in the European Journal of Medicinal Chemistry (Barlow, McHugh *et al.* 2011).

Several novel compounds evaluated in the RPMC assay possessed mast cell stabilising activity superior to that of many therapeutic anti-allergy agents, therefore, any compound which demonstrated potent *in vitro* mast cell stabilising activity, as well as a favourable solubility profile, was assessed *in vivo* using the PCA assay. DF2.05, TZ1.04 TZ1.05 and TZ1.06, which demonstrate mast cell stabilising activity in the *in vitro* RPMC assay, as well as TZ2.01, TZ2.02, TZ2.03, TZ2.04, TZ2.05, TZ2.11 and TZ2.12, which were designed to address the solubility concerns associated with the Group IV series of compounds, were all demonstrated to inhibit the PCA reaction *in vivo*. The most potent compounds identified were TZ1.04, TZ2.01, TZ2.03 and TZ2.04; all of which demonstrated activity comparable to that of the control compound, DSCG. As in Chapter 3 (DSCG 3mg/kg I.V.) other groups have found similar results with DSCG. DSCG demonstrated high levels of inhibition of PCA in rats (96% inhibition of PCA when given at 3.2 mg/kg) when administered I.V. (Kobayashi, Hiroi *et al.* 1993), although this activity was reduced when given P.O. (37% inhibition of PCA) or I.P (48% inhibition of PCA) at 100 mg/kg. Azelastine demonstrates potent inhibition of PCA when administered (25 mg/kg) P.O. (91%) and I.P. (98%). ID₅₀s of both azelastine and DSCG (I.V.) are 0.3 and 1.0 mg/kg, respectively. Thus, azelastine is approximately three times as effective as DSCG (Chand, Harrison *et al.* 1985). Pemirolast (I.V.) demonstrates inhibition of PCA which is more potent than DSCG (Yanagihara, Kasai *et al.* 1988), as does ketotifen (67%, 0.1 mg/kg I.V.; DSCG 63% 3.2 mg/kg I.V.) (Greenwood 1982).

Again, several novel compounds demonstrated activity which appears to be comparable to current anti-allergy therapies, most notably TZ1.04, TZ2.01, TZ2.03, TZ2.04 and TZ2.05 which demonstrated 94%, 84%, 79%, 76% and 88% inhibition of PCA respectively, when administered at 3mg/kg. It appears that TZ1.04,

following results obtained both *in vitro* and *in vivo*, is a very promising mast cell stabilising agent, which demonstrates *in vitro* activity superior to and *in vivo* activity comparable to several therapeutic agents. Unfortunately DF2.02, which showed potent *in vitro* activity, appeared to elicit histamine release *in vivo*.

Following the findings that TZ2.01 and TZ2.04 demonstrated potent *in vivo* mast cell stabilising activity, comparable to that of DSCG, toxicity was determined for both TZ2.01 and TZ2.04 using the SHIRPA behavioural observation study which assessed undisturbed behaviour, active behaviour, restrained behaviour and manipulated behaviour and together assessed the general effects of a drug candidate on the CNS. Both compounds were administered orally to Wistar rats and their behaviour was observed at several time points over 24 hours. Although these compounds appear to be relatively lipophilic, there was no noticeable evidence of toxicity which may indicate that these novel anti-allergy compounds do not exhibit neurotoxicity, CNS stimulating or depressing, or autonomic nervous system modulating effects, at the concentration evaluated (10 mg/kg P.O., actual bioavailable dose 5.7 mg/kg). TZ2.01 and TZ2.04 demonstrated potent inhibition of PCA activity when evaluated at 3 mg/kg indicating that at an effective dose these compounds are not obviously toxic. Although further toxicity evaluations should of course be conducted, these results indicate that compounds which demonstrate promising anti-allergy activity may be safe and therefore, should be considered further for investigations into their use in the treatment of human allergic disease.

The *in vitro* RPMC assay, in which histamine was quantified using fluorescence following derivatisation with OPT, revealed that a number of those compounds designed to address the solubility concerns of the Group IV series, specifically TZ2.04, TZ2.06 and TZ2.08, appeared to cause the release of histamine *in vitro*. Therefore, an alternative LCMS method using the Thermo Accela Liquid Chromatograph (HPLC) with a Thermo LTQ-XL-Orbitrap Discovery Mass Spectrometer (MS) system, with a view to confirming the data obtained using the fluorescence detection method. Although several sets of guidelines exist for the validation of LCMS methods, this method was fully validated according to EMA guidelines. This method complies with validation criteria of LLOQ, accuracy, precision, calibration curve, selectivity, carry-over, dilution integrity and stability. Following on from the RPMC assay, histamine release from mast cells was quantified following derivatisation with dansyl chloride allowing for the detection of dansyl histamine fragment ions by MS/MS.

It is clear that validation of the above method of detection and quantification of dansylated histamine has provided a novel use for this LCMS system which has not previously been reported. The use of this system to quantify histamine released from mast cells is clearly demonstrated by the determination of mast cell stabilising activity of novel compounds using RPMC assay results. Although histamine release from RPMCs has been quantified following derivatisation with dansyl chloride using high performance thin layer chromatography with densitometry (Singh, Periera *et al.* 1993), HPLC following derivatisation with OPT (Arakawa and Tachibana 1986) or fluorecamine (van Haaster, Engels *et al.* 1993) and nano-ESI TOF MS (Sirikatitham, Yamamoto *et al.* 2007), similar methods using large numbers of RPMC derived samples have not been carried out using this system. With the exception of work carried out by Sirikatitham, Yamamoto *et al.* (2007), the method developed here provides increased sensitivity and accuracy compared to other methods and allows for the high-throughput determination of mast cell stabilising activity of novel compounds. This novel method may be used to explore mast cell processes and their manipulation and therefore, may be a highly useful tool in this field of research.

RPMC assay data obtained using this novel LCMS method was in agreement with that obtained by the fluorescence method. Results indicated that these compounds elicit the release of histamine from mast cells and therefore, are likely to have an alternative mechanism of action which may account for their PCA activity which was observed in Chapter 3. This finding does not in any way undermine the fact that these compounds demonstrate potent inhibition of an *in vivo* anti-allergy model.

Following results obtained using the LCMS method, TZ2.13, a zwitterionic analogue of TZ2.01, was synthesised to increase further the hydrophilicity of Group V compounds and to hopefully decrease the histamine releasing properties of this series of compounds. Encouragingly, LCMS data indicated that this compound did not mediate histamine release and that it is a potent mast cell stabiliser at 20, 10, 5 and 1 μ M in the presence of the elicitors calcium ionophore A23187 and compound 48/80. Again, as with many compounds which demonstrated *in vitro* mast cell stabilising activity in Chapter 1, TZ2.13 demonstrated activity superior to that of DSCG.

The evidence gathered at this point indicated that most Group V compounds which exhibited potent inhibition of PCA, did not prevent but actually elicited, the release of histamine from mast cells and therefore, were not mast cell stabilising compounds. These compounds were evaluated for their ability to interact with the H1Receptor, to investigate whether or not this could explain their inhibition of PCA.

Using the GP ileum model for H1R antagonist activity, several compounds emerged as having non-competitive H1R antagonist activity; specifically, TZ2.01, TZ.2.04, TZ2.05 and TZ1.04. TZ2.05 was also evaluated for H1R antagonist activity and affinity by MDS Pharma Services (Appendix 1) and results obtained confirmed the GP ileum H1R antagonist data.

As discussed previously, many agents which inhibit PCA, such as VUF-K-8788, a novel anti-allergy compound (Takizawa, Matsumoto *et al.* 2001) and luteolin (Baolin, Weiwei *et al.* 2005) also demonstrate H1R antagonist activity, while many classic H1R antagonists, such as ebastine and cetirizine (Yakuo, Yabuuchi *et al.* 2001), mepyramine (Braga and Mota 1976) and azelastine (Chand, Harrison *et al.* 1985), can also inhibit PCA indicating the complexities of this field. Results obtained in this study indicate that compounds which were considered to be mast cell stabilising compounds seem to be H1R antagonists, a mechanism of action which accounts for their *in vivo* inhibition of PCA. These compounds remain as promising anti-allergy agents despite the observation they are not mast cell stabilising agents.

Following on from the guinea pig ileum H1R antagonist study, TZ2.05, which was determined to be a non-competitive H1R antagonist, was also evaluated *in vivo* for its anti-pruritic activity. Results obtained for compound 48/80-induced scratching behaviour in mice were extremely encouraging and indicate that this compound is an effective anti-pruritic agent at 60 mg/kg P.O. (actual bioavailable dose 34.2 mg/kg). Its activity is comparable to that of the control compound, terfenadine (30 mg/kg P.O.), in this setting.

As discussed, terfenadine, although demonstrating favourable anti-pruritic activity (Sugimoto, Umakoshi *et al.* 1998; Inagaki, Nakamura *et al.* 1999) was withdrawn from EU markets (IMB 1999) because of its potential to cause cardiac arrhythmia when used in combination with other drugs such as erythromycin and ketoconazole. Fexofenadine, the pharmacologically active metabolite of terfenadine, displays a much more favourable side effect profile than terfenadine (DuBuske 1999). Although it is currently used to treat allergic rhinitis and urticaria, fexofenadine does not inhibit histamine-, PCA- or compound 48/80-induced scratching behaviour (Hossen, Fujii *et al.* 2005; Kobayashi, Takahashi *et al.* 2005). Tranilast, at a dose of 300 mg/kg, inhibits compound 48/80-induced scratching behaviour (Jiang, Tsumuro *et al.* 2005) while bepotastine, a second-generation H1R antagonist, inhibits compound 48/80-induced scratching behaviour at 10 mg/kg in NC/Nga mice (Tanizaki, Kambe *et al.* 2008). Azelastine also demonstrates potent inhibition of compound 48/80-induced scratching behaviour (89%) in BALB/c mice at 10 mg/kg, although evodiamine, a novel agent, requires a higher dose (50 mg/kg) to elicit a

similar response (85% inhibition of scratching events) (Shin, Bae *et al.* 2007). Astemizole (3, 10 and 30 mg/kg) and azelastine (1, 3 and 10 mg/kg) as well as chlorpheniramine (3 and 10 mg/kg) all inhibit compound 48/80-induced scratching behaviour in BALB/c mice while DSCG (30-300 mg/kg) is ineffective (Sugimoto, Umakoshi *et al.* 1998). Results suggest that TZ2.05, having anti-pruritic activity at doses similar to, or slightly higher than, those discussed may be desirable for use as an alternative to current therapies.

It is encouraging to note that TZ2.05 demonstrates potent anti-pruritic activity, while TZ2.04, a compound which degrades naturally over time to form TZ2.05, demonstrates a favourable side effect profile when evaluated using the SHIRPA behavioural observation protocol. The side effects observed upon evaluation of TZ2.05 may be favourable in comparison to other anti-pruritic agents discussed and therefore, this compound should be further evaluated for anti-pruritic and anti-allergic activity. It is extremely encouraging to note that this compound is a strong candidate for future use in the treatment of human allergic conditions, especially those characterised by excessive pruritis, such as allergic dermatitis.

In conclusion, the data obtained over the course of this project indicate that three classes of active compounds have emerged from these studies:

- 1) Those which are simply mast cell stabilisers, such as TZ1.04,
- 2) Those which appear to be non-competitive H1R antagonists, such as TZ2.01 and
- 3) TZ2.05, which possesses not only H1R antagonist activity but also anti-pruritic activity.

Therefore, it is extremely heartening to note that the work carried out in this thesis has established that a number of novel compounds synthesised in this lab possess promising novel anti-allergy activity which may be useful in the treatment of allergic conditions such as allergic rhinitis, allergic conjunctivitis and atopic dermatitis. Accordingly, further research should be carried out to investigate further their anti-allergy activity and to determine their mechanism of action.

6.2 Future Studies

6.2.1 Evaluate Active Compounds Further

6.2.1.1 IgE-Mediated Mast Cell Degranulation *In Vitro*

Compounds were evaluated for *in vitro* mast cell stabilising activity in the presence of both calcium ionophore A23187 and compound 48/80, both of which result in increased calcium influx and subsequent mast cell degranulation. Because studies using IgE-mediated degranulation mimic the precise mechanisms of the onset of an allergic reaction, it would be desirable to determine the activity of promising compounds in this situation.

Compounds would therefore be evaluated using IgE-mediated mast cell degranulation, in a similar manner to that which occurs in the PCA reaction. This study would require sensitisation of rats to CEA and their sacrifice 14 days later. Mast cells would be harvested as previously described (Chapter 2) but instead of addition of calcium ionophore A23187 or compound 48/80 they would be challenged with CEA to cause IgE-mediated mast cell degranulation which would result in histamine release. Inhibition of IgE-mediated histamine release by test compounds would be quantified using either OPT-histamine fluorescence conjugate or LCMS following dansylation of histamine using dansyl chloride. Compounds would be evaluated at a concentration range to determine their IC_{50} values.

Results would indicate whether these compounds work as specific agents which prevent the activation of mast cells by interacting with the IgE-FcεRI complex and would help to determine if they prevent IgE-mediated mast cell degranulation.

6.2.1.2 Scratching Behaviour

Because TZ2.05 demonstrates inhibition of compound 48/80 scratching behaviour at 60 mg/kg P.O., perhaps its activity could be evaluated using different agents to induce scratching, such as substance P, histamine and PCA, as discussed in Chapter 5. These studies would be expanded to include all compounds which demonstrated H1R antagonist activity using the guinea pig ileum protocol (Chapter 5).

Different drug concentrations and administration routes could also be evaluated to determine the circumstances in which its activity is maintained.

An attempt would also be made to determine if a dose-response effect is evident upon treatment of animals with various different doses, as is frequently carried out in the literature.

6.2.1.3 Toxicity

Because TZ2.01 and TZ2.05 do not display any obvious toxicity when evaluated in the SHIRPA behavioural observation protocol (10 mg/kg, P.O.) it would be wise to determine their levels of toxicity at increased doses and also following different routes of administration, using the SHIRPA behavioural observation study.

It would also be useful to conduct histology studies on tissues, such as the liver, following SHIRPA evaluation.

These compounds, although seeming to be non-toxic *in vivo*, cause the release of histamine in the RPMC *in vitro* assay. Therefore, their cytotoxicity could be evaluated, perhaps using the RBL-2H3 or HMC-1 cell lines to determine their activity in the lactose dehydrogenase or MTT assays for cytotoxicity.

6.2.2 Mechanism of Action

Other studies, as well as those investigating the inhibition of IgE-mediated mast cell degranulation discussed above, could be carried out to determine mast cell stabilising mechanism of action. It goes without saying that further studies may be carried out to determine if these compounds demonstrate activity at the receptors of other mast cell mediators, apart from that of histamine at the H1R. It would however, be ideal to primarily unravel the mechanism of action of these compounds with respect to mast cell stabilisation and H1R antagonism, before evaluating them further.

6.2.2.1 Interaction with Granule Exocytosis Machinery; SNARE Proteins and Synaptotagmin

Because many of the novel compounds tested here (e.g. DF2.02, DF2.04, TZ1.04, TZ1.06 etc.) inhibit both calcium ionophore A23187- and compound 48/80-mediated mast cell degranulation it is possible that these compounds display a common mechanism of action.

These compounds appear to be highly lipophilic and therefore, they may remain in close contact with the cell membrane. Perhaps because of this they may interact with certain protein complexes involved in the phases of exocytosis, such as the t-SNARE proteins. If a drug-t-SNARE protein interaction occurs perhaps it is sufficient to prevent interaction between t- and v-SNAREs from occurring, thereby preventing the formation of the SNARE complex, correct granule docking and granule fusion which normally results in exocytosis.

Calcium ionophore A23187 and compound 48/80 ultimately cause an increase in intracellular free calcium, although their mechanisms in achieving this effect differ,

as discussed in Chapter 1. Synaptotagmin, the calcium sensor which plays a vital role in calcium-induced membrane fusion during exocytosis, is expressed in human mast cells and is involved in mast cell exocytosis (Kimura, Shiraishi *et al.* 2001). Although the precise location of in mast cells is unknown, it appears that synaptotagmin I is primarily located in the granules of RBL-2H3 mast cells (Baram, Linial *et al.* 1998). Synaptotagmin II translocates to the plasma membrane in neutrophils during degranulation (Lindmark, Karlsson *et al.* 2002) while synaptotagmin I is localised at the cell membrane following increased intracellular calcium concentration during neuronal development (Schwab, Mouton *et al.* 2001). These novel compounds may also interfere with the calcium sensor synaptotagmin to prevent interaction of synaptotagmin with the SNARE complex to inhibit granule-cell membrane fusion, thereby inhibiting granule degranulation.

To establish the validity of the SNARE and synaptotagmin exocytosis hypotheses, future studies would be carried out to determine the effect of these compounds on the vesicle fusion processes. Experiments which aim to establish whether these compounds have a transient or a lasting effect would perhaps also give an insight into their mechanism of action. Approaches taken could involve the use of patch clamp (Oberhauser and Fernandez 1993), western blot, immunocytochemistry (Morton, Faull *et al.* 2001) as well as studies involving transgenic mice (Han, Rhee *et al.* 2005).

6.3 Possible Further Applications for these Novel Compounds

Although the mechanism of action of these compounds at this stage can only be speculated upon, it is interesting to note that compounds which inhibit mast cell granule exocytosis may also have applications apart from those primarily associated with allergy.

The pro-inflammatory cytokine IL-1 β is implicated in a number of inflammatory diseases such as rheumatoid arthritis (Koch, Lemmermeier *et al.* 1996), coronary artery disease (Weahre, Yndestad *et al.* 2004), Alzheimer's disease and mild cognitive impairment (Forlenza, Diniz *et al.* 2009). IL-1 β is also involved in mediating inflammatory pain hypersensitivity by inducing COX-2 upregulation in the CNS (Samad, Moore *et al.* 2001) while upregulation of IL-1 β in astrocytes is also associated with persistent pain (Guo, Wang *et al.* 2007).

The IL-1 β precursor, pro-IL-1 β , is expressed by monocytes and is secreted via lysosome exocytosis (Gardella, Andrei *et al.* 2002), a process which is not fully

understood but is none the less dependent on increased levels of cytosolic calcium (Andrei, Margicco *et al.* 2004). Pro-IL-1 β is then cleaved by caspase-1 to form active IL-1 β . DCs also secrete pro-IL-1 β by exocytosis of lysosomes, upon CD8⁺ T cell-mediated dendritic cell calcium influx (Gardella, Andrei *et al.* 2001).

Because the release of pro-IL-1 β occurs by exocytosis, if these compounds act by preventing granule fusion and exocytosis in mast cells it is possible that they may also inhibit vesicle fusion in cells which exocytose pro-IL-1 β . If this approach is effective it is reasonable to conclude that the novel compounds evaluated in this project could be used for the localised treatment of inflammatory diseases, to prevent the release of IL-1 β .

Current therapies which modulate IL-1 β are currently used as treatment in inflammatory disease. IL-1 β receptor antagonists, such as Kineret (anakinra) (Biovitrum 2010) which is used in the USA to treat of rheumatoid arthritis, inhibit the activity of IL-1 β at its receptor. IL-1RI and IL-1 β blockers, such as rilonacept which is undergoing phase III clinical trials for used in the treatment of gout (Regeneron 2010), bind to and immobilise IL-1 β , preventing its interaction with its receptor. Research is also being conducted on the use of novel anti-cancer agents, histone deacetylases, in inflammatory conditions. These compounds were found to prevent the exocytosis of pro-IL-1 β containing secretory granules, perhaps by causing disruption of microtubules (Carta, Tassi *et al.* 2006). It is therefore clear that the inhibition of pro-IL-1 β exocytosis is desirable under certain circumstances; perhaps the compounds evaluated here would be effective in this role. It would of course, be necessary to design a drug delivery method which ensures only target cells are affected.

Other, non-allergy cells may be also targets for these novel compounds. Thyroid parafollicular cells, or C cells, degranulate to release calcitonin (Rix, Raue *et al.* 1984), a hormone required for the reduction of calcium in the blood. Perhaps inhibition of the degranulation of calcitonin would be useful in the treatment of hypocalcemia. Pancreatic β cells also release insulin via calcium dependent exocytosis (Huang, Shen *et al.* 1995) although inhibition of this process would obviously not be desirable, except in the case of hyperinsulinemia.

Because these compounds may inhibit granule fusion and exocytosis in mast cells, it is therefore possible that they may exert a similar effect on other vesicle exocytosing cells, such as those discussed above. If a mechanism of action can be determined for these novel compounds it is possible that they may have alternative applications apart from those associated with allergy.

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Appendix I

**MDS Pharma Services and Charles
River Outsourced Studies**

MDS Pharma Services Studies

(Now Ricerca www.ricerca.com)



- **Assay Number 239610: Histamine H1R**

Source: Human Recombinant CHO-K1 Cells

Ligand: 1.2 nM [³H] Mepyramine

Vehicle: 1% DMSO

Incubation Time and Temperature: 3 hours, 25 °C

Incubation Buffer: 50 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 100 mM NaCl, 250 mM Sucrose

Non-Specific Ligand: 1 μM Mepyramine

K_D: 1.1 nM

B_{MAX}: 6.7 pmol/mg Protein

Specific Binding: Result Below

Quantitation Method: Radioligand Binding

Significance Criteria: ≥ 50% of Max Stimulation of Inhibition

- **Assay Number 313200: Histamine H1R, GTP_γS Binding**

Target: Human CHO-K1 Cells Chinese Hamster Ovary

Vehicle: 0.4% DMSO

Inhibition Time and Temperature: 30 minutes, 30 °C

Incubation Buffer: 20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 1mM DTT,
1mM EDTA

Quantitation Method: Quantitation of Bound [³⁵S] GTP_γS

Significance Criteria Antagonist: ≥ 50% Inhibition of Histamine Induced Bound [³⁵s]
GTP_γS

- **Assay Results**

Compound	TZ2.05	TZ2.14	TZ2.05	TZ2.14
Primary Assay	Histamine H1R	Histamine H1R	Histamine H1R, GTP γ S Binding	Histamine H1R, GTP γ S Binding
Cell Line	CHO-K1	CHO-K1	CHO-K1	CHO-K1
Species	Human	Human	Human	Human
n	2	2	2	2
Concentration	10 μ m	10 μ m	10 μ m	10 μ m
Response	94%	92%	85% (Antagonist)	67% (Antagonist)

*Histamine receptor 1 affinity and antagonist activities of TZ2.05 and TZ2.14
evaluated at 10 μ M, n=2*

Pharmacokinetics of TZ2.05 in the Sprague Dawley Rat

- **Summary**

The objective of this study was to obtain plasma samples to determine the pharmacokinetics of TZ2.05 following intravenous and oral administration to surgically prepared male Sprague Dawley rats.

Six male Sprague Dawley rats received either a single intravenous or single oral administration of TZ2.05 at target dose levels of 2 mg/kg and 10 mg/kg, respectively.

Plasma samples were collected up to 24 h post administration and the concentration of TZ2.05 in plasma determined using a method established at Charles River.

Following oral administration of TZ2.05 at 10 mg/kg, maximum plasma concentrations were observed at 4 h post dose. The bioavailability was estimated at 57% and terminal elimination half-lives were similar following oral and intravenous administration (*ca* 11 h and 10 h for oral and intravenous, respectively). Plasma clearance following intravenous administration was high (1946 mL/kg/h).

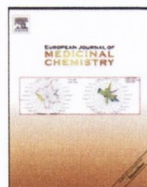
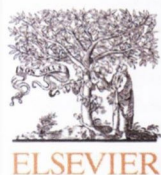
• **Results**

Subject	Actual Dose (mg/kg)	C _{max} (ng/mL)	T _{max} (h)	AUC _{0-t} (ng.h/mL)	AUC _{0-∞} (ng.h/mL)	T _½ (h)	CL/F (mL/h/kg)
004M	9.95	136	4.00	1868	2416	10.57	4119
005M	10.05	140	4.00	2281	3093	12.06	3249
006M	10.15	126	4.00	2020	2577	10.58	3939
N		3	3	3	3	3	3
Mean		134	4.00	2056	2695	11.07	3769
SD		7.21	0.00	208.9	354.0	0.8537	459.3
Min		126	4.00	1868	2416	10.57	3249
Median		136	4.00	2020	2577	10.58	3939
Max		140	4.00	2281	3093	12.06	4119
Geometric Mean		134	4.00	2049	2680	11.05	3750
CV% Geometric		5.4	0.0	10.1	12.9	7.6	12.7
Mean		122	4.00	1704	2098	9.631	2995
90% CI Lower		146	4.00	2408	3292	12.51	4543
Mean							
90% CI Upper							
Mean							

Pharmacokinetic parameters for TZ2.05 following a single oral administration at 10 mg/kg. < 4 data points were used to determine the terminal elimination slope

Appendix II

Published Work



Original article

Synthesis of novel mast cell-stabilising and anti-allergic 1,2,3,4-tetrahydro-1-naphthalenols and related compounds

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ABSTRACT

In a continuation of our studies into indan and tetralin systems as novel modulators of allergic and inflammatory phenomena, we have extended our work to include dimers linked via C–C bonds. Of these compounds, 2-Benzyl-2-[2-phenyl-1-ethenyl]-1,2,3,4-tetrahydro-1-naphthalenol **12** exhibited most promising activity both *in vitro* and *in vivo*.

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1. Introduction

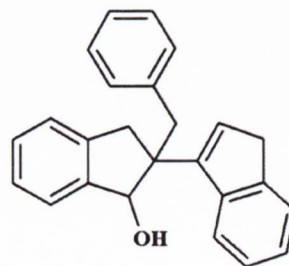
Compounds exhibiting multi-modality in their activity against allergic and inflammatory phenomena are increasingly recognised [1]. The pharmacological axiom of the mast cell stabilizer, as originally attributed to the cromoglycate-like drugs, has been expanded to reflect additional mechanisms of action, including inhibition of release of mediators such as PAF, arachidonate products and cytokines, and also direct anti-histaminic effects and effects related to broader inhibition of target cells, tissues and organs. These multi-modal effects are typified by drugs such as ketotifen, with both H1-receptor antagonist and mast cell stabilizer properties.

Prior work in the area [2a,b,c] to develop molecules with dual anti-allergic/anti-inflammatory properties within a single structural unit has established that indane dimer compounds such as **1** (Fig. 1) exhibit smooth muscle relaxant and/or mast cell-stabilising and/or anti-inflammatory properties. Indan and indanone fragments are present in several molecules showing anti-inflammatory activity. Indan acetic acid derivatives can be viewed as conformationally restricted aryl acetic acid derivatives [3]. 2-[4-(Imidazolin-2-ylideneamino)benzyl]-indan-1-ones have been designed as potent prostacyclin antagonists [4], and alkanesulfonamido-1-indanones

are inhibitors of cyclooxygenase [5]. In this study, we wished to explore whether ring expansion of compound **1** had a positive effect on activity, as we have previously demonstrated that tetrahydronaphthylamines possess anti-allergic activity [6] and that both amine-linked homo- and heterodimers of indan and tetralin exhibit protective effects in both *in vitro* and *in vivo* models of allergy [7].

2. Chemistry

The key step in the synthetic strategy for the target compounds was the directed aldol condensation of the parent ketones, followed by enolate alkylation and subsequent reduction. Initial investigations utilized TMSOTf-catalysis. The silyl enol ether **2** of α -tetralone [8] was obtained by reaction of the parent ketone with TMSOTf. Employing the method of Murata, and different catalyst concentrations (1–10%),

Fig. 1. Biindeyl-1-ol **1**.

Abbreviations: Con A, Concanavalin A; DSCG, disodium cromoglycate; RPMC, rat peritoneal mast cell; PCA, passive cutaneous anaphylaxis; TMSOTf, trimethylsilyl trifluoromethanesulfonate.

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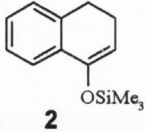
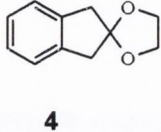
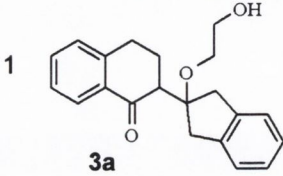
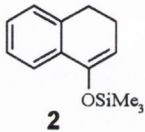
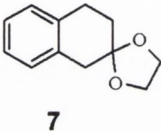
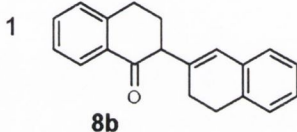
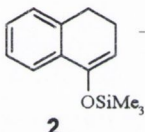
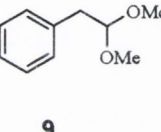
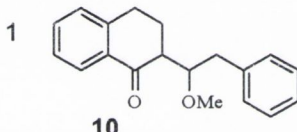
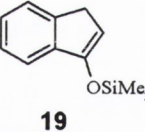
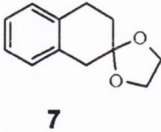
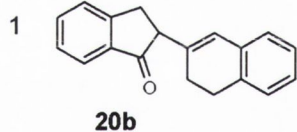
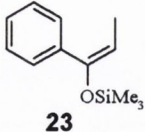
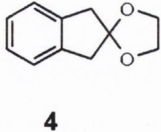
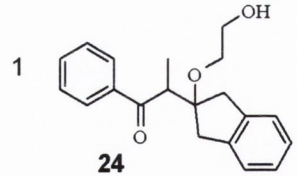
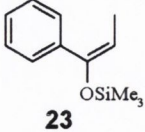
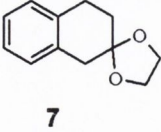
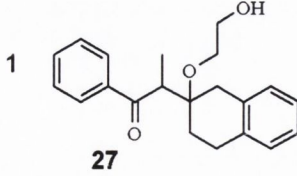
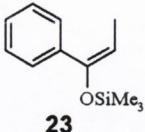
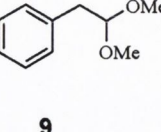
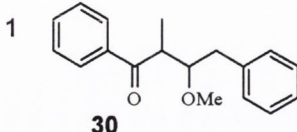
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the cross-coupling reaction between **2** and 2,2-dimethoxy-indan [9] afforded traces of the aldol product after reaction for 24 h at -78°C , yet analysis of the reaction mixture showed the presence of significant quantities of the β , γ -eliminated derivative **3b**. Murata quoted the lack of β -elimination of alcohols as one of the advantages of this reaction; however the potential for conjugation in this system upon

elimination likely explains the ready degradation. Murata's work employed acyclic acetals and ortho esters, and there is less information on the use of TMSOTf-catalysed aldol reactions using cyclic acetals. Therefore, dioxolan **4** [10,11] was prepared using ethylene glycol. Reaction of **2** with **4** was attempted under Murata conditions, again varying the temperature, duration of reaction and catalyst

Table 1
SnCl₄ catalysed Mukaiyama coupling of silyl enol ethers and acetals.^a

Entry	Silyl enol ether	Acetal	Time (hr)	Product(s)	Yield (%)
1			1	 3a	51 (3a)
2			1	 8b	32
3			1	 10	46
4			1	 20b	31
5			1	 24	69
6			1	 27	28
7			1	 30	70

^a Products from entries 1,2 and 4 decomposed readily by β -elimination; in the case of entries 2 and 4 only the β -elimination products were isolated.

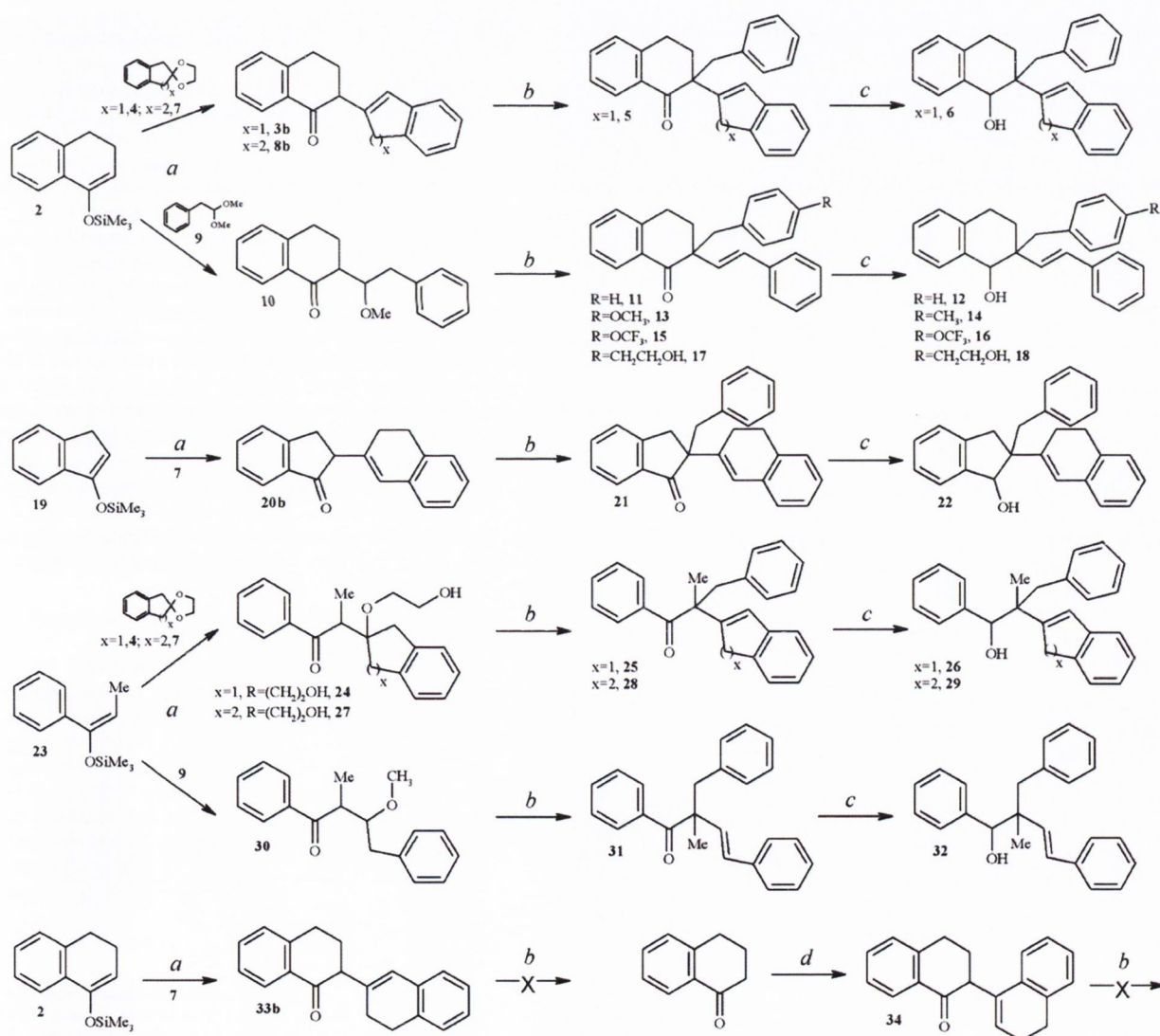
concentration (2.5–5%), yet all efforts to isolate the required dimer in preparative quantities proved futile.

Following the apparent shortcomings of TMSOTf-directed approaches, we decided to employ Mukaiyama conditions [12]. Using a stoichiometric quantity of TiCl_4 in DCM, to which **2** and **4** were sequentially added at -78°C , the reaction afforded a mixture of products. Analysis revealed the correct aldol to be present, in addition to starting material, the β -elimination product, and considerable quantities of an indan dimer. Tin tetrachloride has also been employed as an alternative activating agent, the diastereoselectivity of the reaction dependent on the substrate and the stereochemistry of the alkene [13a,b]. In contrast to the behaviour of TiCl_4 with enolate anions, SnCl_4 reacts to form α -stannyl ketones [14]. Nakamura [15] advised the preliminary formation of a stannyl ketone from the silyl enol ether with subsequent addition of an aldehyde or dimethyl acetal. Following this method led to a 54% yield of the desired product **3a** (Table 1). As a procedural variant, addition of SnCl_4 as a final step to a cooled mixture of the dioxolan and silyl enol ether led also to the desired product. After 24 h

storage at 0°C **3a** decomposed to the elimination product **3b**. Additional compounds (entries 2–7, Table 1) were also obtained via SnCl_4 -catalysed condensation analogously to **3a**. With the exception of **30**, these compounds have not been previously reported; Moorthy et al prepared and separated the *syn* and *anti* isomers of **30** via similar means in 57% yield, utilising TiCl_4 -directed aldol condensation between the trimethylsilylenol ether of propiophenone and phenylacetaldehyde dimethyl acetal [16].

Enolate alkylation of **3** (**a** or **b**) was performed using potassium *tert*-butoxide as base and benzyl bromide as electrophile. The reaction was performed in *tert*-butanol/diethylether at room temperature and the alkylated product **5** was obtained in 23% yield. Reduction of the carbonyl functionality was accomplished using sodium borohydride to afford alcohol **6** as a mixture of isomers (Scheme 1).

Likewise, alkylation and reduction of **10**, **20b**, **24**, **27** and **30** gave **12**, **14**, **16**, **18**, **22**, **26**, **29** and **32**. Compound **33b**, in contrast, could not be alkylated: the only product isolated from the reaction mixture was the α -keto alcohol. Compound **34** was approached via



Scheme 1. Synthetic methods for the preparation of target compounds. (a) SnCl_4 , DCM, -78°C , 1 hr; (b) ArCH_2Br , $\text{K}^+ \text{t-Bu}^-$, *t*-BuOH/ Et_2O (2:1), 24 hr; (c) NaBH_4 , MeOH, 1 hr; (d) $\text{A1 tri-}t\text{-BuO}^-$, PhCH_3 , Δ .

Table 2
Mast cell stabilising activity of novel compounds and DSCG in RPMC.^a

Compound	Elicitor	IC ₅₀ (μM)
6	Compound 48/80	4.1
	Con A	0.75
	Ionophore A23187	10
8b	Compound 48/80	8.0
	Compound 48/80	55.3
12	Compound 48/80	9.4
14	Compound 48/80	ND ^b
16	Compound 48/80	ND
18	Compound 48/80	7.7
22	Compound 48/80	3.6
	Con A	4.7
26	Compound 48/80	82% at 20 μM(±10)
	Compound 48/80	3.3
29	Compound 48/80	70.9
32	Compound 48/80	10% at 20 μM(±3)
DSCG	Compound 48/80	

^a Challenge with compound 48/80 (0.2 μg mL⁻¹), calcium ionophore A23187 (1 μg mL⁻¹) or Con A (20 μg mL⁻¹), 5 min exposure.

^b None determinable.

self-condensation of α -tetralone. In their study of tetralones, Springer et al. reported the dimerization of α -tetralone using anhydrous HCl [17]. Using basic conditions, addition of aluminium tri-*tert*-butoxide to α -tetralone in dry toluene under reflux, the reaction was heated for several hours, and after workup yielded **34**. Like **33b**, attempted α -alkylation of **34** using either potassium *tert*-butoxide or lithium di-isopropylamide as base was unsuccessful. As the rate of enolate alkylation can often be slow, coordinating reagents such as HMPA have been used to enhance reactivity. Goto et al. employed a selection of reagents to enhance the alkylation of α -tetralone, and demonstrated significantly improved yields with several of these [18]. In our hands, HMPA failed to have any impact on the alkylation of **34**. It may be that the considerable steric bulk of the dimer prevented approach of the alkylating agent.

3. Pharmacology

3.1. Mast cell-stabilising activity

Test compounds were evaluated for inhibition of elicitor-induced degranulation of rat peritoneal mast cells (RPMC), isolated as previously reported [19]. Cell populations were of >90% viability and mast cells comprised 1.4% of the total cell count. The results are shown in Table 2, and are compared with the reference compound disodium cromoglycate (DSCG).

3.2. Passive cutaneous anaphylaxis (PCA)

Passive cutaneous anaphylaxis was performed as previously described [20] using an antiserum raised against chicken egg albumin. Following intradermal inoculation with the antiserum, the rats were intravenously challenged with antigen in Evans Blue

Table 3
Protective activity of selected compounds against PCA (n = 5).^a

Compound	PCA	
6	–5	(±7)
12	44*	(±18)
22	2	(±15)
DSCG	87	(±7)

*P < 0.001.

^a Results are expressed as % inhibition [Mean ± S.E.M] of rat PCA induced by IV injection of ovalbumin (1 mg rat⁻¹). Rats were passively sensitized with ID injections of antiserum (0.1 mL site⁻¹) to ovalbumin and challenged IV 48 h later.

dye, with simultaneous injection of vehicle (2.5% Tween 80, positive control) or test compound at a dose of 3 mg kg⁻¹. Thirty minutes after intravenous injection, the animals were sacrificed and the skin reflected. Excised tissue was assessed using UV spectroscopy, following the method of Katayama [21]. Results are shown in Table 3.

3.3. Inhibition of murine ear oedema

Inflammation of murine ears (male Laca mice, 25–30 g) was achieved by topical application of arachidonic acid (AA) using a previously reported method [22]. Test compounds, the control (indometacin), and arachidonic acid (AA) were dissolved in acetone, the AA prepared immediately before use from a stock solution and stored on ice. Compounds were evaluated for ability to inhibit oedema and compared to indometacin. Results are shown in Fig. 2.

4. Results

The mast cell-stabilising activity of the novel compounds is shown in Table 2. Of these compounds, the benzylated dimer alcohols with rigid ring structures **6** and **22** and the dihydronaphthalenyl compound **29** exhibited the most promising activity. In addition to inhibiting mediator release due to provocation by compound 48/80, compounds **6** and **22** were also seen to inhibit release induced by the lectin concanavalin A and also by the calcium ionophore A23187, test compound **6** being more potent in both cases (IC₅₀s of 0.75 and 10 μM versus concanavalin A and ionophore respectively). Opening of the rigid ring structures of **6** and **22** resulted in some reduction in activity. Of the two hydro-aromatic rings in **6** and **22**, a greater loss in activity was observed upon opening of the ring bearing the alkenic substituent rather than the alcohol moiety. This may be seen from a comparison of the IC₅₀ of compound **29** compared with that of **12** (3.3 vs. 55.3 μM). In addition, the value for **32**, where both rings had been opened, was 70.9 μM. Interestingly, **8b** retained mast cell-stabilising activity (8 μM). The results of the PCA experiment as shown in Table 3 show that among the compounds tested, only the less strained analogue **12** possessed any ability to significantly inhibit PCA. Among the compounds tested for anti-inflammatory properties in the murine ear model (Fig. 2), only alcohol **6** showed significant inhibition of

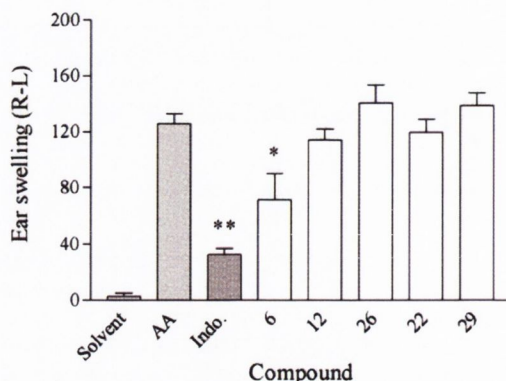


Fig. 2. Inhibition of arachidonic acid-induced mouse ear oedema. Results are expressed as weight differences between untreated (L) and treated (R) ears, oedema having been induced by 1 mg arachidonic acid 1 hr before measurement. Test compounds and indometacin administered at a dose of 300 μg ear⁻¹, arachidonic acid administered at a dose of 1 mg ear⁻¹. Bars represent $\bar{X} \pm$ S.E.M. from 5 animals. **P < 0.0001, *P < 0.05.

arachidonic acid-induced oedema. None of the compounds were comparable to the reference drug indometacin.

5. Discussion

The initial target compound **6**, like its indan dimer analogues [2] exhibited potent mast cell-stabilising activity. Compound **6** inhibited both compound 48/80-, ionophore- and Con A-induced degranulation. Deconstruction of the rigid skeletal framework of **6** revealed that both ring systems were not necessary for activity, although mast cell-stabilising activity was diminished without an indenyl or dihydronaphthalenyl fragment. A similar pattern of activity was seen with compound **22** and its acyclic analogues, where a doubly fused ring system again appeared optimal, yet the presence of just one cycle, a naphthalenyl ring as in **29**, did not impair activity *in vitro*. In contrast, the indenyl analogue **26** was nearly 30-fold less active. In the PCA experiment, the results obtained were not as expected, as alcohols **6** and **22** both significantly inhibited degranulation of mast cells *in vitro*, yet were devoid of activity *in vivo*. One reason for this discrepancy may be the relatively poor solubility of these compounds (log *P* of both compounds 6.30). However, compound **12**, with a similar log *P* of 6.58, despite a poor profile *in vitro*, displayed interesting activity *in vivo*. With regard to anti-inflammatory properties, although compound **6** exhibited weak activity in the mouse ear model, none of the compounds tested showed comparable activity to their indan congeners [2b], where the diastereomers of the analogous dimeric indan showed equipotency to indometacin. Therefore, the most promising candidate from this study is compound **12**, and further studies to improve the physicochemical profile of this compound are underway, with a view to enhancing its anti-allergic activity. This includes a more thorough evaluation of compounds such as **14**, the *para*-methyl derivative, whose *in vitro* profile is more favourable.

6. Conclusions

The novel alcohols described in this study were obtained via the SnCl_4 -catalysed Mukaiyama coupling of silyl enol ethers and acetals. Of the compounds tested, **12** showed the most interesting activity, as it displayed anti-allergic activity both *in vitro* and *in vivo*. Only one of the compounds tested in the *in vivo* model of inflammation showed modest activity. Our results complement those obtained from earlier studies [2b] with dimeric indanes, where anti-inflammatory activity was not linked to mast cell-stabilising activity.

7. Experimental protocols

All commercially available solvents and reagents were used without further purification. Reagents for synthesis and biological assays were purchased from Sigma–Aldrich. IR spectra were generated on a Perkin Elmer Paragon 1000 FT-IR. NMR spectra were generated at Bruker DPX-400 instrument, at 400.13 MHz for proton (^1H) magnetic resonance and 100.61 MHz for carbon (^{13}C) spectra, in chloroform-*d*. Chemical shifts are expressed in δ units (ppm). Low-resolution mass spectra were obtained on a Saturn GC/MS 2000 [CP-3800 Gas Chromatograph], while high-resolution mass spectra (HRMS) were obtained on a Micromass LCT instrument.

7.1. General procedure for silyl enol ethers

7.1.1. (3,4-Dihydro-1-naphthalenyloxy)(trimethyl)silane (**2**)

To a solution of α -tetralone (1 g, 6.84 mmol) in dry DCM (10 mL) at 0 °C was added triethylamine (0.76 g, 7.52 mmol) and trimethylsilyltrifluoromethanesulphonate (TMSOTf, 1.52 g, 6.84 mmol). The reaction was stirred at 0 °C for 30 min, and immediately subject

to flash column chromatography on silica that had been pre-treated with ethyl acetate and petroleum ether. The compound was eluted with petroleum ether, and the silyl enol ether (1.34 g, 95%) was obtained as a colourless oil, and stored in a dry flask under nitrogen at 0 °C until future use. IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$: 2961, 2835, 1339, 1253, 1189, 1094 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 0.51 (9H, s, $\text{Si}(\text{CH}_3)_3$), 2.54 (2H, m, CH_2), 2.98 (2H, dd, $J_1 = 9$ Hz, $J_2 = 7.9$ Hz, ArCH_2), 5.43 (1H, t, $J = 4.5$ Hz, $\text{C}=\text{CH}$), 7.30 (1H, Ar–CH), 7.36 (1H, ddd, $J_1 = 8$ Hz, $J_2 = 7.2$ Hz, $J_3 = 1$ Hz, Ar–CH), 7.42 (1H, dd, $J_1 = 8$ Hz, $J_2 = 7.3$ Hz, Ar–CH), 7.70 (1H, d, $J = 7.6$ Hz, COAr–CH); ^{13}C NMR δ_{C} –0.06 (CH_3), 21.96 (CH_2), 27.97 (CH_2), 104.79 ($\text{C}=\text{CH}$), 121.60 Ar–CH), 125.91 (Ar–CH), 126.68 (Ar–CH), 127.04 (Ar–CH), 133.29 (Ar–C), 136.72 (Ar–C), 147.95 ($\text{OC}=\text{C}$).

7.2. General procedure for acetalization

7.2.1. 1',3'-Dihydro-spiro[[1,3]dioxolane-2,2'-indene] (**4**)

To a solution of 2-indanone (2 g, 15.2 mmol) in ethylene glycol (10 mL) was added triethylorthoformate (5 mL) and a crystal of TsOH. The reaction was stirred at room temperature for 3 h, and the solvent removed *in vacuo*. The residue was purified by flash column chromatography on silica gel (eluant: pet ether:ethyl acetate, 5:1) to yield the dioxane, (2.4 g, 90%), a colourless oil with the following physical properties: IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$: 2955, 2885, 1403, 1331, 1292, 1226, 1104, 1030 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 3.27 (4H, s, $2 \times \text{CH}_2$), 4.06 (4H, s, $2 \times \text{OCH}_2$), 7.23–7.28 (4H, m, $4 \times \text{Ar-CH}$); ^{13}C NMR δ_{C} 42.84 ($2 \times \text{CH}_2$), 64.07 ($2 \times \text{OCH}_2$), 117.11 (OCO), 124.14 ($2 \times \text{Ar-CH}$), 126.25 ($2 \times \text{Ar-CH}$), 139.45 ($2 \times \text{Ar-C}$); MS, *m/z*, (RI) 177 ($\text{M}+1$, 28), 176 (M^+ , 100), 117 (12), 104 (42).

7.3. General procedure for SnCl_4 -catalysed Mukaiyama coupling of silyl enol ethers and acetals

To a solution of the appropriate silyl enol ether (2.13 g, 9.77 mmol) in anhydrous DCM at –78 °C was added the appropriate acetal (1.72 g, 9.77 mmol) and tin tetrachloride (2.55 g, 9.77 mmol). The reaction was stirred at –78 °C under an atmosphere of nitrogen for 1 h, and then quenched by the addition of iced water. The reaction was partitioned between water and DCM and the combined organic extracts (3×20 mL washings) were dried over anhydrous sodium sulphate and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (eluant: pet ether:ethyl acetate, 2:1) to yield the aldol product, and in the case of entries 1, 2 and 4 (Table 1), the β,γ eliminated derivative.

7.3.1. 2-[2-(2-Hydroxyethoxy)-2,3-dihydro-1H-2-indenyl]-1,2,3,4-tetrahydro-1-naphthalenone (**3a**)

Yellow oil (1.62 g, 51%); IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$: 3494, 2932, 2873, 1740, 1684, 1601, 1484, 1456, 1227 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 2.20 (1H, m, 1H of $\text{CH}_2\text{CH}_2\text{CH}$), 2.40 (1H, m, 1H of $\text{CH}_2\text{CH}_2\text{CH}$), 2.81–3.60 ($5 \times \text{CH}_2$, CH and OH), 7.14–7.28 (6H, m, $6 \times \text{Ar-CH}$), 7.43 (1H, ddd, $J_1 = 8.4$ Hz, $J_2 = 7.52$ Hz, $J_3 = 1$ Hz, Ar–CH), 8.01 (1H, dd, $J_1 = 8$ Hz, $J_2 = 1$ Hz, COAr–CH); ^{13}C NMR δ_{C} 24.85 (ArCH_2CH_2), 28.70 (ArCH_2CH_2), 42.23, 42.57 ($2 \times \text{ArCH}_2\text{CH}$), 54.83 (CH), 61.54 (HOCH_2), 63.73 ($\text{OCH}_2\text{CH}_2\text{OH}$), 86.70 (ArCO), 123.56, 123.66, 126.11 ($\times 2$), 126.19, 126.85, 128.16, 132.77 ($8 \times \text{Ar-CH}$), 133.35, 140.46, 141.64, 143.51 ($4 \times \text{Ar-C}$), 198.05 ($\text{C}=\text{O}$); MS, *m/z*, (RI) 260 (35), 146 (100), 103 (13).

Decomposition of **3a** on standing led to the β,γ eliminated derivative 2-(1H-Inden-2-yl)-3,4-dihydro-2H-naphthalen-1-one **3b**, a pale crystalline solid: m.p. 113–115 °C; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$: 3072, 2936, 1687, 1601, 1550, 1460, 1222 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 2.45 (2H, m, ArCH_2CH_2), 3.10 (2H, m, ArCH_2CH_2), 3.55 (2H, s, CH_2 indenyl), 3.79 (1H, dd, $J_1 = 8.4$ Hz, $J_2 = 7.0$ Hz, CH), 6.68

(1H, s, C₁CH), 7.18 (1H, ddd, $J_1 = 7.9$ Hz, $J_2 = 7.28$ Hz, $J_3 = 1.0$ Hz, Ar–CH), 7.25–7.39 (4H, m, 4× Ar–CH), 7.45 (1H, d, $J = 7.5$ Hz, Ar–CH), 7.53 (1H, ddd, $J_1 = 8.1$ Hz, $J_2 = 7.6$ Hz, $J_3 = 1.5$ Hz, Ar–CH), 8.15 (1H, dd, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz, Ar–CH); ^{13}C NMR δ_{C} 27.66 (CH₂), 28.90 (CH₂), 39.94 (CH₂CO), 49.59 (CH), 120.10 (C=CH), 123.07, 123.84, 125.83, 126.32, 127.29, 128.32 (×2C) (7× Ar–CH), 132.07 (quat. C), 133.03 (Ar–CH), 142.92, 143.47, 144.29, 145.99 (4× quat. C), 197.14 (C=O); MS, m/z , (RI) 261 (M+1, 58), 260 (M⁺, 100), 232 (6); HRMS (M + H)⁺ 261.1282, C₁₉H₁₇O requires 261.1279.

7.3.2. 2-Benzyl-2-(1H-2-indenyl)1,2,3,4-tetrahydro-1-naphthalenone (5)

To a solution of **3b** (0.54 g, 1.68 mmol) in *t*-butanol/ether (10 mL, 1:1) was added benzyl bromide (1.0 mL, 8.40 mmol). To this solution was added potassium *tert*-butoxide as a solution in *t*-butanol (5 mL), adding the base dropwise over several hours. The reaction was stirred overnight, and the *t*-butanol removed by ether/water extraction. The combined organic extracts (3 × 20 mL) were concentrated *in vacuo*, and the residue purified by flash column chromatography on silica gel (eluant: pet ether:ethyl acetate, 10:1) to yield the alkylated product (0.14 g, 23%). The product was obtained as yellow crystals: m.p. 125–127 °C; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 2929, 1684, 1550, 1220 cm^{-1} ; ^1H NMR (CDCl₃, 400 Hz) δ_{H} 2.22 and 2.37 (2H, 2× m, ArCH₂CH₂), 2.82 and 3.06 (2× m, ArCH₂CH₂), 3.18 and 3.50 (2H, 2× d, $^2J = 14$ Hz and 13.7 Hz, PhCH₂), 3.25 and 3.59 (2H, 2× d, $J = 22.5$ Hz, CH₂C=CH), 6.53 (1H, s, C=CH), 7.11–7.45, (12H, m, 12× Ar–CH), 8.20 (1H, d, $J = 8$ Hz, COAr–CH); ^{13}C NMR δ_{C} 25.44, 30.88, 38.91, 43.97 (4× CH₂), 52.91 (COCCH₂), 120.26, 123.02, 124.06, 125.90, 125.94, 126.11, 127.40 (×2C), 127.67, 128.11, 129.47, 130.48 (×2C) (C=CH and 12× Ar–CH), 131.91 (quat. C), 132.80 (Ar–CH), 137.17, 142.61, 143.04, 143.89, 146.49 (5× quat. C), 198.0 (C=O); MS, m/z , (RI) 351 (M⁺, 22), 350 (76), 259 (100), 231 (37).

7.3.3. 2-Benzyl-2-(1H-2-indenyl)1,2,3,4-tetrahydro-1-naphthalenol (6)

To a stirred solution of **5** (100 mg, 0.29 mmol) in methanol (5 mL) at 0 °C was added sodium borohydride (0.02 g, 0.58 mmol). The reaction mixture was stirred for 1 h, allowing the reaction to reach room temperature. The solvent was then removed *in vacuo*, and the residue purified by flash column chromatography on silica gel (eluant: pet ether:ethyl acetate, 5:1) to yield the white crystalline alcohol as a mixture of isomers (87 mg, 87%): m.p. 60–62 °C; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 2931, 2361, 2343, 1551, 1496, 1456, 1252 cm^{-1} ; ^1H NMR (CDCl₃, 400 Hz) δ_{H} 1.97–2.13 (1H, m, 1H of ArCH₂CH₂), 2.34 (1H, m, 1H of ArCH₂CH₂), 2.80–3.40 (4H, m, 2× ArCH₂), 3.58 (2H, m, PhCH₂), 4.73 and 4.80 (1H, 2× s, CHOH), 6.33 and 6.69 (1H, 2× s, C=CH), 7.03–7.54 (13H, 4× m, 13× Ar–CH); ^{13}C NMR δ_{C} 24.79 and 27.62 (ArCH₂CH₂), 25.17 and 25.55 (ArCH₂), 38.52 and 38.66 (PhCH₂), 41.51 and 41.72 (ArCH₂), 44.27 and 45.08 (CH₂CCHOH), 119.95 and 120.26, 122.89 and 123.14, 123.48 and 123.88, 125.9 (×3C), 127.5 (×3C), 128.59 and 128.82, 128.84 and 128.97, 129.31 and 129.46, 129.6 (×2C) (13× Ar–CH and C=CH), 135.17, 136.66, 137.24, 142.56, 144.15, 152.17 (5× Ar–C and C=CH); MS, m/z , (RI) 334 (M – H₂O), 261 (100), 243 (34), 215 (13), 145 (28), 117 (74), 91 (68), 65 (19); HRMS (M + Na)⁺ 375.1713, C₂₆H₂₄ONa requires 375.1725.

7.3.4. 2-(3,4-Dihydro-2-naphthalenyl)-1,2,3,4-tetrahydro-1-naphthalenone (8b)

The title compound, the β -elimination product, was obtained as described for **3b**, by the reaction of **2** with **7** [23]. Yield 32%; m.p. 94–96 °C; IR (KBr disc) $\nu_{\text{max}}/\text{cm}^{-1}$ 2941, 1679, 1599, 1453, 1223, 743 cm^{-1} ; ^1H NMR (CDCl₃, 400 Hz) δ_{H} 2.25–2.44 (2H, m, CH₂), 2.93 (2H, m, CH₂), 3.10 (2H, m, CH₂), 3.45 (1H, m, CH), 6.32 (1H, s, C=CH), 7.01 (1H, m, Ar–CH), 7.17 (3H, m, 3× Ar–CH), 7.29 (1H, d, $J = 8$ Hz, Ar–CH), 7.37 (1H, dd, $J_1 = 8.6$ Hz, $J_2 = 7.5$ Hz, Ar–CH), 7.52 (1H, ddd,

$J_1 = 8$ Hz, $J_2 = 7.5$ Hz, $J_3 = 1$ Hz, Ar–CH), 8.13 (1H, d, $J = 8$ Hz, COAr–CH); ^{13}C NMR δ_{C} 25.67 (CH₂), 27.73 (CH₂), 27.94 (CH₂), 28.23 (CH₂), 55.39 (CH), 124.71, 125.43, 125.94, 126.22, 126.28, 126.73, 127.08, 128.30 (C=CH and 7× Ar–CH), 132.40 (quat. C), 132.94 (Ar–CH), 133.87 (quat. C), 134.51 (quat. C), 139.02 (quat. C), 143.56 (quat. C), 197.65 (C=O); MS, m/z , (RI) 274 (M⁺, 100), 257 (60), 146 (21), 117 (23); HRMS (M + Na)⁺ 297.1262, C₂₀H₁₈ONa requires 297.1255.

7.3.5. 2-(1-Methoxy-2-phenylethyl)-1,2,3,4-tetrahydro-1-naphthalenone (10)

The title compound was obtained by the reaction of **2** with commercially available acetal **9** as described for **3b**. Yield 46%; m.p. 61–63 °C; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 2931, 1684, 1601, 1456, 1222, 1098 cm^{-1} ; ^1H NMR (CDCl₃, 400 Hz) δ_{H} 2.13 and 2.39 (2H, 2× m, CH₂), 2.67–3.17 (5H, m, 2× Ar–CH₂ and CH), 3.27 and 3.36 (3H, 2× s, OCH₃), 4.27 and 4.50 (1H, 2× m, CHOCH₃), 7.24–7.41 (7H, m, 7× Ar–CH), 7.49 (1H, m, Ar–CH), 8.07 and 8.11 (1H, 2× d, $J = 7.5$ Hz, COAr–CH); ^{13}C NMR δ_{C} 21.67 and 23.02 (CH₂), 28.54 and 28.77 (CH₂), 37.48 and 37.55 (CH₂), 49.35 and 49.88 (CH), 57.50 and 58.21 (CHOCH₃), 79.28 and 81.97 (CHOCH₃), 125.65 and 125.96, 126.13 and 126.25, 126.82 and 127.04, 127.81 and 128.15 (×2C), 128.24 and 128.38, 128.9 (×2C) (8× Ar–CH), 132.43 and 132.61 (Ar–C), 132.84 and 133.00 (Ar–CH), 138.15 and 139.62, 143.68 and 143.83 (2× Ar–C), 198.00 and 198.39 (C=O); MS, m/z , (RI) 249 (77), 248 (100), 118 (18), 115 (10), 89 (14); HRMS (M + Na)⁺, 303.1346, C₁₉H₂₀O₂Na requires 303.1361.

7.3.6. 2-Benzyl-2-[2-phenyl-1-ethenyl]-1,2,3,4-tetrahydro-1-naphthalenone (11)

The title compound was obtained by the treatment of **10** with benzyl bromide as described for **5**. Yield 40%; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3030, 2927, 1682, 1601, 1455, 1228 cm^{-1} ; ^1H NMR (CDCl₃, 400 Hz) δ_{H} 2.14 (2H, m, ArCH₂CH₂), 2.87 and 3.03 (2H, 2× m, CH₂), 3.23 (2H, 2× d, $^2J = 13.5$ Hz, PhCH₂), 6.28 (2H, 2× s, CH=CH), 7.17–7.40 (12H, m, 12× Ar–CH), 7.45 (1H, m, Ar–CH), 8.18 (1H, dd, $J_1 = 7.5$ Hz, $J_2 = 1.0$ Hz, COAr–CH); ^{13}C NMR δ_{C} 25.46 (ArCH₂CH₂), 31.55 (ArCH₂CH₂), 43.45 (COCCH₂Ar), 51.86 (CH), 125.85 (×2C), 125.90, 126.19, 127.10, 127.42 (×2C), 127.64, 128.06 (×2C), 128.07, 130.42, 130.57 (×2C), 130.91, 132.77 (14× Ar–CH and CH=CH), 131.77, 136.48, 136.87, 143.00 (4× Ar–C), 198.37 (C=O); MS, m/z , (RI) 338 (M⁺, 15), 247 (100), 91 (37); HRMS (M + Na)⁺ 361.1586, C₂₅H₂₂ONa requires 361.1586.

7.3.7. 2-Benzyl-2-[2-phenyl-1-ethenyl]-1,2,3,4-tetrahydro-1-naphthalenol (12)

The title compound was obtained by the treatment of **11** with sodium borohydride as described for **6**. Yield 90%; m.p. 132–134 °C; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3028, 2925, 1550, 1494, 1005 cm^{-1} ; ^1H NMR (CDCl₃, 400 Hz) δ_{H} 1.83–1.96 (2H, m, 1H of ArCH₂CH₂ and OH), 2.19–2.30 (1H, m, 1H of ArCH₂CH₂), 2.87–3.25 (4H, m, 2× ArCH₂), 4.58 and 4.63 (1H, 2× s, CHOH), 6.11 (1H, 2× d, $J = 16.6$ Hz, CH=CH), 6.49 (1H, 2× d, $J = 16.6$ Hz, CH=CH), 7.13–7.56 (14H, m, 14× Ar–CH); ^{13}C NMR δ_{C} 25.16 and 25.55 (CH₂), 26.31 and 26.82 (CH₂), 42.10 and 43.47 (CH₂), 43.69 and 43.84 (CHOHC), 72.36 and 72.60 (CHOH), 125.64, 125.76, 125.84, 125.88, 125.93, 126.64, 127.00, 127.20, 127.29, 127.50, 128.01, 128.16, 128.38, 128.59, 128.69, 129.02, 129.15, 130.42, 130.67, 130.70, 132.77 and 132.90, 10 signals overlapped (14× Ar–CH and CH=CH), 135.19, 136.93, 137.11, 137.53 (4× Ar–C); MS, m/z , (RI) 323 (89), 249 (100), 117 (46); HRMS (M + Na)⁺ 363.1716, C₂₅H₂₄ONa requires 363.1725.

7.3.8. 2-(4-Methylbenzyl)-2-(2-phenyl-1-ethenyl)-1,2,3,4-tetrahydro-1-naphthalenone (13)

The title compound was obtained by the treatment of **10** with 4-methyl benzyl bromide as described for **5**. Yield 39%; m.p. 89–94 °C;

IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3024, 2921, 1678, 1599, 1514, 1455 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} (ppm) 2.17–2.24 (2H, m, ArCH_2CH_2), 2.41 (3H, s, CH_3), 2.88–2.97 (1H, m, 1H of ArCH_2CH_2), 3.05–3.15 (1H, m, 1H of ArCH_2CH_2), 3.24 (1H, d, $J = 13.56$ Hz, 1H of ArCH_2), 3.32 (1H, d, $J = 13.56$ Hz, 1H of ArCH_2), 6.37 (2H, s, $\text{CH}=\text{CH}$), 7.11–7.54 (12H, m, $12 \times \text{Ar}-\text{CH}$), 8.28 (1H, d, $J = 7.52$ Hz, $\text{COAr}-\text{CH}$); ^{13}C NMR δ_{C} (ppm) 20.71 ($\text{Ar}-\text{CH}_3$), 25.57 (CH_2), 31.59 (CH_2), 43.11 (CH_2), 51.95 (quat.C), 125.96 $\times 2$, 126.28, 127.20, 127.68, 128.18 $\times 2$, 128.23, 128.28 $\times 2$, 130.56 $\times 2$, 130.65, 130.85, 132.89 ($13 \times \text{ArCH}$ and $\text{CH}=\text{CH}$), 131.83, 133.76, 135.49, 136.56, 143.14 ($5 \times \text{ArC}$), 198.60 ($\text{C}=\text{O}$); HRMS ($\text{M} + \text{Na}$) $^+$ 375.1713, $\text{C}_{26}\text{H}_{24}\text{ONa}$ requires 375.1713.

7.3.9. 2-(4-Methylbenzyl)-2-(2-phenyl-1-ethenyl)-1,2,3,4-tetrahydro-1-naphthalenol (**14**)

The title compound was obtained by the treatment of **13** with sodium borohydride as described for **6**. Yield 70%; m.p. 68–73 °C; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3430, 3023, 2926, 1736, 1690, 1600, 1492, 1449 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} (ppm) 1.77–1.86 (1H, m, 1H of ArCH_2CH_2), 1.87–1.97 (1H, m, OH), 2.14–2.27 (1H, m, 1H of ArCH_2CH_2), 2.36 and 2.38 (3H, 2 $\times$ s, $\text{Ar}-\text{CH}_3$), 2.77–3.09 (4H, m, 2 $\times$ ArCH_2), 4.55 and 4.60 (1H, 2 $\times$ s, CHOH), 6.01 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$ (minor diastereomer)), 6.18 (1H, d, $J = 17.04$ Hz, 1H of $\text{CH}=\text{CH}$ (minor diastereomer)), 6.41 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$ (major diastereomer)), 6.54 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$ (major diastereomer)), 7.12–7.41 (12H, m, $12 \times \text{Ar}-\text{CH}$), 7.52–7.54 (1H, m, CHOArCH); ^{13}C NMR δ_{C} (ppm) 20.63 and 20.66 (CH_3), 25.13 and 25.52 (CH_2), 26.07 and 26.75 (CH_2), 41.53 and 43.05 (quat.C), 43.60 and 43.79 (CH_2), 72.29 and 72.53 (CHOH), 125.62, 125.73, 125.82 $\times 2$, 126.61, 127.00, 127.18, 127.47, 128.00, 128.03, 128.15 $\times 2$, 128.22 $\times 2$, 128.38, 128.68, 128.82, 129.16, 130.23 $\times 2$, 130.49, 130.65, 132.87, 133.00 ($13 \times \text{ArCH}$ and $\text{CH}=\text{CH}$), 133.86, 134.27, 135.05, 135.19, 135.41, 136.02, 136.86, 137.00, 137.20, 137.48 ($5 \times \text{ArC}$); HRMS ($\text{M} + \text{Na}$) $^+$ 377.1894, $\text{C}_{26}\text{H}_{26}\text{ONa}$ requires 377.1881.

7.3.10. 2-(4-Trifluoromethoxybenzyl)-2-(2-phenyl-1-ethenyl)-1,2,3,4-tetrahydro-1-naphthalenone (**15**)

The title compound was obtained by the treatment of **10** with 4-(trifluoromethoxy)benzyl bromide as described for **5**. Yield 35%; m.p. 55–57 °C; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3345, 3028, 2927, 2860, 1682, 1600, 1508, 1455 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} (ppm) 2.10–2.27 (2H, m, $\text{Ar}-\text{CH}_2\text{CH}_2$), 2.87–2.99 and 3.06–3.21 (2H, 2 $\times$ m, $\text{Ar}-\text{CH}_2\text{CH}_2$), 3.25 (1H, d, $J = 13.56$ Hz, 1H of ArCH_2), 3.38 (1H, d, $J = 13.56$ Hz, 1H of ArCH_2), 6.34 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$), 6.41 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$), 7.17–7.53 (12H, m, $12 \times \text{Ar}-\text{CH}$), 8.29–8.31 (1H, m, $\text{COAr}-\text{CH}$); ^{13}C NMR δ_{C} (ppm) 25.91 (CH_2), 32.03 (CH_2), 43.22 (CH_2), 52.36 (quat.C), 120.42 $\times 2$, 121.89, 126.38 $\times 2$, 126.80, 127.83, 128.14, 128.66 $\times 2$, 128.71, 130.37, 131.88, 132.35 $\times 2$, 133.47 ($13 \times \text{Ar}-\text{CH}$, OCF_3 and $\text{CH}=\text{CH}$), 132.22, 136.23, 137.74, 143.45, 147.99 ($5 \times \text{Ar}-\text{C}$), 198.52 ($\text{C}=\text{O}$); HRMS ($\text{M} + \text{Na}$) $^+$ 445.1393, $\text{C}_{26}\text{H}_{21}\text{F}_3\text{O}_2\text{Na}$ requires 445.1391.

7.3.11. 2-(4-Trifluoromethoxybenzyl)-2-(2-phenyl-1-ethenyl)-1,2,3,4-tetrahydro-1-naphthalenol (**16**)

The title compound was obtained by the treatment of **15** with sodium borohydride as described for **6**. Yield 77%; m.p. 100–104 °C; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3361, 3027, 2930, 2863, 1599, 1508, 1434 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} (ppm) 1.77–1.87 (1H, m, 1H of ArCH_2CH_2), 1.89–2.14 (1H, m, OH), 2.16–2.29 (1H, m, ArCH_2CH_2), 2.80–3.14 (4H, m, 2 $\times$ ArCH_2), 4.59–4.63 (1H, m, CHOH), 5.98 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$ (minor diastereomer)), 6.17 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$ (minor diastereomer)), 6.40 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$ (major diastereomer)), 6.53 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$ (major diastereomer)), 7.09–7.49 (12H, m, $12 \times \text{Ar}-\text{CH}$), 7.54–7.59 (1H, m, CHOHArCH); ^{13}C NMR δ_{C} (ppm) 25.06 and 25.51 (CH_2), 26.08 and 26.79 (CH_2), 41.14 and 42.96 (CH_2),

43.68 and 43.76 (quat.C), 71.99 and 72.56 (CHOH), 119.71, 119.95 $\times 2$, 120.07, 120.20, 120.56, 120.63, 121.40, 125.60, 125.83 $\times 2$, 125.99, 126.25, 126.84, 127.18, 127.36, 127.67, 128.10, 128.24 $\times 2$, 128.27, 128.40, 128.48, 128.53, 128.68, 128.76, 129.31, 129.48, 131.06, 131.64 $\times 2$, 131.90, 132.14 ($13 \times \text{Ar}-\text{CH}$, OCF_3 and $\text{CH}=\text{CH}$), 135.06, 135.84, 135.93, 136.27, 136.66, 136.90, 136.94, 137.26 ($5 \times \text{ArC}$).

7.3.12. Methyl 2-(4-((1,2,3,4-tetrahydro-1-oxo-2-styrylnaphthalen-2-yl)methyl)phenyl) acetate (**17**)

0.18 mL of di-isopropylamine by the dropwise addition of 0.52 mL of a 2.5M solution of butyl lithium (1.73 mmol). The mixture was stirred at -78 °C under an atmosphere of nitrogen for 10 min, resulting in the formation of lithium di-isopropylamide (LDA). 0.32 g (1.1 mmol) of **10** was dissolved in 3 mL of dry THF and added dropwise to the flask, followed by 3 mL of a solution of the methyl-2-(4-bromomethyl phenyl)acetate in THF (1.3 mmol). The reaction was stirred for 4 h at room temperature, after which it was partitioned between diethyl ether and water. The combined organic extracts (3 $\times$ 20 mL washings) were dried over anhydrous magnesium sulphate and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (eluant: petroleum ether:ethyl acetate 8:1), to yield the alkylated product. Yield 0.18 g, 40%; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3401, 3025, 2929, 1736, 1677, 1600, 1514, 1493, 1454, 1434 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} (ppm) 2.11–2.17 (2H, m, ArCH_2CH_2), 2.91–2.93 (1H, m, 1H of ArCH_2), 3.00–3.10 (1H, m, 1H of ArCH_2), 3.16 (1H, d, $J = 13.56$ Hz, 1H of ArCH_2), 3.28 (1H, d, $J = 13.56$ Hz, ArCH_2), 3.62 (2H, s, $\text{ArCH}_2\text{C}=\text{O}$), 3.72 (3H, s, COOCH_3), 6.28–6.29 (2H, d, $J = 1.48$ Hz, $\text{CH}=\text{CH}$), 7.10–7.37 and 7.43–7.49 and 8.18–8.20 (13H, 3 $\times$ m, $\text{Ar}-\text{CH}$); ^{13}C NMR δ_{C} (ppm) 25.47 (CH_2), 31.57 (CH_2), 40.34 (CH_2), 43.06 (CH_2), 51.61 (OCH_3), 51.87 (quat.C), 125.87 $\times 2$, 126.23, 127.17, 127.63, 128.10 $\times 2$, 128.14, 128.37 $\times 2$, 130.31, 130.80 $\times 2$, 130.98, 132.85 ($13 \times \text{Ar}-\text{CH}$ and $\text{CH}=\text{CH}$), 131.56, 131.69, 135.74, 136.40, 143.02 ($5 \times \text{ArC}$), 171.70 ($\text{ArCH}_2\text{COOCH}_3$), 198.37 ($\text{ArC}=\text{O}$); HRMS ($+\text{Na}^+$) 433.1797 $\text{C}_{28}\text{H}_{26}\text{O}_3\text{Na}$ requires 433.1780; error +4.0 ppm.

7.3.13. 2-(4-Hydroxyethylbenzyl)-2-(2-phenyl-1-ethenyl)-1,2,3,4-tetrahydro-1-naphthalenol (**18**)

To a stirred solution of **17** (0.2 g, 0.49 mmol) in dry THF at 0 °C (5 mL) was added lithium aluminium hydride (0.09 g, 2.45 mol). The reaction mixture was stirred under an atmosphere of nitrogen for 3 h, allowing the reaction to reach room temperature, and then quenched by the addition of 2 M HCl. The reaction was partitioned between ether and water, and the combined organic extracts (3 $\times$ 20 mL) were dried over anhydrous magnesium sulphate and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (eluant: petroleum ether:ethyl acetate 3:1) to yield an off-white viscous oil. Yield 90%; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3379, 3056, 3024, 2931, 1724, 1599, 1578, 1512, 1493, 1449, 1432 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} (ppm) 1.67–2.08 (3H, m, 1H of ArCH_2CH_2 and 2 $\times$ OH), 2.11–2.17 (1H, m, 1H of ArCH_2CH_2), 2.71–3.10 (6H, m, 3 $\times$ ArCH_2), 3.79–3.94 (2H, m, CH_2OH), 4.54 and 4.59 (1H, 2 $\times$ s, CHOH), 6.01 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$ (minor diastereomer)), 6.17 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$ (minor diastereomer)), 6.41 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$ (major diastereomer)), 6.53 (1H, d, $J = 16.56$ Hz, 1H of $\text{CH}=\text{CH}$ (major diastereomer)), 7.05–7.46 (12H, m, $12 \times \text{Ar}-\text{CH}$), 7.50–7.59 (1H, m, $\text{Ar}-\text{CH}$); ^{13}C NMR δ_{C} (ppm) 25.14 and 25.53 (CH_2), 26.20 and 26.75 (CH_2), 38.34 and 34.89 (CH_2), 41.58 and 43.06 (CH_2), 43.66 and 43.80 (quat.C), 63.20 and 63.24 (CH_2OH), 72.27 and 72.50 (CHOH), 125.62, 125.74, 125.82 $\times 2$, 125.87, 126.68, 127.04, 127.20, 127.48, 127.96, 128.05, 128.16 $\times 2$, 128.19 $\times 2$, 128.39, 128.60, 128.68, 128.94, 129.09, 129.21, 130.58 $\times 2$, 130.69, 130.83, 131.00, 132.71, 132.89 ($13 \times \text{ArCH}$ and $\text{CH}=\text{CH}$), 135.15, 135.63, 135.98, 136.02,

136.84, 137.05, 137.16, 137.45 ($5 \times \text{Ar-C}$); HRMS ($\text{M} + \text{Na}$)⁺ 407.1987, $\text{C}_{27}\text{H}_{28}\text{O}_2\text{Na}$ requires 407.1987.

7.3.14. 2-(1,2,3,4-Tetrahydro-2-naphthalenylyden)-1-indanone (20b)

The title compound, the β -elimination product, was obtained as described for **3b**, by the reaction of **19** [24], prepared as for **2**, with **7** [23]. Yield 31%; m.p. 109–110 °C; IR (KBr disc) $\nu_{\text{max}}/\text{cm}^{-1}$ 1683, 1597, 1462, 1304, 1222 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 2.45 (2H, m, CH_2), 3.10 (2H, m, CH_2), 3.57 (2H, broad s, CH_2CHCO), 3.79 (1H, t, $J = 7$ Hz, CH), 6.70 (1H, s, $\text{C}=\text{CH}$), 7.21 (1H, dd, $J_1 = 8.4$ Hz, $J_2 = 7$ Hz, Ar-CH), 7.28–7.31 (2H, m, $2 \times$ Ar-CH), 7.35–7.41 (2H, m, $2 \times$ Ar-CH), 7.47 (1H, d, $J = 7$ Hz, Ar-CH), 7.54 (1H, dd, $J_1 = 8.4$ Hz, $J_2 = 7.5$ Hz, Ar-CH), 8.17 (1H, d, $J = 8$ Hz, COAr-CH); ^{13}C NMR δ_{C} 27.70, 28.92 ($2 \times \text{CH}_2\text{Ar}$), 39.98 ($\text{CH}_2\text{C}=\text{C}$), 49.60 (CH), 120.14, 123.11, 123.87, 125.86, 126.34, 127.29, 128.32, 128.37, ($\text{C}=\text{CH}$ and $7 \times$ Ar-CH), 132.10 (quat. C), 133.06 (Ar-CH), 142.97, 143.51, 144.33, 146.07 ($4 \times$ quat. C), 197.12 ($\text{C}=\text{O}$); MS, m/z , (RI) 261 ($\text{M}+1$, 25), 260 (M^+ , 100), 141 (39), 115 (61), 89 (44), 63 (19); HRMS ($\text{M} + \text{H}$)⁺, 261.1301, $\text{C}_{19}\text{H}_{17}\text{O}$ requires 261.1279; ($\text{M} + \text{Na}$)⁺, 283.1081, $\text{C}_{19}\text{H}_{16}\text{ONa}$ requires 283.1099.

7.3.15. 2-Benzyl-2-(3,4-dihydro-2-naphthalenyl)-1-indanone (21)

The title compound was obtained by the treatment of **20b** with benzyl bromide as described for **5**. Yield 54%; white solid, m.p. 130–132 °C; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 2929, 1683, 1602, 1455, 1292, 1231 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 2.23 (1H, m, 1H of CH_2CH_2), 2.36 and 2.40 (1H, $2 \times$ dd, $J_1 = 4.5$ Hz, $J_2 = 3.5$ Hz, 1H of CH_2CH_2), 2.81 and 2.85 (1H, $2 \times$ dd, $J_1 = 5$ Hz, $J_2 \sim 3$ Hz, 1H of CH_2CH_2), 3.07 (1H, m, 1H of CH_2CH_2), 3.35 (2H, $2 \times$ d, $J = 13.5$ Hz, CH_2), 3.43 (2H, $2 \times$ d, $J = 23$ Hz, CH_2), 6.54 (1H, s, $\text{C}=\text{CH}$), 7.12–7.34 (10H, m, $10 \times$ Ar-CH), 7.41–7.45 (2H, m, $2 \times$ Ar-CH), 8.21 (1H, d, $J = 8$ Hz, COAr-CH); ^{13}C NMR δ_{C} 25.45, 30.89, 38.92, 43.98 ($4 \times \text{CH}_2$), 52.92 (CH), 120.27, 123.03, 124.07, 125.91, 125.95, 126.12, 127.41 ($\times 2\text{C}$), 127.68, 128.13, 129.49, 130.49 ($\times 2\text{C}$) ($\text{C}=\text{CH}$ and $12 \times$ Ar-CH), 131.92 (quat. C), 132.81 (Ar-CH), 137.19, 142.63, 143.05, 143.90, 146.50 ($5 \times$ quat. C), 198.02 ($\text{C}=\text{O}$); MS, m/z , (RI) 351 ($\text{M}+1$, 31), 350 (M^+ , 100), 259 (75), 231 (34), 115 (11); HRMS ($\text{M} + \text{Na}$)⁺, 373.1547, $\text{C}_{26}\text{H}_{22}\text{ONa}$ requires 373.1568.

7.3.16. 2-Benzyl-2-(3,4-dihydro-2-naphthalenyl)-1-indanol (22)

The title compound was obtained by the treatment of **21** with sodium borohydride as described for **6**. Yield 94%; white solid, m.p. 74–76 °C; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3408, 3069, 3029, 2930, 1548, 1454, 1379 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 1.73 and 2.06 (1H, s and d, $J_d \sim 7$ Hz, OH), 2.43–2.66 (3H, m, 3H of CH_2), 2.78–3.28 (1H, broad m, 5H of CH_2), 4.96 and 5.30 (1H, s and d, $J_d \sim 7$ Hz, CHOH), 6.16 (1H, s, $\text{C}=\text{CH}$), 6.94–7.07 (3H, m, $3 \times$ Ar-CH), 7.14–7.25 (6H, m, $6 \times$ Ar-CH), 7.28–7.55 (m, $4 \times$ Ar-CH); ^{13}C NMR δ_{C} 24.94 and 25.20 (CH_2), 27.80 and 27.87 (CH_2), 35.94 ($\times 2$) (CH_2), 36.94 and 40.23 (CH_2), 58.12 and 58.48 (alip. quat. C), 80.99 and 82.23 (CHOH), 123.21, 123.90, 124.26, 124.86, 125.37, 125.62 ($\times 2\text{C}$), 125.66, 125.82, 125.97, 126.00, 126.04, 126.09, 126.29, 126.38, 126.50, 126.55, 126.67, 127.25 ($\times 2\text{C}$), 127.29 ($\times 2\text{C}$), 127.85, 128.61, 129.81 ($\times 2\text{C}$), 130.03 ($\times 2\text{C}$) ($13 \times$ Ar-CH and $\text{C}=\text{CH}$), 134.11, 134.32, 134.54, 137.75, 139.69, 142.16 ($6 \times$ quat. C); MS, m/z , (RI) 351 (M^+ , 5), 333 (100), 259 (99), 243 (31), 205 (21), 103 (35); HRMS ($\text{M} + \text{Na}$)⁺ 375.1737, $\text{C}_{26}\text{H}_{24}\text{ONa}$ requires 375.1725.

7.3.17. 2-[2-(2-Hydroxyethoxy)-2,3-dihydro-1H-2-indenyl]-1-phenyl-1-propanone (24)

The title compound was obtained as described for **3b**, by the reaction of **23** [25], prepared as for **2**, with **4**. Yield 69%; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3460, 2939, 1737, 1679, 1596, 1448, 1227, 1086, 1057 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 1.32 (3H, d, $J = 7$ Hz, CH_3),

2.35 (1H, broad s, OH), 3.12–3.46 (6H, m, $2 \times \text{CH}_2$ indan and $\text{OCH}_2\text{CH}_2\text{O}$), 4.15 (1H, q, $J = 7$ Hz, CH), 7.15–7.20 (4H, m, $4 \times$ Ar-CH), 7.47 (2H, m, $2 \times$ Ar-CH), 7.56 (1H, m, Ar-CH), 8.01 (2H, m, $2 \times$ COAr-CH); ^{13}C NMR δ_{C} 13.63 (CH_3), 39.30, 41.08 ($2 \times \text{CH}_2\text{Ar}$), 45.82 (CH), 61.48, 63.69 ($\text{OCH}_2\text{CH}_2\text{OH}$), 87.91 (quat. C), 123.63, 123.72, 126.24 ($\times 2\text{C}$), 127.89 ($\times 2\text{C}$), 128.17 ($\times 2\text{C}$), 132.50 ($9 \times$ Ar-CH), 137.89, 140.60, 140.94 ($3 \times$ Ar-C), 203.00 ($\text{C}=\text{O}$); MS, m/z , (RI) 249 ($\text{M}+1$, enone, 100), 248 (M^+ , enone, 25), 176 (13), 105 (9); HRMS ($\text{M} + \text{Na}$)⁺ 333.1470, $\text{C}_{20}\text{H}_{22}\text{O}_3\text{Na}$ requires 333.1467.

7.3.18. 2-(1H-2-Indenyl)-2-methyl-1,3-diphenyl-1-propanone (25)

The title compound was obtained by the treatment of **24** with benzyl bromide as described for **5**. Yield 40%, pale oil; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3068, 3031, 1681, 1463, 1240, 967, 910 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 1.51 (3H, s, CH_3), 3.21–3.33 (2H, m, CH_2), 3.49 (2H, $2 \times$ d, $J = 12.5$ Hz, CH_2), 6.72 (1H, s, $\text{C}=\text{CH}$), 6.90–6.93 (2H, m, $2 \times$ Ar-CH), 7.16–7.47 (10H, m, $10 \times$ Ar-CH), 7.77–7.93 (2H, m, $2 \times$ COAr-CH); ^{13}C NMR δ_{C} 22.8 (CH_3), 38.9, 44.4 ($2 \times \text{CH}_2$), 53.7 (CH_3CCH_2), 120.5, 123.3, 124.1, 125.9, 126.1, 127.3 ($\times 2\text{C}$), 127.8 ($\times 2\text{C}$), 128.1 ($\times 2\text{C}$), 128.5, 130.2 ($\times 2\text{C}$), 131.4 ($14 \times$ Ar-CH and $\text{C}=\text{CH}$), 137.0, 137.0, 142.6, 144.0, 150.8 ($4 \times$ Ar-C and $\text{C}=\text{CH}$), 202.6 ($\text{C}=\text{O}$); MS, m/z , (RI) 338 (M^+ , 7), 233 (100), 105 (28).

7.3.19. 2-(1H-2-Indenyl)-2-methyl-1,3-diphenyl-1-propanol (26)

The title compound was obtained by the treatment of **25** with sodium borohydride as described for **6** as a white crystalline solid, primarily as one pair of isomers: m.p. 74–76 °C; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3615, 3563, 3066, 3032, 2977, 1604, 1551, 1496, 1455, 1392, 1252, 1206, 1044, 1024 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 1.05 (3H, s, CH_3), 2.08 (1H, broad s, OH), 3.04 (2H, s, CH_2), 3.42–3.60 (2H, $2 \times$ d, $J = 23$ Hz, CH_2), 4.81 (1H, s, CHOH), 6.66 ($\text{C}=\text{CH}$), 7.05–7.35 (13H, m, $13 \times$ Ar-CH), 7.44–7.46 (1H, d, $J = 7.0$ Hz, Ar-CH); ^{13}C NMR δ_{C} 18.83 (CH_3), 39.93 (CH_2), 44.57 (CH_2), 46.20 (CCH_3), 79.76 (CHOH), 120.10, 123.00, 123.70, 125.67, 125.79, 127.18 ($\times 2\text{C}$), 127.31 ($\times 2\text{C}$), 127.67 ($\times 2\text{C}$), 128.63, 129.61, 130.01 ($\times 2\text{C}$) ($\text{C}=\text{CH}$ and $14 \times$ Ar-CH), 137.68, 140.38, 142.94, 144.18, 148.03 ($5 \times$ quat. C); MS, m/z , (RI) 145 (11), 144 (100), 115 (44); HRMS ($\text{M} + \text{Na}$)⁺ 363.1731, $\text{C}_{25}\text{H}_{24}\text{ONa}$ requires 363.1725.

7.3.20. 2-[2-(2-Hydroxyethoxy)-1,2,3,4-tetrahydro-2-naphthalenyl]-1-phenyl-1-propanone (27)

The title compound was obtained as described for **3b**, by the reaction of **23** [25], prepared as for **2**, with **7** [23]. Yield 28%; IR (film) $\nu_{\text{max}}/\text{cm}^{-1}$ 3438, 2938, 2877, 1676, 1449, 1223, 1095, 1051 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 1.35 and 1.39 (3H, $2 \times$ d, $J = 7$ Hz, CH_3), 1.67 and 2.12 (2H, $2 \times$ m, $\text{CH}_2\text{CH}_2\text{Ar}$), 2.44 (1H, $2 \times$ broad s, OH), 2.61–2.76 (2H, m, CH_2Ar), 2.90–3.14 (2H, m, CH_2Ar), 3.36 and 3.54 (4H, $2 \times$ m, $\text{OCH}_2\text{CH}_2\text{OH}$), 4.15 (1H, m, CH), 7.02–7.14 (4H, m, $4 \times$ Ar-CH), 7.46–7.51 (2H, m, $2 \times$ Ar-CH), 7.58 (1H, m, Ar-CH), 8.00 and 8.03 (2H, $2 \times$ d, $J = 7.5$ and 7 Hz, $2 \times$ COAr-CH); ^{13}C NMR δ_{C} 12.03 and 12.84 (CH_3), 25.23 and 25.32 (CH_2), 28.95 and 29.07 (CH_2), 34.92 and 35.21 (CH_2), 44.14 and 45.22 (CH), 61.44 and 61.48 (OCH_2), 62.31 and 62.82 (OCH_2), 76.42 and 76.76 (CH_2CCH_2), 125.15 and 125.37 (Ar-CH), 125.41 and 125.55 (Ar-CH), 127.81 ($\times 2\text{C}$, $2 \times$ Ar-CH), 127.88 and 127.94 (Ar-CH), 128.33 ($\times 2\text{C}$, $2 \times$ Ar-CH), 128.70 and 129.01 (Ar-CH), 132.66 and 132.69 (Ar-CH), 133.74 and 134.05 (Ar-C), 135.28 and 135.89 (Ar-C), 137.98 and 138.08 (Ar-C), 202.77 and 202.95 ($\text{C}=\text{O}$); MS, m/z , (RI) 262 (M^+ enone, 24), 157 (100), 105 (62); HRMS ($\text{M} + \text{Na}$)⁺ 347.1591, $\text{C}_{21}\text{H}_{24}\text{O}_3\text{Na}$ requires 347.1623.

7.3.21. 2-(3,4-Dihydro-2-naphthalenyl)-2-methyl-1,3-diphenyl-1-propanol (29)

The title compound was obtained by the treatment of **27** with benzyl bromide as described for **5**. The crude reaction mixture was monitored by GCMS, revealing a mixture of products, including the desired mass of compound **28**: MS, m/z , (RI) 353 (M^+ , 18), 261

(52), 247 (89), 248 (75), 131 (43), 117 (34), 105 (100). The crude reaction mixture was not purified, but treated with sodium borohydride as described for **6** to yield the alcohol (0.21 g, 35%), a white crystalline solid, primarily as one pair of isomers: IR (film) $\nu_{\max}/\text{cm}^{-1}$ 3557, 3029, 2929, 1453, 1040 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 1.05 (3H, s, CH_3), 2.59 (3H, m, 3H of CH_2), 2.92 (2H, m, CH_2), 3.10 (1H, m, 1H of CH_2), 4.86 (1H, d, $J = 1.5$ Hz, CHOH), 6.35 (1H, s, $\text{C}=\text{CH}$), 7.02 (1H, m, Ar-CH), 7.08 (2H, m, $2 \times$ Ar-CH), 7.14–7.22 (6H, m, $6 \times$ Ar-CH), 7.33–7.41 (3H, m, $3 \times$ Ar-CH), 7.45 (2H, m, $2 \times$ Ar-CH); ^{13}C NMR δ_{C} 16.83 (CH_3), 23.99 (CH_2), 27.81 (CH_2), 41.82 (CCH_2Ph), 48.99 (CH_3CCH_2), 77.76 (CHOH), 125.63, 125.92, 126.04, 126.46, 126.53, 126.55, 127.18 ($\times 2\text{C}$), 127.23, 127.30 ($\times 2\text{C}$), 128.03 ($\times 2\text{C}$), 129.91 ($\times 2\text{C}$) ($14 \times$ Ar-CH and $\text{C}=\text{CH}$), 133.82, 134.36, 137.43, 139.88, 142.37 ($4 \times$ Ar-C and $\text{C}=\text{CH}$); MS, m/z , (RI) 247 (13), 157 (100), 129 (18).

7.3.22. 3-Methoxy-2-methyl-1,4-diphenyl-1-butanone (**30**)

The title compound was obtained as described for **3b**, by the reaction of **23** [25], prepared as for **2**, with commercially available **9**. Yield 70%, 2:1 *syn:anti* diastereomers [16]; IR (film) $\nu_{\max}/\text{cm}^{-1}$ 2936, 1686, 1598, 1496, 1449, 1369, 1223, 1100 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 1.24 and 1.35 (3H, $2 \times$ d, $J = 7.04$ Hz, CH_3), 2.72–3.11 (2H, m, CH_2), 3.17 and 3.30 (3H, $2 \times$ s, OCH_3), 3.54 and 3.74 (1H, $2 \times$ qn, CHCO), 3.86 (1H, 2 overlapping quartets, CHOMe), 7.22–7.35 (5H, m, $5 \times$ Ar-CH), 7.43 (2H, m, $2 \times$ $m\text{COAr-CH}$), 7.54 (1H, m, $p\text{COAr-CH}$), 7.78 and 7.95 (2H, $2 \times$ m, $2 \times$ COAr-CH); ^{13}C NMR δ_{C} 12.47 and 13.12 (CH_3), 36.82 and 38.00 (CH_2), 44.08 and 44.13 (CH_3CH), 57.98 and 58.16 (OCH_3), 83.28 and 83.86 (CH_3OCH), 125.79 and 125.92 (Ar-CH), 127.81 ($\times 2\text{C}$) and 127.84 ($\times 2\text{C}$) (Ar-CH), 127.89 ($\times 2\text{C}$) and 127.91 ($\times 2\text{C}$) (Ar-CH), 128.09 ($\times 2\text{C}$) and 128.16 ($\times 2\text{C}$) (Ar-CH), 129.23 ($\times 2\text{C}$) and 129.26 ($\times 2\text{C}$) (Ar-CH), 132.33 and 132.42 (Ar-CH), 136.29 and 137.16 (Ar-C), 138.12 and 138.20 (Ar-C), 202.52 and 202.99 ($\text{C}=\text{O}$); MS, m/z , (RI) 269 ($\text{M}+1$, 98), 268 (M^+ , 44), 237 (86), 134 (40), 105 (100); HRMS ($\text{M} + \text{Na}$) $^+$ 291.1333, $\text{C}_{18}\text{H}_{20}\text{O}_2\text{Na}$ requires 291.1361.

7.3.23. 2-Benzyl-2-methyl-1,4-diphenyl-3-buten-1-one (**31**)

The title compound was obtained by the treatment of **30** with benzyl bromide as described for **5**. Yield 33%; IR (film) $\nu_{\max}/\text{cm}^{-1}$ 3030, 2933, 1724, 1681, 1598, 1497, 1452, 1271, 1237 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 1.49 (3H, s, CH_3), 3.30 (2H, s, CH_2), 6.50 (2H, $2 \times$ d, $J = 16.4$ Hz, $\text{CH}=\text{CH}$), 7.13 (2H, m, $2 \times$ Ar-CH), 7.22–7.41 (9H, m, $9 \times$ Ar-CH), 7.48 (2H, m, $2 \times$ Ar-CH), 7.87 (2H, m, $2 \times$ COAr-CH); ^{13}C NMR δ_{C} 22.76 (CH_3), 45.04 (CH_2), 53.45 (CH), 125.88 ($\times 2\text{C}$), 125.93, 127.20, 127.36 ($\times 2\text{C}$), 127.64 ($\times 2\text{C}$), 128.22 ($\times 2\text{C}$), 128.65 ($\times 2\text{C}$), 129.89, 130.54 ($\times 2\text{C}$), 131.12, 133.67 ($15 \times$ Ar-CH and $\text{CH}=\text{CH}$), 136.59, 136.98, 137.27 ($3 \times$ Ar-C), 203.88 ($\text{C}=\text{O}$); MS, m/z , (RI) 326 (M^+ , 5), 221 (80), 143 (30), 105 (100), 91 (48).

7.3.24. 2-Benzyl-2-methyl-1,4-diphenyl-3-buten-1-ol (**32**)

The title compound was obtained by the treatment of **31** with sodium borohydride as described for **6**. Yield 75%; IR (film) $\nu_{\max}/\text{cm}^{-1}$ 3029, 1548, 1496 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 0.96 and 1.17 (3H, $2 \times$ s, CH_3), 2.17 (1H, broad s, OH), 2.76–3.06 (2H, m, CH_2), 4.61 and 4.69 (1H, $2 \times$ s, CHOH), 6.12 and 6.35 (1H, $2 \times$ d, $J = 16$ Hz, $\text{CH}=\text{CH}$), 6.20 and 6.54 (1H, $2 \times$ d, $J = 16$ Hz, $\text{CH}=\text{CH}$), 7.18–7.42 (15H, m, $15 \times$ Ar-CH); ^{13}C NMR δ_{C} 18.45 and 18.72 (CH_3), 43.83 and 44.05 (CH_2), 45.60 and 45.65 (CH_3CCH_2), 79.78 and 79.87 (CHOH), 125.60 and 125.65, 125.73 and 125.80 ($\times 2\text{C}$), 126.64 and 126.73, 127.07, 127.16, 127.18, 127.23, 127.26, 127.31, 127.72 ($\times 7\text{C}$), 128.08 and 128.12 ($\times 2\text{C}$), 129.25 and 129.85, 130.35 and 130.39 ($\times 2\text{C}$), 134.17 and 134.38 ($15 \times$ Ar-CH and $\text{CH}=\text{CH}$), 137.26 and 137.30, 137.69 and 137.76, 140.66 and 141.07 ($3 \times$ Ar-C); MS, m/z , (RI) 310 (1), 221 (12), 131 (100), 91 (53), 77 (22); HRMS ($\text{M} + \text{Na}$) $^+$ 351.1709, $\text{C}_{24}\text{H}_{24}\text{O}_2\text{Na}$ requires 351.1725.

7.3.25. 2-(3,4-Dihydro-1-naphthalenyl)-1,2,3,4-tetrahydro-1-naphthalenone (**34**)

To a stirred solution of α -tetralone (5 g, 34.2 mmol) in toluene (50 mL) was added aluminium tri-*tert*-butoxide (8.42 g, 34.2 mmol). The reaction was heated under reflux, with the flask attached to a Dean–Stark apparatus, to enable azeotropic distillation and removal of water. After 12 h of reflux, during which time water removal had been performed periodically, the solvent was removed *in vacuo* and the residue was purified by flash column chromatography on silica gel (eluant: pet ether:ethyl acetate, 10:1) to yield the aldol condensation product (0.75 g, 8%), a white crystalline solid, with the following properties: m.p. 132–136 $^{\circ}\text{C}$; IR (KBr disc) $\nu_{\max}/\text{cm}^{-1}$ 2950, 2003, 1679, 1597, 1459, 1307 cm^{-1} ; ^1H NMR (CDCl_3 , 400 Hz) δ_{H} 2.31 (3H, m, 3H of CH_2), 2.38 (1H, m, 1H of CH_2), 2.79 (2H, m, CH_2), 2.97–3.12 (2H, m, CH_2), 3.89 (1H, dd, $J_1 = 9$ Hz, $J_2 = 4.5$ Hz, CH), 5.84 (1H, t, $J = 4.5$ Hz, $\text{C}=\text{CH}$), 7.13–7.20 (4H, m, $4 \times$ Ar-CH), 7.31 (1H, d, $J = 7.5$ Hz, Ar-CH), 7.38 (1H, dd, $J_1 = 8.5$ Hz, $J_2 = 7.5$ Hz, Ar-CH), 7.53 (1H, dd, $J_1 = 8$ Hz, $J_2 = 7.5$ Hz, Ar-CH), 8.16 (1H, d, $J = 8$ Hz, Ar-CH); ^{13}C NMR δ_{C} 22.75, 27.71, 27.83, 28.35 ($4 \times$ CH_2), 50.36 (CH), 122.68, 125.85, 126.28, 126.34, 126.67, 127.18, 127.37, 128.33, 132.95 ($8 \times$ Ar-CH and $\text{C}=\text{CH}$), 132.63, 133.42, 134.90, 136.52, 143.61 ($5 \times$ quat. C), 198.48 ($\text{C}=\text{O}$); MS, m/z , (RI) 275 ($\text{M}+1$, 73), 274 (M^+ , 100), 256 (24); HRMS ($\text{M} + \text{Na}$) $^+$ 297.1259, $\text{C}_{20}\text{H}_{18}\text{O}_2\text{Na}$ requires 297.1255.

7.4. Statistical analysis

Statistical analysis was performed using GraphPAD Prism software (GraphPAD, San Diego, CA, USA). The RPMC experiments were repeated on the cells of 5 animals. Data is expressed as the mean $\pm$ the standard error of the mean (S.E.M.). Statistical analysis of arachidonic acid-induced mouse ear oedema was performed using Students *t*-test when comparing untreated (positive control) groups with treatment groups. *P* values of <0.05 were considered significant.

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