

Transient Receptor Potential Vanilloid 1 (TRPV1) in Haematological Malignancies

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Dedication

To my parents, husband Asal, son Awsam and my newborn Karam.

With all my love....

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. This study was approved by the Human Research Ethics Committee Network, Tasmania (Approval No. H0011050).

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List of Abbreviations

A-425619	1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)urea
A-778317	1-((R)-5-tert-butyl-indan-1-yl)-3-isoquinolin-5-yl-urea
AA	Arachidonic acid
ACA	N-(p-amylicinnamoyl)anthranilic acid
ADP	Adenosine diphosphate
AEA	N-arachidonoyl ethanolamine (anandamide)
ALL	Acute lymphocytic leukaemia
AMG628	(R)-N-(4-(6-(4-(1-(4-fluorophenyl)ethyl)piperazin-1-yl)pyrimidin-4-yloxy)benzo[d]thiazol-2-yl)acetamide
AML	Acute Monocytic Leukaemia
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AP-1	Activator protein-1
ATL	Adult T-cell leukaemia
ATP	Adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BCTC	N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide.
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BTP2	4-methy-4'-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide
C- terminus	Carboxy terminus
CaM	Calmodulin
CaMKII	Ca ²⁺ -calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
CAP	Capsaicin
CAZ	Capsazepine
CD	Cluster of Differentiation
CDK	Cyclin-dependent kinase
CGRP	Calcitonin gene-related peptide
CLL/SLL	Chronic lymphocytic leukaemia/ small lymphocytic lymphoma
CML	Chronic myelogenous leukaemia
CMML	Chronic Myelomonocytic Leukaemia
CNS	Central Nervous System
COPD	Chronic Obstructive pulmonary disease
CRP	C- reactive protein
DAG	Diacylglycerol
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPTHF	Diphenyltetrahydrofuran
DPBA	Diphenylboronic anhydride
DRG	Dorsal root ganglion

ECL	Enhanced Chemiluminescence
eIF2 α	Eukaryotic translation initiation factor 2, subunit 1 (α , 35kDa)
eIF2 α K3	Eukaryotic translation initiation factor-2 α kinase-3
EIPA	Ethylisopropyl amiloride
ER	Endoplasmic reticulum
ET	Essential thrombocythaemia
ETC	Electron transport chain
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
GADD153	Growth arrest- and DNA damage-inducible transcript 3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GARD	Gastroesophageal reflux disease
GM-CSF	Granulocyte-macrophage colony stimulating factor
GTP γ S	Guanosine gamma thiophosphate
HCL	Hairy-Cell Leukaemia
HEK293	Human Embryonic Kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HL-60	Human myelocytic leukaemia
HRP	Horseradish Peroxidase
ICDA	Inhibitor of caspase activated DNase
IDN	Identification number
IFN- γ	Interferon-gamma
IL-1/ 2/ 6	Interleukin-1/ 2/ 6
ILD	Interstitial lung disease
Ins(1,4,5)P3	Inositol 1,4,5-trisphosphate
IP ₃	Inositol triphosphate
JNJ17203212	4-(3-trifluoromethyl-pyridin-2-yl)-piperazine-1-carboxylic acid (5-trifluoromethyl-pyridin-2-yl)-amide
JYL1421	N-(4-tert-butylbenzyl)-N'-[3-fluoro-4-(methylsulfonylamino)benzyl]thiourea
KB-R7943	2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate
LGH	Launceston General Hospital
LNCaP	Androgen-dependent prostate cancer cells
MAPK	Mitogen-activated protein (MAP) kinases
MFI	Median Fluorescence intensity
ML-9	1-(5-chloronaphtalene-1-sulphonyl) homopiperazine
MM	Multiple myeloma
MPD	Myeloproliferative Disorder
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
M.W	Molecular weight
N- terminus	Amino- terminus
NADA	N-arachidonoyldopamine
NADH	Nicotinamide adenine dinucleotide
NFAT	Nuclear factor of activated T-cells

NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFM	Non-Fat Milk
NGF	Nerve growth factor
NHBE	Normal human bronchial epithelial
NHL	Non-Hodgkin's Lymphoma
OAG	1-oleoyl-2-acetyl-sn-glycerol
<i>p21</i> ^{WAF1/CIP1}	Cyclin-dependent kinase inhibitor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PBSA	Phosphate buffer saline-sodium azide
PBST	Phosphate buffer saline tween- 20
PC3	Androgen-independent prostate cancer cells
PHB2	Prohibitin
PI3K	Phosphatidylinositol 3-kinase
PIP ₂	Phosphatidyl-inositol-4,5-bisphosphate
PKA	Protein kinases A
PKC	Protein kinases C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMT	Photo multiplier tube
PgE ₂	Prostaglandin E ₂
PP	Peyer's patch
PTCL-NOS	Peripheral T-cell lymphoma/ not otherwise specified
PVDF	Polyvinylidene difluoride
Q-PCR	Quantitative real-time PCR
RCF	Relative Centrifugal Force
RHC80267	1,6-di[O-(carbamoyl)cyclohexanone oxime]hexane
ROS	Reactive oxygen species
RT-4	Human well-differentiated low-grade papillary
RT-PCR	Reverse transcription polymerase chain reaction
RTX	Resiniferatoxin
SB366791	N-(3-methoxyphenyl)-4-chlorocinnamide
SDS	Sodium Dodecyl Sulphate
SSC	Side Scatter
STAT	Signal transducer and activator of transcription
TBMC	6-tert-butyl-m-cresol
THC	Δ^9 -tetrahydrocannabinol
TNF- α	tumour necrosis factor-alpha
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TRIM	1-(2-(trifluoromethyl)phenyl) imidazole
trkA	Tyrosine kinase A
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid type 1
URB597	3'-carbamoylbiphenyl-3-yl cyclohexylcarbamate.
UTAS	University of Tasmania
V _a	Varitint-waddler phenotype

WS-12	2-isopropyl-5-methyl-cyclohexanecarboxylic acid (4-methoxy-phenyl)-amide
2-APB	2-aminoethoxydiphenyl borate
4 α -PDD	4 α -phorbol 12,13-didecanoate
5-HT	Serotonin
5(6)-EET	5',6'-epoxyeicosatrienoic acid
5-(S)-HETE	5-(S)-hydroxyeicosatetraenoic acid
12-(S)-HPETE and 15-(S)-HPETE	12- and 15-(S)-hydroperoxyeicosatetraenoic acids
20-HETE	20-hydroxyeicosatetraenoic acid
$\Delta\Psi_m$	Mitochondrial membrane potential

Presentations at Conferences during PhD Candidature

Conference Presentations

- **Omari, S** and Adams, MJ and Khalafallah AA and Mohamed, M and Geraghty, DP, TRPV1 expression in haematological malignancies, Annual Combined ASM of APSA and ASCEPT, 1 - 4 December, Melbourne, Australia (2013) [Conference Extract].
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- **Omari, S** and Kunde, DA and Adams MJ and Geraghty DP, Inhibition of human haematological malignant cell line growth by capsaicin is not TRPV1-mediated, Annual Combined ASM of APSA and ASCEPT, 2-5 December, Sydney, Australia (2012) [Conference Extract].

Presentations related to but not directly arising from this thesis

Conference Presentation

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Abstract

Transient receptor potential vanilloid-1 (TRPV1) is a member of the TRP family of channels that are responsible for nociceptive, thermal and mechanical sensations. It is primarily associated with neuronal cells, but has been detected in different non-neuronal cells, including leukocytes. Capsaicin (CAP), the active ingredient of hot chilli peppers, is one of a number of related endogenous and plant-derived compounds (broadly termed ‘vanilloid-like agents’) that have been shown to induce apoptosis and inhibit cell proliferation in some cancer cells, through both TRPV1-dependent and -independent mechanisms. The expression and function of TRPV1 in haematological malignancies however, has not been extensively investigated. Specific targeting by vanilloid-like agents toward TRPV1 on cancerous cells in patients with haematological malignancies may represent a novel therapeutic approach to treating these diseases.

This thesis investigated the expression and function of TRPV1 in haematological malignancies, using both blood cancer cell lines and blood samples obtained from patients with different blood cancers. The specific aims were to; 1) study the effect of TRPV1 agonists and antagonists on the viability of THP-1, U266B1 and U937 haematological malignant cell lines, 2) validate and optimise Western blotting and flow cytometry protocols to detect TRPV1 expression in leukocytes, 3) investigate TRPV1 expression in THP-1, U266B1 and U937 cells, and 4) compare TRPV1 expression in leukocytes obtained from patients with blood cancers to normal subjects.

The thesis begins with a comprehensive review and discussion on TRPV1 structure and function, as well as its expression and role in health and disease. In particular, there is a focus on the role of TRPV1 in cancer, including haematological malignancies (Chapter 1).

In Chapter 2, the effect of CAP on the metabolic activity of three malignant haematological cell lines, THP-1, U266B1 and U937, was investigated. Metabolic activity assays were performed using the alamarBlue[®] method. CAP induced cytotoxicity in all three cell lines in a concentration-dependent manner. A biphasic effect on metabolic activity was observed on THP-1 cells [EC_{50} , IC_{50} (95% CI) = 32.9 (19.9-54.3), 219 (144-246) μ M]. U266B1 cells were more resistant to CAP-induced death than THP-1 and U937 cells. TRPV1 and CB1 antagonists (SB452533 and AM251, respectively) suppressed the CAP-induced increase in THP-1 cell metabolic activity ($P < 0.001$). These experiments suggest that CAP inhibits the metabolic activity of malignant haematological cells through a non-TRPV1-dependent mechanism.

Chapters 3 and 4 represent the experimental work and trouble-shooting conducted to develop, validate and optimise methods for the detection of TRPV1 expression in human cells. Western blotting (Chapter 3) and flow cytometric (Chapter 4) methods have been previously published, however few have documented the use of appropriate controls for the detection of TRPV1, suggesting that data in the literature may not necessarily be valid. A problem identified in the current study was the correct application of negative controls, particularly to assess the specificity and therefore suitability of the primary antibody used in these methods. These optimised

protocols were then used to investigate the expression of TRPV1 in human malignant haematological cell lines (Chapter 5) and leukocytes obtained from patients with blood cancers (Chapter 6).

Increased expression of TRPV1 protein was observed in THP-1, U266B1 and U937 cells compared to normal leukocytes. Furthermore, a TRPV1 dimer was detected in U266B1 cells. Interestingly, TRPV1 was detected in non-haematological cell lines that have previously been used as TRPV1-negative cells for Western blotting, including untransfected- and TRPV1-transfected (without tetracycline to switch TRPV1 transcription off) HEK293 and RAW264.7 cells. This latter finding highlights the need for appropriate negative (and positive) controls in both flow cytometric and Western blotting studies of TRPV1.

Expression of TRPV1 in leukocytes obtained from patients with a range of haematological malignancies, including multiple myeloma (MM) and B-cell non-Hodgkin's Lymphoma (B-NHL), was then investigated (Chapter 6). TRPV1 expression was detected in all patients and controls using flow cytometry, but not Western blotting. Using flow cytometry, a sub-group of patients ($4/49=8.2\%$, MM=2, B-NHL=2) demonstrated increased expression of TRPV1 relative to the remainder of the cohort. TRPV1 was found to be similar to the control group for 91.8% of all patients. There were no significant differences in TRPV1 expression (assessed using flow cytometry) between patients with MM and B-NHL, or between *de novo* patients and those undergoing treatment. Using Western blotting, TRPV1 (~95kDa) was detected in one MM and four B-NHL patients, although interestingly, a 240kDa band was also detected in both a B-NHL and a MM patient. In addition,

although C-reactive protein was elevated (≥ 5 mg/L) in 25% of all patients, it was not associated with higher TRPV1 expression. These results indicate that TRPV1 expression in leukocytes is relatively increased in a small subset of patients with blood cancers, and is not associated with inflammation. Furthermore, some patients may have a unique isoform of TRPV1 that warrants further investigation.

In summary, this study has generated new data and knowledge on the role of TRPV1 in haematological cells, including those from patients with blood cancers. A number of novel findings have been reported. Firstly, the inhibition of cell metabolic activity by the TRPV1 agonist, CAP, was found to be independent of TRPV1 activation in malignant haematological cell lines. Secondly, optimised Western blotting and flow cytometric methods for the detection of TRPV1 expression were developed and successfully validated. Thirdly, increased TRPV1 expression was demonstrated in the THP-1, U266B1 and U937 malignant haematological cell lines. Finally, increased TRPV1 expression was observed in some patients with MM and B-NHL, but was not associated with inflammation. The results presented in this thesis can be used as a basis for future studies of TRPV1 function in other human cells and cancers.

Chapter 1: Literature Review

1.1 Introduction

Natural compounds have been used over decades for treating various diseases and for relieving symptoms, although the mechanisms explaining their effects have largely not been described. Capsaicin (CAP), the active ingredient of ‘hot chilli peppers’, has been proposed as an anticancer agent. It has been shown to induce apoptosis and inhibit cell proliferation in some cancer cells, including haematological malignancies (Ito et al. 2004; Zhang et al. 2003a).

The CAP receptor, transient receptor potential vanilloid-1 (TRPV1), originally described as an afferent neuron nociceptive receptor, is expressed in neuronal and non-neuronal cells (Cortright and Szallasi 2004; Nagy et al. 2004). However, there are few published studies of TRPV1 expression in haematological malignancies (Bhutani et al. 2007; Gertsch et al. 2002). The focus of this thesis was to therefore investigate the expression and role of TRPV1 in malignant haematological cell lines, as well as in patients with blood cancers and compared to normal subjects.

1.2 Overview of TRP Channels

TRP channels and more specifically, TRPV1, are not only important in many sensory systems, they are crucial components for the function of non-sensory neurons, such as in epithelial, blood and smooth muscle cells (Minke 2006).

The TRP superfamily consists of a large number of non-selective cation channels that are permeable to both monovalent and divalent cations. These channels are seen as universal biological sensors that detect changes in the environment, including

intracellular and extracellular messengers, exogenous chemicals, temperature, and mechanical stress (Table 1-1).

TRP channels are involved in multiple functions, including nociception, temperature, mechanical sensations, renal $\text{Ca}^{2+}/\text{Mg}^{2+}$ handling, lysosomal function, cardiovascular regulation, control of cell growth and proliferation, perception of pungent compounds (e.g., chilli, mustard, garlic), taste perception, smooth muscle tone and blood pressure regulation (Christensen and Corey 2007; Clapham et al. 2003; Ramsey et al. 2006). TRP channels consist of six transmembrane domains segments (S1–S6) and a pore region between S5 and S6, with both the carboxy (C-) and amino (N-) termini located intracellularly (Clapham et al. 2005; Minke 2006). Despite the structural similarities between the TRPs and the voltage-gated K^+ channels, these group of channels are quite different (Clapham et al. 2005). At least 28 different TRP subunit genes have been identified in mammals, comprising six subfamilies, namely the classical or canonical TRPs, TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucolipin). Each subfamily comprises several channel subtypes, which differ in their selectivity for Ca^{2+} , activation mechanisms, and interacting proteins (see Table 1-1) (Clapham 2007; Holzer 2008; Nilius 2007).

The group of thermo-TRP channels, such as TRPV1 and TRPM8 sense a wide spectrum of temperatures from painful cold to painful heat ($\geq 43^\circ\text{C}$, $< 22\text{--}26^\circ\text{C}$, respectively) (Dhaka et al. 2006). Moreover, they are able to detect specific chemical entities including unpleasant and/or painful toxins, whereby TRP channels subserve chemoesthesia, defined as the chemical sensibility of the skin and mucus membranes

Table 1-1: Overview of TRP family subgroups

TRP	P _{Ca} /P _{Na}	Agonists/ Activators	Role	Antagonists	References
TRPC1	-	Depletion of int. Ca ²⁺ stores, PLC, DAG, OAG, orexin-A, TRK-PLC γ BDNF, bFGF, thapsigargin,carbachol, G _{q/11} -coupled receptors, membrane stretch, int. NO-mediated cysteine S-nitrosylation	Vertebrate mechanosensitive Ca ²⁺ permeable channel that is gated by tension developed in the lipid bilayer	2APB, Gd ³⁺ , La ³⁺ , SKF96365, Ca ²⁺ -CaM, GsMTx-4	(Abramowitz and Birnbaumer 2009; Alexander et al. 2007; Bollimuntha et al. 2006; Maroto et al. 2005; Venkatachalam et al. 2003)
TRPC2	2.7	DAG, Ca ²⁺ stores depletion, AA	Pheromone and odours sensing in mice	Unknown	(Abramowitz and Birnbaumer 2009; Gailly 2012; Zufall 2005)
TRPC3	1.6	DAG, OAG, orexin-A, IP ₃ , Ca ²⁺ store depletion, TRK-PLC γ , BDNF, PLC, G _{q/11} -coupled receptors, Ins(1,4,5)P ₃ , Ca ²⁺ , PIP2	BDNF mediated neuronal differentiation, vasomotor function, resistance vessel, airway regulator, antigen(Ag) stimulation lymphocytes	Gd ³⁺ , La ³⁺ , Ni ²⁺ , 2-APB, SKF96365, KB-R7943, BTP2	(Abramowitz and Birnbaumer 2009; Alexander et al. 2007; He et al. 2005; Hofmann et al. 1999; Kiyonaka et al. 2009; Numaga et al. 2010)
TRPC4	1.1	GTP γ S, La ³⁺ (at μ M range), G _{q/11} -coupled receptors, ext.H ⁺ , thapsigargin, F2v peptide and calmidazolium by antagonism of Ca ²⁺ -CaM, NO-mediated cysteine S-nitrosylation,	Vasoregulation, lung microvascular permeability, GABAergic input lateral geniculate nucleus	Pyr3, BTP2, La ³⁺ (high concentrations), 2-APB, SKF96365	(Abramowitz and Birnbaumer 2009; Alexander et al. 2007; Jung et al. 2011; Nilius et al. 2005)
TRPC5	9	GTP γ S, G _{q/11} -coupled receptors, Ins(1,4,5)P ₃ , adenophostin A, thapsigargin, La ³⁺ , Gd ³⁺ , high int.Ca ²⁺ , lysophosphatidyl choline, ext.H ⁺ , Riluzole, lead, genistein, rosiglitazone	Growth cone morphology, brain development, innate fear	La ³⁺ (high concentrations, enhanced at low), 2-APB, SKF96365, KB-R7943, BTP2, flufenamic acid, chlorpromazine	(Abramowitz and Birnbaumer 2009; Alexander et al. 2007; Gross et al. 2009; He et al. 2005; Majeed et al. 2011; Riccio et al. 2009; Richter et al. 2014; Semtner et al. 2007; Sukumar and Beech 2010; Wong et al. 2010; Xu et al. 2005b)
TRPC6	5	DAG, AIF ₄ ⁻ , G _{q/11} -coupled receptors, membrane stretch, GTP γ S, 20-HETE, OAG (independent of PKC) and inhibition of DAG lipase with RHC80267, flufenamate, hyperforin	Vasomotor and cardiac function, airway resistance, platelet aggregation.	2-APB, BTP2, La ³⁺ (IC ₅₀ = 6 μ M), Gd ³⁺ , amiloride, SKF96365, ACA, KB-R7943, ML-9 (independent of MLCK), ext.H ⁺ , GsMTx-4	(Abramowitz and Birnbaumer 2009; Alexander et al. 2007; Clapham 2003; Nilius et al. 2005; Xu et al. 2005b)
TRPC7	2	DAG, exocytosis, Ca ²⁺ store depletion, G _{q/11} -coupled receptors, OAG	Respiratory rhythm activity, conducts mono- and divalent- cations with a preference for divalents	La ³⁺ , SKF96365, amiloride, 2-APB	(Abramowitz and Birnbaumer 2009; Alexander et al. 2007; Ben-Mabrouk and Tryba)
TRPV1	10	CAP, resiniferatoxin, olvanil, PKC, depolarisation, heat (\geq 43C), low pH (\leq 5.9), AEA, eicosanoids, 12, 15-(S)-HPETE, 5-(S)-HETE, leukotriene B4, NADA, adenosine and 2-APB	Selective for Ca ²⁺ and Mg ²⁺ , inflammation, pancreatitis, sensing hot chilli, pain, noxious temperature, bladder distension and more.	Ruthenium red, 5'-IRT, 6-iodo-nordihydroCAP, BCTC, CAZ, A-425619, A-778317, AMG517, AMG628, SB-705498, JNJ17203212, JYL1421, SB366791, SB452533	(Alexander et al. 2004; Caterina and Julius 2001; Nathan et al. 2002; Nilius et al. 2005; Voets et al. 2004a; Vriens et al. 2009)

TRPV2	1-3	Heat (>53°C, rodent), 2-APB (rodent), DPBA, THC, probenecid, cannabidiol, growth factors i.e. IGF-1	Sensing thermal pain, phagocytosis, axon outgrowth regulation	Ruthenium, SKF96365, amiloride, TRIM, La ³⁺	(Caterina et al. 1999; Hu et al. 2004; Link et al. ; Shibasaki et al.)
TRPV3	2.6	Heat (> 23– 39°C), 2-APB, camphor, TBMC, carvacrol, eugenol, thymol, menthol, incensole acetate, DPBA	mechanosensor in vascular smooth muscle cells	Ruthenium red (<1 mM), DPTHF	(Beech et al. 2004; Hu et al. 2004; Moqrich et al. 2005; Smith et al. 2002)
TRPV4	6	Heat (> 24°- 32°C), 4α-PDD, bisandrographolide A, anandamide, AA, 5(6)-EET, cell swelling,	Osmosensing, warm sensing, nociception, pressure sensing in DRG	Ruthenium red (voltage-dependent), La ³⁺ , Gd ³⁺	(Clapham 2003; Harteneck and Schultz 2007; Kanzaki et al. 1999; Nilius et al. 2004; Vriens et al. 2004; Watanabe et al. 2002)
TRPV5	>100	Low [Ca ²⁺] _i , hyperpolarization	Ca ²⁺ reabsorption in the kidney	Ruthenium red, econazole, miconazole, Pb ²⁺ = Cu ²⁺ = Gd ³⁺ > Cd ²⁺ > Zn ²⁺ > La ³⁺ > Co ²⁺ > Fe ²⁺ , Mg ²⁺	(Alexander et al. 2007; den Dekker et al. ; Nilius and Voets 2005)
TRPV6	>100	Low [Ca ²⁺] _i , hyperpolarization, 2-APB	Ca ²⁺ reabsorption in the intestine	Ruthenium, Cd ²⁺ , Mg ²⁺ , La ³⁺	(Alexander et al. 2007; den Dekker et al. ; Nilius and Voets 2005)
TRPA1	0.8–1.4	Cooling (< 17°C), isothiocyanates, THC, allicin, menthol, thymol, cinnamaldehyde (100μM), carvacrol, formalin, 4-HNE, methylparaben, URB597, cyclopentone, prostaglandins, 1,4-dihydropyridines, isoflurane, desflurane, propofol, etomidate, Camphor (100μM)	Mechanically gated transduction channel required for the auditory response in mammals. Reception of pungent painful stimuli (mustard oil, wasabi, horse radish, garlic and onions)	Camphor (1mM), cinnamaldehyde (3mM), ruthenium red, gentamicin, Gd ³⁺ , amiloride, menthol (mouse), HC-030031, blockers of the mechanosensory channels	(Alexander et al. 2007; Alpizar et al. 2013; Corey et al. 2004; Macpherson et al. 2005; Sukharev and Corey 2004)
TRPP2	1-5	Mechanical stress, int.Ca ²⁺ , constitutive activity suppressed by co-expression of TRPP1	Signalling complex with TRPP1, cilia movement, development of heart, skeletal muscle and kidney, fertility.	La ³⁺ , Gd ³⁺ , amiloride	(Delmas 2004; Delmas et al. 2004; Giamarchi et al. 2006; Volk et al. 2003)
TRPP3	4	Int.Ca ²⁺ , low constitutive activity, depolarisation, cell swelling	Hair cell, Kidney, retinal developments	Phenamil, benzamil, EIPA, amiloride, La ³⁺ , Gd ³⁺ , flufenamate	(Delmas 2005; Shimizu et al. 2009)
TRPP5	1-5	Int.Ca ²⁺	Cell proliferation and apoptosis, Ca ²⁺ homeostasis, spermatogenesis	Unknown	(Chen et al. 2008; Guo et al. 2000; Xiao et al. 2010)
TRPML1	~ 1	TRPML1 ^{va} : constitutively active, ext. high H ⁺ (equivalent to Intralysosomal high H ⁺), Ca ²⁺	Role in late endosomes pathway that is necessary for lysosome formation and recycling	Amiloride, low pH	(LaPlante et al. 2002; Piper and Luzio 2004; Raychowdhury et al. 2004; Xu et al. 2007a)
TRPML2	-	TRPML2 ^{va} : constitutively active, ext. high H ⁺ (equivalent to intralysosomal high H ⁺), ADPR, oxidative stress	Mediating cation (Ca ²⁺ /Fe ²⁺) efflux from endosomes and lysosomes	Cu ²⁺ , clotrimazole, flufenamic acid, 2APB, ACA	(Jia et al. 2011; Montell 2005; Moreau et al. 2013; Xu et al. 2007a; Zeng et al. 2012)
TRPML3	-	TRPML3 ^{va} : constitutively active. TRPML3 ^{WT} : activated by Na ⁺ -free ext. solution, depolarization	Hair cell, stereocilia maturation, and int. vesicle transport	TRPML3 ^{va} & TRPML3 ^{WT} : Gd ³⁺ , ext.acidification (intralysosomal acidification), TRPML3	(Di Palma et al. 2002; Grimm et al. 2007; Montell 2005; Xu et al. 2007a)

TRPM1	non-selective cation	Translocation, constitutively active	Tumour suppressor, sensor of cellular redox status, oxidant stress sensor in immune and glia cells, respiratory bursts in neutrophils	La ³⁺ , Gd ³⁺	(Alexander et al. 2007; Duncan et al. 2001; Hara et al. 2002)
TRPM2	0.5–1.6	Oxidative and nitrosative stress, NAD, radicals, oxidative, int. ADPR, cADPR, int. Ca ²⁺ , AA, heat ~ 35°C	Regulates endothelial barrier function, cell proliferation	Clotrimazole, miconazole, econazole, flufenamic acid, ACA, 2-APB, ADPR and cADPR blocked by AMP and 8-bromo-cADPR	(Alexander et al. 2007; Clapham 2003; Fonfria et al. 2004; Hecquet et al. ; Perraud et al. 2005)
TRPM3	1–2	Cell swelling, Ca ²⁺ store depletion, pregnenolone sulphate, nifedipine, depolarisation, D- erythrospingosine, dihydrosphingosine, eicosanoids	Sphingolipid signalling, renal volume regulation (osmosensor)	La ³⁺ , Gd ³⁺ , 2-APB, int. Mg ²⁺ , ext. Na ⁺ (TRPM3α2 only)	(Alexander et al. 2007; Grimm et al. 2003; Grimm et al. 2005; Harteneck and Schultz 2007)
TRPM4	-	Int. Ca ²⁺ , ATP, decavanadate, depolarization, heat, PIP2, BTP2	Negative-feedback regulation of Ca ²⁺ fluctuation, release of IL-2 from T-cell. Impermeable to Ca ²⁺	ATP4, ADP, AMP, AMP-PNP, adenosine, int. spermine, flufenamic acid, ext. clotrimazole, 9-phenanthrol	(Alexander et al. 2007; Launay et al. 2004; Ullrich et al. 2005)
TRPM5	-	G _{q/11} -coupled receptors, Ins(1,4,5)P3, int. Ca ²⁺ , membrane depolarization, heat, PIP2	Taste receptor of the tongue; transduction of sweet, amino acid and bitter stimuli, impermeable to Ca ²⁺	Int. spermine, flufenamic acid, ext. Protons	(Alexander et al. 2007; Ullrich et al. 2005; Zhang et al. 2003b)
TRPM6	-	Reduction of Mg ²⁺ , ext. H ⁺ , 2-APB	High permeability for Mg ²⁺ , influx pathway for divalent cations	Ruthenium red, inward current by monovalent cations blocked by Ca ²⁺ and Mg ²⁺	(Alexander et al. 2007; Voets et al. 2004b)
TRPM7	3	Reduction of Mg ²⁺ , G-proteins, PKA, int. ATP, PIP2, ext. H ⁺	Mg ²⁺ and Ca ²⁺ entry & trace metal ion uptake. Role in neuronal cell death	Spermine (permeant blocker), carvacrol, La ³⁺ , Mg ²⁺ , 2-APB	(Aarts et al. 2003; Alexander et al. 2007; Monteilh-Zoller et al. 2003; Nadler et al. 2001)
TRPM8	1-3	menthol, depolarisation, Ca ²⁺ , cooling (< 22–26°C), PIP2; WS-12, menthol, icilin	Voltage-dependent channel, pain and cold sensor	CAZ, BCTC, and thio-BCTC, low pH, La ³⁺ , clotrimazole, 2-APB, ACA, NADA, anandamide, linoleic acid, cannabinoids	(Alexander et al. 2007; Behrendt et al. 2004; McKemy et al. 2002; Peier et al. 2002; Reid 2005; Voets et al. 2004a)

int.: intracellular; ext.: extracellular; ^{WT}: wild type; AA: Arachidonic acid; ACA: N-(p-amylicinnamoyl)anthranilic acid; ADP: Adenosine diphosphate; ADPR: Adenosine 5'-diphosphoribose; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; BCTC: N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carbox-amide; BDNF: Brain-derived neurotrophic factor; bFGF: Basic fibroblast growth factor; BTP2: 4-methy-4'-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide; cADPR: cyclic ADP Ribose; CaM: calmodulin; CAZ: capsazepine; DAG: Diacylglycerol; DPBA: Diphenylboronic anhydride; DPTHF: Diphenyltetrahydrofuran; EIPA: Ethylisopropyl amiloride; GTPγS: Guanosine gamma thiophosphate; Ins(1,4,5)P3: Inositol 1,4,5-trisphosphate; IP3: Inositol trisphosphate; NADA: N-arachidonoyl-dopamine; NO: nitric oxide; OAG: 1-oleoyl-2-acetyl-sn-glycerol; PIP2: Phosphatidylinositol 4,5-bisphosphate; 4α-PDD: 4α-phorbol 12,13-didecanoate; PKA: Protein kinases A; PLC: Phospholipase C; TRIM: 1-(2-(trifluoromethyl)phenyl) imidazole; TBMC: 6-tert-butyl-m-cresol; THC: Δ⁹-tetrahydrocannabinol; 2-APB: 2-Aminoethoxydiphenyl borate; 4-HNE: 4-Hydroxynonenal; 5(6)-EET: 5',6'-epoxyeicosatrienoic acid; 5-(S)-HETE: 5-(S)-hydroxyeicosatetraenoic acid; 12, 15-(S)-HPETE: 12- and 15-(S)-hydroperoxyeicosatetraenoic acids; 20-HETE: 20-hydroxyeicosatetraenoic acid.

which is distinct from taste and smell (Bandell et al. 2007). TRP channels are opened or closed by conformational changes in the channel protein (Bandell et al. 2007; Dhaka et al. 2006). The ion selectivity differs markedly among the same family of TRP channels, as most of the channels are non-selective cation channels, which is particularly true for TRPV1 although with a preference for Ca^{2+} (Julius and Basbaum 2001).

1.3 Transient Receptor Potential Vanilloid 1 (TRPV1)

Interest in TRPV1 began when pharmacological aspects of CAP were first recognised. CAP, which is derived from *Capsicum spp.*, is responsible for the sensation of ‘hot’ and ‘burning’ when exposed to chilli peppers. It acts specifically on nociceptive afferent neurons (Jancsó 1960). Researchers found that the selectivity of CAP’s action on afferent neurons could only be explained by an action on specific CAP receptors which led to the revelation of TRPV1 as a CAP-specific receptor (Caterina et al. 1997; Nagy et al. 2004; Szolcsányi et al. 1975).

1.3.1 Structure

TRPV1 is composed of six transmembrane domains with a pore-forming hydrophobic span between the fifth and sixth transmembrane domains (Caterina et al. 1997). Four N- terminal extremities interact with the cytosol (Clapham 2003). Like many other TRP channels, TRPV1 has a long N- terminus containing three ankyrin-repeat domains and a C- terminus containing a TRP domain close to the sixth transmembrane domain (Tominaga and Tominaga 2005). The binding site present on the ankyrin repeat domain of TRPV1 has been reported to bind triphosphate nucleotides such as ATP and calmodulin (CaM) in the same site (Lishko et al. 2007; Rosenbaum et al. 2004). By binding to the pointed binding site, these molecules

generate the different sensitivity and regulate the function of TRPV1 (Phelps et al. 2010). The intracellular domain of TRPV1 contains multiple binding sites for phosphatidylinositol-4,5-bisphosphate (PIP₂) on the N- and C-termini (Grycova et al. 2012), and two other sites for a protein-kinase C and Ca²⁺/CaM-dependent protein kinase II (CaMKII) which are important proteins for channel regulation (*see* Figure 1-1) (Ferrer-Montiel et al. 2004; Jeske et al. 2009).

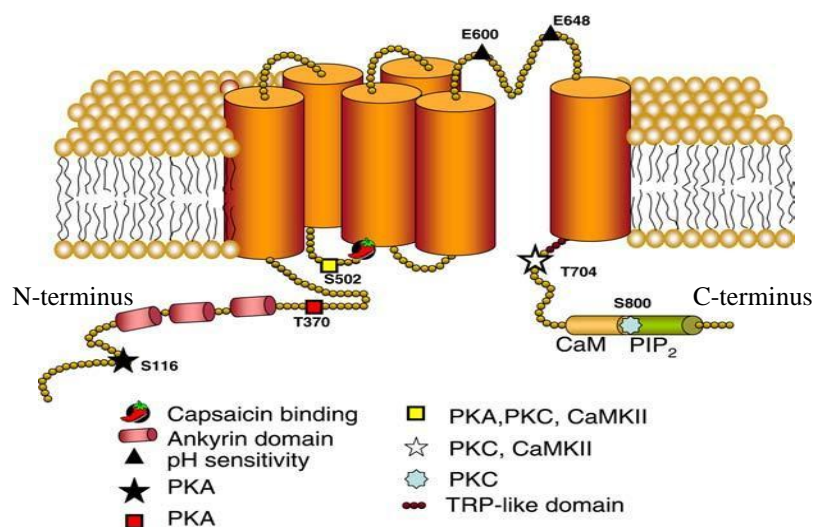


Figure 1-1: Topological model of TRPV1. TRPV1 subunit exhibiting six putative transmembrane domains, an intracellular N-and C-terminal domains, amino acid residues specific for CAP and proton activation, as well as residues important for phosphorylation by PKA, PKC and CaMKII. Kinases are highlighted at both intra-cytoplasmic regions. The TRP-like, CAM- and PIP₂- binding domains are also depicted. Reproduced from Planells-Cases et al (2005).

1.3.2 Activation of TRPV1

The initial detection of noxious signals occurs predominantly at the peripheral terminals of primary afferent neurons, called polymodal nociceptive neurons (Fields, 1987). TRPV1 has been shown to be the polymodal nociceptive receptor, that is also allosterically modulated by many proalgesic agents (Holzer 2008).

The TRPV1 channel is activated by vanilloids, which are present in a variety of plants that are normally ingested through the diet. As previously mentioned, they are

found in spicy foods, such as hot chilli pepper, and belong to the family of *Solanaceae*, genus *Capsicum*, in which the molecules responsible for the ‘burning’ taste are CAP and other capsaicinoids (Monsees et al. 1982). In addition to capsaicinoids, TRPV1 channels are activated by a variety of other plant-derived vanilloids including, camphor and resiniferatoxin (RTX), and putative endogenous vanilloids such as the endocannabinoid, anandamide, some lipoxygenase products of arachidonic acid (AA) such as 12-(S)- and 15-(S)-hydroperoxyeicosatetraenoic acid (12S- and 15S-HPETE), and N-arachidonoyldopamine (NADA) and its congener, N-oleoyldopamine (Chu et al. 2003; Van Der Stelt and Di Marzo 2004; Xu et al. 2005a). Vanilloids bind to intracellular sites on the TRPV1 channel, and the amino acids critical for CAP/RTX binding include Arg-91, Tyr-511, Ser-512, Ile 514, Val-518 and residue 547 (Met in rat, Leu in human), which are part of transmembrane segments 3 and 4 (Jordt and Julius 2002). CAP is a key activator of TRPV1 and acts by lowering the heat threshold required to open the TRPV1 channel (Szallasi and Blumberg 1999). Vanilloid-like agents may affect cellular function through two mechanisms; 1) the interaction with the TRPV1 receptor, or 2) direct interaction with the cell due to their ability to cross the plasma membrane bilayer because of their lipophilicity (Ziglioli et al. 2009).

Other activators of the TRPV1 channel include many noxious stimuli including heat ($\geq 43^{\circ}\text{C}$) (Loyd et al. 2012), acidosis ($\text{pH} \leq 5.9$) (Kaszas et al. 2012), mechanosensing (Inoue et al. 2009) and other chemesthetic (irritant agents) including vanillotoxins 1-3 (tarantula), ginger and ethanol (Bandell et al. 2007; Dhaka et al. 2006; Loyd et al. 2012). The thermal sensitivity of TRPV1 is enhanced by some endogenous modulators such as bradykinin, ATP, nerve growth factor (NGF) (Chuang et al.

2001) and PAR-2 agonists (Amadesi et al. 2004). NGF appears to act via phospholipase C (PLC) to hydrolyse PIP₂, leading to the inhibition of the channel (Chuang et al. 2001). Sustained exposure to agonists increases the Ca²⁺ permeability of TRPV1 and causes pore-dilation (10.1 Å° initial to 12.3 Å° after 3 min of exposure) (Chung et al. 2008). Neurons that express TRPV1 are eventually overloaded by Ca²⁺, which in conjunction with other factors, can result in mitochondrial swelling, long-lasting defunctionalisation or even degeneration of the neurons (Szoke et al. 2002; Szolcsanyi et al. 1975). In addition, TRPV1 allows protons to enter the cell in an acidic environment, which results in intracellular acidification (Hellwig et al. 2004). Researchers have reported that TRPV1^(-/-) null mice were defective in nociceptive, inflammatory and hypothermic responses to vanilloid compounds, supporting the notion that TRPV1 contributes to acute thermal nociception and hyperalgesia after tissue injury (Caterina et al. 2000).

1.3.3 Expression of TRPV1

Many studies have confirmed that the TRPV1 channel is expressed primarily on sensory neurons originating from the CNS, including dorsal root and trigeminal ganglia (Himi et al. 2012; Julius and Basbaum 2001; Schicho et al. 2004). Following synthesis in the neuronal soma, TRPV1 is transported to both the central and peripheral terminals of the primary afferent neurons (Guo et al. 1999). The fibres of these neurons innervate all tissues of the body including skin, muscle, bone, internal organs and vascular system. However, there are regional differences in the relative proportion of sensory neurons that stain positive for TRPV1, e.g., TRPV1-immunoreactive fibres are considerably more prevalent in visceral than in somatic afferent neurones (Brierley et al. 2005; Robinson et al. 2004).

Once believed to be exclusively neuronal, many studies subsequently reported TRPV1 expression in non-neuronal cells in all organs (Cortright and Szallasi 2004; Fernandes et al. 2012), such as urothelium and urinary tract (Avelino and Cruz 2006; Birder et al. 2001); human smooth muscle, keratinocytes (Jaggar et al. 2001); myenteric ganglia, muscle layer, mucosa and epithelial cells of the gastrointestinal tract (Geppetti and Trevisani 2004); mouse epidermis and keratinocytes (Bode et al. 2009); airway epithelial cells (Reilly et al. 2003), human umbilical vein endothelial cells (Himi et al. 2012), leukocytes (Saunders et al. 2009; Saunders et al. 2007) and microglia (Kim et al. 2006b). In addition, elevated TRPV1 expression was identified in some malignancies including, prostatic cancer cell lines, e.g., PC3 and LNCaP (Sánchez et al. 2006; Ziglioli et al. 2009), pancreas (Hartel et al. 2006; Mergler et al. 2012), colon (Domotor et al. 2005) and urinary bladder (Lazzeri et al. 2005). The wide distribution of TRPV1 emphasises its role as a universal sensor of many chemical stimuli.

1.4 TRPV1 and Pain

Pain is an unpleasant physical or emotional sensation associated with actual, or potential, tissue damage or trauma. It can be classified depending on its origin as: 1) nociceptive pain; the pain caused by stimulating the nociceptive receptors and transmitted over intact neural pathways, 2) neuropathic pain; the pain caused by damage to the nerve fibres leading to dysfunction of the sensory nerves to transmit signals correctly to the brain. Pain can be either acute or chronic (Nilius et al. 2007).

1.4.1 TRPV1 and nociceptive pain

The body's reaction to nociceptive stimuli, including thermal, mechanical and chemical, is a natural process that minimises the physical harm to the body, and is not considered as a diseased state (Nilius et al. 2007).

1.4.2 Sensitisation and desensitisation of TRPV1 receptors

There is compelling evidence that nociceptor sensitisation by inflammatory agents is primarily due to TRPV1 channel activity. Sensitisation is an increased responsiveness of neurons to their normal input or recruitment of a response to normally sub-threshold inputs (Fisher 2009). TRPV1 sensitisation by proalgesic agents may be due to either direct activation of the channel or to its potentiation. Direct activation of TRPV1 by endogenous vanilloids and acidosis released by injured tissue leads to increased TRPV1-mediated intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in both heterologous expression systems and in primary sensory neurons (Caterina and Julius 2001; Chu et al. 2003; Huang et al. 2002; Planells-Cases et al. 2005; Tominaga et al. 1998). The efficacy and potency of each single mediator is low. However, in inflammatory conditions, several modulators are simultaneously released and therefore act in collaboration. Interestingly, most TRPV1 agonists act by reducing the threshold for heat activation from 42°C to ~35°C, leading to TRPV1 activation at body temperature, and thus hyperalgesia (Caterina and Julius 2001; Julius and Basbaum 2001; Nagy et al. 2004). TRPV1 increases $[\text{Ca}^{2+}]_i$ that provokes the release of inflammatory mediators at peripheral terminals, further increasing the irritability of nociceptors. This feedback circuit enhances the hyperirritability of the injured tissue (Messeguer et al. 2006).

TRPV1 potentiation by proalgesic substances also involves the activation of intracellular signalling pathways (Figure 1-2) (Planells-Cases et al. 2005). Two major mechanisms have been found to be responsible for TRPV1 potentiation that leads to nociceptor sensitisation, (1) chemical modification of the intracellular domains of the channel, which is induced by protein kinases or phosphatases, and/or by hydrolysis of phosphoinositides produces a higher TRPV1 activity, and (2) an increase of TRPV1 receptor expression in peripheral terminals which enhances nociceptor responses. Both mechanisms are believed to occur in parallel (Figure 1-2) (Planells-Cases et al. 2005).

Sensitisation of the ion channel by phosphorylation of TRPV1 by PKA, PKC and other kinases is of particular importance (Premkumar and Ahern 2000; Tominaga et al. 2001). This is triggered by activation of receptors that respond to products of inflammation such as prostaglandins, leukotrienes, bradykinin, ATP, serotonin, and calcitonin-gene-related peptide (CGRP) (Nilius 2007) (Figure 1-2). Prostaglandins and 8-bromo-cyclic AMP trigger TRPV1 sensitisation through a PKA-mediated pathway (Bhave et al. 2002), whereas bradykinin and ATP sensitise TRPV1 by a PKC-mediated pathway (Premkumar and Ahern 2000; Tominaga et al. 2001). In addition, stimuli that activate PLC, such as bradykinin, NGF and anandamide, can also sensitise TRPV1 through hydrolysis of PIP₂, which normally inhibits the channel (Chuang et al. 2001; Prescott and Julius 2003). It is well established that activation of PLC sensitises TRPV1 channels to the three most studied activators: CAP, H⁺ and heat (Rohacs et al. 2008). In contrast, several laboratories have shown that NGF increases the number of TRPV1 channels in the plasma membrane of DRG neurons and may contribute to pain associated with tissue repair in a

phosphatidylinositol 3-kinase (PI3K) dependent pathway (Nilius 2007; Stein et al. 2006; Zhang et al. 2005). Furthermore, the release of neuropeptides such as substance P and CGRP from central and peripheral neurons also have a role in TRPV1 activation (Planells-Cases et al. 2005). Another sensitisation mechanism is TRPV1 pore dilation following prolonged exposure to agonists (Chung et al. 2008). However, repeated or prolonged application of CAP evokes a transient current leading to insensitivity of TRPV1 to subsequent noxious stimuli, the phenomenon of desensitisation (Rohacs et al. 2008). TRPV1 desensitisation protects cells from potential excitotoxicity that arises from excessive activation of the receptor that may lead to Ca^{2+} overload (Bhave et al. 2002). Although phosphorylation causes sensitisation, dephosphorylation promotes desensitisation of TRPV1, which is a major mechanism of inhibitory regulation by protein phosphatases (Mohapatra and Nau 2005).

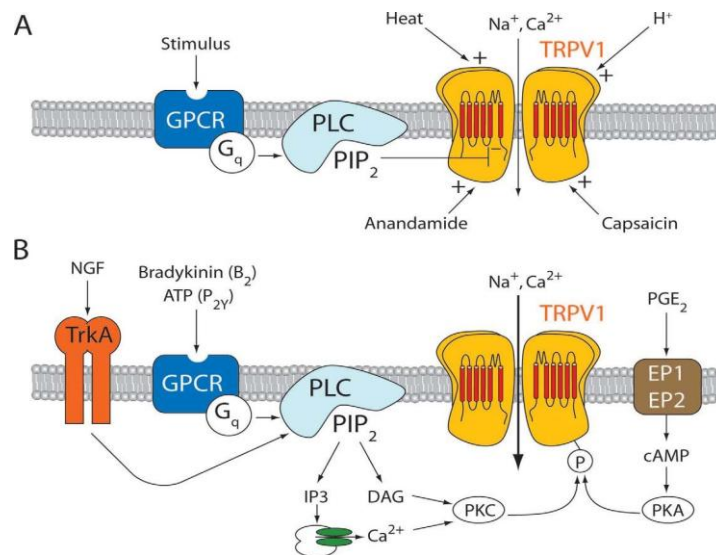


Figure 1-2: TRPV1 signal transduction. A) TRPV1 is activated by noxious heat ($\geq 43^\circ\text{C}$), low pH (≤ 5.9), eicosanoids, anandamide, and CAP. Ligands of guanine nucleotide (G) binding GPCR that activate PLC leading to hydrolysis of PIP₂ disinhibit the TRPV1 channels. (B) TRPV1 sensitisation resulting in neuropathic pain depends on channel phosphorylation by PKC or PKA and is triggered by products of inflammation, including prostaglandin E₂ (PGE₂), bradykinin, ATP, and serotonin (5-HT). Adapted from Benarroch (2008).

Desensitisation of TRPV1 to CAP is a Ca^{2+} -dependent process (Koplas et al. 1997; Mohapatra and Nau 2003). The Ca^{2+} sensor CaM activates the protein phosphatase calcineurin leading to TRPV1 desensitisation (Docherty et al. 1996). Calcineurin greatly reduces CAP-induced desensitisation of sensory neurons (Jung et al. 2004). It has been suggested that a rise in cytosolic Ca^{2+} levels caused by TRPV1 activation results in the activation of Ca^{2+} /CaM-dependent protein phosphatases that mediate channel desensitisation (Docherty et al. 1996). Furthermore, desensitisation of TRPV1 to CAP involves a number of intracellular components including PKA, ATP and CaM (Bhave et al. 2002; Lishko et al. 2007; Mohapatra and Nau 2003). It appears that there is a dynamic balance between phosphorylation/dephosphorylation of TRPV1 by CaMKII and calcineurin, respectively. This balance controls the activation/desensitisation state of the channel by regulating ligand binding (Figure 1-3, A), as CaMKII recovers TRPV1 from the desensitised state (Docherty et al. 1996; Jung et al. 2004; Mohapatra and Nau 2005; Suh and Oh 2005). TRPV1 has five phosphorylation sites for CaMKII, in which Ser 502 and Thr 704 are targeted for phosphorylation (Jung et al. 2004).

Aside from calcineurin desensitisation of TRPV1, other mechanisms have been proposed. Some studies suggested that the Ca^{2+} passing through TRPV1 activates PLC and subsequently leads to PIP_2 depletion resulting in desensitisation (Liu et al. 2005; Rohacs et al. 2008; Stein et al. 2006) (Figure 1-3, B). Therefore, PIP_2 have a dual function in sensitisation and desensitisation of TRPV1 (Lukacs et al. 2007). Moreover, some researchers found that stimulation of PKA eliminates the desensitisation of TRPV1 to repeated application of CAP. Whilst TRPV1 is highly

phosphorylated in the resting state, PKA can phosphorylate TRPV1 more obviously in the desensitised state at Ser116 amino acid (Bhave et al. 2002).

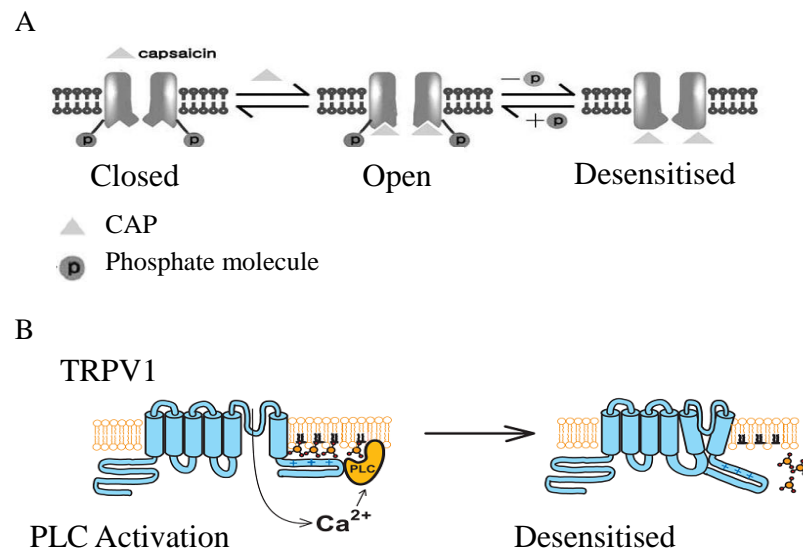


Figure 1-3: TRPV1 status, A) Binding of the CAP leads to TRPV1 activation, but prolonged exposure causes desensitisation, adopted from Suh, YG & Oh U (2005). **B)** Model of desensitisation of TRPV1. Calcium entry through TRPV1 activates a calcium sensitive PLC isoform leading to the depletion of PIP2 and inhibits TRPV1 activity. Adapted from Rohacs T (2008).

1.5 TRPV1 and Disease

TRPV1 appears to be involved in either the pathogenesis or progress of many diseases and conditions. Here, the role of TRPV1 in different diseases is reviewed.

1.5.1 Neuropathic Pain Syndromes

Neuropathic pain syndromes are a group of heterogeneous conditions caused by a lesion or disease that affects the peripheral or CNS and are often clinically characterised by spontaneous burning pain. In some patients, the nerve lesion enhances molecular dysfunction that evokes pathological activity of nociceptive neurons and thus, hypersensitivity of TRPV1 that causes pain (Baron 2006). The hyperactivity in nociceptors induces secondary changes in processing neurons in the

spinal cord and brain, whereby input from normal mechanical stimuli is sensed as pain.

1.5.2 Neurogenic Inflammation

Neurogenic inflammation is a process where inflammation is provoked by the nervous system, leading to the release of inflammatory mediators (Meggs 1993). The most common features of neurogenic inflammation are redness and warmth (secondary to vasodilation), swelling (secondary to plasma extravasation), and hypersensitivity (Richardson and Vasko 2002). It is triggered by overstimulation of peripheral nociceptor terminals as a consequence of injury, or by non-neurogenic inflammation (Proudfoot et al. 2006). Many studies have reported the role of TRPV1 in the neurogenic inflammatory process. Tissue damage induces inflammation, which is characterised by hypersensitivity to noxious stimuli or even allodynia (pain due to a stimulus which does not normally provoke pain) (Julius and Basbaum 2001). This provokes the release of neurotransmitters such as substance P and CGRP, and in the case of tissue injury, ATP and H⁺ from damaged cells. This in turn leads to vasodilation, increased capillary permeability at the site of injury and ultimately TRPV1 sensitisation (Nilius 2007).

1.5.3 Systemic Diseases

TRPV1 receptor was reported to be involved in a number of systemic diseases (Nilius et al. 2007). Table 1-2 summarises the role of TRPV1 in some systemic diseases. The role of TRPV1 as a potential therapeutic target has also been described. Desensitisation of the receptor by CAP and RTX has been reported to work as a pain relief (Kissin 2008; Knotkova et al. 2008). TRPV1 has a protective role as its role in

Table 1-2: Summary of studies investigating the role of TRPV1 in systemic diseases and conditions

TRPV1 in Systemic Diseases	TRPV1 role/effect	References
Urinary tract:		
• Bladder cystitis	• Hyperalgesia and hyperreflexia, increase anandamides concentration.	(Avelino and Cruz 2006; Dinis et al. 2004; Nilius et al. 2007)
• Painful bladder syndromes.	• Inactivation of bladder nociceptors i.e. treatment approach	(Habler et al. 1990; Sengupta and Gebhart 1994)
• Urinary incontinence due to bladder overactivity caused by spinal cord lesions	• Vanilloids suppresses involuntary bladder contractions leading i.e. treatment approach	(de Groat 1997)
• End-stage kidney disease	• Activation of the channel leads to PBMC death	(Saunders et al. 2009)
Gastrointestinal tract:		
• Gastrointestinal inflammation	• Aids in the mechanism of intestinal inflammation	(Massa et al. 2005; Sanger 2007)
• Erosive esophagitis	• Increase TRPV1 expression	(Matthews et al. 2004)
• Functional dyspepsia	• CAP therapy	(Allescher 2006; Rosch et al. 2006)
• Inflammatory bowel disease	• Regulates neurogenic inflammation and protect from colon cancer.	(Vinuesa et al. 2012)
Cardiovascular system:		
• Salt-sensitive hypertension	• Prevention action through vasodilatory and natriuretic–diuretic actions	(Inoue et al. 2006; Watanabe et al. 2009)
• Depressor effect on cardiac tissue	• Changes in heart rate and contractility	
• Myocardial ischemia	• Protect the heart through the release of substance P, CGRP and other neurokines	
Respiratory tract:		
• Cough and airway obstruction.	• Causes cough through endogenous inflammatory mediators such as PgE2, bradykinin, and histamine	(Fox et al. 1996; Jia et al. 2002)
• Acid- induced cough in guinea pigs.	• CAP cough hypersensitivity	(Higenbottam 2002; Laloo et al. 1998; Niimi et al. 2004; O'Connell et al. 1996; Plevkova et al. 2006)
• Chronic obstructive pulmonary disease, interstitial lung disease, gastroesophegal reflux disease, upper airway cough syndrome, bronchiectasis and airway infections.		
• Guinea pig and rat ozone-induced airway inflammation and hyperreactivity	• Reduction by CAP	(Kaneko et al. 1994; Koto et al. 1995)
• Airway response to formaldehyde, cigarette smoke in rats	• CAP decreased airway response to such substances.	(Lundberg and Saria 1983; Lundblad and Lundberg 1984; Morris et al. 1999)
• Guinea pigs sulphur dioxide-provoked bronchoconstriction	• Reduction by CAP	(Bannenberg et al. 1994; Laloo et al. 1995)

CAP: capsaicin; TRPV1: transient receptor potential vanilloid-1; CGRP: Calcitonin gene-related peptide; PgE2: Prostaglandin E₂; PBMC: peripheral blood mononuclear cell.

protecting the heart in myocardial ischemia (Inoue et al. 2006), or is directly involved in the inflammatory process (Table 1-2).

1.5.4 Vanilloid-induced Apoptosis and Cancer

Vanilloids, including CAP and their role in tumoral growth and cell transformation have been of great interest over the past few years. It has been shown that vanilloids play a role in cell death (apoptosis) as well as the inhibition of tumour proliferation through TRPV1-dependent and -independent mechanisms (Ziglioli et al. 2009). Several vanilloids have been reported to induce cell death in a variety of cell types (Table 1-3). Apoptosis, defined as a programmed cell death, is an important function in the cell as it controls cell proliferation (Khan et al. 2007). However, the apoptosis pathway is often dysfunctional in tumour cells (Thompson 1995). The mechanisms of apoptosis are potentially active in every cell, either tumoral or normal, though some studies have demonstrated the ability of vanilloids to target apoptosis in tumour cells rather than normal cells (Ito et al. 2004; Macho et al. 1999).

There is general agreement in the literature that prolonged contact time between normal cells such as TRPV1-transfected HEK293 and CAP, does not induce pre-apoptotic events such as vacuolisation or DNA damage (Caterina et al. 1997), whereas a shorter periods of contact between tumoral cells and CAP results in pre-apoptotic events. Following exposure of normal thymocytes (Amantini et al. 2004) and neoplastic cells to low concentrations of CAP, TRPV1-dependent apoptosis occurs. In contrast however, exposure to high concentrations induces cell-death through TRPV1-independent pathways (Amantini et al. 2004; Macho et al. 1999). Tumour cells are known to represent non-controlled cellular activity. In these cells, the electron transport chain (ETC) is constitutionally activated. The interaction of

Table 1-3: Some non-haematological cell lines that undergo vanilloid-induced cell death

Cell type	Vanilloid	Mechanism of apoptosis and/or associated observation	TRPV1-mediated	References
Glioma cell lines: Ge227, U87,G2258, U251 A172	AEA	Unknown	Yes	(Contassot et al. 2004b)
	CAP	Down-regulation of Bcl-2 and activation of caspase-3, reduce ROS level, upregulation of DR5	No	(Gil and Kang 2008; Kim et al. 2010; Lee et al. 2000)
Rat C6 glioma cells	AEA	Influx of Ca^{2+} , activation of the ceramide pathway, TRPV1 involve with CB1&2	Partially	(Jacobsson et al. 2001)
	CAP	Peroxyntirite – mediated apoptosis	No	(Qiao et al. 2005)
U373 (III)	CAP	Ca^{2+} influx and p38 MAPK activation	Yes	(Amantini et al. 2007)
Hepatic cells: Chang liver cell (non-tumour)	AEA	Lipid rafts interaction: p38/JNK , ceramide pathways, AP-1, caspase3 activity	No	(Giuliano et al. 2006)
HepG2 (hepatoma)	CAP	Mediated by NADPH oxidase-mediated increase in ROS or by activation of the PLC-dependent $[Ca^{2+}]_i$ release pathway	No	(Huang et al. 2009; Kim et al. 2005; Lee et al. 2004; Reilly et al. 2003)
Bronchiolar cells: BEAS-2B, BEAS-2B (TRPV1 ^{+/+}), NHBE, A549	CAP	Unknown	Yes	(Reilly et al. 2005; Reilly et al. 2003; Thomas et al. 2007)
	Nonivamide	Activation of ER-bound TRPV1 stimulates GADD153 expression via EIF2 α K3/EIF2 α pathway		
	RTX, AEA			
Neuroblastoma: CHP100	AEA	Increase $[Ca^{2+}]_i$, mitochondrial uncoupling, and cytochrome c release	Yes	(Maccarrone et al. 2000)
SHSY-5Y	CAP	Inhibited protein synthesis IC50=60mM, induced DNA strand breaks	Unknown	(Richeux et al. 1999)
Squamous cell carcinoma: COLO16, SRB-12	CAP, RTX	Rapid induction of hydroperoxide generation, inhibition of mitochondrial respiration	Unknown	(Hail Jr and Lotan 2002)
Embryonic kidney cells: TRPV1-HEK293	CAP	Increase in $[Ca^{2+}]_i$, apoptosis mechanism unknown	Unknown	(Grant et al. 2002)
Cervical cancer: CC299, caski, HeLa cells	AEA	Cleavage of caspase-7	Yes	(Contassot et al. 2004a)
KB cells	CAP	Disruption of mitochondrial potential and activation of caspase 9, 3 and poly-(ADP-ribose) polymerase	Unknown	(Lin et al. 2013)
Spermatogenic stem: Gc-5spg, Rat Gc-6spg	CAP	Caspase-3 activation, DNA fragmentation	Unknown	(Mizrak et al. 2008)
Urothelial cell cancer: RT4	CAP	Fas/CD95 clustering, caspase-3, -8 activation, BID cleavage	Yes	(Amantini et al. 2009)
Pancreatic cancer: BxPC-3, AsPC-1	CAP	Mediates ROS production which leads to the inhibition of mitochondrial complex-I and III activity	Unknown	(Pramanik KC 2011; Zhang et al. 2008)
Melanoma: A375 (mouse), A375-S2	CAP	Inhibition of NADH oxidase, high nitric oxide, down- regulate the ICDA	Unknown	(Gong et al. 2005; Jun et al. 2007; Kim 2012; Morre et al. 1996)
B16-F10 (murine)		Down-regulation the Bcl-2 and caspase-3 activation		

AEA: N-arachidonylethanolamine (anandamide); AP-1: activator protein-1; CAP: capsaicin; Bcl-2: B-cell lymphoma 2; CB1&2: cannabinoid receptor 1&2; eIF2- α : eukaryotic initiation factor 2- α ; eIF2 α K3: eukaryotic translation initiation factor-2 α kinase-3; ER: endoplasmic reticulum; MAPK: Mitogen-activated protein kinases; NADH: Nicotinamide adenine dinucleotide; PLC: Phospholipase C; ROS: Reactive oxygen species; RTX: resiniferatoxin; TRPV1^{+/+}: TRPV1- overexpressed; RT4: Human well-differentiated low-grade papillary.

tumoral cells with CAP, which antagonises coenzyme Q, inhibits the run of the ETC from complex I to complex II and produces more reactive oxygen species (ROS) in tumour cells than in normal cells (Ziglioli et al. 2009).

1.6 TRPV1 Expression and Function in Immune System Cells

Few studies have investigated TRPV1 expression in immune cells. TRPV1 mRNA has been detected in the peripheral blood mononuclear cells (PBMCs) of rats (Schumacher et al. 2000), and humans using RT-PCR (Saunders et al. 2007). In the next few pages, TRPV1 expression and role in the leukocytes have been extensively reviewed.

1.6.1 Lymphocytes

TRP channels have been shown to play an important role in Ca^{2+} increase in lymphocytes (Pedersen et al. 2005). A variety of TRP channels are known to be expressed and functional in lymphocytes such as TRPM4 and TRPC7 (Launay et al. 2004; Lievremont et al. 2005; Mori et al. 2002). TRPV1 mRNA has been found to be expressed in murine lymph nodes and DRG, but weakly in the spleen, using RT-PCR (Inada et al. 2006). An increase in $[\text{Ca}^{2+}]_i$ is essential for lymphocyte activation, proliferation and differentiation (Gallo et al. 2006). In B- and T-lymphocytes, the reaction between antigen and receptor results in a dual phase Ca^{2+} response, a transient $[\text{Ca}^{2+}]_i$ increase due to Ca^{2+} release from endoplasmic reticulum (ER) stores, and a subsequent prolonged $[\text{Ca}^{2+}]_i$ increment through Ca^{2+} influx from the extracellular environment across the plasma membrane. Both phases lead to increased $[\text{Ca}^{2+}]_i$ and subsequently activation of many transcription factors such as nuclear factor of activated T-cells (NFAT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). These in turn lead to cell proliferation and the

production of various cytokines (Inada et al. 2006). In the transient $[Ca^{2+}]_i$ increase phase, the IP₃-receptor is activated by IP₃ cleaved from PIP₂, causing the release of Ca^{2+} from the ER into the cytoplasm. In the subsequent phase, different ion channels induce Ca^{2+} influx across the plasma membrane and maintain high $[Ca^{2+}]_i$ levels (Inada et al. 2006).

NF- κ B is a protein complex that controls the transcription of DNA. It involves cellular response to stimuli such as stress, cytokines, free radicals, bacterial or viral antigens, and inflammation (Gilmore 2006). In inactive lymphocytes, NF- κ B dimers are isolated in the cytoplasm in an inactive form by association with a suppressor I κ B subunit, mainly I κ B α . Upon activation, multiple kinases lead to phosphorylation of I κ B α and proteasome-mediated degradation. Thus, active NF- κ B complex is released and translocated to the nucleus. In the nucleus, NF- κ B activates many genes that participate in inflammation, immune responses and cellular proliferation regulation (Baldwin 1996). It also causes blockade of the nuclear translocation of p65, leading to the inhibition of apoptosis (Han et al. 2002; Singh et al. 1996). CAP has been reported to inhibit the release of the inflammatory cytokines (Han et al. 2002; Sancho et al. 2002), and this effect is TRPV1-mediated (Chen et al. 2003). Two human transcription factors, NF- κ B and activator protein 1 (AP-1), which have been implied in many inflammatory diseases and apoptosis were inhibited by CAP and RTX (Han et al. 2002; Portis et al. 2001; Sancho et al. 2002; Singh et al. 1996).

Furthermore, substance P, an indirect TRPV1 activator which is produced by TRPV1 stimulation, has been demonstrated to involve in regulating the functions of

lymphocytes. Its gene expression and its receptor have been detected on human lymphocytes (Lai et al. 1998). CAP has been shown to have immunomodulatory effects through its ability to modulate lymphocyte proliferation and immunoglobulin A, E and G production (Nilsson et al. 1991; Takano et al. 2007). In addition, CAP inhibits T-helper cell cytokine production in cultured murine Peyer's patch (PP) cells, *in vitro*, whereas oral injection of capsicum extract and CAP enhances the production of T-helper 1 cytokines such as IL-2 and interferon-gamma in response to the mitogen, concanavalin A (Takano et al. 2007). Capsicum extracts and CAP had an opposite effect on immune responses *in vitro* and *ex vivo*, indicating the presence of an indirect effect *in vivo*. Furthermore, direct treatment of PP cells with 3 and 30 μ M of CAP reduced cell viability (Takano et al. 2007). TRPV1 has been detected on T-lymphocytes in PP and it mediates CAP-induced T-cell reduction which was partly inhibited with capsazepine. A study found that CAP, if administered subcutaneously to neonatal rats, it would cause a marked reduction in thymus weight and a lack of thymocyte differentiation by triggering apoptosis (Santoni et al. 2000). This indicates a negative effect of CAP on the thymus and lymphocytes which contributes to apoptosis in these cells.

1.6.2 Macrophages

There have been few studies investigated the CAP effects on macrophages. The ROS species generated by activated macrophages play a crucial role in the initiation of inflammation and have harmful effects on neuroinflammatory diseases. Treating macrophages with 10 μ M CAP suppresses superoxide anion, hydrogen peroxide and nitrite radical production completely *in vitro* (Joe and Lokesh 1994). Furthermore, CAP inhibited store-operated Ca^{2+} entry in rat peritoneal macrophages (Joe and Lokesh 1994). In the CNS, ROS production was substantially inhibited upon TRPV1

suppression in microglia, the tissue macrophages function mainly in the CNS. In addition, membrane depolarisation, the main characteristic of NADPH oxidase activity in phagocytes, was reported to promote TRPV1 activity in microglia (Schilling and Eder 2009a).

1.6.2.1 Dendritic cells

Dendritic cells (DCs) are a set of leukocytes widely distributed in all tissues, especially in those that interface with the external environment. They are potent antigen presenting cells, that have the ability to stimulate T-cells and differentiate B-cells (Satthaporn and Eremin 2001).

The myeloid and lymphoid-derived subsets of DCs perform specific stimulatory functions. They are produced in the bone marrow from haemopoietic stem cells and circulate in the peripheral blood as immature precursors prior to migrating into peripheral tissues. DCs differentiate within different tissues and become able to capture and process antigens and present them to other cells of the immune system (Satthaporn and Eremin 2001). Moreover, DCs can secrete cytokines, migrate to lymph nodes after activation and activate lymphocytes (Shortman and Liu 2002). The expression of TRPV1 in DCs is controversial, as TRPV1 protein has been detected in human (Toth et al. 2009) and murine DCs (Basu and Srivastava 2005). Basu and Srivastava (2005) detected TRPV1 in murine DCs using Western blot and RT-PCR, and found that TRPV1 activation could lead to the activation and maturation of the DCs. However, O'Connell and co-workers (2005) failed to detect TRPV1 mRNA in murine DCs and criticised the lack of reported controls and potential genomic contamination in Basu and Srivastava's studies. Toth et al (2009)

have shown that human peripheral blood monocytes, immature and mature DCs express functional TRPV1 using flow cytometry, Ca^{2+} -imaging and quantitative real-time PCR (Q-PCR). In addition, TRPV1 expression dramatically increased during cytokine-induced *in vitro* differentiation of monocytes to immature DCs. CAP has been reported to suppress phagocytosis by a TRPV1-mediated pathway, and did not induce apoptosis in immature DCs. In contrast to mouse DCs, CAP did not also promote DC maturation in the absence of cytokines, in humans (Toth et al. 2009).

1.6.3 Neutrophils

Neutrophils play a central role in the inflammatory response and comprise an essential part of the innate immune system (Wang et al. 2005). An increase in cytosolic $[\text{Ca}^{2+}]_i$ is attributed to the neutrophils in response to chemoattractants (Krause et al. 1990), and induces synthesis and release of prostaglandins (Krump et al. 1995). CAP affects neutrophil function and increases $[\text{Ca}^{2+}]_i$ in rat neutrophils by inducing extracellular Ca^{2+} influx and Ca^{2+} release from the internal Ca^{2+} pool (Wang et al. 2005). TRPV1 mRNA has been detected in human and rat neutrophils and in HL-60 (neutrophilic promyelocytic leukaemia) cell line using RT-PCR, however the role of TRPV1 in these cells remains poorly understood (Heiner et al. 2003; Wang et al. 2005).

1.7 The TRPV1: Role in Haematological Malignancies

A limited number of studies have investigated TRPV1 expression and function in haematological malignancies. Furthermore, TRPV1 expression by malignant haematological cell lines has not been extensively studied (*see summary* Table 1-4).

Table 1-4: Summary of TRPV1-expression and function in malignant haematological cell lines

Haematological cancer cell line	TRPV1 expression	Vanilloid tested	Vanilloid effect	TRPV1-mediated?	References
Acute myeloid leukaemia:					
• Kasumi-1	Unknown	CAP	Apoptosis	Unknown	(Ito et al. 2004)
• THP-1	Expressed	CAP	Stimulation at low, lethal at high concentrations	Yes	(Kunde et al. 2010)
Acute lymphoid leukaemia:					
• CEM	Unknown	THC	Apoptosis	Unknown	(Powles et al. 2005)
• HPB-ALL	Unknown	CAP	Apoptosis	Unknown	(Zhang et al. 2003a)
• Jurkat T-cell line	Expressed	Arvanil,	Apoptosis	No	(Dou et al. 2011; Gertsch et al. 2002; Macho et al. 1999; Sancho et al. 2003; Zhang et al. 2003a)
		CAP	Immunomodulatory and anti-inflammatory, apoptosis is preceded by a reduction of $\Delta\Psi_m$ and increase in ROS	Unknown	
Human promyelocytic leukaemia:					
• HL-60	Unknown	CAP THC	>50mM induced Apoptosis, cytotoxicity Apoptosis	Unknown	(Tsou et al. 2006)
• UF-1, NB4		CAP	Apoptosis		(Ito et al. 2004; Powles et al. 2005; Roy et al. 2002)
ATL cells: HPB-ATL-T, HPB-CTL-I, HUT-102	Unknown	CAP	Apoptosis, down-regulates Tax and Bcl-2 expression, inhibits NF-κB	Unknown	(Zhang et al. 2003a)
Erythroblastic leukaemia: HEL-92	Unknown	THC	Apoptosis	Unknown	(Powles et al. 2005)
CML: KU812, K562	Unknown	CAP	Apoptosis	Unknown	(Ito et al. 2004; Roy et al. 2002)
MM cells: U266, MM.1S	Unknown	CAP	> 5mM inhibited cell proliferation, Apoptosis, inhibits STAT3, potentiates chemotherapy	Unknown	(Bhutani et al. 2007)
Lymphoma: U937	Expressed	AEA	Apoptosis	Yes	(Ito et al. 2004; Maccarrone et al. 2000)
		CAP	Apoptosis	Unknown	

CAP: capsaicin; STAT: Signal transducer and activator of transcription; THC: Δ^9 -tetrahydrocannabinol; AEA: Anandamide; ATL: adult T-cell leukaemia; CML: chronic myeloid leukaemia; MM: multiple myeloma. $\Delta\Psi_m$: mitochondrial transmembrane potential.

1.7.1 Leukaemic cell lines

Few studies have investigated the expression and function of TRPV1 and its agonists in malignant haematological cells. As TRPV1 has been reported to be expressed in monocytes (Schilling and Eder 2009b), Ca^{2+} influx via TRPV1 was important for strengthening the adhesion between THP-1 cells (acute monocytic leukaemia cell line), and human umbilical vein epithelial cells. Mechanical stress caused by monocyte adhesion might activate TRPV1 channels (Himi et al. 2012).

Some groups have studied the effect of CAP on leukaemic cell lines *in vitro* and *in vivo*. Ito et al (2004) have reported that CAP suppressed the growth of leukemic cell lines i.e. Kasumi-1, UF-1 and NB4, but not normal bone marrow mononuclear cells, via induction of G_0 - G_1 phase cell cycle arrest and apoptosis. This effect was associated with increased ROS production, therefore CAP-induced cell death signalling is thought to be mediated by a mitochondrial-dependent pathway (Table 1-4) (Ip et al. 2012; Ito et al. 2004).

The tumour suppressor protein p53 is an important protein that mediates cell death (Ko and Prives 1996). CAP promotes the activation of p53 through phosphorylation, since nullification of p53 expression significantly reduced CAP-induced cell cycle arrest. CAP-sensitive leukaemic cells, such as NB4 and Kasumi-1, express wild-type p53 (Ito et al. 2004). The activation p53 by CAP leads to the up-regulation of some genes including the tumour suppression gene cyclin-dependent kinases (CDK) inhibitor ($p21^{WAF1/CIP1}$), and the pro-apoptotic Bcl-2-associated X (*Bax*) genes (Ito et al. 2004; Lakin and Jackson 1999; Miyashita and Reed 1995). This leads to the production of BAX protein which in turn, moves from the cytosol to the outer

mitochondrial membrane, causing the release of cytochrome c and caspases cascade activation leading to apoptosis (Green and Reed 1998; Ito et al. 2004; Sarkaria et al. 1998). Whilst cell cycle activation involves many molecules including cyclins, CDKs, and inhibitors, the upregulation of such molecules leads to cell cycle arrest. This concept has been considered in anticancerous drug studies (Paschka et al. 1998).

1.7.2 Adult T-cell leukaemia

Adult T-cell leukaemia (ATL) is an aggressive form of human T-cell malignancy caused by human T-cell leukaemia virus type-1 (Hinuma et al. 1981). There have been few studies demonstrating the role of CAP in inhibiting ATL cells proliferation. Direct injection of CAP into laboratory mice dramatically inhibited the growth of tumours in these mice (Morre et al. 1996). Exposure of ATL cells to CAP provoked G₁ phase and decreased S phase, where cell division occurs, in a dose-dependant manner. However, normal peripheral blood T-cells showed neither apoptosis nor necrosis (Zhang et al. 2003a). NF- κ B activation, an essential apoptosis inhibitor (Portis et al. 2001) by Tax, which in turn a human T-cell leukaemia virus type-1 transactivator protein, plays a crucial role in the pathogenesis of ATL (Arima et al. 1999). As noted, CAP can inhibit the activation of NF- κ B and DNA synthesis by decreasing NF- κ B binding activity of p65, resulting in the suppression of ATL (Table 1-4). The growth-inhibitory potential of CAP on ATL cells was reported to be mainly due to G₁/cell cycle arrest and apoptosis (Zhang et al. 2003a). G₁ cell cycle arrest has been demonstrated to be a common apoptosis mechanism of many anticancerous drugs (Choi et al. 2001; Fukuoka et al. 2000).

In addition to the role of decreased NF- κ B activity in CAP-induced apoptosis, down-regulation of B-cell lymphoma-2 protein (Bcl-2) may also be responsible

for apoptosis. As in contrast to Bax, Bcl-2 and B-cell lymphoma-extra large (Bcl-xL) are essential factors which act to protect the cell against apoptosis (Yang and Korsmeyer 1996). Indeed, the Bcl-2/Bax ratio is considered to be an important determinant of apoptosis (Reed et al. 1996). CAP has the ability to reduce the expression of Bcl-2 as well as the Bcl-2/Bax ratio, leading to apoptosis in ATL cell lines (Zhang et al. 2003a).

1.7.3 Multiple Myeloma

There have been few studies that have investigated the effect of CAP on multiple myeloma (MM) cells. Signal transducer and activator of transcription (STAT) is a well known family which regulates the expression of gene products (Darnell 2002). Among the STATs, STAT3 is the member mostly related to tumorigenesis. It is constitutively active in tumour cells and can be activated by growth factors, such as interleukin-6 (IL-6), and oncogenic kinase such as Src. STAT3 regulates the expression of genes that mediate proliferation (e.g., c-myc and cyclin D1), suppress apoptosis (e.g., Bcl-xL) and encourage angiogenesis (Bhutani et al. 2007).

It has been found that CAP can cause apoptosis in MM cells through the inhibition of IL-6-inducible STAT3 activation (Bhutani et al. 2007). STAT3 has been demonstrated to be linked to a chemoresistance and radioresistance, therefore inhibition of STAT3 activation may play a role in prevention and treatment of cancer (Aggarwal et al. 2006).

Velcade (PS341) and thalidomide are common therapeutic agents for the treatment of MM patients (Cavo 2006). Prolonged exposure to these compounds is associated with toxicity and development of chemoresistance. Up-regulation of different anti-

apoptotic proteins such as Bcl-2, and Bcl-xL has a critical role in the mechanism of the chemoresistance (Chauhan et al. 2007; Mitsiades et al. 2002). CAP was demonstrated to enhance the apoptotic effect of velcade and thalidomide (Bhutani et al. 2007). However, the role of TRPV1 in this effect has not been determined.

1.8 Project Aims

The *overall aim* of this project was to investigate the expression and role of TRPV1 in haematological malignancies. The *specific aims* were to:

1. Investigate the viability/activity of cells cultured from the haematological malignancies, following the exposure to the TRPV1 agonist, CAP (Chapter 2);
2. Investigate the effect of TRPV1 and cannabinoid receptor antagonists on malignant haematological cell lines (Chapter 2);
3. Optimise Western blotting and flow cytometry protocols to detect TRPV1 expression in human white blood cells (Chapter 3 and 4);
4. Investigate the expression of TRPV1 protein in PBMCs obtained from healthy adult donors, malignant haematological cells and other cell lines (Chapter 5);
5. Assess whether TRPV1 expression is altered in patients with haematological malignancies, compared to gender-matched healthy controls (Chapter 6).

1.9 Hypotheses

1. CAP induces a reduction in the viability of cells cultured from haematological malignancies cell lines, and this effect is TRPV1-mediated.
2. TRPV1 receptor is expressed in normal leukocytes and it is overexpressed in malignant haematological cell lines.

3. TRPV1 expression is increased in leukocytes obtained from patients with haematological malignancies compared to healthy subjects.

**Chapter 2: Capsaicin-Induced Death of Human Haematological
Malignant Cell Lines is Independent of TRPV1 Activation**

2.1 Abstract

Capsaicin (CAP), the major active component of hot chilli peppers, has previously been shown to have anticancer properties. The precise mechanism(s) by which CAP acts on malignant cells however, have yet to be determined. The aims of this study were to; 1) investigate the effect of CAP on the metabolic activity (viability) of human malignant haematological cell lines, and 2) to determine if any effect was mediated through its cellular receptor, transient receptor potential vanilloid-1 (TRPV1). THP-1 (acute monocytic leukaemia), U266B1 (myeloma) and U937 (histiocytic lymphoma) cell lines were exposed to increasing concentrations of CAP (8-1000 μM). Cell metabolic activity was measured after 24hr in the presence and absence of TRPV1, CB1 and CB2 antagonists (0.1-100 μM) using the alamarBlue[®] method (resazurin reduction). CAP reduced metabolic activity in THP-1, U266B1 and U937 cells in a concentration-dependant manner. A biphasic effect was observed in THP-1 cells ($\text{EC}_{50}/\text{IC}_{50}$ (95% CI) = 32.9(19.9-54.3)/ 219(144-246) μM). Blocking TRPV1 using SB452533 and CB1 receptors using AM251 (100 μM) suppressed the CAP-induced increase in THP-1 metabolic activity ($P < 0.001$). U266B1 cells were more resistant to CAP than THP-1 and U937 cells. Metabolic activity was significantly inhibited by CAP in U937 compared to U266B1 cells (IC_{50} : 197 vs. 431 μM , respectively, $P < 0.008$). AM251 and SB452533 appeared to act as partial agonists and displayed a synergistic effect with CAP in U937 cells. In conclusion, THP-1, U266B1 and U937 cell lines responded differently to CAP. TRPV1, CB1 and CB2 antagonists did not affect CAP-induced metabolic activity in U266B1 cells, although TRPV1 or CB1 receptors appeared to mediate an increase in metabolic activity in THP-1 cells. This data strongly suggests that CAP inhibits the metabolic activity of malignant haematological cells through TRPV1-independent mechanisms.

2.2 Introduction

Vanilloids are naturally-occurring substances found in many spicy foods. CAP is the major vanilloid present in hot chilli peppers (*Capsicum spp.*) (Monsereenusorn et al. 1982) and is responsible for the ‘burning’ sensation when consumed (Knotkova et al. 2008). The actions of CAP are mediated by the non-selective cation channel, transient receptor potential vanilloid-1 (TRPV1) (Nagy et al. 2004).

TRPV1 belongs to the TRP superfamily, which consists of a number of non-selective cation channels that are permeable to both monovalent and divalent cations (Alexander et al. 2007). TRP channels are involved in many functions, including nociception, temperature and mechanical sensation, renal $\text{Ca}^{2+}/\text{Mg}^{2+}$ handling, cardiovascular and respiratory regulation, and the control of cell growth and proliferation (Christensen and Corey 2007; Ramsey et al. 2006).

The TRPV1 channel was originally believed to be expressed primarily on sensory neurons originating from the central nervous system (CNS), including dorsal root and trigeminal ganglion neurons (Julius and Basbaum 2001; Schicho et al. 2004). However, TRPV1 message and/or protein has been detected in non-neuronal cells such as the urothelium (Birder et al. 2001), smooth muscle and keratinocytes (Jaggar et al. 2001), epithelial cells of the gastrointestinal (Geppetti and Trevisani 2004) and respiratory tracts (Reilly et al. 2003), and leukocytes (Saunders et al. 2009; Saunders et al. 2007).

CAP has been reported to have anticancer properties. It promotes apoptosis and inhibits proliferation of a number of cancer cell lines, including glioma (Amantini et

al. 2007; Contassot et al. 2004a; Gil and Kang 2008), hepatoma (Huang et al. 2009; Lee et al. 2004; Reilly et al. 2003), A549 lung adenocarcinoma (Thomas et al. 2007), squamous cell carcinoma (Hail Jr and Lotan 2002), cervical cancer (Contassot et al. 2004a), urothelial and bladder cell cancer (Amantini et al. 2009; Chen et al. 2012a) and pancreatic neuroendocrine tumour cells (Skrzypski et al. 2014). In addition, CAP, in combination with other drugs such as cisplatin, induces apoptosis in cisplatin-resistant gastric cancer cell lines (Huh et al. 2011). Several mechanisms of action have been proposed for CAP-induced apoptosis of cancer cells, depending on cell type and method used to assess the cell death (Amantini et al. 2009; Chen et al. 2012a; Morré et al. 1995). Furthermore, many studies have investigated the effect of CAP on leukaemic cell lines *in vitro* and patient cells *ex vivo*. These have shown that CAP causes apoptosis in a concentration- and time-dependant manner in Kasumi-1 (Contassot et al. 2004a), KU812 (Roy et al. 2002), U266 (Bhutani et al. 2007), U937 (Contassot et al. 2004b; Maccarrone et al. 2000), HPB-ALL and HPB-CTL-I (Zhang et al. 2003a), HEL-92 (Powles et al. 2005), UF-1, NB4 (Contassot et al. 2004b; Powles et al. 2005) and HL-60 cells (Tsou et al. 2006).

TRPV1 expression by, and CAP-induced apoptosis of, human peripheral blood mononuclear cells (PBMCs) have been previously reported (Saunders et al. 2007). However, the role of TRPV1 in malignant haematological cell lines has yet to be extensively investigated. Therefore, the aims of this chapter were to determine; 1) the effect of CAP on the metabolic activity (an indicator of cell viability) of THP-1, U266B1 and U937 cells, and 2) whether any observed effects were TRPV1-mediated.

2.3 Materials and Methods

2.3.1 Materials

Cells and solutions

The human acute monocytic leukaemia cell line, THP-1, was obtained from the European Collection of Cell Cultures (UK). The human multiple myeloma cell line, U266B1, and human histiocytic lymphoma cell line, U937, were purchased from the American Type Culture Collection (Manassas, VA, USA). Table 2-1 summarises the characteristics of the malignant haematological cell lines used in this study. RPMI1640 was purchased from Life Technologies (Grand Island, USA) and ATCC (VA, USA). Foetal bovine serum (FBS) was purchased from Life Technologies, Grand Island, USA, and L-Glutamine-Penicillin-Streptomycin solution was purchased from Sigma-aldrich, St. Louis, USA.

Reagents

The following reagents were purchased from Tocris Bioscience, Ellisville, USA: (E)-Capsaicin (CAP), SB452533 (TRPV1 antagonist), AM251 (CB1 antagonist) and AM630 (CB2 antagonist). Trypan Blue (0.4%) was purchased from Life Technologies, Grand Island, USA.

2.3.2 Methods

THP-1 Cell Culture

THP-1 cells were cultured in RPMI1640 (Life Technologies, Grand Island, USA) supplemented with 20% heat-inactivated FBS (Life Technologies, Grand Island, USA), 2 mM glutamine, 100 U/mL Penicillin and 100 µg/mL Streptomycin, all

Table 2-1: Characteristics of the studied haematological malignant cell lines

	THP-1	U266B1	U937
Species	Human	Human	Human
Original disease	Acute monocytic leukaemia	Multiple Myeloma	Lymphoma
Tissue	Peripheral blood	B- lymphocyte; plasmacytoma; myeloma	Histiocytic lymphoma
Age/ gender	1 year/ male	53 years/ male	37 years/ male
Cellular products	Lysozymes, IL-1	Igs, monoclonal antibody and IL-6	Lysozyme, beta-2 microglubulin and TNF
Morphology	Monocytes	Lymphoblasts	Monocytes
Growth property	suspension	suspension	suspension
Passage	5-10	4-9	4-9
Concentration	5 x 10 ⁵ viable cell/ mL		

IL-1: interlukin-1; IL-6: interlukin-6; Igs: immunoglobulins; TNF: Tumor Necrosis Factor

prewarmed to 37°C prior to use. A frozen vial of THP-1 cells was thawed quickly in the water bath at 37°C and transferred drop wise to 5 mL of warm complete media. Cells were then centrifuged at 150 x g for 5 min. The supernatant was discarded and the cell pellet resuspended at a density of $3-5 \times 10^5$ cells/mL. A 100µL aliquot of cell suspension was placed into an Eppendorf tube for cell counting. Twenty microlitres of cell suspension was mixed with 20µL of Invitrogen Countess 0.4% Trypan Blue and 10µL of the mix was loaded on a slide for cell counting using Countess[®] automated cell counter (Life Technologies, Grand Island, USA). Cells were then seeded at 2×10^5 /mL and transferred to a T25 culture flask and incubated in vertical position at 37°C, 5% CO₂ in a Heraeus HERecell 150 incubator (Thermo Fisher Scientific, Walham, USA) for 7 days and then repositioned horizontally for another 7 days. Once the culture was established, the FBS was reduced to 10%. To keep cells in the exponential growth phase, cells were maintained between $3-8 \times 10^5$ cells/mL. Cell viability was maintained >90%, otherwise were discarded.

U266B1 and U937 Cell Culture

U266B1 and U937 cell lines were cultured in RPMI1640 (ATCC, VA, USA), containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate and supplemented with 15% and 10% FBS, respectively. 100,000 IU/mL Penicillin and 100,000 µg/mL of Streptomycin were used in primary cultures only, according to supplier's instructions. Frozen vials of U266B1 and U937 cells were thawed quickly in the water bath at 37°C (< 2 min) and transferred drop wise to 9 mL of warm complete media, centrifuged at 150 x g for 5 min and resuspended slowly in 5 mL of fresh warm media. Cell counts were performed as previously described. The cell suspensions were then transferred to T25

culture flasks and incubated in horizontal position at 37°C, in 5% CO₂ in a Heraeus HEREcell 150 incubator (Thermo Fisher Scientific, Walham, USA) for 3 days before subculturing. Culture was maintained at 2 x 10⁵ cell/mL and was not allowed to exceed 1 x 10⁶ cell/mL. Every subculture was considered as one passage.

2.3.3 Cryopreservation

For U937 and U266B1, 1-5 x 10⁶ cells were resuspended in 1 mL of complete media containing 5% (v/v) dimethyl sulfoxide (DMSO). THP-1 cells, however, were harvested at 1-3 x 10⁶ cells and resuspended in a cryopreservation vial containing 1 mL of RPMI1640 (Life Technologies, Grand Island, USA) supplemented with 20% FBS, 2 mM glutamine, 100 U/mL Penicillin, 100 µg/mL Streptomycin and 10% glycerol. Aliquots were stored at -80°C using a NalgeneTM Mr. FrostyTM Cryo 1°C freezing container (Thermo Scientific, MA, USA) for 24 hr, which allows for a freezing rate of 1°C/min before transferring to the nitrogen phase.

2.3.4 Cell metabolic activity assays

CAP and SB452533 were reconstituted in ethanol, whereas AM251 and AM630 were reconstituted in DMSO. Cell metabolic activity was measured using the alamarBlue[®] method according to manufacturer's instructions. Briefly, resazurin, the active ingredient of alamarBlue[®] reagent, is taken into the cells and reduced to resorufin, producing bright red fluorescence. The extent of reduction is related to cell viability and metabolic activity (Nakayama et al. 1997).

Cells were exposed to either increasing concentrations of CAP alone or to 125µM of CAP in the absence and presence of the TRPV1, CB1 or CB2 antagonists for 22 hr at 37°C in 5% CO₂. The cells were then incubated with alamarBlue[®] reagent for

another 2 hr. Fluorescence was measured at 590 nm using an excitation wavelength of 545 nm on a Tecan GENios plate reader (Zurich, Switzerland). The relative fluorescence of treated cells was normalised to untreated cells (vehicle) and metabolic activity expressed as a percentage of vehicle.

Effect of CAP on THP-1, U266B1 and U937 cell metabolic activity

Cells were harvested by centrifugation at $150 \times g$ for 5 min then fresh media was replaced by aspiration. Cells were seeded in 96 wells flat-bottom plates at a density of 5×10^5 cells/ 100 μ L/ well and incubated at 37°C, 5% CO₂ for 10 min. Increasing concentrations of CAP (8-1000 μ M) were then added to the cells. Cell metabolic activity was then measured using the alamarBlue[®] method as described earlier.

Effect of TRPV1, CB1 and CB2 antagonists on CAP-induced changes in cell metabolic activity

In preliminary experiments, 50 μ L of the TRPV1 antagonist, SB452533 (0.1, 1.0 and 10 μ M) was added to THP-1, U266B1 and U937 cells and incubated at 37°C, 5% CO₂ for 30 min. Increasing concentrations of CAP (8-1000 μ M) were then added to the cells and assessed for viability using the alamarBlue[®] method. Test cells were compared to a vehicle containing cells treated with ethanol only.

In subsequent studies, cells were incubated with 0.1-100 μ M SB452533, or the CB1 and CB2 antagonists, AM251 and AM630, independently, for 30 min, and then with 125 μ M CAP. The effect of each antagonist alone on metabolic activity was also investigated and used as a control. Cell metabolic activity was then assessed using

the alamarBlue[®] method. Metabolic activity was compared to vehicle (complete media containing ethanol for SB452533 and DMSO for AM251 and AM630).

Data analysis

Data analysis was performed using GraphPad Prism (Graphpad Software Inc, V5.01, CA, USA). Data is expressed as the mean \pm SEM of *n* experiments. EC₅₀/ IC₅₀ for CAP-induced changes in viability assays were obtained by fitting data using the nonlinear regression curve fit (log [antagonist] vs. response-variable slope (four parameters)) function and expressed as the mean and 95% confidence interval (95% CI). A two-way ANOVA followed by Bonferroni post hoc test was performed to determine the P-value. P<0.05 was considered statistically significant.

2.4 Results

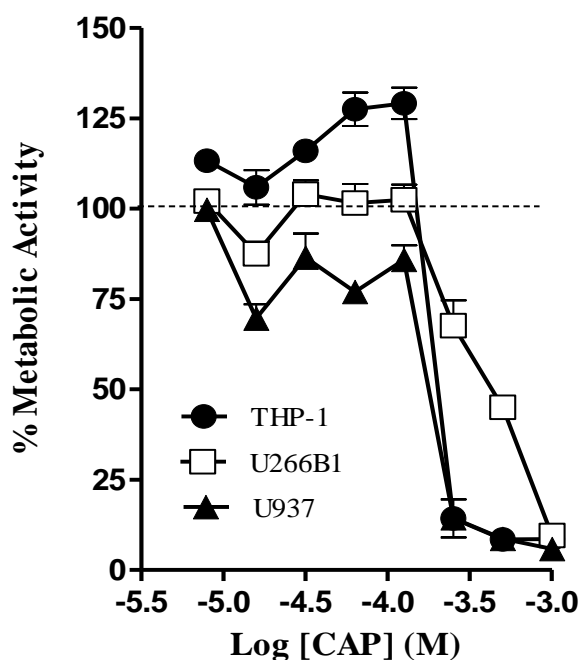


Figure 2-1: Differential response of THP-1, U266B1 and U937 cells to CAP. Cells were treated with increasing CAP concentrations for 24 hr. Metabolic activity was measured using the alamarBlue[®] method (resazurin reduction) and readings were normalised to a pre-exposure metabolic activity (expressed as a percentage). Each point represents the mean \pm SEM (*n* = 10).

CAP decreases THP-1, U266B1 and U937 cell metabolic activity. THP-1 cells responded to CAP with an initial increase in metabolic activity by 29% with the highest increase reached at 125µM of CAP, followed by a decline at a CAP concentration greater than 125µM, indicative of reduced viability and cell death (Figure 2-1). The metabolic activity concentration-response curve for THP-1 cells was more bell-shaped. The stimulatory and inhibitory phases were analysed separately to generate the respective EC₅₀ and IC₅₀ values (Table 2-2). U266B1 cells preserved their activity (104%) under the effect of sub-lethal concentration of CAP (8-125 µM) (Figure 2-1). However, at CAP concentrations greater than 125µM, metabolic activity decreased, but required higher concentrations compared to THP-1 and U937 cells. Furthermore, U937 cell metabolic activity was inhibited by ~1-14% when exposed to the sub-lethal concentrations (8-125 µM) of CAP (Figure 2-1) before declining rapidly by 93% at the highest concentration of CAP (1000µM). The IC₅₀ in U937 cells was significantly lower than compared to IC₅₀ of U266B1 cells (Table 2-2).

SB452533 has no effect on CAP-induced metabolic activity in THP-1, U266B1 and U937 cells. Pre-treatment of the three cell lines with SB452533 (0.1-10 µM) had insignificant effect in the metabolic activity response curve of CAP (Figure 2-2).

Table 2-2: EC₅₀/IC₅₀ for CAP-induced metabolic activity in THP-1, U266B1 and U937 cells

Cell line	*EC ₅₀ (95% CI) (µM)	**IC ₅₀ (95% CI) (µM)
THP-1	32.9 (19.9-54.3)	219 (144-246)
U266B1	N/A	431 (382-489)
U937	N/A	197 (139-278)

*EC₅₀: the concentration of CAP that gives half-maximal increase in response; **IC₅₀: the concentration of CAP where the response is reduced by 50%. Results are expressed as mean (95% confidence interval, CI), n=10; N/A: non-available.

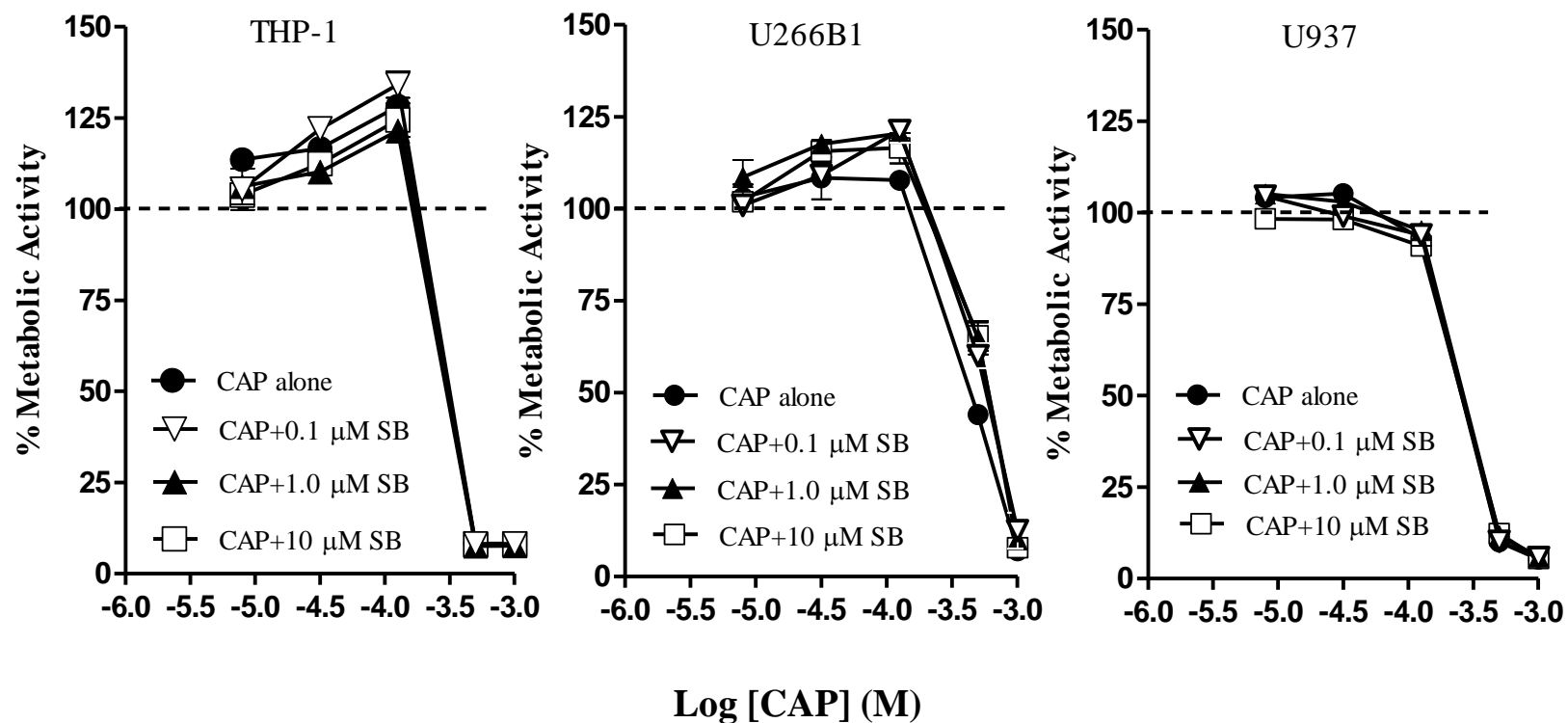


Figure 2-2: Effect of CAP and the TRPV1 antagonist, SB452533, on the metabolic activity (resazurin reduction) of THP-1, U266B1 and U937 cells. Cells were incubated with increasing concentration of SB452533 for 30 min before exposure to CAP for 24 hr. For each cell line, the experiment was performed twice in triplicate for each CAP concentration (mean \pm SEM, $n=6$). Metabolic activity (resazurin reduction) was expressed as percentage.

High concentrations of SB452533 and AM251 enhance the cytotoxic effect of CAP on THP-1 cells. In the experiments with CAP alone (Figure 2-1) and CAP in the presence of SB452533 (Figure 2-2 and Figure 2-3, A), an increase in THP-1 cell metabolic activity (increase resazurin reduction) at 125 μ M CAP of ~29% was observed. However, in subsequent experiments with AM251 and AM630, this increase was not observed (Figure 2-3, B and C). SB452533, AM251 and AM630 (0.1-10 μ M) alone had no effect on the metabolic activity of THP-1 cells. Pre-treatment of THP-1 cells with lower concentrations of SB452533 (0.1, 1.0 and 10 μ M) had a similar effect as CAP alone on the metabolic activity ($P > 0.05$, Figure 2-3, A). In contrast, the highest concentrations of SB452533, AM251 and AM630 (100 μ M) significantly decreased metabolic activity in the presence of CAP (125 μ M) by ~20%, 65% and 55% respectively ($P < 0.001$, compared to CAP alone, Figure 2-3). However, AM630 (100 μ M) alone appeared to have an effect on reducing the metabolic activity independently, with this effect considered non-specific. In addition, pre-exposure of THP-1 cells to AM251 or AM630 (0.1-10 μ M) significantly increased the metabolic activity in the presence of CAP for up to 25% (Figure 2-3: B vs. C, $P < 0.01$ vs. $P < 0.05$, respectively compared to CAP alone).

No significant effect of TRPV1 and CB antagonists on CAP-induced cytotoxicity on U266B1 cells. Similar to THP-1 cells, 125 μ M CAP had little or no effect on the metabolic activity of U266B1 cells (Figure 2-1). However, metabolic activity increased by 125 μ M CAP in the subsequent experiments with antagonists (by ~20%). Pre-treatment of U266B1 cells with SB452533 (0.1-10 μ M) increased CAP-induced metabolic activity, but these results were not statistically significant ($P > 0.05$, compared to CAP alone). In addition, at the highest concentration of

SB452533 (100 μ M), metabolic activity was significantly decreased by 25% compared to CAP alone, but as SB452533 alone at this high concentration appeared to inhibit the metabolic activity, this effect was considered non-specific (Figure 2-4, A). Paradoxically, AM251 (0.1-1.0 μ M) increased the metabolic activity by ~25% compared to CAP. However, AM251 alone appeared to increase the metabolic activity independently, therefore this effect was also considered non-specific (Figure 2-4, B). Furthermore, pre-treatment of U266B1 cells with AM630 had an additive effect to CAP-induced metabolic activity, however this effect was not statistically significant compared to CAP alone (Figure 2-4, C).

TRPV1 and CB1 antagonists enhanced CAP-induced cytotoxicity on U937 cells.

Exposure of U937 cells to CAP alone decreased metabolic activity by 14% (Figure 2-5). All three antagonists alone (0.1-10 μ M) had no effect on cell metabolic activity. However, at the highest concentration (100 μ M) of each of the three antagonists alone or with post-exposure to CAP, completely abolished the metabolic activity of U937 cells. Furthermore, pre-treatment of these cells with SB452533 (10 μ M) or AM251 (0.1-10 μ M), significantly decreased the metabolic activity in the presence of CAP ($P < 0.001$, compared to CAP alone). However, when the cells were pre-treated with SB452533 (0.1-1.0 μ M) or AM630 (0.1-10 μ M), then exposed to CAP, a similar effect to CAP alone on the metabolic activity was observed ($P > 0.05$) (Figure 2-5).

THP-1

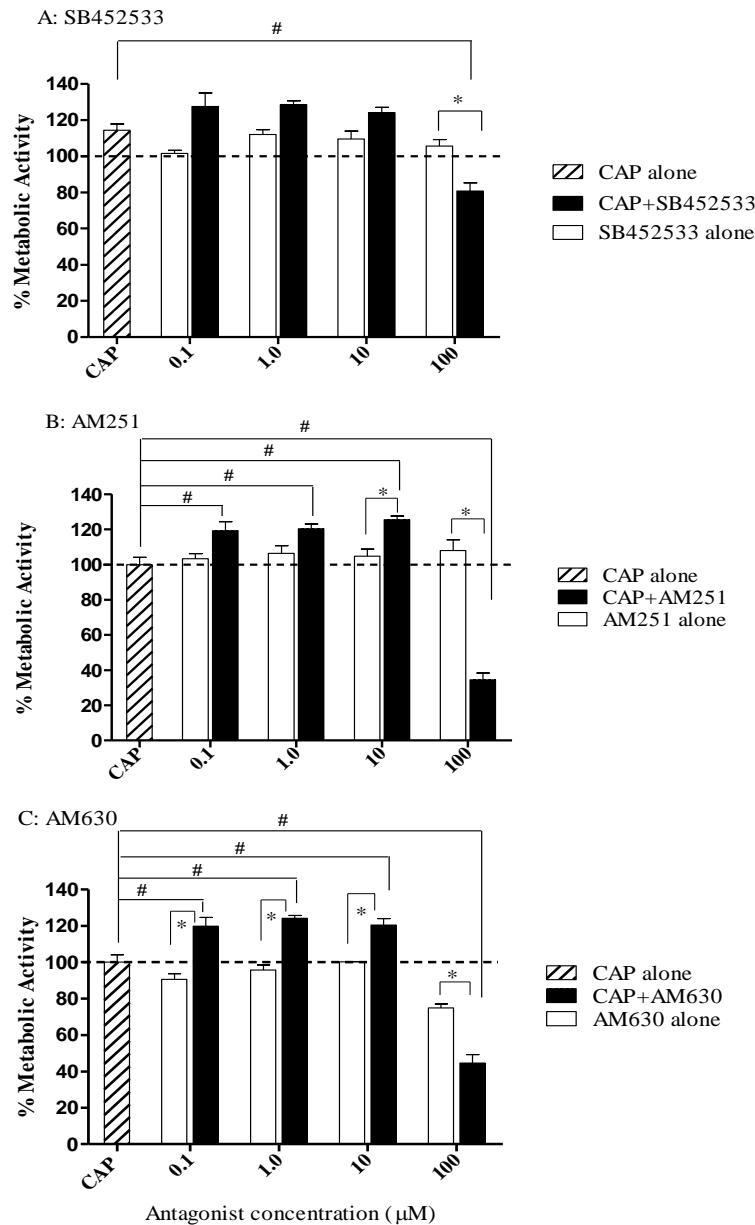


Figure 2-3: Effect of SB452533, AM251 and AM630 on CAP-induced change in metabolic activity (resazurin reduction) in THP-1 cells. Cells were pre-treated with antagonists for 30 min prior to exposure to 125µM CAP (black bars) for 24hr. The effects of the antagonists alone were also determined (white bars). Dotted bars indicate the effect of CAP alone (125µM). Bars represent the mean \pm SEM (n=3). *denotes $P < 0.05$ compared with vehicle; # denotes $P < 0.05$ compared with CAP alone. THP-1 cell metabolic activity increased at 125µM CAP by ~29%. Lower concentrations of SB452533 (0.1, 1.0 and 10 µM) had a similar effect as CAP alone on metabolic activity. The highest concentrations of SB452533, AM251 and AM630 (100µM) significantly decreased metabolic activity in the presence of CAP (125µM) by ~20%, 65% and 55%, respectively. AM251 or AM630 (0.1-10 µM) significantly increased metabolic activity in the presence of CAP for up to 25%.

U266B1

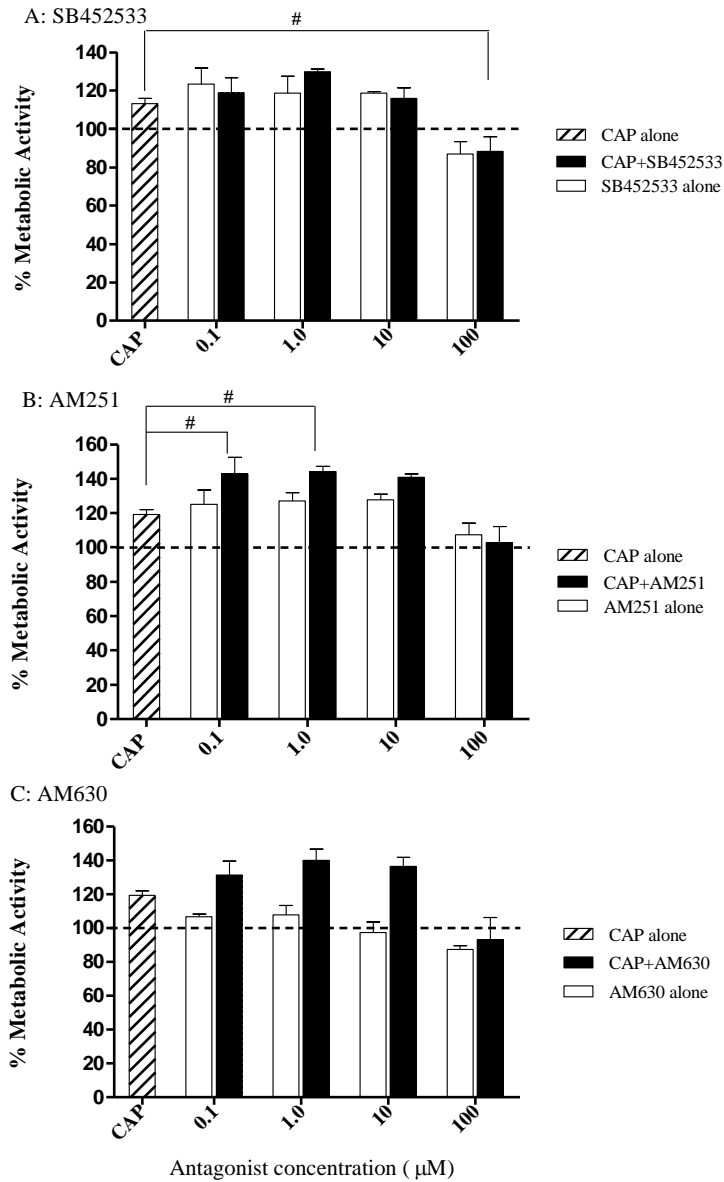


Figure 2-4: Effect of SB452533, AM251 and AM630 on CAP-induced metabolic activity (resazurin reduction) of U266B1 cells. Cells were pre-treated with antagonists for 30 min prior to exposure to 125 μM CAP (black bars) for 24hr. The effects of the antagonists alone were also determined (white bars). Dotted bars indicate the effect of CAP alone (125 μM). Bars represent the mean \pm SEM (n=3); # denotes $P < 0.05$ compared with CAP alone. No significant effect of TRPV1 and CB antagonists on CAP-induced cytotoxicity was observed.

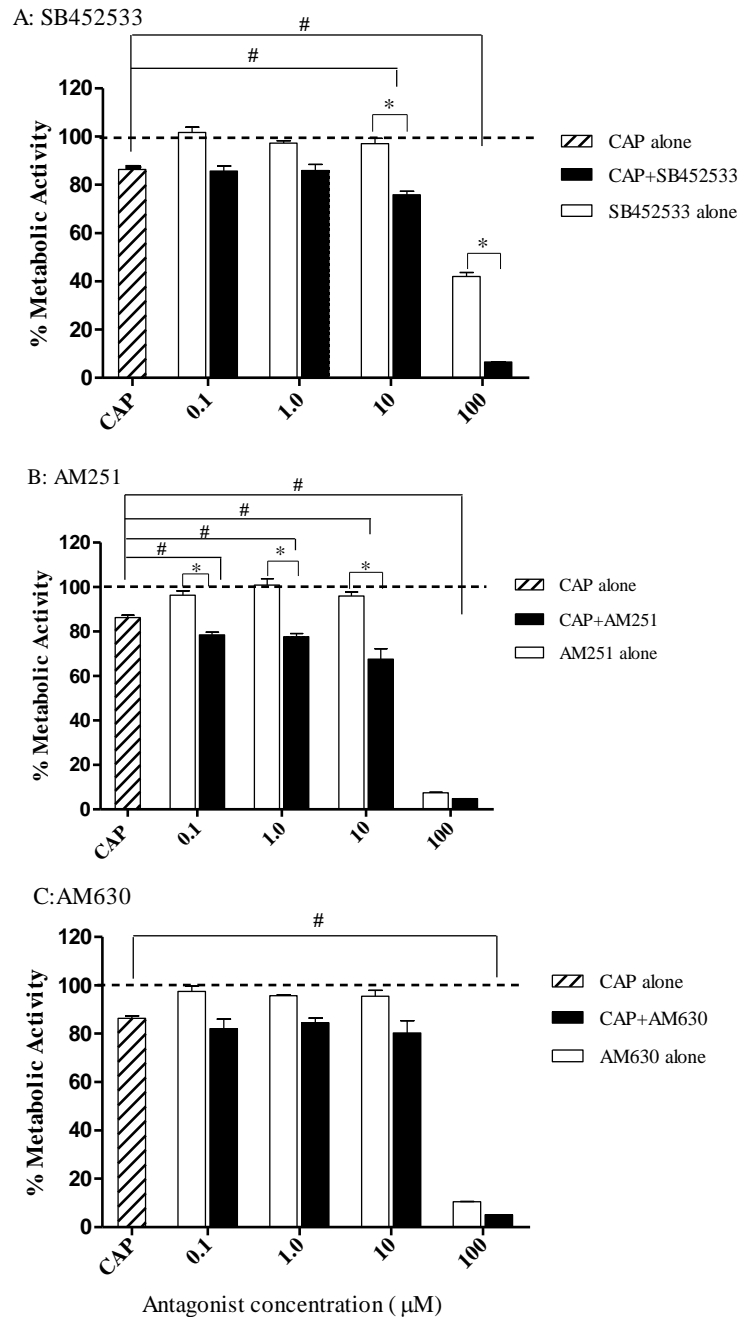


Figure 2-5: Effect of SB452533, AM251 and AM630 on CAP-induced metabolic activity (resazurin reduction) of U937 cells. Cells were pre-treated with antagonists for 30 min prior to exposure to 125µM CAP (black bars) for 24hr. The effects of the antagonists alone were also determined (white bars). Dotted bars indicate the effect of CAP alone (125 µM). Bars represent the mean \pm SEM (n=3). * denotes $P < 0.05$ compared with vehicle; # denotes $P < 0.05$ compared with CAP alone. CAP alone decreased metabolic activity by 14%. The three antagonists at 100µM alone or post-exposure to CAP, completely abolished the metabolic activity. SB452533 (10µM) or AM251 (0.1-10 µM) significantly decreased the metabolic activity in the presence of CAP.

2.5 Discussion

CAP has previously been shown to induce apoptosis through a TRPV1-mediated mechanism in some malignant cells including glioma (Amantini et al. 2007), urothelial cell cancer (Amantini et al. 2009) and non-malignant cells such as bronchiolar cells (Reilly et al. 2005). The effect of vanilloid-like agents, such as CAP on different malignant haematological cell lines has been poorly studied. Therefore, the aim of this chapter was to investigate the effect of CAP on the metabolic activity (resazurin reduction) of malignant haematological cell lines THP-1, U266B1 and U937 cells. The specified cell lines were selected because they cover the main categories of haematological malignancies i.e., leukaemia (THP-1), myeloma (U266B1) and lymphoma (U937). The role of the CAP receptor, TRPV1, and cannabinoid CB1 and CB2 receptors, in CAP-induced effects was also investigated.

CAP inhibited metabolic activity, and induced cell death, in a concentration-dependant manner in all three malignant haematological cell lines. This effect was consistent with previous studies in myeloid leukaemia (Ito et al. 2004) and myeloma cell lines (Bhutani et al. 2007). Furthermore, CAP was found to increase metabolic activity (resazurin reduction) in THP-1 cells in a concentration-dependent manner (up to 125 μ M). This effect was not antagonised by pre-exposure to low concentrations of SB452533 (0.1-10 μ M). In contrast, at the highest concentration of SB452533 or AM251 (100 μ M), the effect of CAP appeared to be antagonised and metabolic activity was significantly inhibited. The possible explanations for this suppression are; 1) TRPV1 and CB1 receptors mediate the CAP-induced increase in metabolic activity in THP-1 cells, and as high antagonist concentrations completely block these receptors, cell death ensues; and/or 2) THP-1 cell death observed at the

high concentrations of antagonists used in these experiments combined with CAP, might be a non-specific action. Moreover, low concentrations (0.1-10 μ M) of AM251 or AM630 appeared to act as agonists to further increase the CAP-induced metabolic activity. As a result, these findings suggest that neither CB1 nor CB2 mediate the CAP-induced metabolic activity in THP-1 cells.

U266B1 cells were found to be more resistant to CAP than THP-1 and U937 cells. This finding is consistent with the fact that U266B1 cells (myeloma) are resistant to apoptosis and express high levels of the antiapoptotic protein, Bcl-xL (Preta and Fadeel 2012). Also in this study an increase in CAP-induced metabolic activity (4-20%) in U266B1 cells has been demonstrated (at 125 μ M), and found to be neither TRPV1-, CB1-, nor CB2-mediated. A receptor-independent mechanism of cannabinoid-induced cell-death has been previously demonstrated in cultured human B lymphoblastoid cells (Chen and Buck 2000). The action of cannabinoids did not correlate with their binding affinity to the CB1 and CB2 receptors (Chen and Buck 2000). Thus, TRPV1, CB1, as well as CB2 receptors, appear not be involved in CAP-induced cell death in lymphoblastoid cells, including U266B1.

In addition, interleukin 6 (IL-6) which is the major survival factor for myeloma cells, stimulates myeloma cell growth and promote resistance to therapy (Tricot 2002), signals through STAT proteins, especially STAT3 (Akira et al. 1994). It has emerged that CAP inhibits IL-6 leading to the inhibition of STAT3 and its gene products (i.e., cyclin D1, Bcl-2, Bcl-xL and VEGF), and the induction of caspase 3-dependent apoptosis pathway (Bhutani et al. 2007).

In U937 cells, unlike THP-1 and U266B1 cells, CAP alone appeared to decrease the cell metabolic activity by 14%. In addition, pre-treatment with 100µM SB452533, AM251, or AM630, alone or with subsequent exposure to CAP, completely abolished the metabolic activity of U937 cells. This may be due to the high sensitivity of these cells to high concentrations of CAP and antagonists leading to cell death. Another possible explanation is that all three receptors, TRPV1, CB1 and CB2 might be constitutively active, as the CB1-selective AM251, and the CB2-selective AM630 appear to behave as 'inverse agonists' (Pertwee 2006). Moreover, Reilly et al. (2005) have reported data that highlight the existence of a TRPV1-mediated process that ultimately leads to cell death in a TRPV1 over-expressing bronchial epithelial cell line. In this mechanism, cell death may occur through the activation of intracellular, ER-bound TRPV1, accessible by the potent TRPV1 antagonist, iodoresiniferatoxin, but not the less potent antagonist, capsazepine, to promote cell death. Similar findings were reported by Johansen et al. (2006) in BEAS-2B cells pre-treated with three TRPV1 antagonists, LJO-328, SC0030 and capsazepine, which produced greater toxicity when treated with the TRPV1 agonist, nonivamide, and have suggested that the binding of antagonists to the plasma membrane increases TRPV1 translocation from the ER to the cell surface, resulting in increased sensitisation of the cells, leading to increased cytotoxicity and Ca^{2+} flux (Johansen et al. 2006). Whether SB452533 is able to access the cell interior and block intracellular TRPV1 has yet to be investigated.

Synergistically with CAP, pre-treatment of the U937 cells with lower concentrations of SB452533 (10µM) or AM251 (0.1-10 µM) further inhibited the metabolic activity of these cells. CAP-induced inhibition in metabolic activity was not affected by the

pre-exposure to AM630 (0.1-10 μ M) nor SB452533 (0.1-1.0 μ M) in U937 cells. Altogether, these findings indicate that CAP-induced inhibition in U937 cell metabolic activity (resazurin reduction) was neither TRPV1- nor CB1- or CB2-mediated. Other receptors and mechanisms should be investigated (Contassot et al. 2004b; Maccarrone et al. 2000).

The effect of TRPV1 antagonists alone on cell activity has been previously documented. Capsazepine was shown to be a mitochondrial inhibitor and to sensitise tumour cells to the death receptor, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), which is able to activate apoptosis via non-TRPV1-mediated mechanisms (Athanasίου et al. 2007; Sung et al. 2012). Idoresiniferatoxin was found to decrease Meth A cancer cell activity (Ghosh and Basu 2010). Similarly, a cytotoxic effect of the CB1 antagonist, AM251, on leukocytes has also been reported (Saunders et al. 2009).

The specific pathway by which CAP induces cell death in malignant haematological cell lines is still unclear. Some previously reported studies have suggested some receptor-dependent and -independent mechanisms. Anandamide, the endogenous CB1 and CB2 agonist, interacts with membrane lipid rafts in a non-receptor pathway (Sarker and Maruyama 2003). The membrane rafts in the plasma membrane of mammalian cells are enriched with cholesterol and glycosphingolipids, and play an important role in delivering a number of intracellular signals (Lajoie et al. 2009). CAP is a highly lipophilic substance that has the ability to cross the plasma membrane bilayer (Ziglioli et al. 2009), and may therefore interact directly and pass through the cell membrane in malignant haematological cells. Moreover, other non-

receptor pathways involving cyclooxygenase and peroxisome proliferator-activated receptors have also previously been reported (Pisanti and Bifulco 2009). Other receptors which might have a role in vanilloid-mediated apoptosis is through death receptors; CAP has been reported to induce apoptosis in glioma cells via DR5 upregulation and survivin downregulation, leading to TRAIL-induced apoptosis (Kim et al. 2010). One study reported that low concentrations of CAP induced TRPV1-dependant apoptosis in thymocytes, but at high concentration, cell necrosis was observed (Amantini et al. 2004).

Furthermore, CAP, as a vanilloid, has the ability to increase intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) through the activation of TRPV1 (Dedov et al. 2002; Ito et al. 2013). Excessive Ca^{2+} influx triggered by vanilloid exposure can perturb Ca^{2+} homeostasis and mitochondrial function to promote excitotoxicity in nociceptive neurons (Dedov et al. 2001; Orrenius et al. 2011). An elevation in $[\text{Ca}^{2+}]_i$ induces apoptosis via the activation of Ca^{2+} -dependent enzymes such as phospholipases, proteases and endonucleases (Zhivotovsky and Orrenius 2011). However, increased $[\text{Ca}^{2+}]_i$ does not seem to mediate CAP-induced apoptosis in some cells such as A172 human glioblastoma and transformed cells (Lee et al. 2000; Macho et al. 1999). Thus, whether increased $[\text{Ca}^{2+}]_i$ mediates CAP-induced cell death in haematological malignant cell lines needs to be investigated.

A major limitation of the present studies are that the findings may not necessarily be reproducible in other malignant *in vitro* cell lines or in blood cells obtained from patients with similar haematological malignancies. Another limitation was the inconsistent response in metabolic activity of the three cell lines at 125 μM of CAP.

Furthermore, the high concentrations of vanilloid and antagonists used in the experiments might have indirect and/or non-specific toxic effects, causing changes in metabolic activity. Future studies are warranted to investigate the role of membrane lipid rafts in apoptosis in these cells, as well as the combined effects of TRPV1, CB1 and CB2 receptor antagonists in the cell lines.

In conclusion, CAP promotes cell death in THP-1, U266B1 and U937 cell lines in a concentration-dependent manner, which suggested the presence of TRPV1 in these cells. In addition, CAP increased metabolic activity (resazurin reduction) in THP-1, and this effect was inhibited by high concentration of the SB452533 or AM251. Hence, TRPV1 and/or CB1 receptors may be involved in CAP-induced increase in the metabolic activity in THP-1 cells. Furthermore, it was found that neither TRPV1 nor CB1 or CB2 were involved in the CAP-induced metabolic activity in U266B1 cells. In contrast, synergistically with CAP, pre-treatment of U937 cells with SB452533 or AM251 had a further cytotoxic effect on cells. Thus, these findings indicate that CAP-induced inhibition in U937 cell metabolic activity (resazurin reduction) was neither TRPV1- nor CB1- or CB2- mediated. Altogether, this data suggests that CAP inhibits the metabolic activity of malignant haematological cell lines through a TRPV1-independent mechanism. Whether TRPV1 is (aberrantly) expressed in blood cancer cell lines and/or in blood cells from patients with *de novo* haematological malignancies, has yet to be determined, and will be investigated later in this thesis.

**Chapter 3: Validation and Optimisation of a Western Blotting
Method to Detect TRPV1 Protein in Human Peripheral Blood
Mononuclear Cells and Malignant Haematological Cell Lines**

3.1 Abstract

Transient receptor potential vanilloid-1 (TRPV1) is a non-selective cation channel activated by a variety of endogenous and exogenous stimuli. Methods previously used for the detection of TRPV1 at UTAS were quantitative real-time RT-PCR and immunocytochemistry (Saunders et al. 2007). The aim of this study was to develop and optimise a Western blotting protocol for the detection of TRPV1 in human leukocytes. Protein samples were extracted from peripheral blood obtained from healthy donors. Following denaturation, samples were run in polyacrylamide gels and transferred to a PVDF membrane. Four blocking solutions were assessed, as were three rabbit anti-TRPV1 antibodies and two goat anti-rabbit-HRP antibodies as part of method optimisation. A *WesternDot*TM625 biotin-streptavidin kit and an Enhanced chemiluminescence (ECL) method were also investigated to determine the optimal detection system. The Western blotting protocol was optimised to detect TRPV1 (~95kDa) by using a mixture of 3% BSA and 5% NFM as a blocking solution, Lifespan Biosciences rabbit anti-TRPV1 (diluted 1:10000), and Cell Signalling Technology goat anti-rabbit-HRP (diluted 1:5000) as a secondary antibody. Anti-GAPDH (diluted 1:3000, Cell Signalling Technology) was used as an internal control and THP-1 cells as a positive control. TRPV1 was then detected using the ECL detection method. Considerable inconsistencies were encountered with the staining properties of the different anti-TRPV1 antibodies assessed. However, TRPV1 was detected with significantly better specificity using the Lifespan Biosciences anti-TRPV1 antibody. In conclusion, a Western blotting protocol to detect TRPV1 band in PBMCs was validated and optimised. This method was applied in later chapters to investigate TRPV1 protein in malignant

haematological cell lines, as well as in leukocytes from healthy volunteers and patients with blood cancers.

3.2 Introduction

Detection of TRPV1 by Western blotting has previously been attempted at UTAS using the Biotin-Streptavidin method in THP-1 cells. However, the need for improved reproducibility, with cleaner blots and reduced background, to detect TRPV1 in haematological malignant cell lines and patient samples (see Chapters 5 and 6), warranted the re-development of the Western blotting protocol for human cells using an ECL method.

Few papers have reported TRPV1 expression using Western blotting on human PBMCs or on cells obtained from patients with haematological malignancies (Amantini et al. 2007; Lee et al. 2009; Sun et al. 2013), although data has been published using cells from other species (Amantini et al. 2009; Caprodossi et al. 2011; Huang et al. 2010; Zhang et al. 2007). Thus, the aim of this chapter was to validate and optimise a Western blot protocol for the detection of TRPV1. This method would be later applied to detect TRPV1 in normal human PBMCs, haematological malignant cell lines and cells obtained from patients with haematological malignancies (see chapter 5 and 6).

3.3 Materials and Methods

3.3.1 Materials

BD Vacutainer® CPT™ Tubes were purchased from BD Biosciences, San Jose, USA. Mammalian cell lyses kit (MCL-1) was obtained from Sigma Aldrich, St.

Louis, USA. PageRuler™ Plus Prestained Protein (Fermentas, Thermo Scientific, Burlington, Canada) and MagicMark™ XP (Life Technologies, Grand Island, USA) were used as ladders. Bio-Rad DC Protein Assay kit, protease inhibitor cocktail, mini-Protein® TGX™ Precast Gels (Any kDa gels), Laemmli sample buffer, running buffer, blotting grade blocker non fat dry milk (NFM), and extra thick blot paper were purchased from Bio-Rad Laboratories, CA, USA. Immobilon polyvinylidene difluoride (PVDF) membranes, 0.2µm pore size, were obtained from Millipore, MA, USA. Two detection systems were tested, Biotin-streptavidin (WesternDot™ 625 kit, Life Technologies, Grand Island, USA), and the ECL kit (Immobilon™ Western, Millipore, MA, USA) were used for Western blotting.

3.3.2 Cells

THP-1, U266B1 and U937 cell lines were cultured as previously described (see section 2.3.1). PBMCs were obtained from blood drawn from healthy donors using BD Vacutainer® CPT™ Tubes as described below (see section 3.3.5.1).

3.3.3 Antibodies

Three anti-TRPV1 primary antibodies, two anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Table 3-1) and two secondary antibodies were evaluated (Table 3-2). GAPDH was used as a loading control.

3.3.4 Ethical Approval

All patients and subjects had been consented before participating in this study. The study was approved by the Human Research Ethics Committee Network, Tasmania (H0011050).

Table 3-1: Characteristics of the primary rabbit anti-TRPV1 and anti-GAPDH antibodies

Protein	Company	Catalogue #	Epitope	Reactivity	Purity	Recommended Dilution
TRPV1	Alomone Labs	ACC-030	Polyclonal anti C-terminus (aa 824-838)	Human, rat, mouse	Affinity purified on immobilised antigen	1: 200
	Santa Cruz Biotechnology	sc-20813	Polyclonal anti N-terminus (aa 1-50)	Human	Proprietary techniques	1: 100-1: 1000
	LifeSpan Biosciences	LS-C150735	Polyclonal anti N-terminus (1-50)	Rat, human, monkey, mouse	Affinity purified	1: 1000
GAPDH	Santa Cruz Biotechnology	sc-25778	polyclonal anti 1-335	Human	Proprietary techniques	1: 100-1000
	Cell signalling Technology	14C10	Monoclonal anti C-terminus	Human	Affinity purified	1: 1000

aa: amino acids; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; N- / C-terminus: amino/ carboxy terminus; TRPV1: transient receptor potential vanilloid-1

Table 3-2: Characteristics of secondary antibodies used in Western blot

Company	Catalogue number	Host	Purity
Santa Cruz Biotechnology	sc-2004	Goat anti-rabbit IgG-HRP	Affinity purified
Cell signalling Technology	7074P2	Goat anti-rabbit IgG (H&L)-HRP	Affinity purified

IgG: immunoglobulin-g; HRP: horseradish peroxidase; H&L: heavy & light chain.

3.3.5 Cell Processing

3.3.5.1 Blood Collection and Cell Isolation

PBMCs were isolated using the BD Vacutainer[®] CPT[™] Tubes according to manufacturer's instructions. Briefly, 6 mL of peripheral whole blood was obtained from the median or cephalic vein of adult donors. Blood was collected and stored into an upright CPT tube at room temperature. Samples were then mixed by inversion several times and centrifuged at 1700 x g for 30 min (within two hours of collection). Plasma was aspirated, the cell layer collected, and immediately transferred to a 15 mL conical centrifuge tube. Cultured cells were obtained at a concentration of $3-6 \times 10^6$ cells and the cell count calculated using Invitrogen Countess, as described previously (see *section 2.3.1*).

3.3.5.2 Cell Washing

Phosphate buffered saline (PBS) was added to PBMCs and inverted several times. Tubes were then centrifuged for 5 min at 420 x g at 4°C. The supernatant was discarded, the pellet resuspended in PBS, and the wash step repeated. The supernatant was then discarded and the tube placed on ice prior to protein extraction.

3.3.5.3 Protein Extraction

The cell pellet was lysed using the Sigma Mammalian Cell Lysis kit (MCL-1) (Sigma Aldrich, St. Luis, USA) containing Tris-EDTA buffer, deoxycholic acid sodium salt, Igepal CA 630, sodium dodecyl sulphate (SDS), sodium chloride (at 1:1:1:1:1 ratio), and 10 $\mu\text{L/mL}$ of protease inhibitor cocktail. The cells were homogenised by a 25 gauge needle for 15 seconds, then incubated on an orbital mixer at 4°C for 15 min. The cell lysate was then centrifuged at $12000 \times g$ for 10 min. The supernatant was transferred to a chilled 1.5 mL Eppendorf tube for storage at -20°C, or assayed immediately for protein.

3.3.6 Protein Assay

Protein concentration obtained from the cell lysate was measured using the Bio-Rad DC Protein Assay kit according to the manufacturer's instructions. Twenty microlitres of Reagent S (surfactant solution) were added for every 1 mL of Reagent A (alkaline copper tartrate solution), to make the working Reagent A. Five dilutions of protein standard (0.2 mg/mL - 1.54 mg/mL) were prepared to construct the standard curve. A 500 μL of the working Reagent A was added to 100 μL of each sample and standard. Four millilitres of reagent B (a dilute Folin reagent) were added to each tube and incubated for 15 min at room temperature. Absorbance was measured at 750 nm using a spectrophotometer (Biochrom Libra S12, Cambridge, UK). Protein lysate was then aliquoted and stored at -20°C.

3.3.7 Blocking Solutions Optimisation

To block non-specific binding, and increase the specific reaction, 3% bovine serum albumin (BSA) or 5% NFM, both dissolved in PBST (PBS + 0.05% Tween-20), a mixture of both, as well as 2% goat serum with 5% NFM were assessed as blocking

solutions. Blocking solutions were tested as diluents as well for the primary and secondary antibodies along with testing the PBST alone (Table 3-3).

3.3.8 Protein Quantity Optimisation

Different protein quantities (5-50 μ g) were loaded to optimise the quantity of protein suitable for each cell type and probed antibodies.

3.3.9 Western Blotting (Optimised Protocol)

SDS-PAGE gels in the form of Mini-Protean[®] TGX[™] Precast Gels (Any kDa gels, Bio-Rad Laboratories, CA, USA) were used in all Western blotting experiments. Protein samples were prepared by diluting the protein lysate sample with Laemmli sample buffer containing β -mercaptoethanol in a 1:1 ratio. Samples were then heated for 5 min at 95°C and loaded onto the gels. Pre-stained and unstained ladders were loaded onto the same gel well.

The protein samples were electrophoresed at 200 V for 45 min using a running buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 (Bio-Rad Laboratories, CA, USA) in the cold room at 4°C. PVDF membranes were submerged in 100% methanol for 10 min on an orbital shaker to activate the membranes. Methanol was then equalised with an equal volume of transferring buffer (25mM Tris, 0.2M glycine and 5% methanol) for 10 min. The mixture was then discarded and the membranes submerged only in the transferring buffer and re-incubated for 10 min on the orbital shaker.

After running the protein samples, gels were removed, rinsed with molecular grade distilled water and equilibrated in the transferring buffer for 15 min. Two pieces

Table 3-3: Western blotting protocols tested to detect TRPV1 in human malignant cell lines and PBMCs

Rabbit Anti-TRPV1	Detection Method	Condition	Results	Figure
Santa Cruz Biotechnology (anti-human TRPV1)	Western Dot™ 625 kit (Biotin-Streptavidin)	<ul style="list-style-type: none"> • New lot kit. Protein quantity study (40, 30 and 20µg). • Secondary Ab alone control blot. Blocking, dilution: 3% BSA. 	<ul style="list-style-type: none"> • Non-specific binding in the secondary alone blot. • High background, dim bands with 20µg, decent with 30µg. • Clearer bands with dry membrane detection. 	Figure 3-1
		<ul style="list-style-type: none"> • Blocking, 1°Ab and 2°Ab dilution: 2% goat serum+ 5% NFM. 	<ul style="list-style-type: none"> • Neither signal nor ladder. 	-
	ECL	<ul style="list-style-type: none"> • Loading 30µg protein. • Blocking and 1°Ab dilution: 3% BSA. 2°Ab: in 5% NFM. • 2°Ab alone blot. 2°Ab titration (1:5000 vs. 1:10000). 	<ul style="list-style-type: none"> • Negative 2°Ab alone control blot. Non-specific binding in TRPV1 and GAPDH blots. Cleaner background. Better TRPV1 signal with 1:5000 2°Ab. 	Figure 3-2
		<ul style="list-style-type: none"> • Protein quantity study (30, 20, 10µg). 2°Ab 1:5000 diluted in 5% NFM. Blocking & 1°Ab: 3% BSA. 	<ul style="list-style-type: none"> • Non-specific bands. Strong bands are down the gel comparing to the ladder. 	Figure 3-3
Alomone Labs. (anti-human TRPV1)	ECL	<ul style="list-style-type: none"> • Blocking and 1°Ab dilutions: 3% BSA. 2°Ab 1:5000 in 5% NFM. GAPDH: cell signalling Tech., 1:1000. 	<ul style="list-style-type: none"> • Non-specific bands. TRPV1 bands: clear and in place comparing to ladder. GAPDH: clear with low-background. 	Figure 3-4, A
		<ul style="list-style-type: none"> • Blocking, 1° and 2° Abs dilutions in 5% NFM in PBST. 	<ul style="list-style-type: none"> • Low-background, no non-specific bands. TRPV1: weak. GAPDH: sharp& specific. 	Figure 3-4, B
		<ul style="list-style-type: none"> • Blocking and antibodies dilutions: 5% NFM. Biotinylated ladder was used to compare. 	<ul style="list-style-type: none"> • TRPV1 bands: weak. GAPDH: faint non-specific bands. 	Figure 3-4, C
LifeSpan Biosciences (anti-rat/human TRPV1)	ECL	<ul style="list-style-type: none"> • 1°Ab titration study (1:1000- 10000) • Blocking in 5% NFM. Dilutions: PBST or 5% NFM. 	<ul style="list-style-type: none"> • TRPV1: ~4-5 bands with the highest dilution 1°Ab. • Diluting 1°Ab in PBST gave multi bands. 	Figure 3-6
		<ul style="list-style-type: none"> • Anti-TRPV1 titre = 1:10000. • Blocking, 1°Ab and 2°Ab: 3% BSA or 5% NFM or 3% BSA+ 5% NFM. 	<ul style="list-style-type: none"> • TRPV1 bands: single band was achieved at 5 µg of THP-1 protein with 1:10000 1°Ab, but 4 bands with more protein. 	Figure 3-5
		<ul style="list-style-type: none"> • Secondary antibody titration study: 1:5000-10000. 	<ul style="list-style-type: none"> • Similar results using both dilutions. Titre: 10000. 	Figure 3-7
		<ul style="list-style-type: none"> • Test Cell signalling secondary antibody:1:5000-10000 	<ul style="list-style-type: none"> • Clear background and less non-specific bands. Titre: 5000. 	Figure 3-8

ECL: Enhanced Chemiluminescence; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; PBST: phosphate buffered saline-tween20; NFM: non-fat milk; 1°Ab: primary antibody; 2°Ab: secondary antibody. BSA: bovine serum albumin; TRPV1: transient receptor potential vanilloid-1; M.W: molecular weight.

of extra thick blot papers and two foam pads for each gel were submerged in the transferring buffer before assembling the transferring cascade in the following order; foam pad, blot paper, gel, membrane, blot paper and finally a foam pad on the black side of the cassette. To remove any air bubbles trapped between layers, gentle strokes with a plastic roller were performed after adding each layer.

The cassette was then closed firmly and placed inside the transferring tank, black side of the cassette to the black side of the central core. A frozen cooling unit was then inserted on the rear of the cassettes to keep the transferring unit cool during the process. The unit was filled with the transferring buffer, closed and run at 95 V for 1 hr at 4°C in the cold room. Once the protein transferral to the membrane was completed and verified using the pre-stained ladder, the membranes were removed and submerged in 100% methanol for 10 min to fix the transferred protein bands. To remove methanol, membranes were resubmerged in water.

The membranes were then incubated in a blocking solution containing 3% BSA with 5% NFM, dissolved in the PBST for 1 hr, using a gentle motion on an orbital shaker at room temperature. The membranes were then washed with PBST for 5 min using a gentle motion on an orbital shaker. After discarding the washing solution, the blot was then incubated with anti-TRPV1 (LifeSpan Biosciences, WA, USA) diluted 1:10000 in the blocking solution and incubated in the cold room, 4°C overnight (on a very gentle motion) on an orbital shaker.

The following day, the blots were washed on a fast motioned orbital shaker with PBST 4 times (2 times for 10 min each, and 2 times for 5 min each). The membranes

were then incubated with 1:5000 goat anti-rabbit IgG-HRP-conjugated (Cell signalling Technology, MA, USA) diluted in the blocking solution for 1 hr, and again washed as described earlier. The membranes were stained with the ImmobilonTM Western detection kit (Millipore, MA, USA) for 5 min and visualised using a LAS-3000 Image reader (Fuji, Tokyo, Japan).

The membranes were then re-washed and re-blocked for 20 min and incubated with anti-GAPDH (Cell signalling Technology, MA, USA), diluted for 1:3000 in the blocking solution, for 2 hr. Subsequently, membranes were re-washed, stained and visualised as described earlier.

3.4 Results

3.4.1 Optimisation of the Western Blotting Protocol

Protein samples were isolated from THP-1, U266B1, U937 cells and PBMCs from healthy subjects. In initial experiments, high background and several non-specific bands predominated. Therefore, alternative blocking solutions, antibodies and detection methods were tested and documented. All protocols tested are summarised in Table 3-3.

3.4.1.1 Detection System Method Selection

The *WesternDot*TM 625 biotin-streptavidin kit produced high background, with blots not evenly uniformed and patchy (Figure 3-1) compared to the ECL detection method, which produced cleaner blots and less background (Figure 3-2). When the biotin-streptavidin system was used, non-specific binding caused by the secondary antibody was greater compared to the ECL system (Figure 3-1 vs. Figure 3-2).

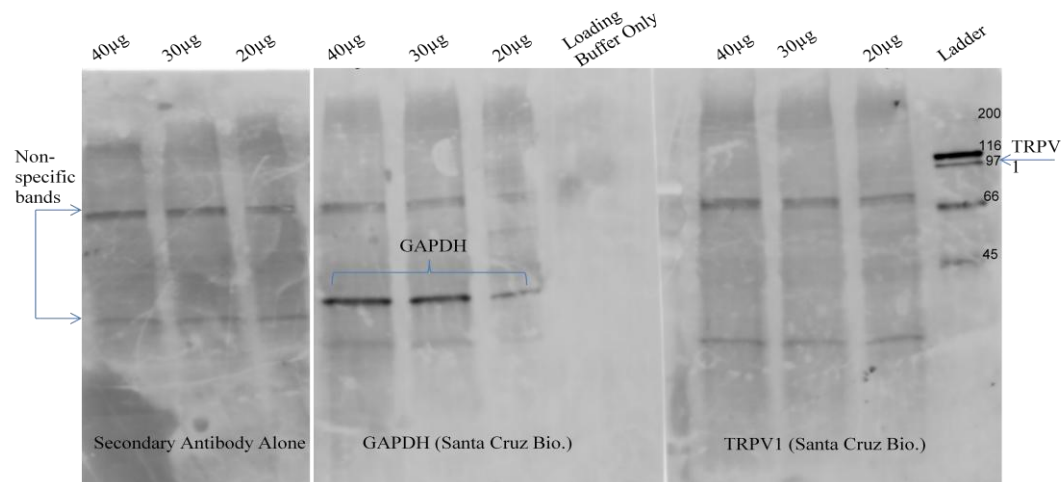


Figure 3-1: TRPV1 detection attempt using the Biotin-Streptavidin detection system in THP-1 cells. All antibodies used were supplied by Santa Cruz Biotechnology. Blocking step and the antibody incubation were performed in 3% BSA. Different amounts (40, 30 and 20 µg) of THP-1 cell lysate were investigated. Multiple non-specific (non-TRPV1) bands were observed at secondary alone, TRPV1 and GAPDH blots (at ~70 & 20 kDa) with no TRPV1 band (~95 kDa). High- background in all blots was observed.

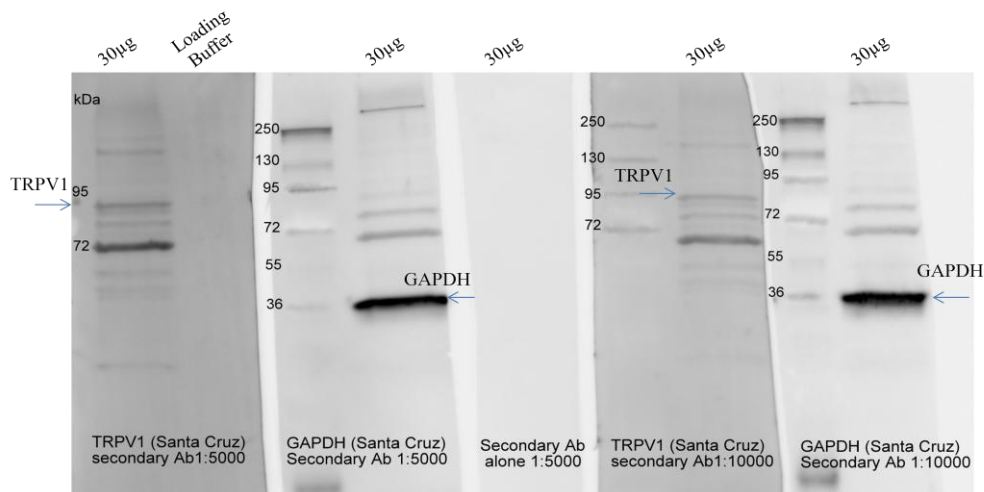


Figure 3-2: ECL detection method with secondary antibody dilution study, 1:5000 vs. 1:10000 of Santa Cruz Biotechnology in THP-1 cells. Blocking and primary antibody dilution performed in 3% BSA, secondary antibody in 5% NFM. A clean secondary alone control blot (tested at 1:5000) was noticed. TRPV1 was detected at ~95 kDa, however many non-specific bands detected in TRPV1 (~20, 55, 65, 80 and 200 kDa) and GAPDH blots (~65, 80, 90, and 300 kDa). Cleaner background was obtained using this method. A sharper band was observed using 1:5000 secondary antibody.

3.4.1.1 Anti-GAPDH Antibodies Optimisation

The anti-GAPDH (Cell Signalling Technology) antibody produced specific, discrete bands compared to anti-GAPDH antibody obtained from Santa Cruz Biotechnology,

with reduced non-specific bindings (Figure 3-3 and Figure 3-4). Therefore, the Cell Signalling Technology anti-GAPDH was used in subsequent experiments.

3.4.1.2 *Ladders*

All protein bands were shown in kilodaltons (kDa). Loading the biotinylated ladder (Cell Signalling Technology, MA, USA) as a molecular weight reference resulted in non-specific bands (Figure 3-4, C). Therefore, it was demonstrated that unstained MagicMark™XP (Life Technologies, Grand Island, USA), which produced better visualisation with no cross-interaction with any antibody was used. It is not biotinylated or composed of two components and ready to use without boiling. TRPV1 molecular weight was detected ~95-100 kDa according to each anti-TRPV1 supplier's sheet.

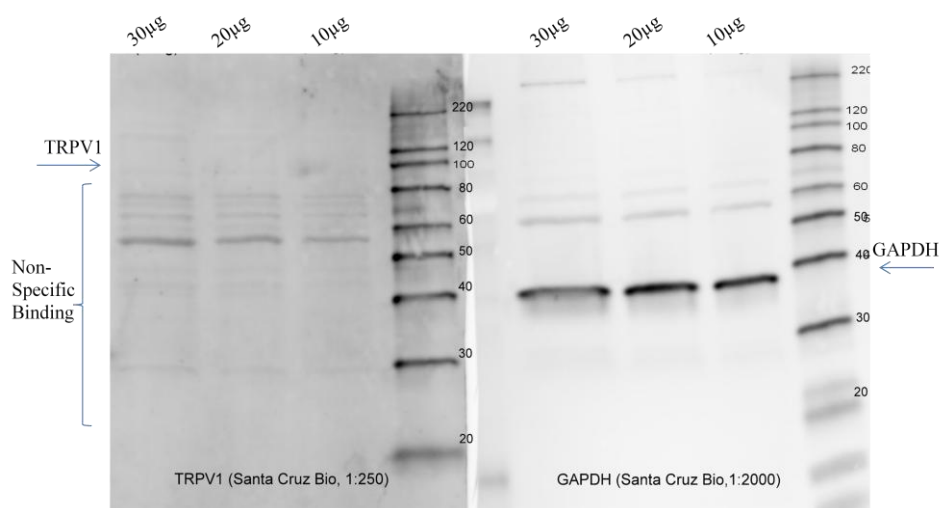


Figure 3-3: Protein quantity study (10, 20 and 30 µg) of THP-1 cell lysate to detect TRPV1 with Santa Cruz anti-TRPV1 using the ECL method. Blocking step and primary antibody dilutions were performed in 3% BSA and secondary antibody dilution in 5% NFM. Very weak TRPV1 band at ~95 kDa with lots of stronger non-specific bands (~55-75 kDa and 30 kDa) were observed for the TRPV1 antibody and ~250, 55 and 70 kDa for the GAPDH control.

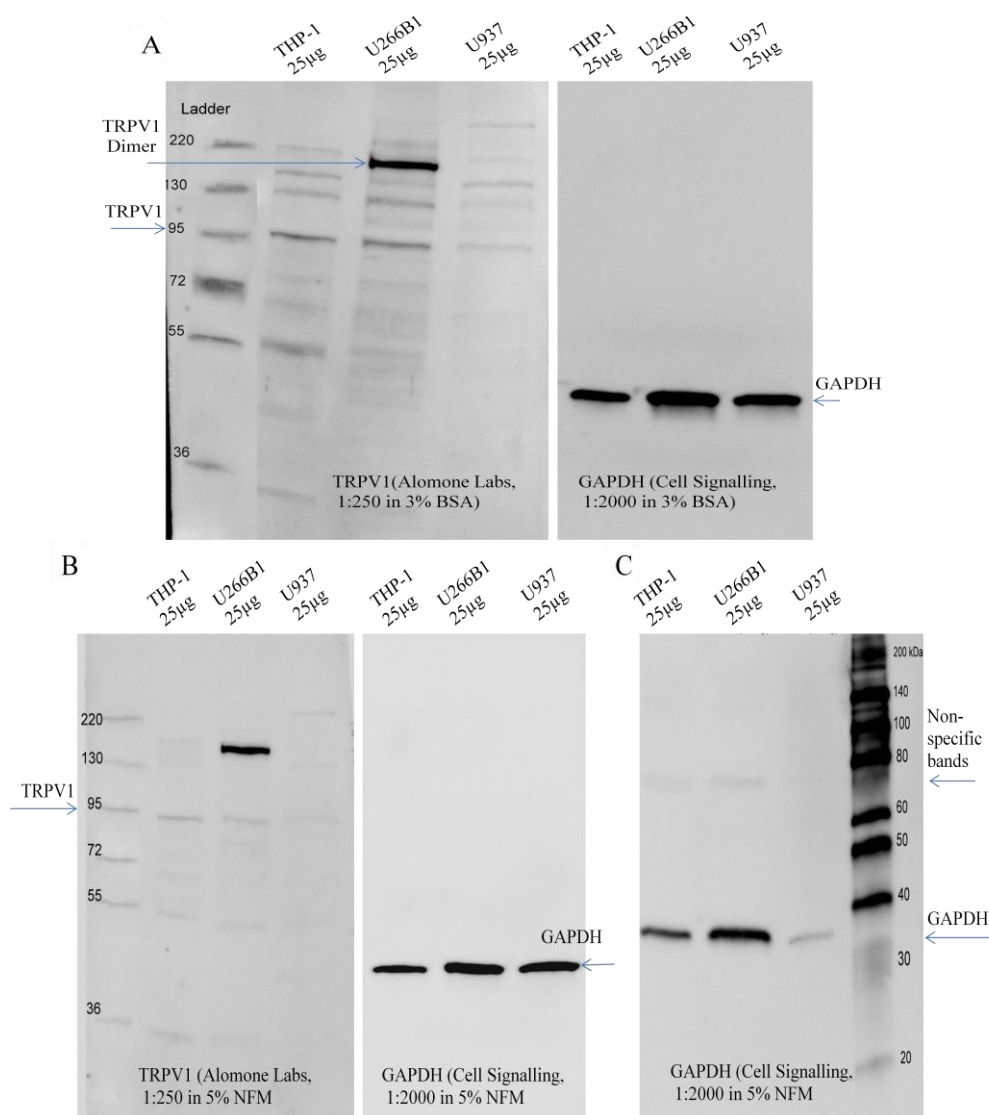


Figure 3-4: TRPV1 detection using Alomone Labs anti-TRPV1 antibody. The blocking and the primary antibodies dilution were performed in **A**: 3% BSA. **B&C**: 5% NFM. The secondary antibody was diluted in 5% NFM. TRPV1 band was detected (~95 kDa) with other extra bands ~60, 55, 30 kDa. A TRPV1 dimer was detectable in U266B1 cells. GAPDH showed a clean background with sharp strong bands. **C**: Biotinylated ladder was used and showed faint non-specific bands.

3.4.1.3 Anti-TRPV1 Antibodies Optimisation

Weak TRPV1 signal was detected using the Santa Cruz Biotechnology antibody (Figure 3-1 to Figure 3-3). Increasing the amount of protein increased the non-specific binding, with negligible improvement in TRPV1 signal (Figure 3-3). Improved TRPV1 signal was observed using the Alomone Labs antibody. However, many non-specific bands were also detected despite testing different blocking

reagents. NFM minimised the background and the non-specific binding significantly, but also minimised the intensity of the TRPV1 band (Figure 3-4). Detection of TRPV1 protein was optimised using the LifeSpan Biosciences primary antibody (MA, USA) that produced minimal non-specific binding (Figure 3-5). Optimal results were produced by diluting the primary and secondary antibodies in a blocking solution containing a mixture of 3% BSA, 5% NFM in PBST (Figure 3-5, B). This antibody was subsequently titred to determine optimal dilution, which ended up to be set on 1:10000 (Figure 3-6).

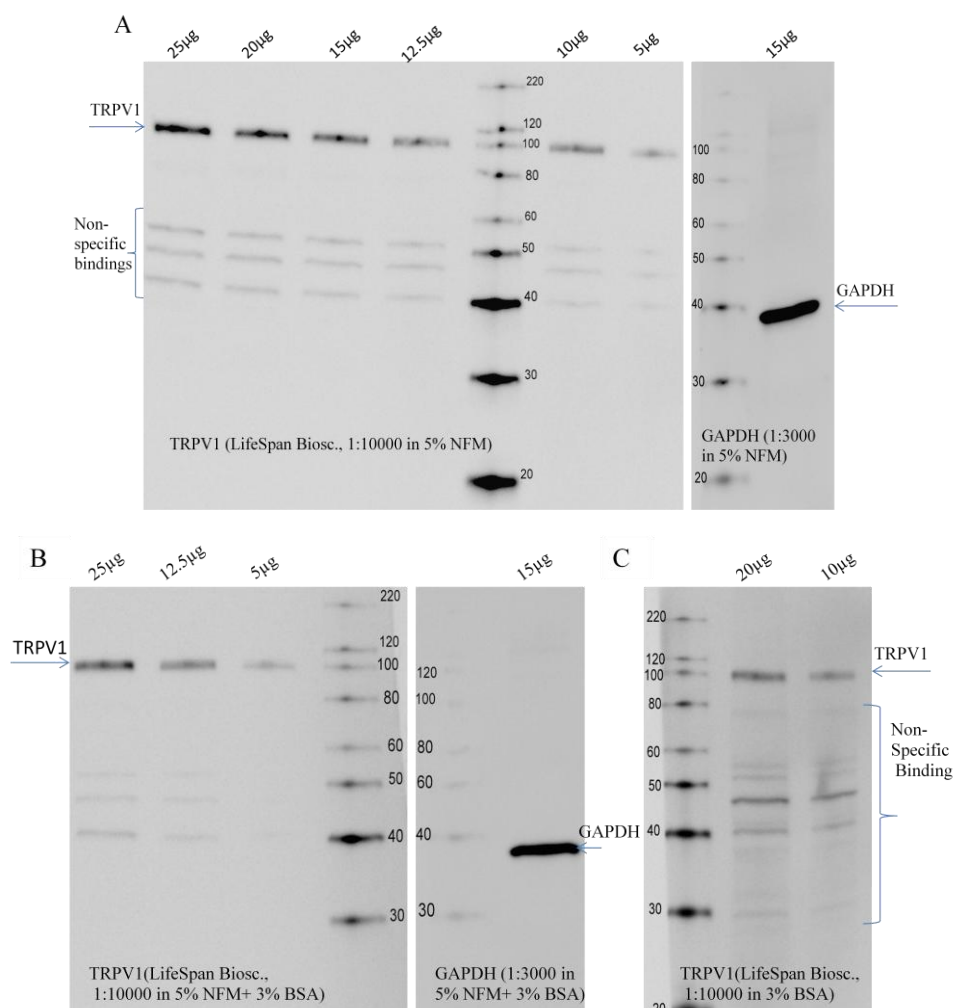


Figure 3-5: Detecting TRPV1 using LifeSpan Biosciences antibody. A blocking optimisation and protein quantity were also performed. The blocking and the dilution of the primary and secondary antibodies were performed in: **A:** 5% NFM, **B:** double blocking with 3% BSA+ 5% NFM in PBST wash. TRPV1 band was strong at ~100 kDa. Three extra bands with less intensity (~40, 47 and 52 kDa) were observed. **C:** 3% BSA with so many non-specific bands.

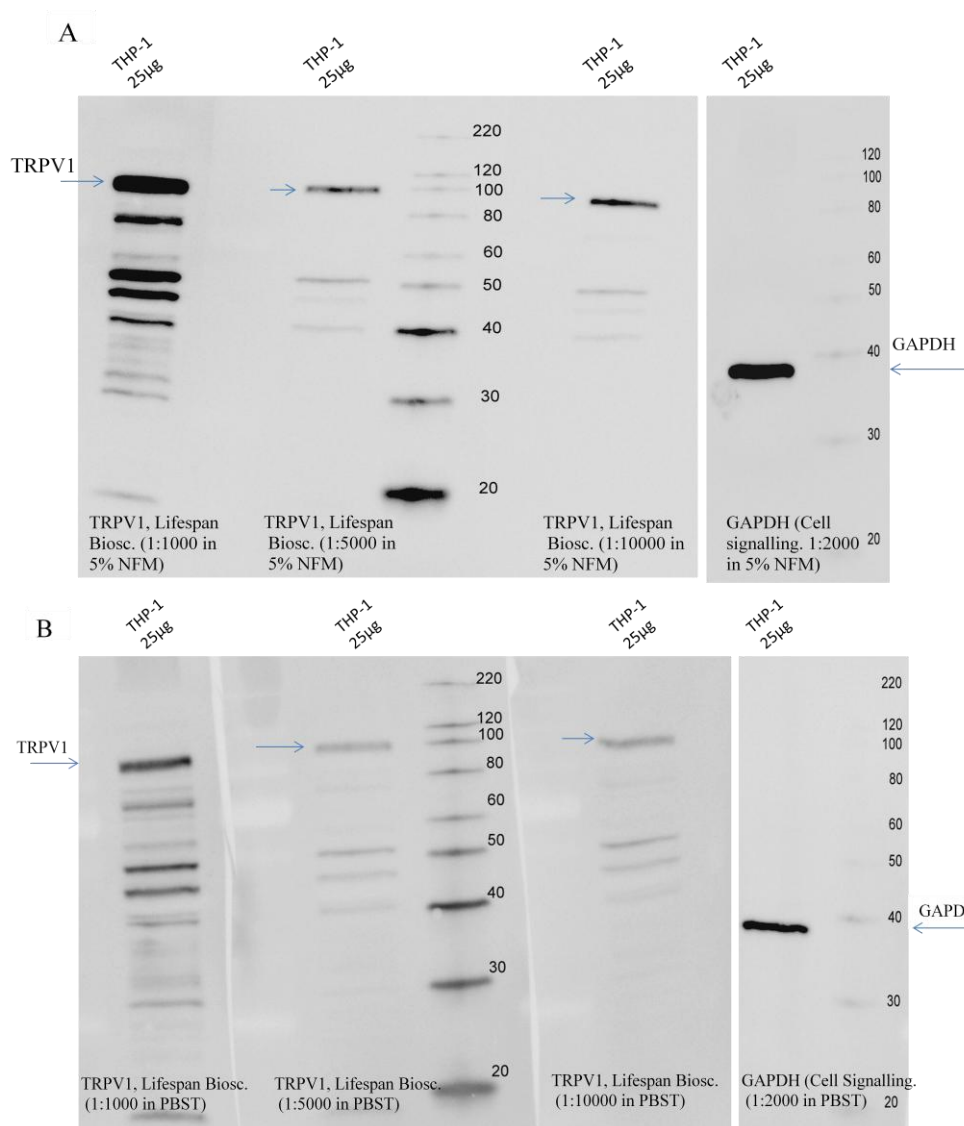


Figure 3-6: Titration & blocking studies for LifeSpan Biosciences Anti-TRPV1. Three dilutions tested: 1:1000, 1:5000 and 1:10000 in different blocking solutions. The blocking step was performed in 5% NFM. The antibodies dilutions were performed in **A**: 5% NFM, **B**: PBST. The titre is 10000. Diluting the antibodies in 5% NFM produced less non-specific binding and less background than PBST.

3.4.1.1 Blocking Optimisation

Blocking with a mixture of 2% goat serum and 5% NFM blocked all specific and non-specific bindings (data not shown). Five percent NFM (Figure 3-4) produced less non-specific binding and cleaner backgrounds than PBST (Figure 3-6) or 3% BSA (Figure 3-5, C). However, blocking with a mixture of 3% BSA and 5% NFM in

PBST produced less non-specific binding and cleaner blots using the LifeSpan Biotechnologies anti-TRPV1 (Figure 3-5, B and Figure 3-7).

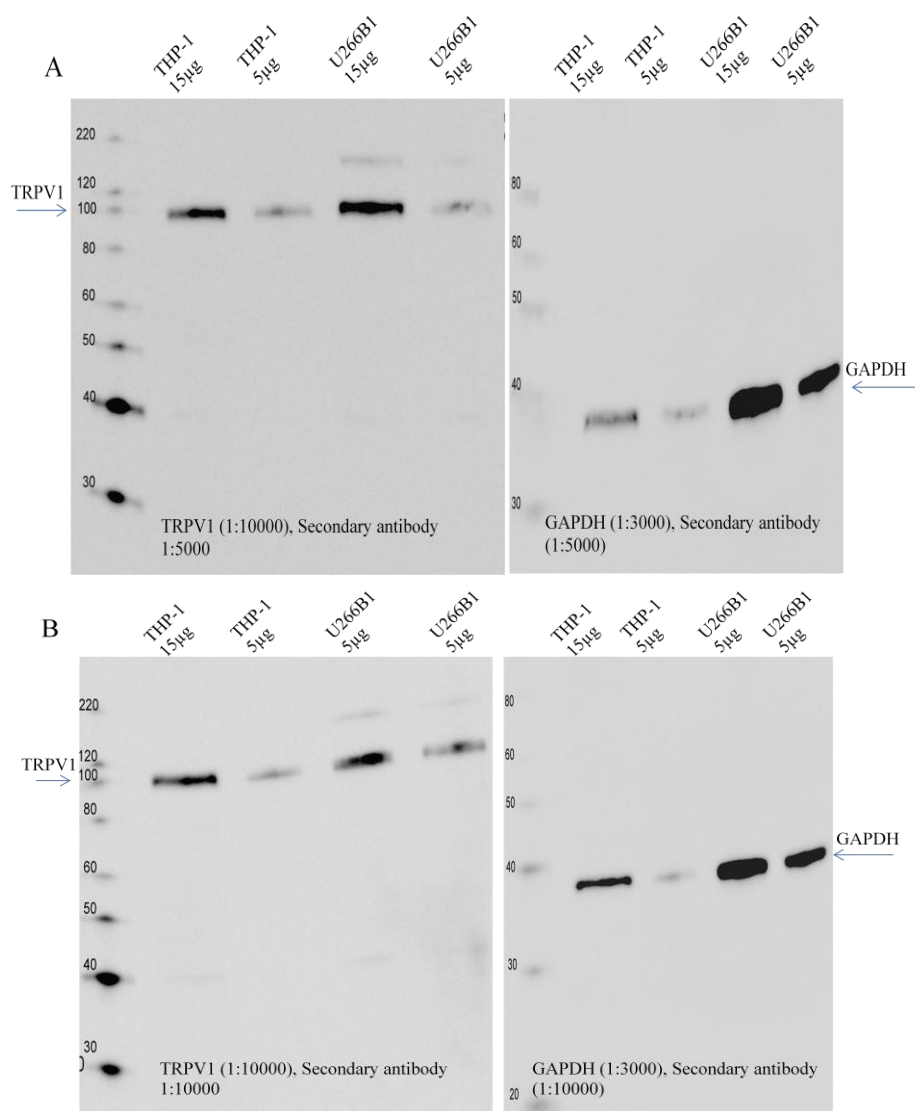


Figure 3-7: Secondary antibody (Santa Cruz Biotechnology) titration study. Two dilutions tested **A:** 1: 5000 or **B:** 1:10000 with 1:20000 of anti-TRPV1. Similar results were obtained from both blots; thus the titre is 1:10000.

3.4.1.2 Secondary Antibody Optimisation

Two goat anti-rabbit HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, CA, USA and Cell signalling Technology, MA, USA) were assessed (Figure 3-7 and Figure 3-8). Two dilutions were tested, 1:5000 and 1:10000. The optimum dilution was 1:10000 for Santa Cruz antibody (Figure 3-7) and 1:5000 for

the Cell Signalling antibody (Figure 3-8). The Cell Signalling Technology secondary antibody produced less background and cleaner blots (Figure 3-8), and therefore was used for patients' samples experiments (Chapter 6). No secondary antibody cross-reaction was observed when using secondary antibody alone as a control for both antibodies, unless used with the Biotin-streptavidin detection system (Figure 3-1).

3.4.1.3 Protein Quantity Optimisation

TRPV1 was found to be detected in THP-1 and U266B1 cell lysate samples containing protein as low 5 μ g (Figure 3-7). However, even using 50 μ g of normal human PBMCs protein produced weak expression of TRPV1 (Figure 3-9).

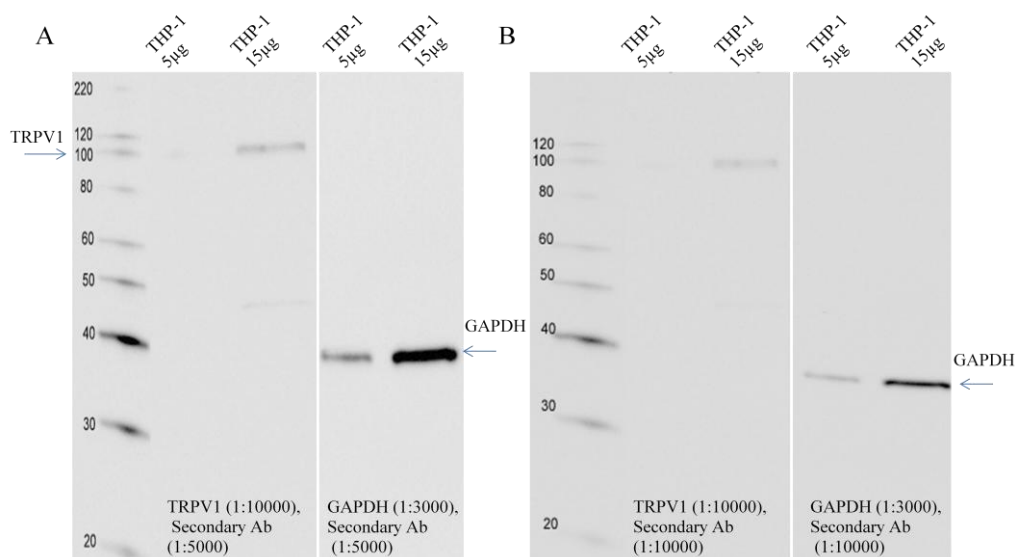


Figure 3-8: Secondary antibody (Cell Signalling Technology) dilution Study. Secondary Antibody was diluted at **A:** 1:5000 and **B:** 1:10000. Blocking and antibody dilutions were performed in 3% BSA and 5% NFM. Stronger bands were observed with 1:5000 dilution, with minimal non-specific binding. The titre was thus set to 1:5000.

3.4.2 Detection of TRPV1 in Human PBMCs using the Optimised Method

When testing the optimised protocol on normal PBMCs, background was negligible, the ladder was distinct and the blots were clear. TRPV1 was detectable in THP-1 protein and barely detectable in some normal PBMCs (Figure 3-9).

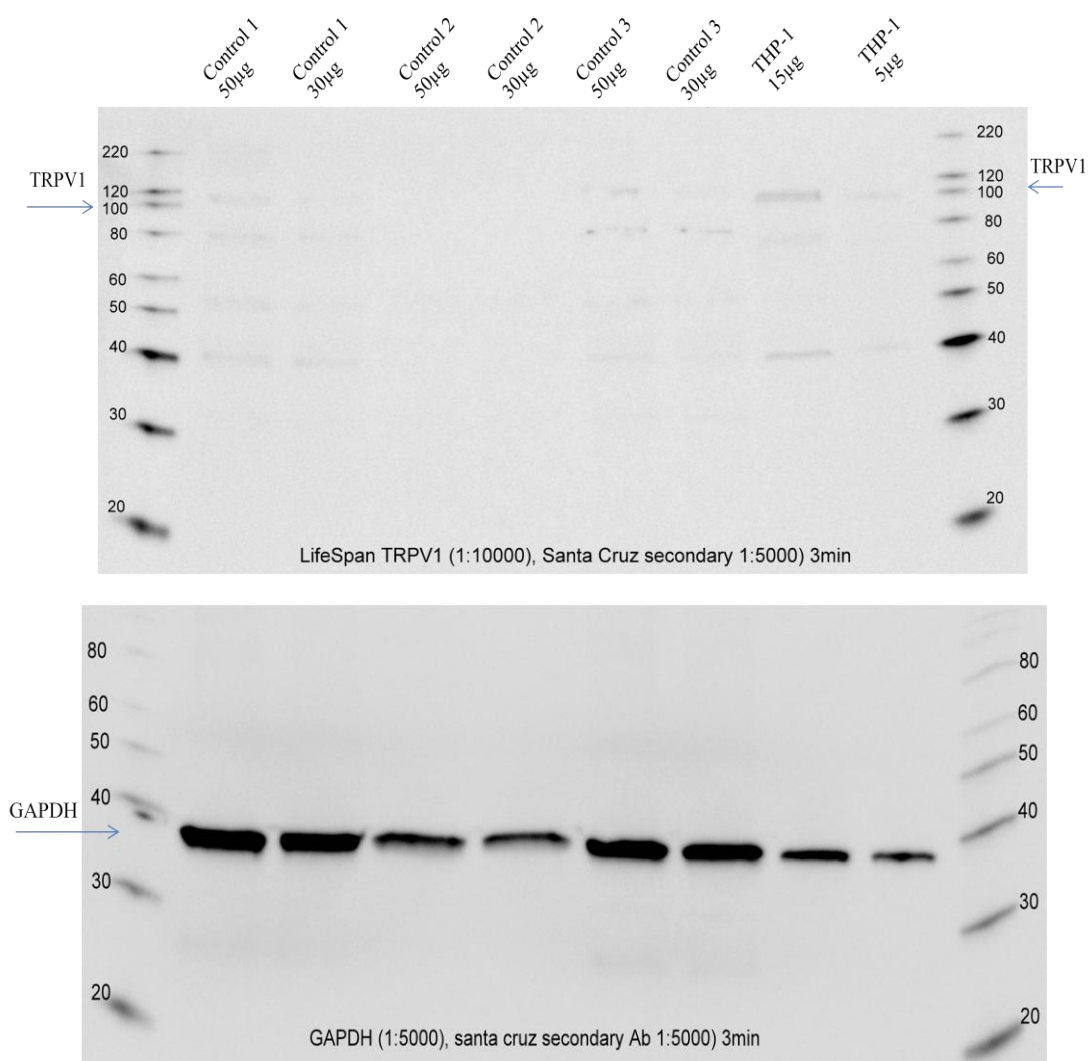


Figure 3-9: TRPV1 detected in normal human PBMCs protein. TRPV1 was successfully detected, though faintly in normal PBMCs protein. Cell Signalling Technology GAPDH was used. Blocking and antibody dilutions were performed in 3% BSA and 5% NFM. Approximately 30-50µg of protein was loaded. THP-1 protein was used as a positive control. Santa Cruz Biotechnology secondary antibody was used.

3.5 Discussion

The aim of this chapter was to validate and optimise a Western blotting method to detect TRPV1 in leukocytes. The optimised method would later be used to detect TRPV1 protein in human PBMCs and haematological malignant cell lines. TRPV1 detection (~95-100 kDa) using Western blotting with optimised blocking, antibody titrations and ECL detection was achieved. Significant inconsistency was encountered with the staining properties of the different anti-TRPV1 antibodies assessed. These differences make the antibody specificity, available in the market, questionable. Rabbit Santa Cruz Biotechnology and Alomone Labs anti-TRPV1 antibodies were evaluated (Table 3-1), which produced weak TRPV1 bands along with lots of non-specific bindings of similar, or in many cases, stronger intensity. However, with the Lifespan Biosciences anti-TRPV1 antibody, TRPV1 was detected with significantly better specificity, although sometimes with some faint extra bands of lower molecular weight. Western blotting conditions were subsequently optimised including blocking, primary and secondary antibodies titres and diluent, respectively. Furthermore, non-specific binding caused by excessive protein loading was avoided by testing different protein quantities for each cell type (Tsang et al. 1989).

The Lifespan Biosciences antibody is affinity purified and used at a very low concentration (1:10000). To the knowledge of the author, some other Lifespan Biosciences antibodies have previously been used to detect other TRP channels, but not TRPV1 (Ryskamp et al. 2011; Yee et al. 2012). The occurrence of some less intensely stained extra bands of lower molecular weight might be caused by protein degradation, isoforms of the receptor, or alternative splicing (Fausson-Pellegrini et al. 2005). When using the rabbit Santa Cruz Biotechnology or Alomone Labs

antibodies, the TRPV1 band itself was of less intensity than the extra bands, and multiple higher and lower molecular weight bands were obvious in the blots.

Another explanation might be due to the difference in the cell type tested compared to this study, as cells were obtained from different origins. A summary table of the previous studies using the antibodies employed in the present study and potential (technical) issues with these previous studies are summarised in Table 3-4.

Some of these studies used cells from experimental animals and it has been found that TRPV1 composition is not 100 percent identical between human and other species, which might affect the reactivity of the anti-TRPV1 used and the occurrence of non-specific binding (Correll et al. 2004; Gavva et al. 2004). Moreover, no previous studies used human PBMCs or haematological malignant cells, which makes the optimisation of Western blotting in this chapter both unique and essential for the further work in this thesis (Chapters 5 and 6).

TRPV1 was previously detected in some cell lines using Western blotting. Of the twelve studies that employed Santa Cruz Biotechnology (CA, USA) antibodies, the affinity purified goat anti-TRPV1 antibodies were used in eleven. In ten studies where TRPV1 bands were detected, the entire blots were not shown, therefore whether non-specific binding was produced is unknown. These studies detected TRPV1 in different animal and human cell lines, including human urothelial cancer cell lines (Amantini et al. 2009; Caprodossi et al. 2011), rabbit corneal epithelial (Zhang et al. 2007), human keratinocytes cell line HaCaT (Huang et al. 2010), glioma cell lines NHA, U87, U373 and normal PBMCs (Amantini et al. 2007),

Table 3-4: Summary of some studies detecting TRPV1 by Western blot

Cell Line	Rabbit Anti-TRPV1	Issues	Study
Human corneal fibroblasts	Santa Cruz Biotechnology	<ul style="list-style-type: none"> • Whole blot not included • No negative control • Loading control and positive control only 	(Yang et al. 2013)
Human: TRPV1-transfected SH-SY5Y, skin and brain Mice: DRG, colon, trigeminal ganglion	Alomone Labs	<ul style="list-style-type: none"> • Whole blot not included. • Loading control only 	(Chen et al. 2012b; Lee et al. 2009; Lilja et al. 2007a; Shinoda et al. 2011; Sun et al. 2013; Wang et al. 2012)
Human bladder cancer T24 cell line	Alomone Labs	<ul style="list-style-type: none"> • Whole blot not included. • No negative control. • Loading control and positive control only. 	(Yang et al. 2010)
Mice colon	Alomone Labs	<ul style="list-style-type: none"> • TRPV1 band at 150kDa not 95kDa • Whole blot not included • Loading control only 	(Wang et al. 2012)
Human focal cortical dysplasia and tuberous sclerosis	Double stained with both	<ul style="list-style-type: none"> • Whole blot not included • Loading control only 	(Shu et al. 2013)

human and mouse mature dendritic cells (Basu and Srivastava 2005; Toth et al. 2009), rat thymocytes and cerebral cortex (Amantini et al. 2004), mouse embryonic fibroblasts and CMS5 cells (Ghosh and Basu 2010), human corneal fibroblasts (Yang et al. 2013) and microtubules (Goswami et al. 2006). Only one paper included the entire blot which showed multiple extra bands of lower molecular weight than TRPV1 in human stomach mucosa (Fausson-Pellegrini et al. 2005). Therefore, it is possible that multiple bands have been also detected in the previous listed studies using the goat anti-TRPV1 antibodies obtained from Santa Cruz Biotechnology (CA, USA) and are present in the initial whole blots before cutting the desired specific sections.

In this study, a Santa Cruz rabbit anti-TRPV1 antibody which was purified by a proprietary rather than by affinity technique, did not detect TRPV1 using the streptavidin-biotin detection system (Figure 3-1). However, it detected TRPV1 and multiple extra bands using the ECL detection system (Figure 3-2). It was thought that antibodies which were not affinity purified have less specificity to the targeted protein (Couchman 2009). Yang (2013) used the Santa Cruz Biosciences rabbit antibody without including the whole blot to show if non-specific or extra bands were present. Thus it is difficult to compare the results. In this chapter, the affinity purified anti-TRPV1 antibody produced better binding to and specificity for TRPV1 protein with less non-specific bands and cleaner blots.

Ten papers which used Alomone Labs anti-TRPV1 antibodies to detect TRPV1 were reviewed (Chen et al. 2012b; Lee et al. 2009; Lilja et al. 2007a; Lilja et al. 2007b; Ma et al. 2011; Shinoda et al. 2011; Shu et al. 2013; Sun et al. 2013; Wang et al.

2012; Yang et al. 2010). Two studies only included the entire blot (Lilja et al. 2007b; Ma et al. 2011), whereas, the other studies cut around the TRPV1 bands, which makes it difficult to conclude if there was any non-specific binding in these studies. Table 3-4 summarises the studies detected TRPV1 by Western Blot using the same anti-TRPV1 used in this study. Shu et al. (2013) double stained human focal cortical dysplasia and tuberous sclerosis with both the Alomone Lab antibody and the rabbit Santa Cruz Biotechnology anti-TRPV1 without including the entire blot or any control. Similar extra bands were reported by Lilja et al. (2007b) and a strong extra band was detected at 65kDa (Shinoda et al. 2011), without specifying the nature of the bands. Ma et al. (2011) included the entire blot using mice cells without extra bands, though did not mention the specific blocking or diluent solution used. Another study detected the TRPV1 band at 150kDa (Wang et al. 2012), whereas non-glycosylated TRPV1 is supposed to be detected at ~95kDa, and glycosylated TRPV1 at ~113kDa (Vetter et al. 2006). Subsequently, for all of the above, it was difficult to rely on this antibody for future experiments, though extra bands, whether specifically or non-specifically bound, were also demonstrated with other anti-TRPV1 antibodies at ~75kDa (Tian et al. 2006) and ~65kDa (Mandadi et al. 2006). However, the goal was to find an anti-TRPV1 antibody with better specificity and less cross-reactivity and non-specific binding.

The anti-GAPDH antibody obtained from Cell signalling Technology produced very discrete specific bands with clear background, compared to the antibody obtained from Santa Cruz Biotechnology. Similar results using the Cell Signalling Technology anti-GAPDH antibody was published previously by Sun et al. (2013) with the same neat, clean bands. This makes this antibody highly recommended for Western

blotting experiments. In contrast, the Santa Cruz Biotechnology anti-GAPDH antibody was also tested by Ma et al. (2011) without including the whole blot to check for possible non-specific binding. This hindered the comparison with the GAPDH results described in this chapter.

Loading a biotinylated ladder as a molecular weight reference when using milk powder as a blocking or diluting agent resulted in non-specific bands due to the interaction between the milk's endogenous biotin with avidin (Jeon et al. 2008). To avoid this type of cross-reactivity, MagicMark™XP was used, which produced better visualisation with no cross-interaction with any antibody.

Compared to previous work performed on TRPV1 in the UTAS laboratory, the following were achieved; a) cleaner blots using the ECL method compared to the Biotin-streptavidin method used previously, b) three anti-TRPV1 antibodies to detect TRPV1 were compared and assessed for immunoreactivity, c) two anti-GAPDH antibodies were compared and assessed for reactivity, d) an optimal blocking solution was chosen after a series of optimisation experiments, and e) two secondary antibodies were evaluated for optimal titre and reactivity.

Optimisation of the blocking step came in two stages; 1) blocking free sites on the membrane, and 2) as a diluent for the primary and secondary antibodies, thus minimising the cross-reaction between the untargeted protein and the antibodies (Johnson et al. 1984). By the use of the double blocking mixture containing NFM with BSA, only specific antibody reactions were clear.

In conclusion, a Western blotting protocol to detect TRPV1 was validated and optimised. This method will be applied in later chapters to investigate TRPV1 in haematological malignant cell lines and patients' samples. For such variations in binding specificity between anti-TRPV1 primary antibodies provided, a necessary step to monitor any antibody-antigen reaction is to use positive and negative controls (Sawicka M et al. 2013). In most papers reviewed above, only a few studies reported positive or negative control to control the specificity of their primary antibody (Yang et al. 2013; Yang et al. 2010; Zhang et al. 2007). Only affinity purified antibodies should be used in Western blotting experiments, as antibodies purified by other methods such as proprietary techniques showed less specificity and lots of non-specific bindings through the blots. Besides, most studies did not include the entire blot in their study, making it difficult to compare results or judge the reactivity of the antibodies (Table 3-4). Data published using Western blotting should be more open to the fact that many antibodies cause extra bands other than the targeted ones and these bands should be acknowledged and investigated.

**Chapter 4: Development and Optimisation of a Flow
Cytometric Method for the Detection of TRPV1 Expression in
Human Leukocytes**

4.1 Abstract

Transient receptor potential vanilloid-1 (TRPV1) is a non-selective cation channel that detects and integrates noxious and inflammatory signals. TRPV1 immunoreactivity has previously been demonstrated at UTAS using immunocytochemistry (Saunders et al. 2009). The aim of this study was to develop and validate a flow cytometric protocol to detect TRPV1 expression in human leukocytes. Immunophenotyping, accompanied with indirect intracellular detection of TRPV1, was performed on leukocytes obtained from healthy donors. Three different rabbit anti-TRPV1 primary antibodies, various blocking protocols and two isotype controls were assessed. Samples were investigated using the Attune[®] Acoustic Focusing Cytometer (Life Technologies, Grand Island, USA), and TRPV1 signal was measured as the median fluorescence intensity (MFI) of each experiment. Non-specific binding was blocked using a mixture of 10% group AB serum, 1% Bovine Serum Albumin and 0.05% sodium azide. The Lifespan Biosciences primary anti-TRPV1 antibody produced the most specific binding, using 0.5µg of antibody per one million cells. Cells were stained with 1:25 goat anti-rabbit-FITC secondary antibody. Two types of negative controls, an isotype and secondary antibody alone (Santa Cruz Biotechnology, CA, USA), were used to distinguish positive from negative signals. It is recommended that only affinity purified antibodies should be considered in flow cytometry experiments to detect TRPV1, as antibodies purified by other methods, such as proprietary techniques, showed less specificity and false negative results. In conclusion, this study describes for the first time an optimised flow cytometric method for the detection of TRPV1 in human leukocytes. This method was applied to the detection of TRPV1 in human leukocytes, and was used in Chapters 5 and 6 to detect TRPV1 in haematological malignancies.

4.2 Introduction

The focus of this chapter was to develop and optimise a method for the detection of TRPV1 in human PBMCs using flow cytometry. The flow cytometry work was performed in two stages. Preliminary experiments were conducted at the Pathology Department, Launceston General Hospital (LGH) using a FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, USA). Later experiments were performed at the School of Human Life Sciences, University of Tasmania using an Attune® Acoustic Focusing Cytometer (Life Technology, Grand Island, USA). In this chapter only the results obtained from the Attune® Cytometer are presented. FACSCalibur™ cytometer data are presented in Appendix I.

Although several studies have reported and discussed TRPV1 expression detected using flow cytometry in blood cells from experimental animals (Amantini et al. 2004; Basu and Srivastava 2005; Caprodossi et al. 2011), only one flow-cytometric study of TRPV1 in human blood cells has been published (Amantini et al. 2007). This lack of studies reporting TRPV1 in human blood cells, and the use of inappropriate controls for the flow cytometry experiments in most published animal studies, specifically a lack of negative controls, meant that reliable detection of TRPV1 in blood cells from patients with haematological malignancies would require the development of an entirely new flow cytometric protocol.

As a result, the aim of this chapter was to develop and optimise a flow cytometry protocol to validate TRPV1 detection in human leukocytes. This method would then be used to investigate TRPV1 expression in human PBMCs, and later in human

haematological malignant cell lines (Chapter 5), and cells from patients with haematological malignancies (Chapter 6).

4.3 Materials and Methods

4.3.1 Materials

Immunophenotyping

All fluorochrome-conjugated CD markers were purchased from Becton Dickinson (San Jose, USA). Fluorochromes which were detected by blue and violet lasers, including CD3-Brilliant Violet™ 421, CD14-V500, CD45-PerCP, CD19-PE, CD13-Brilliant Violet™ 421, CD33-PE, CD7-PE, CD45-PE-Cy™5, CD20-Brilliant Violet™ 421, CD38-Brilliant Violet™ 605.

Antibodies

An indirect detection method was used for measuring expression of TRPV1 in leukocytes. Three TRPV1 primary antibodies (see Table 3-1), and two secondary antibodies were tested [polyclonal IgG goat anti-rabbit-FITC (Santa Cruz Biotechnology, CA, USA); polyclonal IgG goat anti-rabbit Alexa Flour® 488 (Life Technologies, Grand Island, USA)]. Variations in staining properties with different lot numbers may occur with polyclonal antibodies (Saper 2009), therefore same lot number of each antibody was confirmed.

Controls

Two types of controls were used for method development; 1) isotype and (2) secondary antibody (no primary antibody) controls. Leukocytes obtained from a healthy subject were used as a control to blood cancers patients WBCs (see Chapter

6). Two isotype controls were assessed (Table 4-1) and used in the same quantity/dilution as the primary anti-TRPV1 in all experiments.

Table 4-1: Characteristics of the isotype controls used in the study

Company	Catalogue number	Host	Purity
Santa Cruz Biotechnology	sc-3888	Normal rabbit IgG	Affinity purified
Cell signalling Technology	2729	Normal rabbit IgG	Affinity purified

Data were provided by the manufacturers' data sheets. IgG: immunoglobulin-G.

4.3.2 Methods

4.3.2.1 Blood Collection

Peripheral whole blood was obtained from the median or cephalic vein of adult donors into EDTA collection tubes (Becton Dickinson, San Jose, USA), ethical approval H0011050. Full blood counts were performed using a Sysmex XS whole blood analyser (Sysmex Corporation, Kobe, Japan).

4.3.2.2 Flow Cytometer Calibration and Electronic Optimisation

Calibration was set using Attune™ Performance Tracking Beads (Life Technology, Grand Island, USA). Compensation and photomultiplier tube (PMT) voltages optimisation step was performed to eliminate fluorescence spectral overlap. Forward scatter (FSC) and side scatter (SSC) were adjusted for each experiment during the live acquisition before events recording.

4.3.2.3 FSC and SSC Optimisation

FSC is considered to be an indirect measure of overall cell size, whereas SSC represents a direct measure of the cell's granularity and complexity (Romano et al. 2003). Unstained cells were processed along the stained cells throughout all experiments and run before the stained cells to set the cell populations (lymphocytes, monocytes and granulocytes) on the scale and adjust the FSC and SSC (linear) values. FSC threshold was adjusted to minimise debris.

4.3.2.4 Gating Scheme

Lymphocyte and monocyte populations were gated using a conjugated-CD45 vs. SSC plot. 1.0×10^6 cells were stained with CD45-PerCP or CD45-PE-CyTM5 to facilitate gating the main types of leukocytes (granulocytes, monocytes and lymphocytes), and to gate out debris.

4.3.2.5 PMT Voltage Optimisation and Fluorescence Compensation

Fluorescence spillover is not uncommon in most multicolour experiments. Therefore, fluorochrome compensation is a crucial step to override the fluorescence emission spectral overlap (Shapiro and Leif 2003). The compensation was performed using AbCTM Anti-Mouse Bead Kit (Life Technologies, Grand Island, USA), as per manufacturer's instructions. Briefly, 'component A' beads were stained with each fluorochrome and incubated for 15 min at room temperature in the dark. The beads were then washed with PBS for 5 min at 200 x g, and one drop of 'component B' (negative beads) were added to each tube, mixed and analysed. PMT voltages were adjusted during the compensation setup using unstained beads in each of the single stained samples to separate signal (positive) from noise (negative) (Maecker and Trotter 2006). When starting data acquisition, the related detector voltages were

adjusted during the live setup phase of data acquisition. The PMT voltages were adjusted to position the unstained negative cells within the first decade of the histogram (Maecker and Trotter 2006). Compensation was automatically calculated by the Attune[®] Acoustic Focusing software (Life Technologies, Grand Island, USA).

4.3.2.6 Optimisation of Flow Cytometry Protocol to Detect TRPV1

Method optimisation was conducted for fixation/ permeabilisation, blocking, and the TRPV1 immuno-detection reaction (primary, isotype and secondary antibodies).

Fixation and Permeabilisation

Fixation and permeabilisation procedures were required to allow the TRPV1 primary antibody to bind to its intracellular epitope. Two commercially available kits CALTAG[™] (Life Technologies, Grand Island, USA) and Cytofix/Cytoperm[™] (Becton Dickinson, San Jose, USA) were investigated to determine the staining patterns of TRPV1. The fixation/permeabilisation protocols for both kits were applied according to suppliers' instructions, with slight modifications including staining the CD markers for 10 min and centrifuging cells at 300 x g. Cells were fixed, permeabilised and washed using a saponin-based wash reagent to ensure cells permeabilised as the permeabilisation step is reversible.

Assessment of Blocking Reagents

The following general blocking reagents were tested: human AB serum (obtained from a healthy AB blood group donor), 10% goat serum (Life Technologies, Grand Island, USA), and FcR blocking reagent (Miltenyi Biotechnology, Cologne, Germany). The blocking solution was modified in some instances and described in the results.

Assessment of the Primary Antibodies

Three anti-TRPV1 primary antibodies were tested to separate signal from isotype control. To optimise the primary antibody concentration, a titration study was conducted for both TRPV1 antibody and the isotype control. Cells were incubated with 0.5, 1 and 2 µg of primary antibody or isotype control (1:25), diluted in saponin-based wash (Becton Dickinson, San Jose, USA) to determine the best separation between signal and background.

Secondary Antibody Titration

Secondary antibody optimisation involved titration studies for both Alexa Flour[®] 488-conjugated (see Appendix I), and FITC-conjugated polyclonal goat anti-rabbit secondary antibody. Dilution was performed in the saponin-based wash (Becton Dickinson, San Jose, USA) to keep the cells permeabilised. Dilution ranged between 1:25- 1:1000 to choose the optimal titre.

4.3.2.7 Data Acquisition and Analysis

Data acquisition and analysis were completed using the Attune[®] Acoustic Focusing software (CA, USA). 10,000 events were acquired for each sample. Median Fluorescence intensity (MFI) was expressed in arbitrary units on a logarithmic scale.

4.3.2.8 Detection of TRPV1 in Normal Human Leukocytes (Optimised Protocol)

One million cells were surface stained with each of CD3-Brilliant Violet[™] 421, CD14-V500, CD45-PerCP, CD19-PE, CD56-Qdot[®] 605 as per manufacturer's instructions, and incubated for 10 min in the dark at room temperature. Red blood cells were then lysed by incubating cells in 2 mL FACS Lyse solution (BD

Biosciences, San Jose, USA) for 10 min in the dark at room temperature. Cells were centrifuged at 350 x g for 5 min, then washed in 3 mL PBSA (PBS, 10% FBS, 0.1% sodium azide) and re-centrifuged for a further 3 min. Cells were fixed and permeabilised using Cytofix/Cytoperm™ (Becton Dickinson, San Jose, USA) according to manufacturer's instructions by adding 250 µL/tube of BD Cytofix/Cytoperm™ solution then incubated for 20 min at 4°C. Cells were then washed twice (1mL/tube/wash), centrifuged for 3 min at 350 x g and the supernatant was discarded. Non-specific binding sites were then blocked using 50 µL of 10% human AB blood serum, supplemented with 1% BSA, 0.05% sodium azide and made up with the saponin-based wash, and incubated for 15 min at room temperature. After blocking, cells were ready for intracellular staining by incubating the cells with 0.5 µg of Lifespan Biosciences (WA, USA) TRPV1 primary antibody or 0.5 µg of isotype control (Santa Cruz Biotechnology, CA, USA) (1:25) for 45 min at 4°C. Cells were then washed twice and stained with 50µL of FITC-conjugated anti-rabbit secondary antibody (1:25) (Santa Cruz Biotechnology, CA, USA) for 20 min at 4°C, and again washed twice. Finally, 2.5 mL of cold Ca²⁺/ Mg²⁺ free PBS was added to each tube prior to acquisition. Cells were run immediately after sample preparation.

4.4 Results

4.4.1 Flow Cytometry Optimisation setup

Compensation and PMT values were adjusted for all fluorochromes used. Spectral overlap of the fluorochromes was corrected by the Attune® Acoustic software when adjusting the PMT voltages (Figure 4-1). FSC and SSC were adjusted for each sample and the three leukocytes populations were located on the linear scale (Figure 4-2).

Channel	PMT Voltage
FSC-A	2,400
SSC-A	3,800
BL1-A	1,250
BL2-A	1,200
BL3-A	1,150
VL1-A	650
VL2-A	650
VL3-A	650
Name	Threshold
FSC	396,000

Figure 4-1: Example of optimised Attune® Cytometer settings using AbC™ beads. Each fluorochrome is detected in a different channel. PMT values were adjusted for each channel; threshold is the value where debris was excluded.

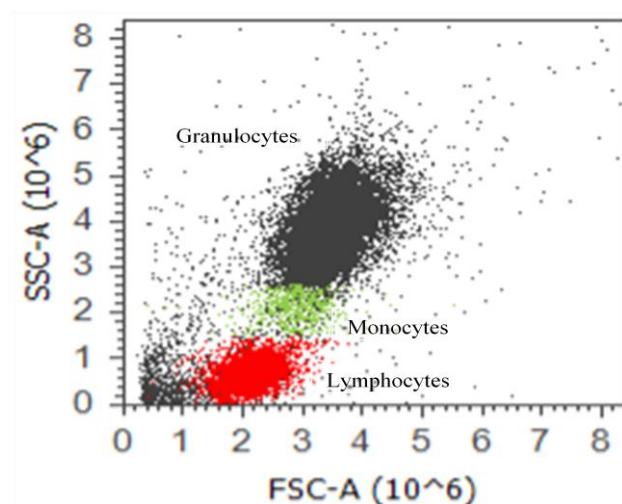


Figure 4-2: FSC and SSC electronic optimisation for fixed/permeabilised cells using the Attune® Flow Cytometer. Optimisation of the FSC and SSC leads to a clear distinction between the three main leukocyte populations, lymphocytes, monocytes and granulocytes, and the exclusion of the debris.

4.4.2 Fixation and Permeabilisation Optimisation

There was no major difference in TRPV1 signal using either the CALTAG™ (Life Technologies, Grand Island, USA) and Cytofix/Cytoperm™ kits (Becton Dickinson, San Jose, USA) (see Appendix I).

4.4.3 Assessment of the Primary Antibodies

Rabbit anti-human TRPV1 antibody #1 (Santa Cruz Biotechnology)

This antibody produced a similar or weaker signal than the isotype control (Figure 4-3). Several blocking solutions were therefore investigated for their potential to minimise background staining. Blocking with 10% goat serum resulted in the best separation between TRPV1 signal (positive) and secondary antibody alone (negative) compared to human AB serum or Fc receptors reagents with Santa Cruz antibody (Figure 4-4). There was no significant change in TRPV1 signal when using fresh or frozen AB serum, or when incubating for 30 min or at 4°C. Complete loss of TRPV1 signal was observed at 37°C (data not shown).

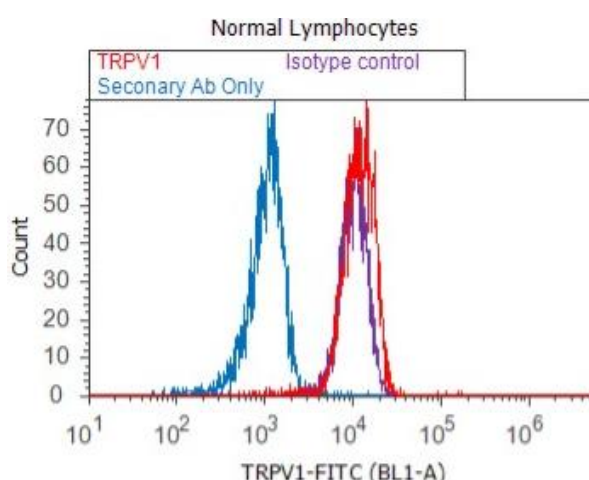


Figure 4-3: Isotype control overlapping with TRPV1 signal in human leukocyte. Data demonstrates staining pattern for isotype control compared with TRPV1 signal and secondary antibody only (goat anti-rabbit-FITC) (all antibodies were obtained from Santa Cruz biotechnology, USA). No separation between isotype control and TRPV1 signal was demonstrated.

The dilution study of the Santa Cruz Biotechnology anti-TRPV1 antibody was performed on the FACSCalibur™ Cytometer. The optimum TRPV1 quantity was 1µg of anti-TRPV1 antibody per one million cells (See Appendix I). There was no

significant difference in TRPV1 signal when the primary antibody was incubated at room temperature or 4°C (data not shown). There was no non-specific binding caused by secondary antibody, but still no separation between TRPV1 and isotype control signals (Figure 4-3). The isotype control obtained from Cell Signalling Technology (MA, USA) produced a little improvement in the signal, still not completely separated from the anti-TRPV1 signal (Figure 4-5).

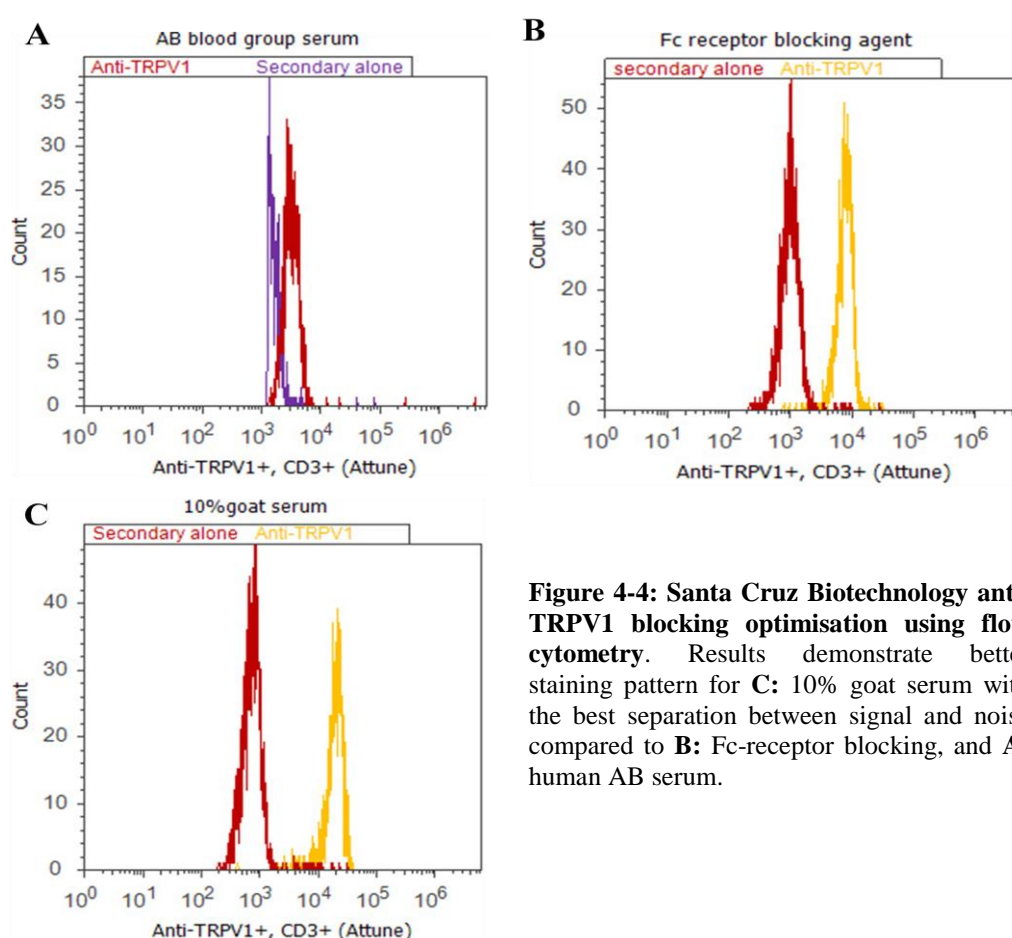


Figure 4-4: Santa Cruz Biotechnology anti-TRPV1 blocking optimisation using flow cytometry. Results demonstrate better staining pattern for **C**: 10% goat serum with the best separation between signal and noise compared to **B**: Fc-receptor blocking, and **A**: human AB serum.

Rabbit anti-human TRPV1 antibody # 2 (Alomone Labs)

A second primary antibody was tested in an attempt to separate TRPV1 and isotype control signals. As with antibody #1, 1µg of anti-TRPV1 antibody per one million cells was tested. Non-specific binding was blocked using 10% goat serum. No

separation between isotype control and TRPV1 signal was observed with this antibody (Figure 4-6).

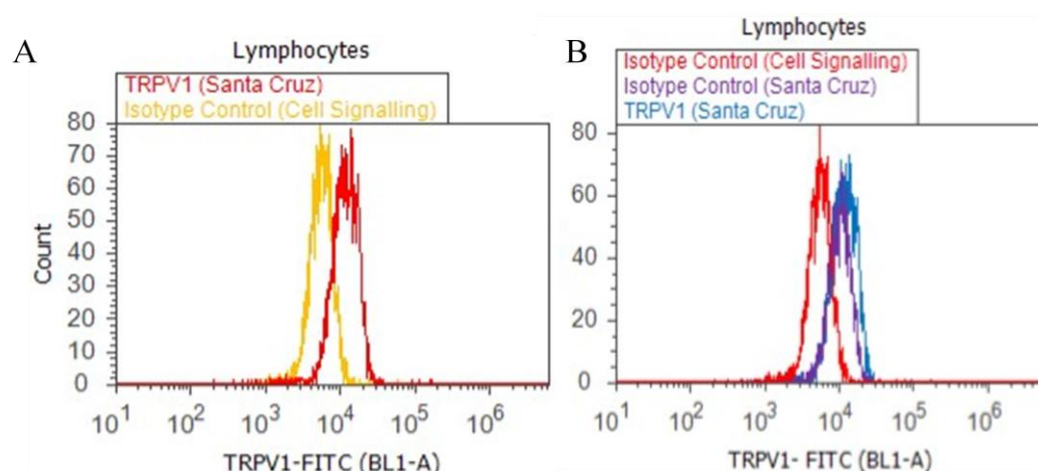


Figure 4-5: Comparison between two isotype controls vs. Santa Cruz Biotechnology anti-TRPV1. **A:** A slight shift to the left was observed with the Cell Signalling Technology isotype control, but not sufficient to cause separation from the TRPV1 signal. **B:** Santa Cruz biotechnology isotype vs. Cell Signalling Technology isotype histograms overlapping with TRPV1 signal.

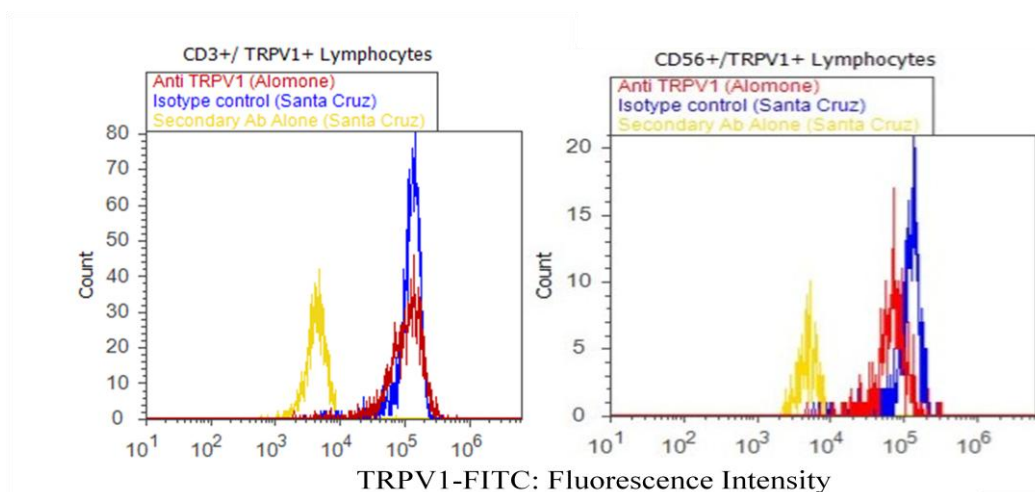


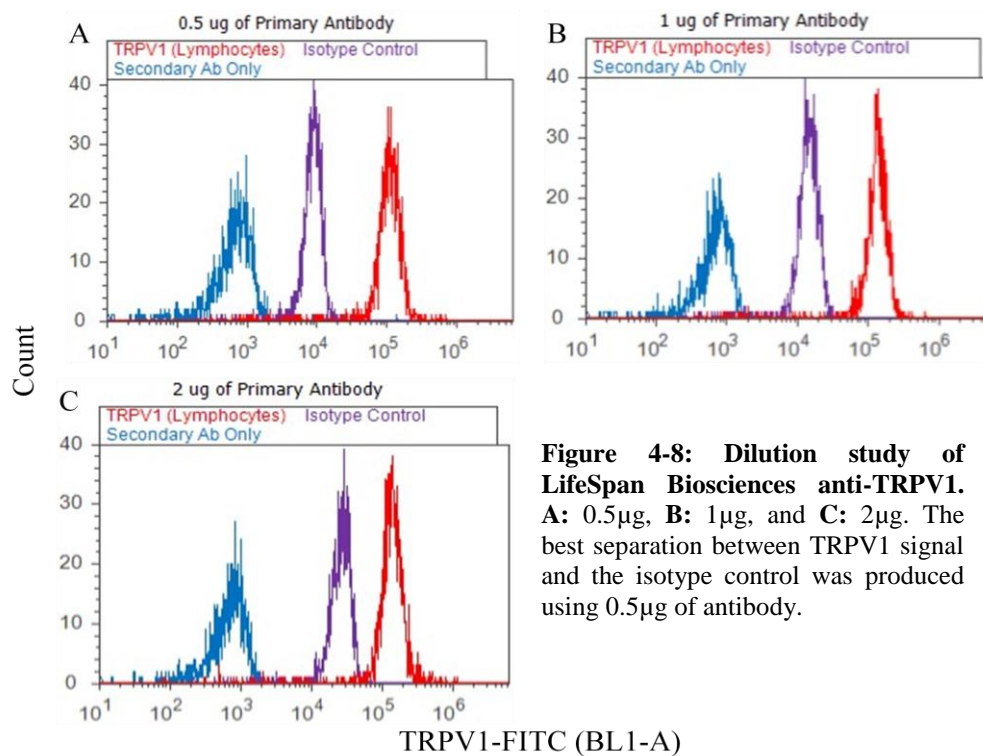
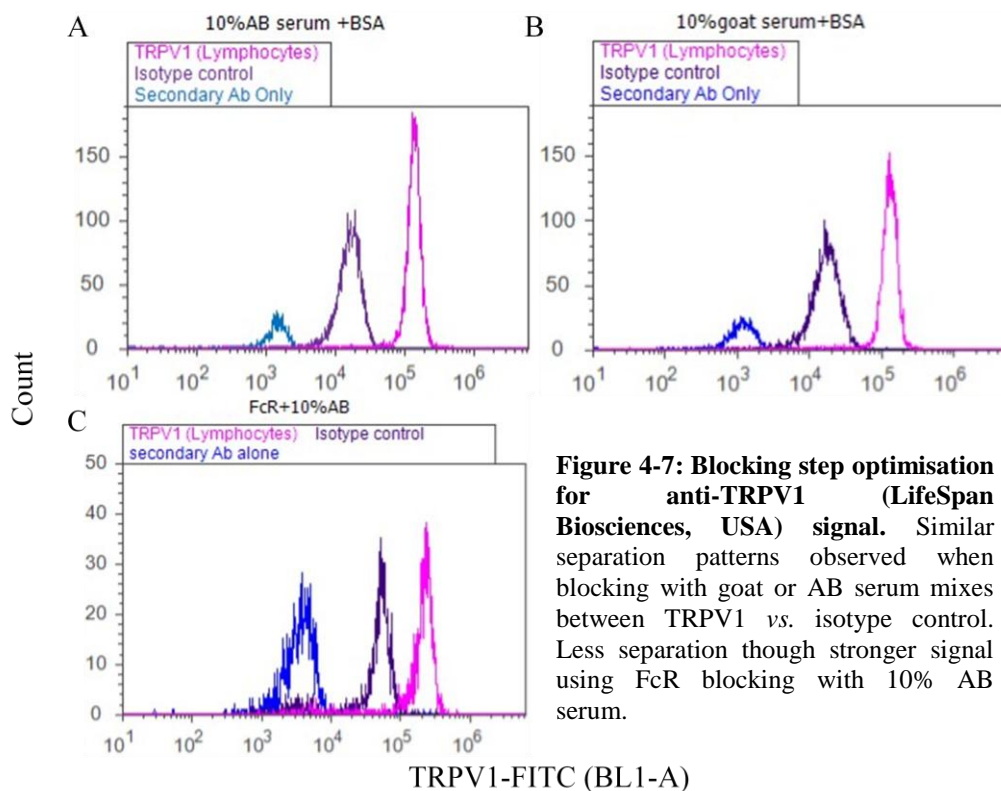
Figure 4-6: Alomone Labs anti-TRPV1 signal. Cells were blocked with 10% goat serum for 15 min at room temperature, stained with $1\mu\text{g}/1$ million cells anti-TRPV1 and 1:25 secondary antibody. No separation between isotype control and the primary antibody signals was achieved. The isotype control signal overlapped with the TRPV1 signal.

Rabbit anti-human TRPV1 antibody # 3 (LifeSpan Biosciences)

Separation between TRPV1 signal, isotype control and secondary antibody control was achieved using the LifeSpan Biosciences anti-TRPV1 primary antibody. To optimise the separation, the same multiple blocking reagents were assessed, including an AB serum mix (10% human AB serum + 0.1% BSA + 0.05% sodium azide), goat serum mix (10% goat serum + 0.1% BSA + 0.05% sodium azide) and a Fc receptor blocking reagent (Miltenyi Biotechnology, Cologne, Germany) mixed with 10% AB serum. All were diluted in the saponin-based wash (Becton Dickinson, San Jose, USA). A similar signal pattern when blocking with AB blood serum mix or goat serum mix were demonstrated (Figure 4-7). However, there was less separation, although stronger signal, using the FcR blocking reagent with 10% AB serum (Figure 4-7). Therefore, the AB blood serum mix was chosen to be the blocking reagent as due to its affordability. It was found that 0.5µg of anti-TRPV1 per one million cells generated the optimal separation between TRPV1 signal and isotype control (Figure 4-8).

4.4.4 Secondary Antibody Assessment

Titration studies were performed for Alexa Flour[®] 488-conjugated secondary antibody using the FACSCalibur[™] (See Appendix I), and FITC-conjugated polyclonal goat anti-rabbit secondary antibody (Figure 4-9). There was no difference between both antibodies, therefore FITC-conjugated polyclonal goat anti-rabbit secondary antibody was chosen for further experiments due to reduced costs.



There was no difference in signal when incubating at room temperature or 4°C (data not shown). There was no significant difference between background staining for FITC-conjugated secondary antibody controls at dilutions ranging between 1:25 and 1:150. Titre was found to be 1:25 with the brighter TRPV1 signal and optimum separation from the secondary antibody alone (Figure 4-9).

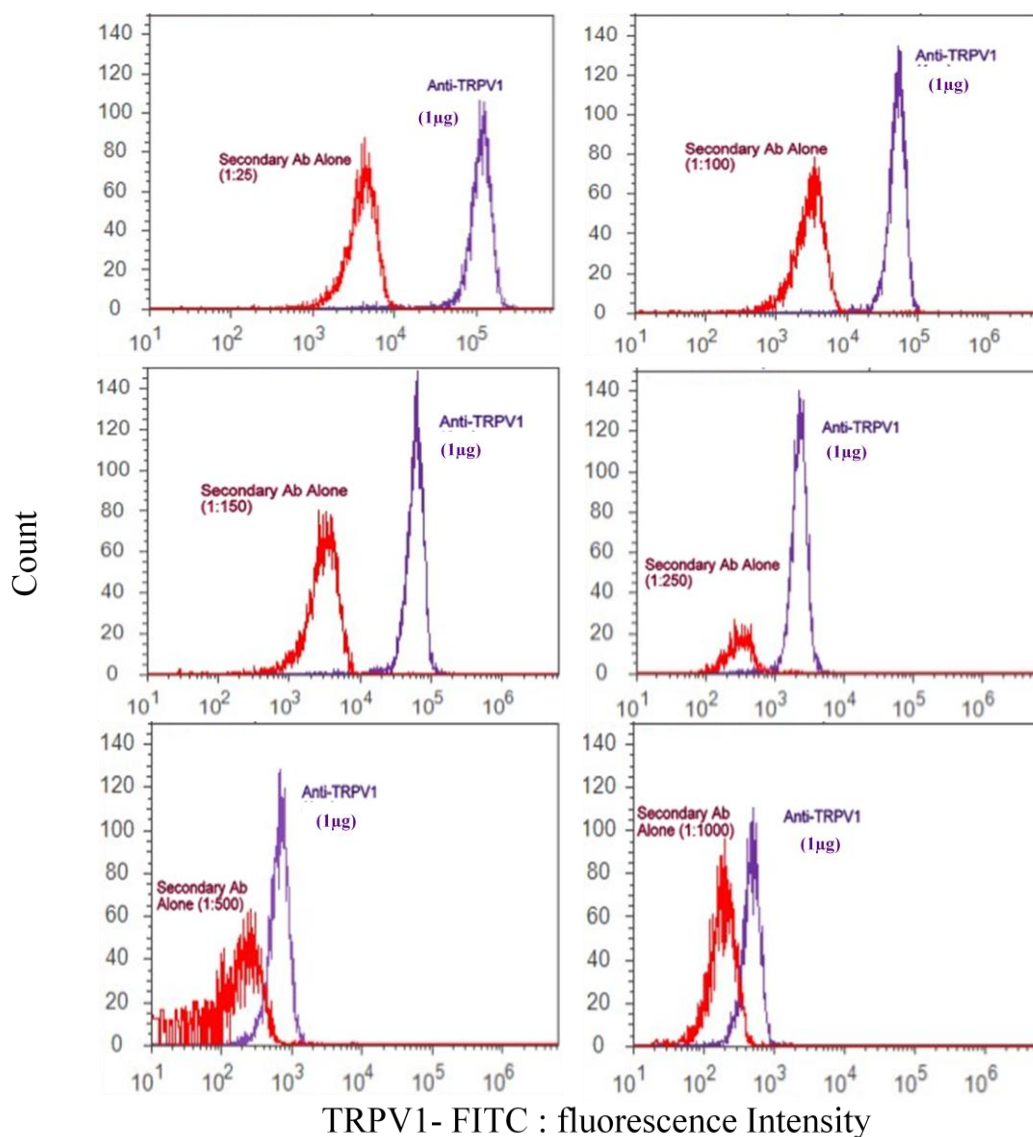


Figure 4-9: Secondary antibody titration for FITC-goat anti rabbit (Santa Cruz Biotechnology) using the Attune® Cytometer. Red histogram represents the secondary antibody alone (negative control), whereas purple histogram represents the secondary antibody with the addition of Santa Cruz primary antibody (positive signal). Dilution range was tested between 1:25-1000. Decreased secondary antibody concentration was accompanied with decreased positive signal. Brighter signal with separation from the negative signal was demonstrated at 1:25 secondary antibody dilution.

4.4.5 Detection of TRPV1 in Human Normal Leukocytes using the Optimised Method

When testing the optimised protocol on normal blood obtained from healthy donor, a separation between TRPV1 signal, isotype control and secondary antibody control was clearly achieved and reproduced. Typical results of the histograms (Figure 4-10), and the mean of the MFI of leukocytes (Table 4-2) show that TRPV1 is expressed in the following order: CD19-B-cells> CD14-monocytes> CD3-T-cells.

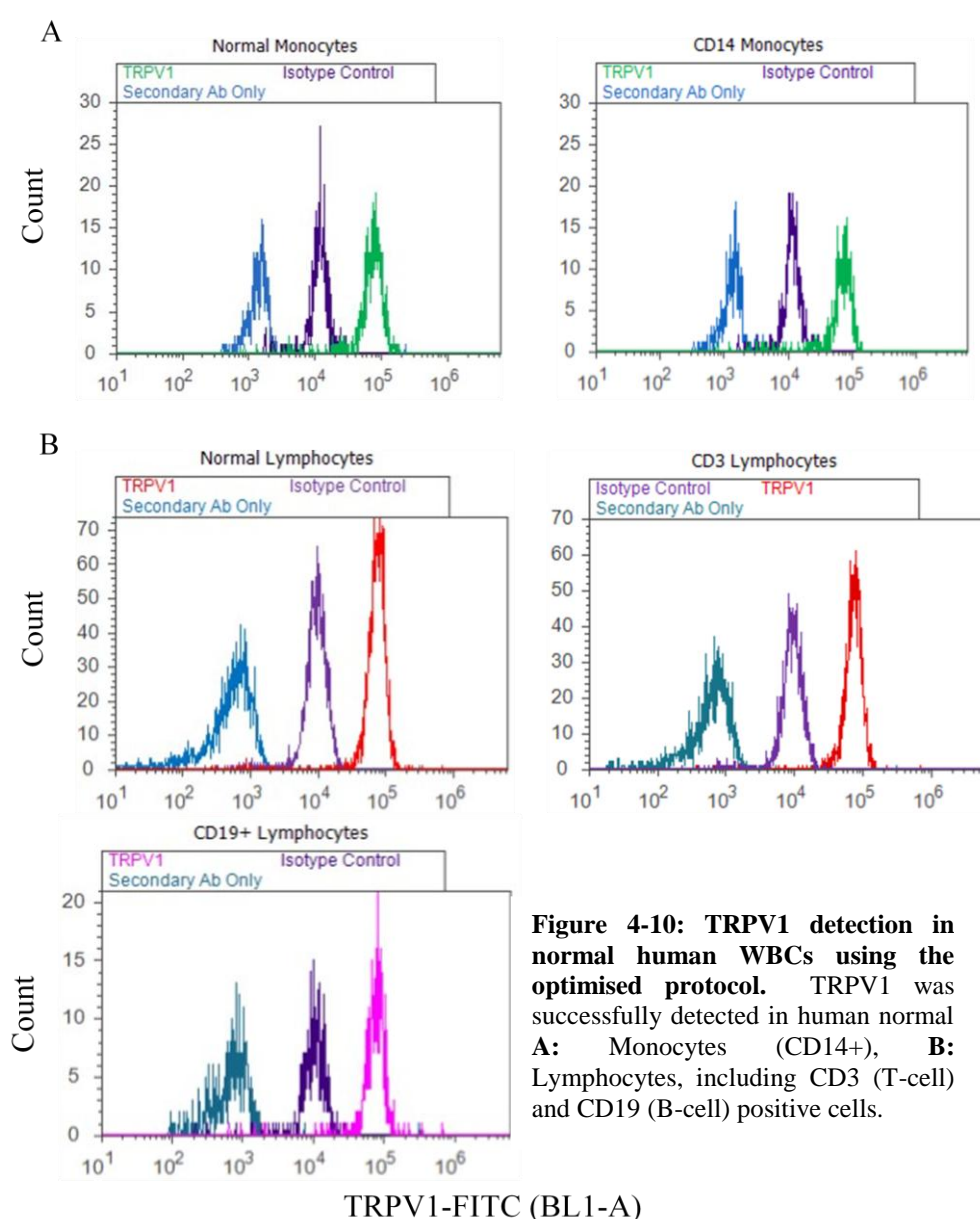


Figure 4-10: TRPV1 detection in normal human WBCs using the optimised protocol. TRPV1 was successfully detected in human normal **A:** Monocytes (CD14+), **B:** Lymphocytes, including CD3 (T-cell) and CD19 (B-cell) positive cells.

Table 4-2: Mean-MFI values for different normal leukocytes subpopulations

Cells	n	Mean-MFI (10^5)
CD14-Monocytes	5	1.12
CD19-B-cells	6	1.36
CD3-T-cells	5	0.96

CD: cluster of differentiation; MFI: median fluorescence intensity; n= number of conducted experiments.

4.5 Discussion

The aim of this chapter was to validate and optimise a flow cytometry method to detect TRPV1 in PBMCs. The optimised method would later be used to detect TRPV1 in haematological malignant cell lines (Chapter 5) and patients samples (Chapter 6). TRPV1 was successfully detected in human PBMCs using the Attune[®] Acoustic Focusing Cytometer.

The data in this chapter casts doubt on other studies that have detected TRPV1 using immunofluorescence techniques i.e., flow cytometry, which might impact on the conclusions previously generated from these studies (Table 4-3). Most published studies reported either few or inappropriate controls, especially the negative control. Using a secondary antibody only, or normal serum as a control, as noted in some studies, is insufficient to control the flow cytometry experiment and test the TRPV1 antibody specificity (Amantini et al. 2004; Amantini et al. 2007; Caprodossi et al. 2011; Zhang et al. 2007). Whether data generated using flow cytometry data is a true positive, false positive, or false negative, is therefore questionable (Figure 4-3). Primary antibodies sometimes bind ‘non-specifically’ to different types of antigens, especially to Fc-receptors, which are expressed on a wide range of human cells, e.g.,

Table 4-3: Some published studies on TRPV1 expression detected by flow cytometry

#	Cell Lines	Anti-TRPV1	Issue	Paper
1	Urothelial cancer cells (pCMV-5637)	Goat anti-human TRPV1, Santa Cruz Biotechnology	<ul style="list-style-type: none"> • Insufficient control. Secondary antibody alone was used as a negative control only. No primary antibody control or isotype controls used. 	(Caprodossi et al. 2011)
2	HEK293T-TRPV1, HEK293T mock and HaCaT cells	Goat anti-human TRPV1, Santa Cruz Biotechnology	<ul style="list-style-type: none"> • TRPV1 was detected on cell surface. • Secondary antibody alone was used as an isotype control. This is a miss-concept of the term 'isotype control'. • Insufficient primary antibody control. 	(Huang et al. 2010)
3	Urothelial cancer cell lines	Goat anti-human TRPV1, C-terminus, Santa Cruz Biotechnology	<ul style="list-style-type: none"> • Insufficient control. No primary antibody control or isotype controls used. • Conflict between method description and figure legend; secondary antibody alone was mentioned as the negative control in the legend, whereas normal goat serum was described in the methods instead. 	(Amantini et al. 2009)
4	NHA, U87 and U373 glioma cells	C-terminus, Chemicon International	<ul style="list-style-type: none"> • Insufficient control by using a normal goat serum only as a negative control. 	(Amantini et al. 2007)
5	Rabbit corneal HCEC	Anti-TRPV1 (SC-12498), Santa Cruz Biotechnology	<ul style="list-style-type: none"> • TRPV1 surface staining, though the TRPV1 epitope is intracellular. 	(Zhang et al. 2007)
6	Mice dendritic cells	VR1 (P-19) and PA1-747, Santa Cruz Biotechnology, Affinity BioReagents	<ul style="list-style-type: none"> • Rabbit IgG concentration (isotype control), which should match the anti-TRPV1 concentration, was not mentioned in the text, nor in the figures, whereas anti-TRPV1 dilutions were illustrated clearly. 	(Basu and Srivastava 2005)
7	Rat thymocytes	Goat anti rat, C-terminus anti-VR1, Santa Cruz Biotechnology	<ul style="list-style-type: none"> • Insufficient control by using a normal goat serum only as a negative control. 	(Amantini et al. 2004)

HEK: human embryonic kidney cells; NHA: normal human astrocytes; C-terminus: carboxy terminus; VR1/ TRPV1: transient receptor potential vanilloid-1; HCEC: human corneal epithelial cells.

neutrophils, monocytes, macrophages, natural killer cells, B-cells and some T-cell subsets (Capel et al. 1994; O'Gorman and Thomas 1999). The term 'non-specific binding' is used to identify any antibody binding to an epitope other than the one that the antibody is generated to bind (Hulspas et al. 2009). However, an epitope can be present in different antigens from different species, therefore 'cross-reactivity' occurs in which the antibody binds to the antigen 'specifically', though un-preferably (Hulspas et al. 2009; Kim et al. 2006a; Oliveira et al. 2001). Blocking Fc-receptors and using the isotype control to rule out this sort of background is essential to distinguish 'false positive' from true positive signal, especially when the targeted antigen is expressed in low levels (Hulspas et al. 2009; O'Gorman and Thomas 1999).

An isotype control is an antibody of the same immunoglobulin type, subclass (isotype) and species as the primary antibody, with either unknown specificity or raised against an antigen that is known not to be expressed in the cells of interest (Stewart et al. 1994). It should be produced by the same manufacturing process, and be of the formulation, concentration, purification and conjugation as the primary antibody (Lowdell 2001). An isotype control is used to determine if unwanted Fc-receptor binding occurs (O'Gorman et al. 1993). Some studies misinterpret the term 'isotype control' by using the secondary antibody alone as an isotype control (Huang et al. 2010). Another study used a fixed dilution of isotype control against multiple dilutions of the primary antibody, without specifying the dilution used for the isotype control (Basu and Srivastava 2005). The isotype control must be used as a substitute for the primary antibody, not the secondary antibody, and be tested in the same dilution as the primary antibody (Stewart et al. 1994). Furthermore, some studies

measured TRPV1 expression externally (surface staining). However, the TRPV1 C- and N- termini are both intracellular (see Chapter 1), and the antibodies used in these studies were designed to detect TRPV1 on one of these epitope sites (Huang et al. 2010; Zhang et al. 2007). Based on the lack of proper controls discussed previously, the results obtained from these studies need to be re-evaluated.

In this chapter, separating the isotype control from the TRPV1 signal in normal PBMCs was challenging. In order to resolve this problem, a series of experiments were conducted. These involved adding a *blocking step* to block Fc-receptors, *evaluating different isotype controls*, *testing different primary antibodies* to enhance the TRPV1 positive signal by more specific antibody-antigen binding, thus separating the TRPV1 from isotype and secondary antibody signals, and finally, *performing dilution studies for the primary and secondary antibodies* to determine optimum separation between the isotype control, TRPV1 and secondary antibody histograms.

Titration of primary and secondary antibodies was essential to obtain the highest positive signal with the lowest negative signal possible on the logarithmic scale (Srivastava et al. 1992). In the present study, different primary antibodies warrant variable staining properties and specificity and as a result produce different results. In flow cytometry, the primary antibody that demonstrates the same staining pattern as the isotype control arises difficulties in separating specific from non-specific signals.

Santa Cruz Biotechnology and Alomone Labs anti-TRPV1 antibodies tested in this study overlapped with the isotype control indicating non-specific staining and low specificity. Similar non-specific binding was previously reported with immunohistochemistry using the same antibodies when testing TRPV1^{-/-} mice bladder tissue, resulting in false positive results (Everaerts et al. 2009). Similar to results obtained by Western blot (Chapter 3), the LifeSpan Biosciences anti-TRPV1 antibody was found to produce more specific binding than the other two antibodies. It has been suggested that antibodies which are not affinity purified have less specificity to the targeted protein (Couchman 2009). The Santa Cruz Biotechnology anti-TRPV1 antibody was purified using proprietary techniques, whereas the isotype control from the same manufacturer was affinity purified. This difference in the antibody purity might have an impact and caused the variation in the binding specificity of the two antibodies, affecting the final result.

Although the Alomone Labs anti-TRPV1 antibody was affinity purified, according to the manufacturer, its binding capacity was insufficient to separate the TRPV1 signal from the isotype control, indicating other unknown factors causing the lack of specificity for TRPV1 by some antibodies compared to others. To the best of our knowledge, no studies have published TRPV1 expression by flow cytometry using the LifeSpan Biosciences anti-TRPV1, which appeared to be a superior antibody.

In order to check the specificity of the primary antibody, knockdown/knockout cells should be used as a negative control (Couchman 2009; Shim et al. 2007). Unfortunately, its not always affordable/applicable to find positive and negative subpopulations of the same cell type, which might be a limitation for these types of

studies (O'Gorman et al. 1993). This is more convenient for animal experiments than for human studies. Another alternative is to find a human tissue or cell type that does not express TRPV1. In an attempt to find a TRPV1-negative cell line to use as a negative control, RAW264.7 cells, TRPV1-transfected (Tetracycline on/off system) and un-transfected human embryonic kidney cells (HEK293) were investigated. However, they were all found to express TRPV1 (*see section 5.4.2*). Thus, in this chapter, two types of negative controls for flow cytometry experiments, i.e. secondary antibody alone and isotype control, were employed.

TRPV1 was detected in normal human leukocytes using the flow cytometry technique optimised in this chapter. The expression level varied between sub-populations of PBMCs (CD19-B-cells > CD14-monocytes > CD3-T-cells). The difference in TRPV1 expression has been previously reported in healthy PBMCs donors using immunocytochemistry (Lai et al. 1998; Saunders et al. 2007). This suggests a unique role for TRPV1 in each of the leukocyte subsets (Saunders et al. 2009).

In conclusion, a flow cytometric method to measure TRPV1 expression in human PBMCs was optimised by evaluating a number of primary, secondary antibodies, isotype controls, and blocking solutions. As described in Chapter 3, only affinity purified antibodies should be considered in flow cytometry experiments to detect TRPV1, as antibodies purified by other methods such as proprietary techniques showed less specificity and false negative results, compared to the isotype control. Two types of negative controls were used to distinguish positive from negative

results. This method was applied to detect TRPV1 on various cells and patients blood samples in the remaining two results chapters of this thesis.

Chapter 5: TRPV1 Expression in Human Haematological Malignancy Cell Lines

5.1 Abstract

TRPV1 is a non-selective cation channel activated by a variety of stimuli. Increased expression of TRPV1 is associated with several cancers. The aim of this study was to determine the level of TRPV1 expression in malignant haematological cell lines as this has previously not been investigated. Three cell lines, THP-1, U266B1 and U937, were investigated using flow cytometry (Attune[®] Acoustic Focusing Cytometer, Life Technologies, Grand Island, USA) and Western blotting. Other cell lines which had previously been used as a source of TRPV1 negative cells, untransfected, hTRPV1-transfected HEK293 (tetracycline off) and RAW264.7, were also reassessed for TRPV1 expression. Increased TRPV1 expression was shown in the malignant haematological cell lines compared to normal peripheral blood mononuclear cells (PBMCs) (Ratio of median fluorescence intensity (MFI) = (MFI cell line) / (MFI normal PBMCs) as follows: THP-1= 2.5, U266B1= 2.3, U937= 1.5. TRPV1 was also surprisingly detected in untransfected, hTRPV1-transfected (tetracycline off) HEK293 and RAW264.7 cells. A TRPV1 monomer (~95kDa) was found to be strongly expressed in both THP-1 and U266B1 cells, but weaker in U937 cells using Western blotting. A TRPV1 dimer was also detected in U266B1 cells, which might be due to the presence of an unknown TRPV1 isoform, or as a consequence of insufficient denaturation time during the Western blotting process. These results strongly suggest that TRPV1 is not uniformly expressed in different cell lines. In conclusion, increased expression of TRPV1 was detected in THP-1, U266B1 and U937 malignant haematological cells, compared to PBMCs from healthy subjects. Furthermore, it is strongly recommended that HEK293 and RAW264.7 cells should not be used as negative controls for experiments involving anti-TRPV1 as they appear to express TRPV1.

5.2 Introduction

There are few previously published studies that have investigated the effect of ion channel antagonists on the functional properties of the malignant haematological cell lines, THP-1, U266B1 and U937 (Himi et al. 2012; Maccarrone et al. 2000). The types of channels expressed in these cells and their precise role remain to be determined (Kim et al. 1996). The TRPV family, specifically TRPV1, with their widespread expression in tissues and diverse functions, may be responsible for many cellular functions (Fernandes et al. 2012; Gunthorpe et al. 2002). Therefore, an alteration in TRPV1 expression may have potential in the treatment of various cancers, as disease-related changes in TRPV1 levels have been described previously in several cancers including cervical cancer and bladder carcinoma (Contassot et al. 2004a; Lazzeri et al. 2005) and reviewed by Gkika and Prevarskaya (2009).

Weak expression of TRPV1 in healthy human PBMCs was demonstrated in Chapters 3 and 4. In this chapter, the optimised flow cytometry and Western blotting protocols were used to investigate TRPV1 expression in the malignant haematological cell lines, THP-1, U266B1 and U937. Furthermore, three cell lines, previously reported to be negative for TRPV1, RAW264.7, hTRPV1-transfected human embryonic kidney (HEK293, tetracycline off), and untransfected HEK293 cells (Chen et al. 2003; Correll et al. 2004; Inoue et al. 2006; Sarker and Maruyama 2003), were also investigated by flow cytometry and Western blotting for their suitability to be used as a negative control for anti-TRPV1 primary antibody.

5.3 Materials and Methods

5.3.1 Cells and Cell Culture

Untransfected HEK293 cells were purchased from Sigma-Aldrich (St. Louis, USA). THP-1, U266B1 and U937 cells were obtained and cultured as previously described (*see section 2.3.1*). RAW264.7 mice macrophage cells were a kind gift from Dr. Rajaraman Eri (University of Tasmania, Launceston, Australia). Human TRPV1-transfected HEK293 cells were a kind gift from Prof. Peter McIntyre (RMIT University, Melbourne, Australia), and were transfected according to the tetracycline on/off system (Gossen and Bujard 1992; Rennel and Gerwins 2002).

5.3.1.1 *TRPV1-transfected HEK293 Cell Culture*

TRPV1-transfected HEK293 cells were cultured in complete media consisting of Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, USA) and supplemented with 10% tetracycline-free FBS (Clontech, CA, USA). In addition, 5 µg/mL blasticidin and 100 µg/mL hygromycin (InvivoGen, CA, USA) were added to select for tetracycline repressor protein and TRPV1 plasmid-containing cells, respectively.

Frozen cells were thawed and cultured in the complete media as described in *Section 2.3.2*. Cell harvesting started with the removal of the old media from the flask and washing of cells by pipetting 5 mL of Hank's Balanced Salt Solution (Life Technologies, Grand Island, USA) onto the side of the flask, gently agitating to wash, and the solution discarded. To detach the cells, 2 mL of Hank's Balanced Salt Solution and 1 mL of TrypLE™ Express (Life Technologies, Grand Island, USA) were added to the T-75 flask, incubated for 1 min at 37°C, and the cells detached by

hitting the flask against a hand with force. Seven millilitres of the complete media were then added and gently aspirated, covering the base of the flask to dislodge remaining cells. The cell suspension was transferred to a 15 mL tube, and centrifuged at 250 x g for 5 min. The supernatant was then discarded and the pellet resuspended in 10 mL of complete media and washed again. Finally, 5 mL of complete media was added and the cells counted prior to each experiment.

Tetracycline Treatment

Cells were seeded into a six-well plate at a density of 3×10^5 cells per well and incubated for 48 hr. Tetracycline was added to a final concentration of 1 $\mu\text{g/mL}$ and incubated for 5 hr, or 24 hr for protein expression to occur. Cells were lysed, protein collected, and then measured as described in *section 3.3.5.3*.

5.3.1.2 Untransfected HEK293 and RAW264.7 Cell Culture

Both types of cell were cultured in DMEM supplemented with 10% heat-inactivated FBS (Life Technologies, Grand Island, USA). RAW264.7 cells were cultured in CELLSTAR[®] hydrophobic cell culture flask (Greiner bio-one, Frickenhausen, Germany), due to their weak adherence to the standard T-75 flask. Cells were detached with 3 mL of TrypLE[™] Express (Life Technologies, Grand Island, USA) and continued as described in *section 5.3.1.1*.

5.3.2 Western Blotting and Flow Cytometry Experiments

The Western blotting (optimised in Chapter 3) and flow cytometry (optimised in Chapter 4) protocols were applied to THP-1, U266B1, U937, RAW246.7 and HEK293 (transfected and untransfected) cell lines. THP-1 cells were tested first, and then used as a positive control for Western blotting experiments (Kunde et al. 2009).

5.3.3 Data Collection and Analysis

Median fluorescence intensity (MFI) was measured by flow cytometry using the Attune[®] Acoustic Focusing software (Carlsbad, USA). A normal EDTA blood sample containing the same number of leukocytes was run in parallel with each cell line experiment as a control. Arbitrary MFI ratio was generated by dividing MFI (cell line) by MFI (normal cells). The TRPV1 expression was designated as either; increased (MFI ratio >1.2), decreased (<0.8) or similar (0.8-1.2), compared to normal leukocytes.

5.4 Results

5.4.1 TRPV1 Expression in Malignant Haematological Cell lines

TRPV1 expression was successfully detected in malignant haematological cell lines using flow cytometry; THP-1 (Figure 5-1), U266B1 (Figure 5-2) and U937 (Figure 5-3). Stronger expression of TRPV1 was observed for THP-1 and U266B1 cells compared to U937, and confirmed by Western blotting. TRPV1 monomer was detected in protein lysate, as low as 5 µg, in THP-1 and U266B1. In addition, a TRPV1 dimer was also detected using 25 µg of U266B1 protein lysate. However, TRPV1 was not detected in U937 cells using Western blotting. A combination of the three malignant haematological cell lines histogram layouts showed that THP-1 cells produced the strongest signal compared to U266B1 and U937 cells (Figure 5-4, Table 5-1). Dithiothreitol, was also tested instead of β-mercaptoethanol as a reducing agent during protein sample preparation, and did not improve the band quality (data not shown).

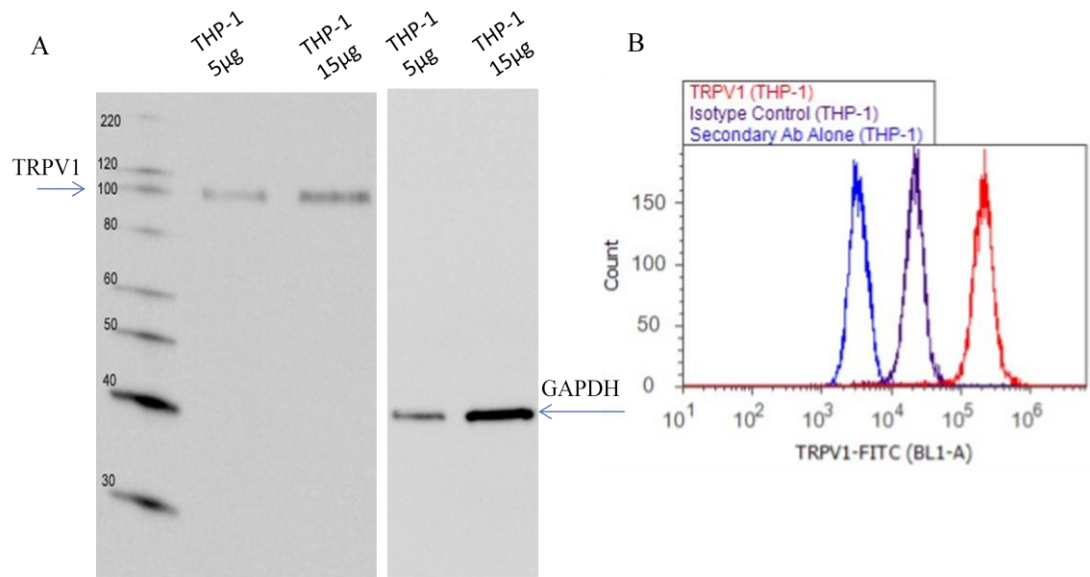


Figure 5-1: TRPV1 expression was detected in THP-1 cells using Western blotting and flow cytometry. A: TRPV1 band was detected in protein lysate from THP-1 cells (5 µg & 15 µg). GAPDH was used as an internal control, **B:** TRPV1 in THP-1 cells vs. isotype control and secondary antibody alone.

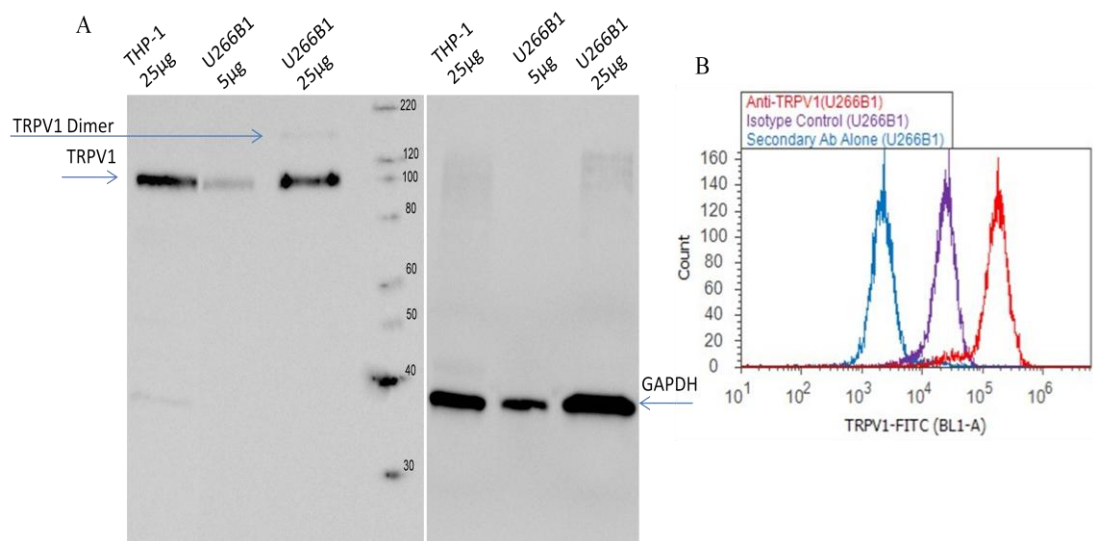


Figure 5-2: TRPV1 expression was detected in U266B1 cells by A: Western blotting, TRPV1 band was detected in protein lysate from U266B1 cells (5 µg). A TRPV1 dimer was also weakly detected at ~200kDa in U266B1 cells protein. Note: band visibility might be affected by printing resolution, **B:** Flow cytometry, TRPV1 in U266B1 cells vs. isotype control and secondary antibody alone.

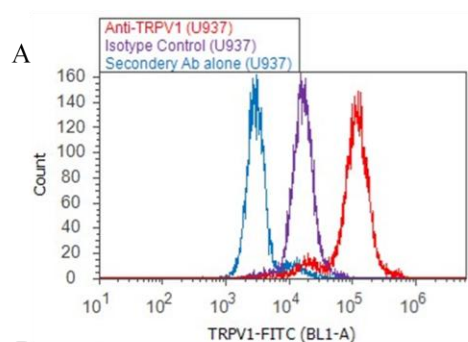


Figure 5-3: TRPV1 expression was detected in U937 lymphoma cells by flow cytometry but not Western blotting. A: TRPV1 in U937 cells vs. isotype control and secondary antibody alone, **B:** No TRPV1 band was detected in up to 30 μ g of protein from U937 cells.

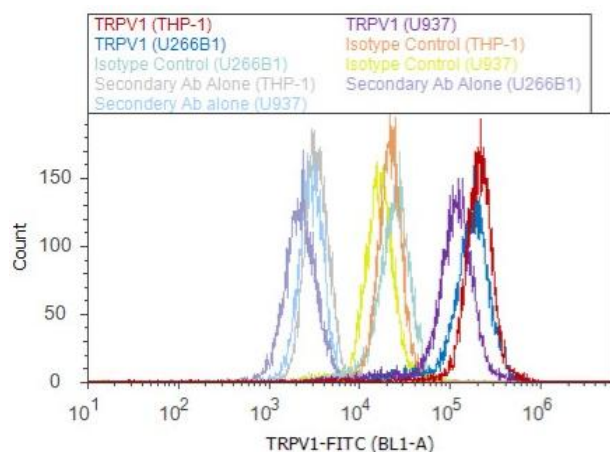
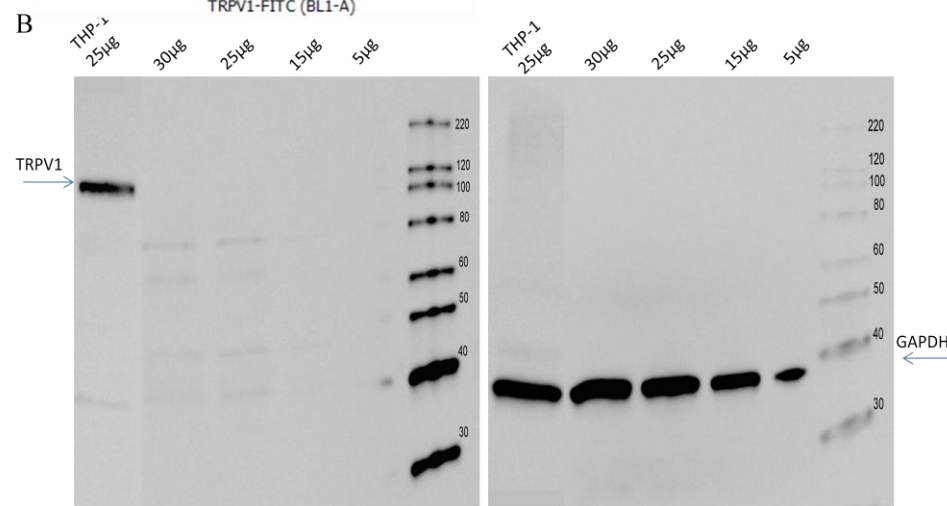


Figure 5-4: Relative expression of TRPV1 in three haematological malignant cell lines. THP-1, U266B1 and U937 TRPV1 flow cytometry histograms vs. isotype controls and secondary Ab alone. Order of TRPV1 signal strength: THP-1> U266B1> U937 cell (shift to the right).

Table 5-1: Comparison of TRPV1 Mean-MFI in THP-1, U266B1 and U937 cell lines and normal leukocytes

Cell line	n	Mean-MFI (10^5) (cell line)	Mean-MFI (10^5) (normal leukocytes)	Ratio	TRPV1 Expression (Relative to normal leukocytes)
THP-1	4	2.38	0.97	2.5	Increased
U266B1	1	1.75	0.76	2.3	Increased
U937	1	1.15	0.76	1.5	Increased

MFI: Median Fluorescence Intensity; n= number of experiments.

5.4.2 TRPV1 Expression in Other Cell lines: A Control Study

5.4.2.1 HEK293 Cells Strongly Express TRPV1

Human TRPV1-transfected HEK293 cells produced similar positive expression of TRPV1, with or without Tetracycline, with a slight increase in TRPV1 signal for tetracycline off HEK293 cells (Figure 5-5). Results were confirmed by Western blotting in which ‘tetracycline off’ transfected-HEK293 cells demonstrated strong TRPV1 expression (Figure 5-6).

Furthermore, untransfected HEK293 cells were found to strongly express TRPV1 (Figure 5-7). Both transfected (Tetracycline off) and untransfected HEK293 cells histograms were plotted by flow cytometer demonstrating same TRPV1 positive pattern (Figure 5-8).

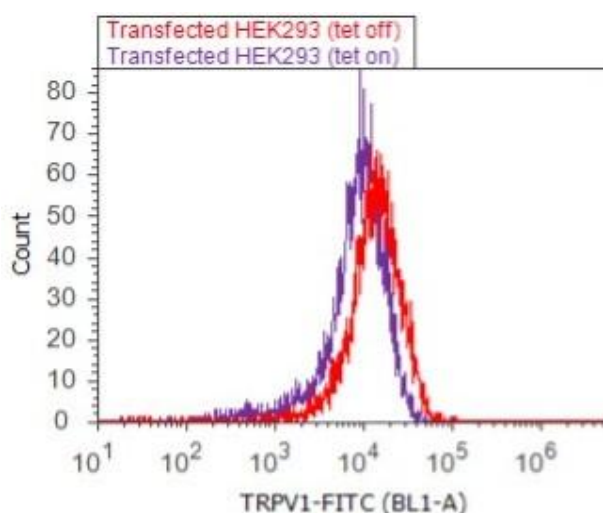


Figure 5-5: TRPV1 expression in TRPV1-transfected HEK293 cells (tetracycline (tet) on/off) by flow cytometry. A slight shift to the right was observed in Tetracycline off HEK293 cells.

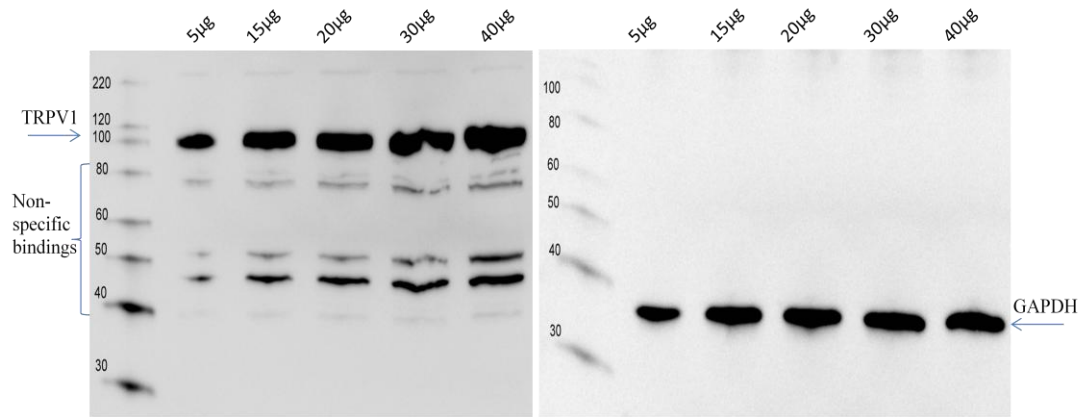


Figure 5-6: TRPV1 expression in TRPV1-transfected HEK293 cells (tetracycline off) by Western blotting. Different quantities of protein were tested. TRPV1 is strongly expressed in transfected HEK293 even without the addition of tetracycline.

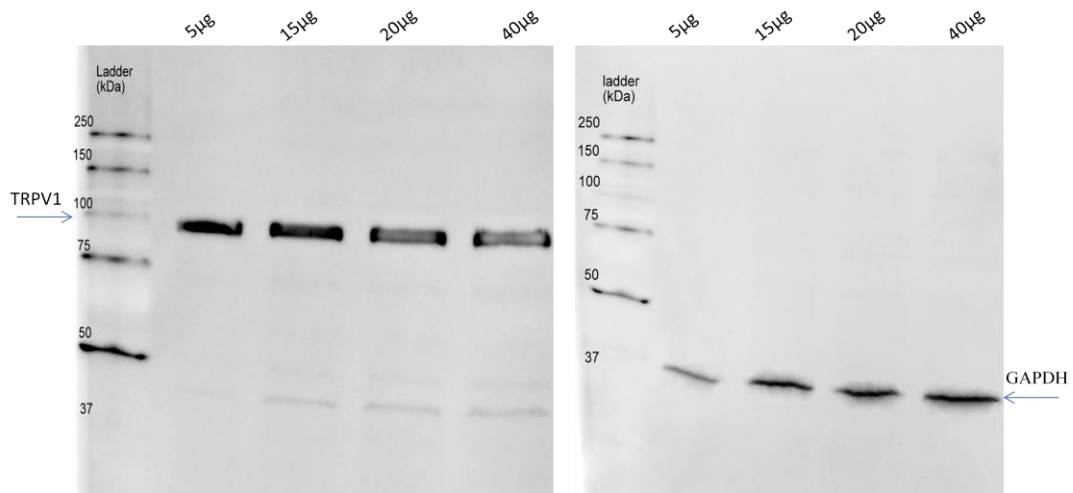


Figure 5-7: TRPV1 expression in untransfected HEK293 cells by Western blotting. Different quantities of protein were tested for confirmation. Results prove that TRPV1 is expressed strongly in untransfected HEK293 cells.

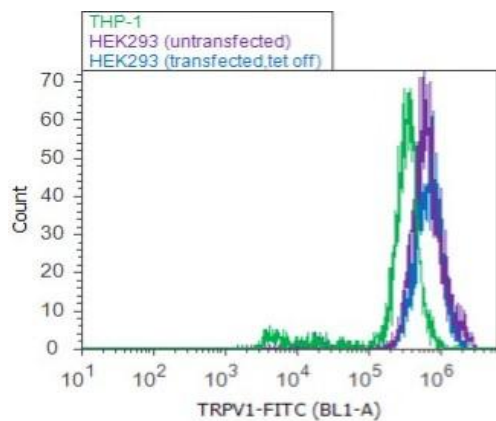


Figure 5-8: TRPV1 expression in HEK293 cells was confirmed by flow cytometry. Transfected (tetracycline off) and untransfected HEK293 cells demonstrate similar signal fluorescence intensity compared to THP-1 cells.

5.4.2.2 RAW264.7 Cells Weakly Express TRPV1

Flow cytometry experiments showed a positive TRPV1 expression in RAW264.7 cells. Western blotting showed weak expression of TRPV1 band using 40 µg of cell lysate (Figure 5-9).

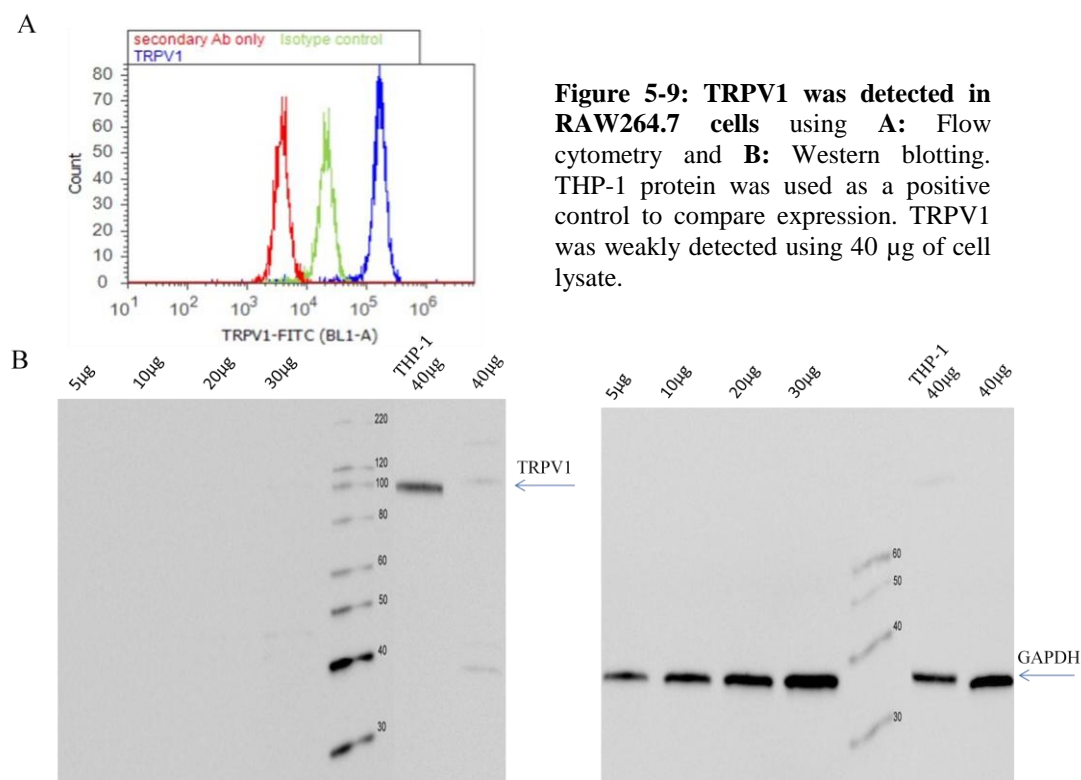


Table 5-2: TRPV1 MFI in some cell lines compared to normal leukocytes from healthy control

Cell line	MFI (10 ⁵) (cell line)	MFI (10 ⁵) (normal cells)	MFI Ratio	TRPV1
t-HEK293 (Tet on)	0.094	0.085	1.11	Similar
t-HEK293 (Tet off)	0.140	0.085	1.6	Increased
RAW264.7	1.675	0.523	3.2	Increased

t-HEK293: TRPV1-transfected human embryonic kidney-293 cells; MFI: Median Fluorescence Intensity; Tet on/off: cells treated/ untreated with Tetracycline.

5.5 Discussion

In this chapter TRPV1 was successfully detected in the malignant haematological cell lines, THP-1, U266B1 and U937, using Western blotting and flow cytometry protocol. Other cell lines were also found to express TRPV1, including untransfected, hTRPV1-transfected HEK293 (tetracycline off, which switches TRPV1 transcription off) and RAW264.7 mice macrophages. Previous studies have reported flow cytometric detection of TRPV1 in human astrocytes (Amantini et al. 2007), human urothelial cancer cells (Amantini et al. 2009), murine dendritic cells (Basu and Srivastava 2005) and rat thymocytes (Amantini et al. 2004), but not human leukocytes. To the knowledge of the author, this is the first study to demonstrate flow cytometric analysis of TRPV1 expression in haematological malignant cell lines.

There are few published papers that report the investigation of TRPV1 in leukocytes, TRPV1 has been detected in rat blood neutrophils (Wang et al. 2005), microglia, the brain-resident macrophage (Kim et al. 2006b; Raivich and Banati 2004) and human blood leukocytes (Saunders et al. 2007; Spinsanti et al. 2008). TRPV1 protein was detected in human (Toth et al. 2009) and mice dendritic cells (Basu and Srivastava 2005), though not in mice mRNA (O'Connell et al. 2005). Therefore, these reports promoted the further investigation of TRPV1 expression in human white blood cells.

In this chapter, three malignant haematological cell lines were investigated. THP-1, an acute monocytic leukaemia cell line, is most often used as a model for human monocytes due to the resemblance in their physiological properties (Kim et al. 1996).

U937 is a histiocytic human lymphoma cell line (Okimura et al. 2007), whereas U266B1 is a plasmacytoma (myeloma) of B-cell origin (Schwabe et al. 1994).

The activation of TRPV1 and its role in THP-1 and U937 cells using TRPV1 antagonists have been previously studied (Himi et al. 2012; Maccarrone et al. 2000; Schilling and Eder 2009b). In the present study, TRPV1 was detected for the first time using flow cytometry in addition to Western blotting in THP-1, U266B1 and U937 cells. A TRPV1 monomer (~95kDa), which represents the non-glycosylated form of the molecule (Vetter et al. 2006) was found to be strongly expressed in both THP-1 and U266B1 cells using a small amount (5 µg) of protein from cell lysate. Furthermore, a TRPV1 dimer (~200kDa) was also detected in U266B1 cells. TRPV1 expression in U937 cells was weaker compared to THP-1 and U266B1 cells using Western blotting. These results strongly suggest that TRPV1 is not uniformly expressed in different haematological malignant cell lines.

The TRPV1 dimer has previously been reported in synaptic junctional protein fraction and root ganglia (Goswami et al. 2010; Prevarskaya et al. 2007). The role of TRPV1, monomer and dimer, in haematological malignant cell lines has yet to be investigated. This phenomenon might be due to the presence of an unknown TRPV1 larger isoform which requires a longer period for adequate denaturation (i.e., >5 min).

Whilst TRPV1 protein may be involved in a variety of normal physiological processes (as discussed in Chapter 1), studies have demonstrated that TRPV1 mRNA and/or protein expression is increased in a variety of inflammatory diseases (Akbar et

al. 2008; Contassot et al. 2004a; Geppetti and Trevisani 2004; Jia et al. 2005; Sadofsky et al. 2012). This raises the possibility that expression of TRPV1 is up-regulated in some haematological malignancies as a result of a systemic inflammation.

IL-6, a pro-inflammatory cytokine secreted by the U266B1 myeloma cells, has a crucial role in inflammation and the stimulation of the immune system (Gabay 2006; Schwabe et al. 1994; Tanabe et al. 2011). It promotes myeloma cell growth and consequently, resistance to therapy (Tricot 2002). There is an accumulating evidence that IL-6 mediates the increase in TRPV1 expression, and conversely, activation of TRPV1 leads to the release of IL-6 and IL-8 (Malagarie-Cazenave et al. 2011; Sappington and Calkins 2008; Toth et al. 2009; Zhang et al. 2007). Similarly, IL-1 is an inflammatory mediator which is also produced by THP-1 cells (Marchand et al. 2005; Yang et al. 2008). TRPV1 mRNA was reported to be produced in the presence of IL-1 α (Sadofsky et al. 2012), whereas IL-1 β can act directly to sensitise TRPV1 and increase its susceptibility to noxious heat (Obreja et al. 2002). Furthermore, tumour necrosis factor-alpha (TNF- α), a major mediator of inflammation, modulates the activity of TRPV1 and is produced by U937 cells (Grivennikov and Karin 2011; Jin and Gereau 2006; Moon et al. 2010). Whether TNF- α provokes overexpression of TRPV1 is unknown. Ultimately, these reports collectively suggest that increased expression of TRPV1 in U266B1 and THP-1 and U937 cells might be due to the secretion of IL-6, IL-1 and TNF- α , respectively, from these cells which play a pivotal role in inflammation and may activate the TRPV1 transcription mechanism. As a result, inhibiting the production of these inflammatory mediators (Anderson

2005; Gupta et al. 2001; Marchalant et al. 2009; Smith et al. 2001) may dampen the expression and activation of TRPV1.

TRPV1 expression varies during carcinogenesis (Gkika and Prevarskaya 2009). Overexpression of TRPV1 was reported in other cancers including glioma (Contassot et al. 2004a) and human tongue epithelium cancer (Marincsák et al. 2009), whereas lower expression was reported in urothelial cancer (Kalogris et al. 2010; Lazzeri et al. 2005). CAP has been reported to induce TRPV1-dependent apoptosis in cancer cells, such as prostate cancer (Ziglioli et al. 2009). Therefore, increasing TRPV1 expression in blood cancers would create a new therapeutic target to treat such conditions.

The variations in ion channel expression in malignancies are either due to transcriptional or translational factors, such as hormones, growth factors or alternatively spliced isoforms (Reynolds and Kyprianou 2006). This causes changes to the amount of Ca^{2+} entering the cell, leading to alterations in essential processes of tumour growth, such as proliferation, apoptosis and migration (Gkika and Prevarskaya 2009). Sustained high Ca^{2+} influx promotes the activation of proteases, phospholipases and the loss of mitochondrial function (Lin et al. 2004; Orrenius et al. 2007; Skryma et al. 2000). For example, the decrease in TRPV1 expression in aggressive tumours such as urothelium transitional cell carcinoma reduces the release of Ca^{2+} , resulting in increased resistance to apoptosis (Gkika and Prevarskaya 2009; Lazzeri et al. 2005).

TRPV1 expression levels may correlate with malignancy progression although evidence suggests this may be either positively, as in prostate cancer (Sánchez et al. 2006), negatively as in bladder carcinoma (Lazzeri et al. 2005) and glioma (Amantini et al. 2007), or with no change as in tongue squamous cell carcinoma (Marincsák et al. 2009). Furthermore, a negative prognostic factor and a short patient survival rate were described with TRPV1 downregulation in bladder (Kalogris et al. 2010), and hepatocellular carcinoma (Miao et al. 2008). Moreover, a protective role of TRPV1 was exhibited in some malignancies, such as skin carcinoma (Bode et al. 2009) and urothelial cancer (Caprodossi et al. 2011). The role of TRPV1 as a prognostic factor in haematological malignancies has yet to be investigated. In this chapter, TRPV1 was found to be overexpressed in malignant blood cell lines compared to normal cells. This may suggest a positive correlation between TRPV1 and blood cancer progression. Whether the same effect is valid for patients, has yet to be investigated (see Chapter 6).

The other major finding of this chapter was that TRPV1 expression was observed in untransfected and human TRPV1-transfected HEK293 cells (tetracycline off) and RAW264.7 cells. This is in direct contrast to other studies that reported TRPV1 to be absent from these cells (Chen et al. 2003; Correll et al. 2004; Inoue et al. 2006; Ma et al. 2011; Sarker and Maruyama 2003; Tsuji et al. 2010). Furthermore, expression of TRPV1 was found to be variable in these cells, using flow cytometry and Western blotting.

HEK293 cells are a broadly used epithelial cell line derived from human embryonic kidney cells and is used for many years in different experimental models, due to its

easy growing and transfecting properties (Thomas and Smart 2005). HEK293 cells are usually transfected with TRPV1 to study its functionality in human cells (Mandadi et al. 2006; Mergler et al. 2012; Riera et al. 2007; Tian et al. 2006; Wen et al. 2012; Xu et al. 2007b). TRPV1-transfected HEK293 cells, either tetracycline on or off, were found to be strongly positive to TRPV1. Some extra bands of lower molecular weight were also detected by Western blotting. These may be due to protein degradation or potentially yet to be described TRPV1 isoforms (Fausone-Pellegrini et al. 2005). To further investigate TRPV1 expression in HEK293 cells, untransfected HEK293 cells, which have been previously reported to be negative for TRPV1 (Correll et al. 2004), also demonstrated strong TRPV1 expression. The reasons for discrepancies with earlier studies is unknown, but may be due to the variation in the specificity of the anti-TRPV1 antibodies used to detect TRPV1, as different primary antibodies were used in previous studies (e.g., Alomone Labs, Jerusalem, Israel; Abcam, Cambridge, UK) compared to the antibody used in the present study (Lifespan Biosciences, WA, USA). Moreover, it may be that TRPV1 expression detected by Western blotting is significantly and gradually increased with accelerated growth rate and high confluence of the cell culture (Marincsák et al. 2009). Therefore, the time when the cells were harvested and used might impact the TRPV1 detection levels. In this chapter, HEK293 and RAW264.7 cells were harvested as recommended when they reached more than 80% confluence. Untransfected HEK293 cells were used within 9 passages, TRPV1-transfected HEK293 within 17 passages and RAW264.7 cells within 6 passages. Further tests, including Na^+ and/or Ca^{2+} response to CAP in these cells warranted to prove functional TRPV1 expression in HEK293 cells.

Previous Western blotting studies (20 µg of protein lysate) reported that RAW264.7 cells do not express TRPV1 (Chen et al. 2003; Ma et al. 2011). Interestingly, in the current study, TRPV1 was not detected using 20 µg of protein lysate, however was weakly expressed using 40 µg. These data confirm the ubiquitous distribution of TRPV1, as there appear to be no cells that do not express TRPV1 (Cortright and Szallasi 2004; Fernandes et al. 2012). Hence, negative controls are still a major problem. Screening a wide range of potentially TRPV1-negative cells to be used as negative controls is costly and time-consuming. Thus, TRPV1 knock down cells should be considered for human studies.

In conclusion, increased expression of TRPV1 was detected in THP-1, U266B1 and U937 malignant haematological cells, compared to normal PBMCs. Therefore, a therapeutic approach should be considered to investigate the implications of such increased expression on these cells and consequently in patients. Furthermore, some cell lines which were reported as TRPV1-negative were found to express TRPV1. As a consequence, HEK293 and RAW264.7 cells should not be used as negative controls for anti-TRPV1 antibody experiments without further examination of TRPV1 expression and function in these cells using other techniques such as PCR and Ca^{2+} imaging.

Chapter 6: TRPV1 Expression in Patients with Haematological Malignancies

6.1 Abstract

Increased expression of TRPV1 is associated with several cancers, including those of the pancreas, tongue and glioma. The aim of this study was to investigate TRPV1 expression and its association with inflammation in patients with haematological malignancies. TRPV1 expression was measured using flow cytometry (Attune® Acoustic Focusing Cytometer, Life Technologies, Grand Island, USA) and Western blotting in 49 patients with haematological malignancies. The patient population (age 31-85 yrs; M:F, 28:21) was divided into three groups: B-cell Non-Hodgkin's lymphoma (B-NHL, n=28), multiple myeloma (MM, n=12) and others (n=9), comprising myeloproliferative disorder (MPD, n=4), peripheral T-cell lymphoma not otherwise specified (PTCL-NOS, n=1), and other leukaemias (n=4). Blood and protein samples from gender-matched healthy controls (n=21) were analysed in parallel with patient samples. C-reactive protein (CRP) was used as a marker of inflammation. TRPV1 was detected in all patients and healthy controls using flow cytometry, but not Western blotting. Using flow cytometry, a sub-group of patients (4/49=8.2%, MM=2, B-NHL=2) demonstrated increased expression of TRPV1 relative to the remainder of the cohort. TRPV1 was found to be similar to the control group for 91.8% of all patients. There were no significant differences in TRPV1 expression between MM and B-NHL patients, or between *de novo* (untreated) patients and those undergoing treatment. Using Western blotting, TRPV1 (~95kDa band) was only detected in one MM and four B-NHL patients. A 240kDa band was also detected in both a B-NHL and a MM patient. C-reactive protein was elevated (≥ 5 mg/L) in 25% of patients, but was not associated with higher TRPV1 expression. In conclusion, inflammation may increase TRPV1 expression in some diseases, but does not appear to do so in the blood cancers.

6.2 Introduction

TRPV1 expression and activity have been extensively studied in various malignancies over the last decade. Interestingly overexpression of TRPV1 has been reported in some cancers, including glioma (Contassot et al. 2004a) and human tongue epithelium cancer (Marincsák et al. 2009). Furthermore, increased expression of TRPV1 has been demonstrated in THP-1, U266B1 and U937 malignant haematological cell lines, compared to normal PBMCs (see Chapter 5). In this chapter, samples were collected from patients with haematological malignancies (*de novo* and under treatment), and investigated for TRPV1 expression using optimised Western blotting and flow cytometry protocols.

Changes to the expression of TRPV1 can provide a better understanding on the therapeutic and the physiological understanding of the TRPV1 and its relationship to cancer mechanisms (Gunthorpe and Chizh 2009; Khairatkar-Joshi and Szallasi 2009; Pal et al. 2009; Tsuji et al. 2010). It may become a target for the development of new drugs to treat patients with blood cancers. Thus, the aim of this chapter was to compare the TRPV1 expression between patients with different haematological malignancies and normal subjects.

6.3 Materials and Methods

Blood collection (see *sections 3.3.5.1 and 4.3.2.1*), sample processing procedure (*sections 3.4.2 and 4.3.2.8*), ethical approval (*section 3.3.4*), materials and methods (*sections 3.3 and 4.3*) were all previously outlined in chapters 3 and 4.

Blood samples were collected at the Launceston General Hospital. Patients with haematological malignancies were consented to participate in this study, with each patient and control subject was given a unique identification number (IDN). For this part of the study, sample processing commenced in the Haematology Laboratory at UTAS within 2 hr following collection. Protein samples were isolated (*Sections 3.3.5.3 and 3.3.6*) and stored at -20°C to perform Western blotting (*Section 3.3.9*). Samples used for flow cytometry were processed, run and data was collected in the same day of blood collection (within 6-8 hr of collection). Controls were normal subjects with no history of malignancies, age- and gender-matched with the patient for each experiment. Control samples were run in parallel with patients' samples.

For gating purposes using flow cytometry, approximately 3000 events were acquired for the control and the patient samples in the desired gate, then MFI value was obtained by the Attune[®] Acoustic Focusing software (Carlsbad, USA). Arbitrary MFI ratio was calculated by dividing the MFI (patient) by MFI (control).

C-reactive protein (CRP) was used as an indicator of inflammation and was performed at the Launceston General Hospital Pathology Department using a latex particle enhanced turbidimetric immunoassay method (CRP Vario, Abbott Laboratories, Irving, USA). The CRP normal range using this method is <5 mg/L.

6.4 Results

6.4.1 General Characteristics of Patients and Controls

Forty-nine samples from patients with haematological malignancies and 21 samples from age- and gender-matched control subjects were obtained (Table 6-1). Two

control samples were excluded, one because of a clotted sample and the other due to the subsequent diagnosis of haemochromatosis. Patients diagnosed with myeloproliferative disorders (MPD) included (n=1 in each case) myelocytic leukaemia (CML), chronic myelomonocytic leukaemia (CMML) and essential thrombocythaemia (ET). CRP values were obtained for 44/49 patients, (range: 1-106 mg/L). CRP was elevated (≥ 5 mg/L) in 25% of the patients.

Table 6-1: General characteristics of patients with haematological malignancies

Characteristics		
Age	Years (range)	31- 85
Patient Gender	Male	28
	Female	21
Subjects	<i>De novo</i>	12
	Under treatment	37
	Control	21
Diagnosis	B-NHL	28
	MM	12
	Acute Monocytic Leukaemia	2
	MPD	4
	HCL	1
	ALL	1
	PTCL-NOS	1

MM: multiple myeloma; B-NHL: B- Cell Non-Hodgkin's lymphoma; MPD: Myeloproliferative Disorder; HCL: Hairy-Cell Leukaemia; ALL: Acute Lymphoblastic Leukaemia; PTCL-NOS: Peripheral T-cell lymphoma/ not otherwise specified.

6.4.2 Detection of TRPV1 using Flow cytometry

Based on the distribution of the MFI ratio for all patients (Figure 6-1), TRPV1 expression was arbitrarily considered to be increased when the MFI ratio was >2 .

Increased TRPV1 expression was detected in 4/49 patients. Of these, 2 had MM and 2 B-NHL. Of the 24 under treatment and four *de novo* B-NHL patients, TRPV1 was found to be similar (n=25), and increased (n=2, under treatment patients) compared to healthy controls (Table 6-2).

Furthermore, with the 9 under treatment and 3 *de novo* MM patients, TRPV1 expression was found to be similar (n=10), and increased (n=2, a *de novo* and an under treatment patient) compared to controls (Table 6-3). TRPV1 in patients with other haematological malignancies (n=9) were all similar to normal controls (Table 6-4). TRPV1 was found to be similar to the control group in 91.8% of patients. The four patients with increased TRPV1-MFI ratios have the highest MFI values (mean \pm SD; 308783 \pm 145474), relative to other patients (mean \pm SD; 154178 \pm 100242) and accounted for 8.2% of the patients.

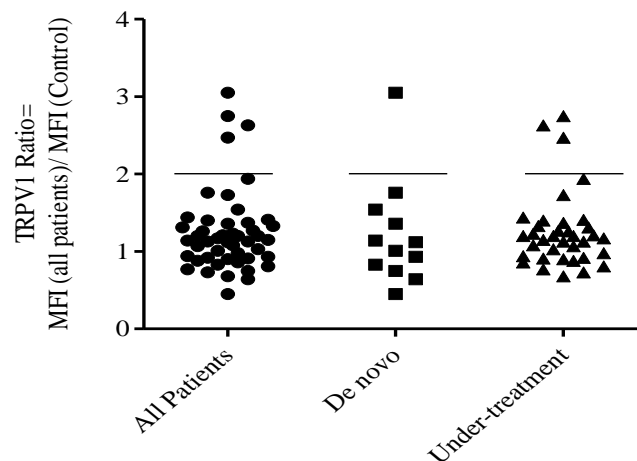


Figure 6-1: TRPV1 expression in patients with haematological malignancies. TRPV1 is expressed relative to a control for each patient. TRPV1 expression was considered increased when MFI ratio >2.

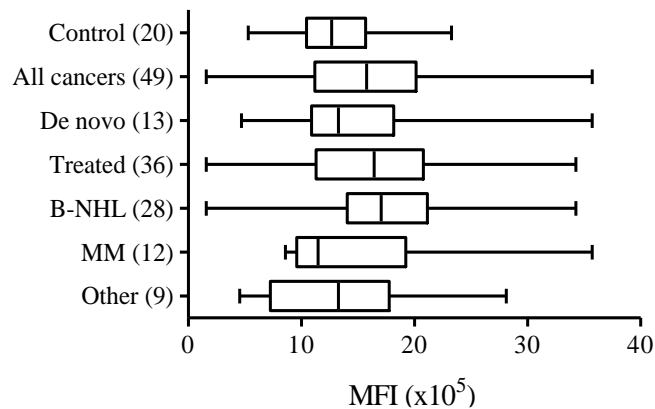


Figure 6-2: TRPV1 expression for all patients with haematological malignancies vs. controls. Boxes represent mean-MFI with the lines representing the minimum and maximum values. The y-axis represents the collected patients cases (number). There were no significant differences in TRPV1 expression between MM and B-NHL patients, or between *de novo* patients and those undergoing treatment.

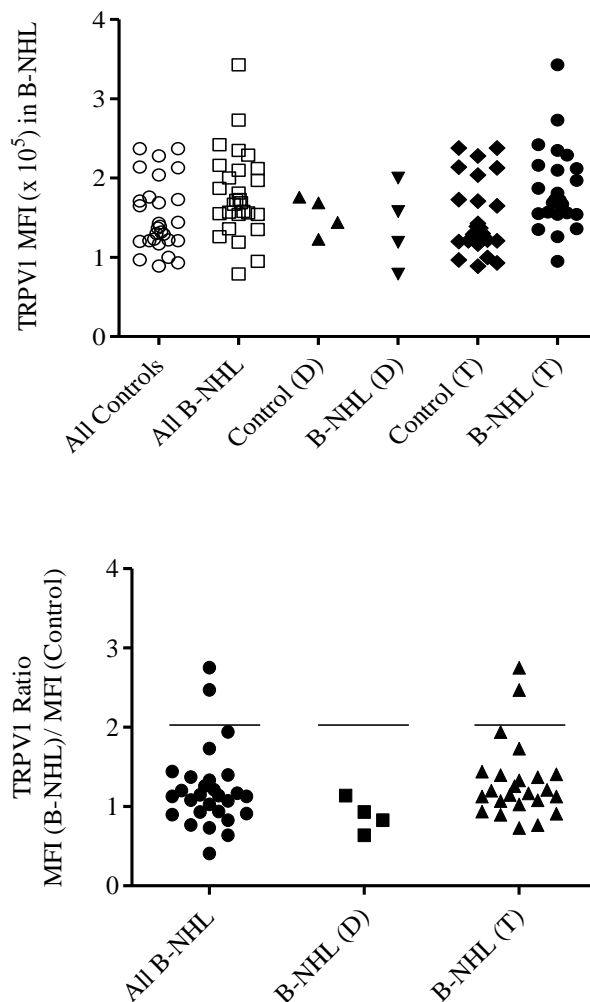


Figure 6-3: TRPV1 expression in patients with B-NHL. TRPV1 is expressed relative to a control for each patient. TRPV1 expression was considered to be increased when MFI ratio >2. D: *de novo*, T: under treatment patients

Table 6-2: Characteristics of B-NHL patients compared to control subjects used for analysis of TRPV1 expression by flow cytometry

Status	Patients					Control Subjects		TRPV1 Expression
	IDN	Age (Years)	Gender	MFI (10 ⁵)	CRP (mg/L)	IDN	MFI (10 ⁵)	MFI Ratio
<i>De novo</i>	P20	74	M	2.00	58	C10	1.76	1.14
	P31	55	M	0.79	2	C14	1.23	0.64
	P33	62	F	1.58	2	C12	1.69	0.93
	P49	83	F	1.19	3	C21	1.44	0.83
Under-treatment	P03	80	F	0.95	8	C02	0.89	1.07
	P10	71	F	1.54	4	C07	1.32	1.17
	P11	70	F	1.69	1	C07	1.20	1.41
	P12	31	M	1.87	NA	C09	1.30	1.44
	P13	29	M	1.56	2	C09	1.29	1.21
	P14	41	M	1.36	4	C09	1.21	1.13
	P15	45	F	1.55	1	C09	1.17	1.33
	P19	68	M	1.67	60	C09	1.22	1.37
	P22	65	M	1.97	7	C10	1.71	1.15
	P23	85	F	1.72	1	C11	1.43	1.20
	P25	77	F	3.43	19	C11	1.39	2.47*
	P27	70	M	1.35	3	C13	0.97	1.40
	P29	77	M	1.81	15	C13	0.93	1.94
	P30	56	M	2.10	4	C14	1.21	1.73
	P32	65	F	1.26	3	C12	1.73	0.73
	P34	80	F	1.54	4	C12	1.65	0.94
	P36	79	M	2.16	1	C16	2.38	0.91
	P37	79	F	2.73	3	C15	1.00	2.73*
	P38	72	M	2.12	1	C17	2.38	0.89
	P40	46	M	2.35	4	C17	2.28	1.03
	P41	76	M	2.42	3	C16	2.14	1.13
	P42	78	M	2.29	1	C16	2.13	1.08
	P44	65	F	1.57	1	C19	2.04	0.77
	P45	73	M	1.73	5	C18	1.37	1.26

IDN: patient/control identification number (P: patient, C: control); MFI: Median Fluorescence Intensity; CRP: C-reactive protein; NA: not available data; *: increase in TRPV1; M: male, F: female.

Table 6-3: Characteristics of MM patients compared to control group subjects used for analysis of TRPV1 expression by flow cytometry

Status	Patients					Control Subjects		TRPV1 Expression
	IDN	Age (Years)	Gender	MFI (10 ⁵)	CRP (mg/L)	IDN	MFI (10 ⁵)	MFI Ratio
<i>De novo</i>	P09	82	M	1.13	4	C05	1.12	1.01
	P21	73	M	1.16	3	C10	1.55	0.75
	P35	72	F	3.57	5	C12	1.17	3.05*
Under-treatment	P02	57	M	0.92	1	C01	1.04	0.88
	P04	67	F	0.86	1	C03	0.68	1.27
	P05	57	F	1.20	1	C06	1.31	0.92
	P06	69	F	1.08	3	C06	1.33	0.81
	P07	68	F	0.89	2	C06	1.31	0.68
	P08	71	M	1.11	3	C04	1.28	0.86
	P26	66	M	2.03	NA	C10	1.68	1.20
	P28	58	M	1.62	1	C14	1.24	1.31
	P39	73	F	2.62	7	C15	1.00	2.62*

IDN: patient/control identification number (P: patient, C: control); MFI: Median Fluorescence Intensity; CRP: C-reactive protein; NA: non available data; *: increase in TRPV1; M: male, F: female.

Table 6-4: Other haematological malignant cancers patients compared to control subjects used for analysis of TRPV1 expression by flow cytometry

Status	Patients						Control Subjects		TRPV1 Expression
	IDN	Disease	Age (Years)	Gender	MFI (10 ⁵)	CRP (mg/L)	IDN	MFI (10 ⁵)	MFI Ratio
<i>De novo</i>	P16	MPD (CMML)	71	M	1.61	NA	C09	1.45	1.12
	P17	HCL	43	M	0.47	NA	C09	1.05	0.45
	P18	PTCL -NOS	63	M	1.63	106	C09	1.20	1.36
	P47	ALL	68	M	2.81	63	C20	1.60	1.76
	P48	AML (M4)	77	F	1.05	NA	C21	0.68	1.54
Under-treatment	P24	MPD	40	F	1.92	1	C11	1.57	1.22
	P43	MPD (ET)	64	F	1.98	1	C19	2.02	0.98
	P01	MPD (CML)	66	M	0.46	4	C03	0.38	1.21
	P46	AML (M4)	43	M	1.33	3	C18	1.25	1.06

IDN: patient/control identification number (P: patient, C: control); MFI: Median Fluorescence Intensity; CRP: C-reactive protein; NA: non available data; M: male, F: female; HCL: Hairy-Cell Leukaemia; PTCL-NOS: Peripheral T-cell lymphoma/ not otherwise specified; ALL: Acute Lymphocytic Leukaemia; AML (M4): Acute Myelomonocytic Leukaemia; MPD: Myeloproliferative Disorder; CML: chronic Myelocytic Leukaemia; CMML: Chronic Myelomonocytic Leukaemia; ET: Essential thrombocythaemia.

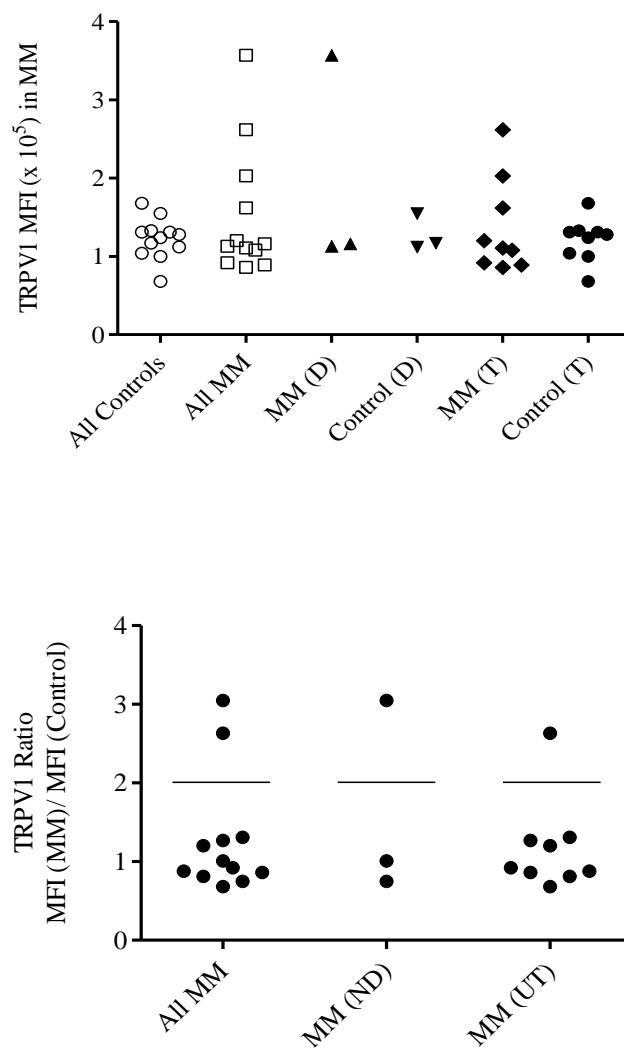


Figure 6-4: TRPV1 expression in patients with MM. TRPV1 is expressed relative to a control for each patient. TRPV1 expression was considered to be increased when MFI ratio >2. D: *de novo*, T: under-treatment.

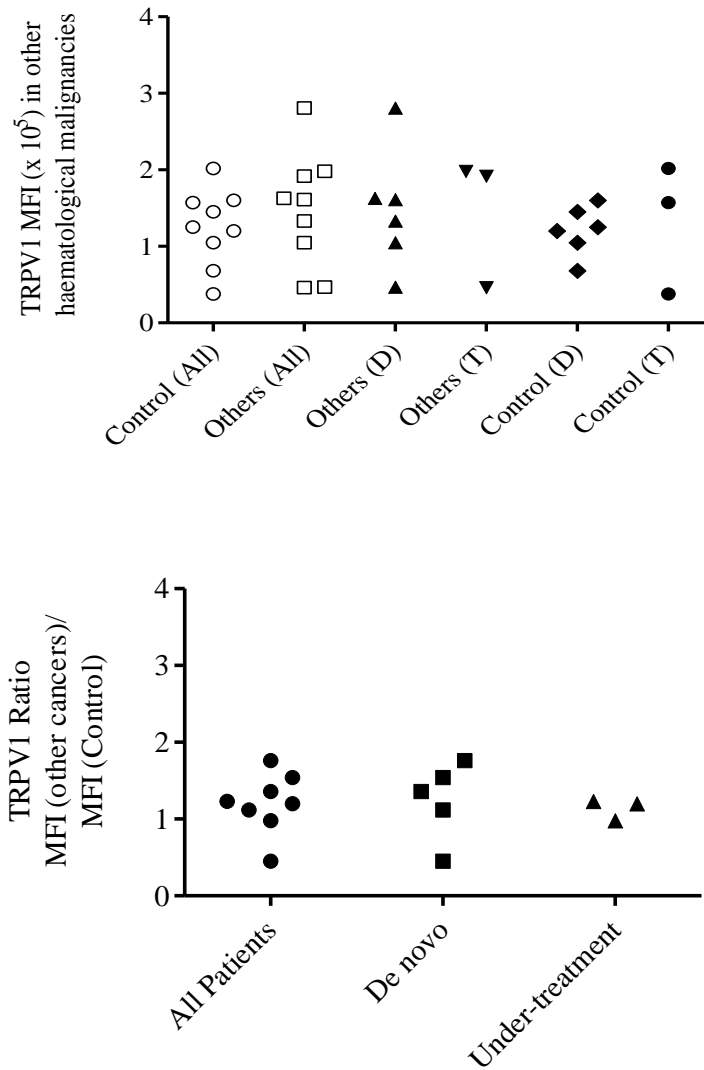


Figure 6-5: TRPV1 expression in patients with other blood cancers. TRPV1 is expressed relative to a control for each patient. TRPV1 expression was considered to be increased when MFI ratio >2. D: *de novo*, T: under-treatment.

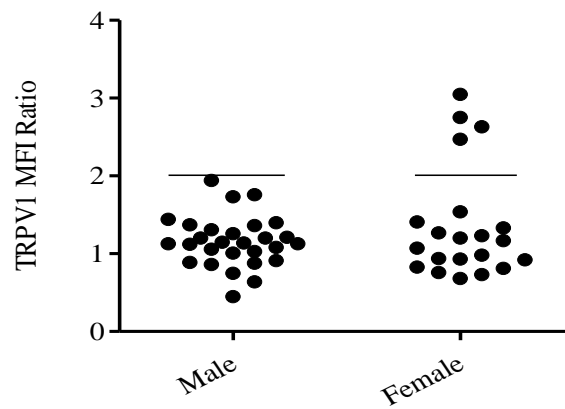


Figure 6-6: TRPV1 MFI ratio between males and females. TRPV1 expression was considered increased when MFI ratio >2 or similar ≤2.

6.4.3 TRPV1 Detection Using Western blotting

Western blotting data was not obtainable from three samples due to insufficient isolation of protein (patients 10, 16 and 19). Forty micrograms of protein lysate was loaded for all control and patient samples. TRPV1 monomer (~95kDa) was detected in five patients (MM=1, B-NHL=4), whereas another TRPV1 specific band was detected at ~240kDa in 1 MM and 1 B-NHL patient (Figure 6-7, Table 6-5). TRPV1 was not detected in the remaining patient or control samples.

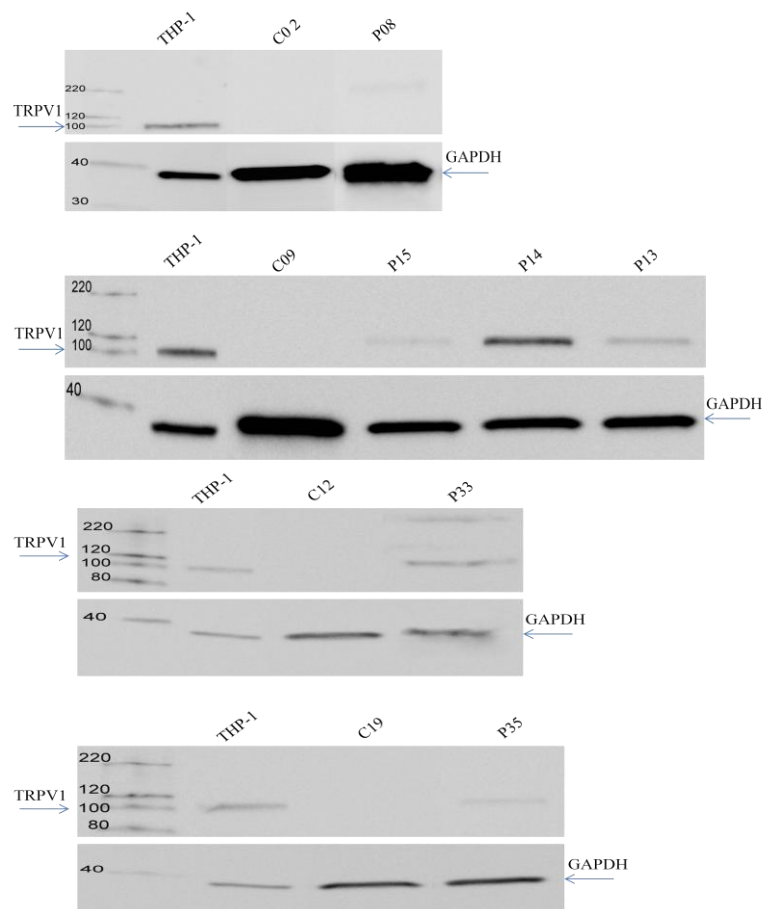


Figure 6-7: TRPV1 expression in PBMCs protein samples of patients with haematological malignancies using Western Blotting. Samples were tested against normal control. Forty micrograms of protein were loaded into each well. GAPDH was used as an internal control. THP-1 protein was used as a positive control. TRPV1 monomer (~ 95kDa) was detected in five patients, whereas another band at ~240 kDa was detected in two patients.

Table 6-5: Patients with detected TRPV1 bands on Western Blotting

TRPV1 band detected at ~ 240kDa						
IDN	Disease	Status	Age	Gender	CRP (mg/L)	Treatment
P08	MM	Under treatment	71	M	3	Clexane, Zometa, IVIG
P33	B-NHL (CLL/SLL)	<i>De novo</i>	62	F	2	-
TRPV1 monomer at ~ 95kDa						
IDN	Disease	Status	Age	Gender	CRP (mg/L)	Treatment
P13	B-NHL (Diffuse Large B-Cell)	Under treatment	29	M	2	Rituximab
P14	B-NHL (Intestinal)	Under treatment	41	M	4	Vinblastine
P15	B-NHL (CLL/SLL)	Under treatment	45	F	1	Rituximab
P33	B-NHL (CLL/SLL)	<i>De novo</i>	62	F	2	-
P35	MM	<i>De novo</i>	72	F	5	-

IDN: patient/control identification number; CLL/SLL: Chronic lymphocytic leukaemia/small lymphocytic lymphoma, MM: Multiple myeloma.

6.5 Discussion

In this chapter, and for the first time, TRPV1 was detected in white blood cells obtained from all controls, and from patients with haematological malignancies using flow cytometry. However, only a small percentage of patients and no controls demonstrated TRPV1 expression using Western blotting. TRPV1 expression was found to be increased compared to controls in four out of the 49 patients as detected by flow cytometry. Using Western blotting, TRPV1 was detected in six out of the 49 patients, but not in any of the controls. To the knowledge of the author, this is the first study to demonstrate TRPV1 expression in leukocytes of patients with haematological malignancies using both detection methods.

TRPV1 expression has been reported to be variable during carcinogenesis (Gkika and Prevarskaya 2009). TRPV1 is overexpressed in glioma (Contassot et al. 2004a), tongue epithelium cancer (Marincsák et al. 2009) and the haematological malignancy cell lines (see Chapter 5), and ‘under’ expressed in urothelial cancer (Kalogris et al. 2010; Lazzeri et al. 2005). In this chapter, TRPV1 expression by PBMCs of the majority (~92%) of patients with haematological malignancies was similar to controls. There were no significant differences in TRPV1 expression between patients from the same specific diagnosis group or compared to other blood cancers, i.e. MM and B-NHL, *de novo* or under treatment, male or female. The variation in TRPV1 expression in malignancies is either due to transcriptional or translational factors, such as hormones, growth factors and alternative splicing isoforms (Reynolds and Kyprianou 2006), or due to the presence of chronic inflammation,

dietary activation of TRPV1 by CAP, which may have a role in the up- or down-regulation of TRPV1 expression (Akbar et al. 2008; Geppetti and Trevisani 2004).

In Western blotting, a TRPV1 monomer (~95kDa) was detected in four B-NHL and one MM patients. Additionally, a TRPV1 band at ~240kDa was demonstrated in two patients, a MM and a B-NHL patient. These TRPV1 bands have also been produced in a *de novo* (~95 and 240kDa) and an under treatment (~95kDa) CLL/SLL patients blots. The reason why TRPV1 was only detected in these cases using Western blotting is unclear. However, these unexpected results may be due to the cell gating process. MFI values were obtained from gating specific groups of cells depending on the diagnosis of each patient i.e., lymphocytes for B-NHL. In contrast, protein obtained from a mixture of PBMCs was used with Western blotting, rather than a disease-specific cell type. Whilst flow cytometry is a more sensitive method to detect protein than Western blot, it is considered that the low levels of TRPV1 expression in the rest of the patients group and control subjects were beyond detection using Western blotting. In addition, there is a possibility that the ~240kDa TRPV1 band detected in two patients is an artefact or a TRPV1 isoform consisting of extra polypeptides. Further investigation through cross-linking techniques or additional sample processing procedures is warranted.

There was no significant difference in TRPV1 expression between females and males. Other studies are in agreement with this finding, e.g., in end-stage kidney disease patients (Saunders et al. 2009), and in healthy subjects (Spinsanti et al. 2008). The four patients with increased TRPV1-MFI ratios and the three of six patients with TRPV1 bands were females older than 70 years. The treatment, the CRP values and

the specific diagnosis of the patient, were all considered in speculating the results. Two possible causes were believed to lead to these findings, 1) genetic variability in some ethnicity i.e., TRPV1 polymorphisms. All patients and control subjects recruited in this study were Caucasian, and it has been reported that TRPV1 Val⁵⁸⁵ Val allele was detected in European-American females which affects their pain perception (Kim et al. 2004); 2) methodological, due to the shortage of patients recruited in this study. Higher numbers of each patients group should be investigated and further assessment should be undertaken.

In general, surgery, chemotherapy, and radiation are currently the available options for cancer treatment. Local or systemic inflammation is provoked by all three, caused mainly by tissue injury and cancer cell apoptosis. Surgery causes the activation of infection- or stress-sensing pathways, whereas chemotherapy and radiotherapy lead to cancer cell necrosis, a pro-inflammatory form of cell death (Vakkila and Lotze 2004). CRP is used as an indicator of inflammation (Sidoroff et al. 2010). TRPV1 plays a role in pain sensation and is associated with inflammation-induced thermal hyperalgesia (Yu et al. 2008). Therefore, TRPV1 is increased in some inflammatory-associated processes and diseases, such as irritable bowel syndrome, airway diseases and end-stage kidney diseases (Akbar et al. 2008; Jia et al. 2005; Sadofsky et al. 2012; Saunders et al. 2009). In this chapter, high inflammatory status was not associated with high TRPV1 levels in haematological malignancy patients. Hence, inflammation may alter TRPV1 expression (increase) in some inflammatory-associated diseases, but not in the blood cancers.

The MFI values of TRPV1 obtained from the leukocytes of each patient and control using flow cytometry varied considerably. This variability might be due simply to individual patient characteristics, differences in the proportions of T- and B-cells and monocytes (which express different levels of TRPV1) between individuals. The variation in TRPV1 expression is not uncommon and has been previously reported in healthy PBMCs donors (Saunders et al. 2007).

In addition, as some blood cancers elicit an abnormal growth of one or more of the white blood cell components, treatments of these disorders are designed to target mainly the immune system (Cohen et al. 2006; Kwong 2010; Singhal and Mehta 2002). Most drugs used to treat blood cancers patients are immunomodulatory and anti-inflammatory, e.g., thalidomide (Singhal and Mehta 2002), rituximab (Cohen et al. 2006; Salvi et al. 2007), aspirin (Ikonomidis et al. 1999; Kopp and Ghosh 1994; Yamamoto and Gaynor 2001) and dexamethazone (De Bosscher et al. 1997). The anti-inflammatory effect is through the inhibition of cytokines, the crucial proteins necessary to the inflammation process. Either thalidomide or lenalidomide was commenced on MM patients included in this study. Both medications are classified as immune-modulating drugs class (IMiDs) with anti-angiogenic activity that inhibits the production of interleukin (IL)-6 and accordingly, activates T- cells to produce IL-2 which alters the number and function of natural killer cells (Khalafallah et al. 2010). Furthermore, most of the B-NHL patients included in this study did receive rituximab as part of their chemotherapy regimen. Rituximab is a humanised chimeric monoclonal anti-CD20 which in turn, expressed primarily on the surface of B-cells. This antibody is used in combination with chemotherapy for the treatment of B-NHL patients, because of its ability to inhibit the B-cell production (Salvi et al. 2007).

Table 6-6 summarises some of the drugs which were administered to the patients who participated in this study upon the time of sample collection. Thus, the alteration in the proportions of the blood cell components may have an effect on the TRPV1 levels expressed in each patient compared to the control and subsequently lead to the inhibition of TRPV1 as previously discussed in Chapter 5 (Chaulet et al. 2011; Choe et al. 2010; Deng et al. 2003; Rafiee et al. 2010).

Although the increased expression of TRPV1 has been detected in haematological malignant cell lines (Chapter 5), no alteration in the channel expression has been demonstrated in patients with blood cancers compared to the normal control, in the present study. These differences between TRPV1 expression in malignant cell lines and cells from patients with malignancy might be due to years of passaging the cell lines, which in turn may lead to changes in the expression characteristics of some receptors (Marincsák et al. 2009). In addition, CAP-induced apoptosis in haematological malignant cell lines was not TRPV1-mediated (see Chapter 2). Therefore, whether the TRPV1 channel is functional in these cells warrants further investigation. Finally, these findings indicate that TRPV1 expression might be increased in association with inflammation in some diseases, but this is not the case in blood cancers. Whilst flow cytometry is a sensitive way to detect protein, Western blotting is relatively less sensitive. Therefore, false negative findings might actually be low levels of TRPV1 expression that were beyond the sensitivity of detection. In addition, the 240kDa band detected in some patients might be an artefact or represents a true TRPV1 isoform, which warrants further investigation. Furthermore, the PMBCs used in this study were obtained from patients/control blood, therefore testing disease-appropriate cells, such as lymph node PBMCs from B-NHL, may lead

Table 6-6: Features of some drugs used to treat haematological malignancies

Drug	Drug-induced cytokines inhibition	Application	References
Thalidomide/ Lenalidomide	TNF and IL-6	MM	(Khalafallah et al. 2010; Singhal and Mehta 2002; Singhal et al. 1999)
Rituximab	Anti-CD20 and TNF- α	B-NHL	(Tamimoto et al. 2008).
Aspirin	IL-1 β , Plasma macrophage colony stimulating factor, CRP and NF- κ B	General anti-inflammatory	(Ikonomidis et al. 1999; Kopp and Ghosh 1994; Yamamoto and Gaynor 2001).
Dexamethasone	IL-6 and NF- κ B	Blood cancers	(De Bosscher et al. 1997).

TNF: Tumor necrosis factor; MM: Multiple myeloma; CD20: Cluster of differentiation; IL-1: interleukin-1; NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells; B-NHL: B-cell Non-Hodgkin's lymphoma; CRP: C-reactive protein.

to different TRPV1 results and conclusions.

In conclusion, leukocytes from patients with haematological malignancies were found to express TRPV1 at similar levels to normal subjects, except for four patients with increased expression. TRPV1 level was not affected by the specific diagnoses, gender or treatment status of the patients. The high inflammatory status did not seem to have a direct effect in increasing the TRPV1 expression. This might be due to the anti-inflammatory therapies that are used to treat patients with blood cancers. Therefore, the specific function of the TRPV1 channel in human leukocytes may be restricted to cation transportation and not associated with tumorigenesis (Samways and Egan 2011). Finally, these findings indicate that in contrast to other conditions, inflammation does not seem to increase TRPV1 expression in patients with blood cancers.

Chapter 7: Conclusions and Future Studies

The primary reasons for undertaking the experiments described in this thesis were to determine whether TRPV1 is expressed in human malignant haematological cells and that TRPV1 plays a significant role in the function/dysfunction of these cells. Based on differences in TRPV1 expression in some neoplastic compared to normal tissues, it was also hypothesised that the expression of TRPV1 in leukocytes would be increased in patients with haematological malignancies compared to healthy controls. Targeting of vanilloid receptors might therefore represent a novel therapeutic approach to treating haematological malignancies.

Three broad series of experiments were conducted; 1) pharmacological (Chapter 2), where malignant cells were treated with the TRPV1 activator, CAP, and TRPV1, CB1 and CB2 antagonists; 2) methodological optimisation (Chapters 3 and 4) of flow cytometry and Western blotting protocols to detect TRPV1, and 3) clinical haematology (Chapters 5 and 6), measurement of TRPV1 in haematological malignant cells (cell lines and cells from patients) using optimised methods for flow cytometry and Western blotting. In this chapter, the major outcomes from these studies are highlighted and discussed.

The effect of CAP on the metabolic activity of the THP-1, U266B1 and U937 malignant haematological cells was investigated, as CAP has previously been shown to induce apoptosis through TRPV1 in other cancer cells. Using the resazurin reduction (AlamarBlue[®]) method to measure cell metabolic activity, it was found that the cell metabolic activity of THP-1, and for the first time U266B1 and U937 cells, was inhibited in a concentration-dependent manner by CAP. These experiments were then extended to investigate the mechanism of CAP-induced inhibition in metabolic

activity in these cells. It was demonstrated for the first time that TRPV1, CB1 and CB2 receptors are not involved in CAP-induced changes in the metabolic activity of U266B1 (myeloma) cells. Furthermore, TRPV1 and CB1 receptors appear to mediate the concentration-dependent increase in metabolic activity (resazurin reduction) in THP-1 cells produced by CAP, whereas TRPV1 (SB452533) and CB1 (AM251) receptor antagonists appear to act synergistically with CAP to inhibit U937 cells metabolic activity.

These cell line studies have raised the interesting question as to whether blood cancer cells from patients might be susceptible to CAP-induced cell death, and indeed, whether this would be TRPV1-mediated. Moreover, the role of membrane lipid rafts and potentially other pathways and receptors, which might facilitate apoptosis in cells from patients with blood cancer warrants further investigation. Finally, the effect of CAP on BCL-2 gene and FAS mRNA levels, which modulate the apoptotic process, should be investigated in malignant haematological cell lines.

Significant effort was devoted to developing reliable Western blotting and flow cytometry methods to detect TRPV1. Exhaustive testing of blocking protocols, primary and secondary antibodies, and permeabilisation techniques, resulted in reproducibly clear Western blots and excellent separation of TRPV1 signal from isotype and secondary antibody controls using flow cytometry. The use of the negative control to avoid false positive results emerged as the key issue for reliable Western blotting and flow cytometric detection of TRPV1. The negative control is a pivotal tool to assess the specificity of primary and secondary antibodies, and the non-specific binding caused by Fc-receptors. Cell lines that have been previously

reported to be devoid of TRPV1 and then used as negative controls were in fact found to express TRPV1. Thus, untransfected and TRPV1-transfected (tetracycline off, which switches TRPV1 transcription off) HEK293 and RAW264.7 cells, at least in our hands, could not be used as controls. Many studies have used methods to detect and report TRPV1 expression. However, the lack of appropriate controls, particularly the negative control, raises doubts of the validity of data reported in these studies, and the associated conclusions. The protocols developed in this thesis are important because the appropriate negative controls were used to optimise the TRPV1 detection methods. Isotype and secondary antibody controls were used as negative controls in parallel with a positive control (THP-1 cells). In future studies, one might consider using TRPV1 knockdown/ knockout cells as a negative control. Finally, testing a number of antibodies, as performed in this study, is recommended due to variability in binding capacity and specificity between different primary, and to a lesser extent, secondary antibodies. An appropriate affinity-purified anti-TRPV1 antibody was found to be crucial for TRPV1 detection by Western blot and flow cytometry. It is hoped that the current study will generate constructive discussions regarding the use of appropriate controls when using flow cytometry and Western blotting methods. Critically, consensus needs to be reached with regard to what constitutes appropriate controls, which potentially impact the outcomes of research.

The optimised Western blot and flow cytometry protocols were then applied to detect and quantitate TRPV1 in malignant haematological cell lines, as well as in leukocytes from normal individuals and patients with blood cancers. In this study, TRPV1 was detected for the first time in THP-1, U266B1 and U937 malignant haematological cell lines, using flow cytometry in addition to Western blotting.

Significantly, TRPV1 expression was found to be increased in all three cell lines, compared to leukocytes from healthy individuals (Chapter 5). U266B1 cells expressed both the TRPV1 monomer (~95kDa) and an additional dimer (~200kDa). The detection of a dimer might be due to the presence of an unknown TRPV1 isoform. U937 cells demonstrated increased TRPV1 expression by flow cytometry compared to normal cells, but not by Western blotting, with significantly weaker TRPV1 expression than THP-1 and U266B1 cells. These results strongly suggest that TRPV1 is not uniformly expressed in different haematological malignant cell lines.

A possible explanation for the apparent increased expression of TRPV1 by malignant haematological cell lines may be due to years of passaging the cells, which could lead to changes in the expression of some receptors (Marincsák et al. 2009). Moreover, whether the TRPV1 channel is functional when expression is increased in these cells requires further investigation, as TRPV1 antagonists in the present study had minimal effect on CAP-induced changes in cell activity (Chapter 2).

To the knowledge of the author, this is the first time TRPV1 has been investigated in the leukocytes of patients diagnosed with a range of haematological malignancies, including multiple myeloma (MM) and B-cell non-Hodgkin's lymphoma (B-NHL), using flow cytometry and Western blotting (Chapter 6). It emerged that TRPV1 expression was detected in all patients and controls using flow cytometry, but not Western blotting. Increased expression of TRPV1 was detected using flow cytometry in a sub-group of patients (4/49=8.2%, MM=2, B-NHL=2) compared to the remainder of the group. Interestingly, most patients with haematological malignancies recruited in this study (91.8%) were found to express TRPV1 at similar

levels to the normal controls. There were no significant differences in TRPV1 expression (assessed using flow cytometry) irrespective of gender, the haematological malignancy diagnosed, or whether patients were *de novo* or under treatment.

In contrast, using Western blotting, a TRPV1 monomer (~95kDa) was detected in four B-NHL patients and one with MM. A TRPV1 band at ~240kDa was observed in both a MM and a B-NHL patient, and in two patients with chronic lymphocytic leukaemia/small lymphocytic lymphoma, including patients that were *de novo* (~95 and 240 kDa) and under treatment (~95kDa). The reason why Western blotting detected TRPV1 only in these specific cases is unclear. Whilst flow cytometry is a sensitive method to detect protein expression, Western blotting is relatively less sensitive. Therefore, the low levels of TRPV1 detected in the remainder of the patients and all control subjects, may be beyond the limit of detection using Western blotting. In addition, there is a possibility that the ~240kDa TRPV1 band detected in two patients is an artefact. Further sample processing procedures, such as increasing the concentration of SDS/ β -mercaptoethanol and performing cross-linking techniques, should be considered to determine whether the oligomers causing the higher molecular weight bands in U266B1 cells (dimer, ~200kDa) and the two patients with blood cancers (~240kDa) are true TRPV1 bands (Milligan and Koshland 1988). The leukocytes used in this study were obtained from patient/control blood, whereas testing disease-appropriate cells, such as lymph node cells from B-NHL patients, would provide additional information on TRPV1 expression at the 'disease source'. Indeed, this may explain why the apparent

increase in TRPV1 expression in malignant haematological cell lines (THP-1, U266B1 and U937), was not observed in the leukocytes of cancer patients.

As PBMCs from healthy adults and patients with haematological malignancies were shown to express TRPV1, and as there is an apparent positive association between inflammation and TRPV1 expression in some conditions (Akbar et al. 2008; Geppetti and Trevisani 2004; Saunders et al. 2009), this relationship was explored. Elevated C-reactive protein level (≥ 5 mg/L) was detected in 25% of patients, however it was not associated with higher levels of TRPV1 expression. The lack of association may be explained by anti-inflammatory therapies that are often used to treat these patients. Thus, it may be that the specific function of the TRPV1 channel in human leukocytes is restricted solely to cation transportation, without any involvement with tumorigenesis (Samways and Egan 2011). These findings indicate that whilst inflammation may increase TRPV1 expression in some conditions, this may not be the case in blood cancers.

A major limitation of this study was that relatively few patients were recruited. This was primarily due to; 1) the restricted time frame of that component of the study (~six months), 2) the small number of patients that are diagnosed with blood cancers in Tasmania each year (Stokes B 2013), and 3) the experimental conditions, as blood samples should be processed within 2 hours of collection to preserve the viability of leukocytes, as there were unsuccessful attempts to store the cells for later processing. Nonetheless, the results presented in this thesis represent novel and important pilot data on the expression of TRPV1 in haematological malignancies, not previously reported. Increasing the overall number of patients, particularly the *de novo* patients,

would allow more robust statistical analysis to be performed. Similarly, it would be interesting to investigate the expression of specific types of blood cancers, although given the relative incidence of these diseases in general, may require multi-centre recruitment. Finally, larger cohort studies may also allow the specific effects of blood cancer drugs on TRPV1 expression to be studied.

In conclusion, this thesis has contributed new knowledge on the expression and potential role of TRPV1 in haematological cells, including those from healthy individuals and patients with blood cancers. A number of novel findings have been reported. Firstly, CAP was found to inhibit the metabolic activity of THP-1, U266B1 and U937 malignant haematological cell lines, independent of TRPV1 activation. TRPV1 and CB1, however, appear to mediate a CAP-induced increase in the metabolic activity in THP-1 cells. Secondly, Western blotting and flow cytometry protocols were developed and optimised for the detection of TRPV1 in human leukocytes. Thirdly, cell lines that have previously been used in other studies as a TRPV1 negative control, including untransfected and TRPV1-transfected (tetracycline off) HEK293 and RAW264.7 cells, were reassessed using the optimised Western blotting and flow cytometry protocols and found to express TRPV1. The data presented in this thesis strongly suggests that these cells should not be used as negative controls for investigations of TRPV1. Finally, increased TRPV1 expression was observed only in some patients with MM and B-NHL, and was not associated with inflammation. Altogether, this thesis provides a fundamental contribution to the knowledge and understanding of TRPV1 measurement protocols, as well as the expression of this important cation channel in malignant and non-malignant blood

cells. The results presented can be used as a basis for future studies of TRPV1 function in other human cells and cancers.

Appendix I: Preliminary Flow Cytometry Optimisation Using BD FACSCalibur™

The preliminary experiments stage of detecting TRPV1 using flow cytometry was executed at the Launceston General Hospital (LGH) FACSCalibur™ flow cytometer (Becton Dickinson Biosciences, San Jose, USA). Thirty one experiments were conducted in the preliminary stage for method optimisation.

Immunophenotyping

All fluorochrome-conjugated CD markers are purchased from Becton Dickinson (San Jose, USA) unless other wise stated. A combination of peridinin chlorophyll protein (PerCP), fluorescein isothiocyanate (FITC), allophycocyanin (APC), and phycoerythrin (PE) conjugated antibodies were selected. CD3-APC (T-cells), CD14-APC (monocytes), CD19-PE (B-Cells) and CD56-PE (natural killer cells) were used for leukocyte immunophenotyping (surface staining) using the FACSCalibur™.

Flow Cytometer Calibration

The FACSCalibur™ cytometer was set to lyse/wash method and the calibration was performed using BD Calibrite™ beads (Becton Dickinson Biosciences, San Jose, USA).

Electronic Optimisation

Four tubes each containing 1×10^6 white blood cells, obtained from normal subject, were stained with CD45-FITC, CD3-PE, CD45-PerCP, or CD45-APC, separately,

and two tubes each containing 1×10^6 white blood cells were subjected to sample processing, with equivalent volumes of PBS in place of the CD markers (unstained cells) for electronic optimisation. All incubations were performed at ambient temperature, unless otherwise stated. Cells were stained with CD markers for 10 minutes, in the dark. Cells were then incubated in 2 mL FACS Lyse solution (BD Biosciences, San Jose, U.S.A) for 10 min, in the dark. Cells were then centrifuged at $300 \times g$ for 5 min, washed in 2 mL PBSA, and re-centrifuged at $300 \times g$ for 3 min.

Forward and Side Scatter Optimisation

Forward scatter (FSC) amplification (amp) gain and side scatter (SSC) voltage were adjusted to position the three main leukocyte subpopulations; lymphocytes, monocytes and granulocytes on the linear scale (Figure I). FSC threshold was adjusted to minimise debris appearing on the scale. FSC and SSC were optimised for all samples under all conditions (Figure II).

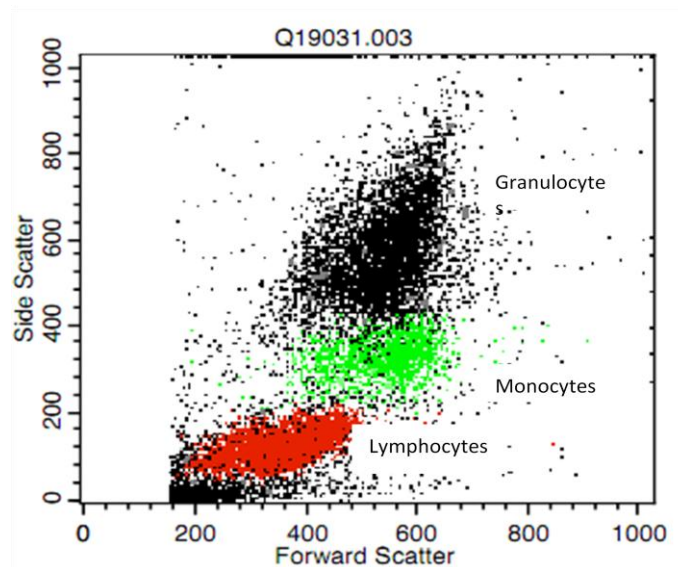


Figure I: FSC and SSC electronic optimisation for fixed and permeabilised cells using FACSCalibur™. A successful optimisation of FSC and SSC, leads to a clear distinction between the three main leukocyte populations, lymphocytes, monocytes, granulocytes and the exclusion of debris.

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Cytometer Type: FACSCalibur

Detectors/Amps:
Param      Detector Voltage  AmpGain  Mode
P1         FSC      E00      1.11    Lin
P2         SSC      431      1.00    Lin
P3         FL1      808      1.00    Log
P4         FL2      812      1.00    Log
P5         FL3      868      1.00    Log
P6         FL1-A     1.00    Lin
P7         FL4      742      1.00    Log

Threshold:
Primary Parameter: FSC
Value: 150

Secondary Parameter: None

Compensation:
FL1 - 0.9 % FL2
FL2 - 38.2 % FL1
FL2 - 0.1 % FL3
FL3 - 10.8 % FL2
FL3 - 2.0 % FL4
FL4 - 10.0 % FL3

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Figure II: Example of optimised FACSCalibur™ settings for lyse/wash method of a normal blood sample. Detectors are the channels in which each fluorochrome is detected; voltages are the PMT values for each channel; threshold is the value where debris was excluded, and compensation matrix showing calculated percentages of corrected spillover of one channel into the other.

PMT Voltage Optimisation

Photomultiplier tube (PMT) voltage optimisation was performed to separate signal (positive) from noise (negative). PMT voltages were optimised before the compensation optimisation step, using two-three drops of unstained cells in each of the single stained samples (Maecker and Trotter 2006). Subsequently, the sample was placed on the FACSCalibur™ sample injection port (SIP). Data acquisition was started and the related detector voltages were adjusted during the live set-up phase of data acquisition. The PMT voltages were adjusted to position the unstained negative cells within the first decade of the histogram. However, the positive cells were positioned within the third decade (Maecker and Trotter 2006).

Fluorescence Compensation

Compensation optimisation step was performed to eliminate fluorescence spectral overlap. For the compensation, single tubes each containing 10 μ L of each CD45-FITC, CD45-PerCP, CD3-PE and CD45-APC antibodies, separately, for electronic optimisation (Maecker and Trotter 2006). Unstained cells (negative population) were added to each of the single stained tubes. Compensation optimisation was performed during the set-up phase of live data acquisition after setting up the PMT voltage (Figure III). Cellular autofluorescence was also investigated, and the data showed negative cell populations located within the first quadrant, indicating minimal autofluorescence of the target cells under test conditions (Figure III, C-E).

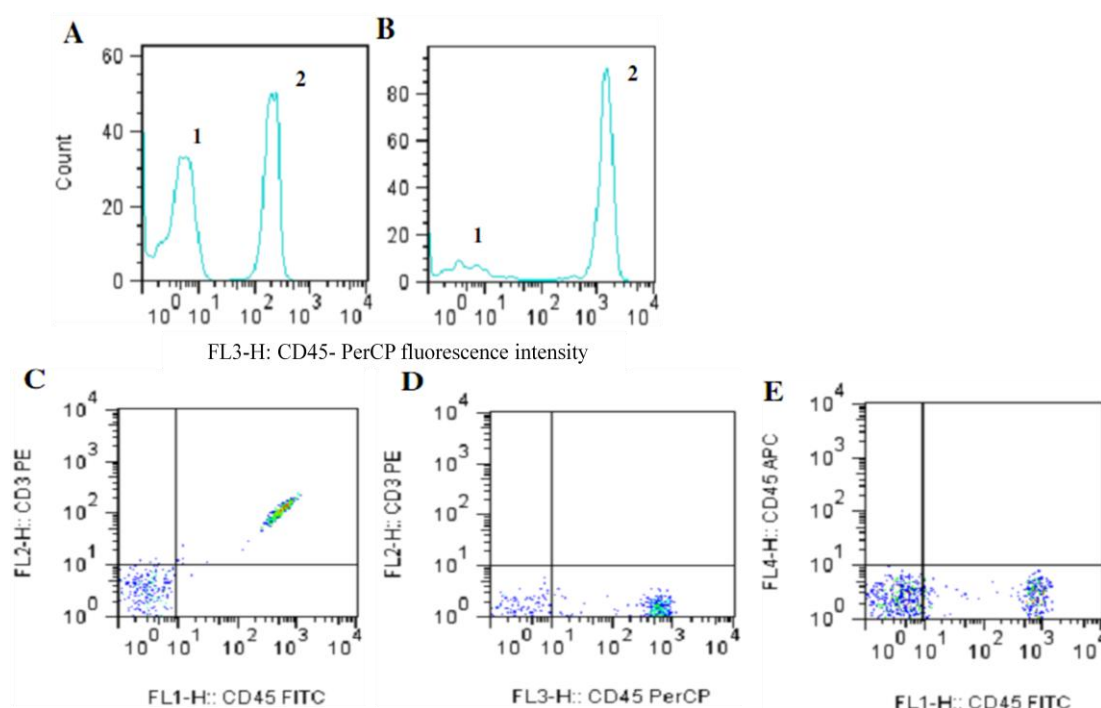


Figure III: Example of compensation optimisation. (A) Data demonstrate PerCP voltage before optimisation, (B) PerCP voltage after electronic optimisation. CD45-PerCP negative population (A1 and B1) positioned within first decade, and CD45-PerCP positive population from second to third decade have increased with optimisation (A2 and B2), (C) Example of over-compensation, (D) under-compensation, (E) Properly compensated data.

Fixation and Permeabilisation

Fixation and permeabilisation procedures were required to allow the TRPV1 primary antibody to bind to its intracellular epitope. Two commercially available kits CALTAG™ (Life Technologies, Grand Island, USA) and Cytofix/Cytoperm™ (Becton Dickinson, San Jose, USA) were investigated to determine the staining patterns of TRPV1. The fixation/permeabilisation protocols for both kits were applied according to suppliers' instructions, with slight modifications including staining the CD markers for 10 min and centrifuging cells at 300 x g. Cells were fixed, permeabilised and washed using a saponin-based wash reagent to ensure cells permeabilised as the permeabilisation step is reversal. There was no significant difference in TRPV1 signal using either the CALTAG™ (Life Technologies, Grand Island, USA) and Cytofix/Cytoperm™ kits (Becton Dickinson, San Jose, USA) (Figure IV).

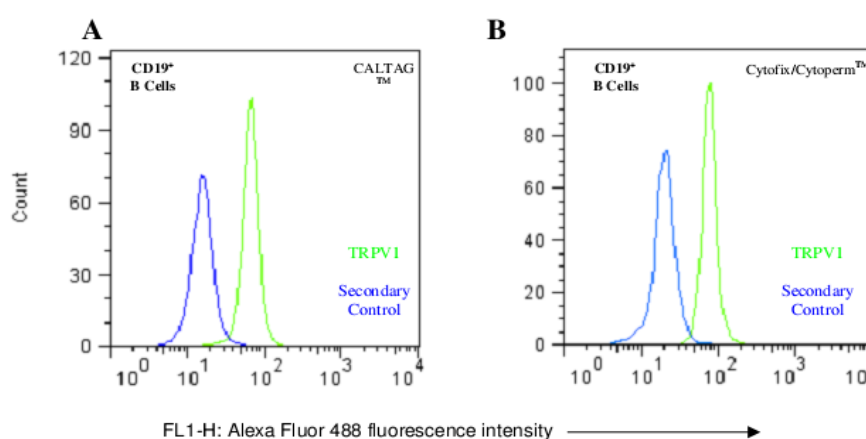


Figure IV: Staining patterns for two fixation/permeabilisation kits tested (A) CALTAG™ kit and (B) BD Cytofix/Cytoperm™ kit. Results demonstrate same efficiency of both kits.

Secondary Antibody Titration

Secondary antibody optimisation involved titration studies for both FITC- and Alexa Fluor[®]488-conjugated polyclonal goat anti-rabbit secondary antibodies (Figure V). The variation between Alexa Fluor[®]488 background staining was observed at

dilutions ranging from 1:50 to 1:250 (Figure V, B). Therefore, optimal dilution for goat anti-rabbit- Alexa Fluor®488- conjugated secondary antibody was 1:50 and 1:25 for FITC-conjugated secondary antibodies. Alexa Fluor®488 as a fluorochrome looks brighter than FITC, however separation between signal and noise was similar for both.

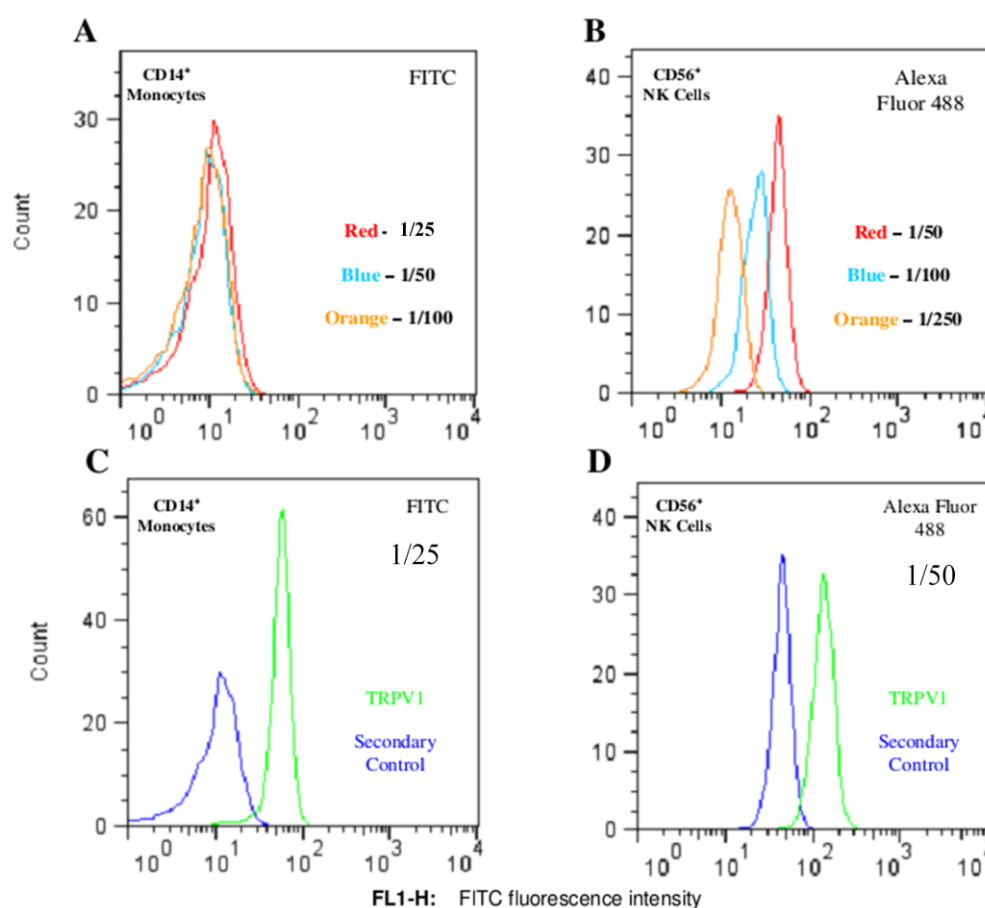


Figure V: FITC- and Alexa Fluor®488- conjugated secondary antibody dilution study using the FACSCalibur™. (A) Secondary antibody alone titration for FITC, and (B) Alexa Fluor®488: an increase in TRPV1 signal with decreasing the secondary antibody titre was noticed. The brightest signal was obtained using 1:50 dilution. (C) Optimised working dilutions with (green) and without (blue) the Santa Cruz anti-TRPV1 antibody for FITC and (D) Alexa Fluor®488. Both secondary antibodies demonstrated similar staining patterns between secondary alone control (Blue) and TRPV1 signal (Green).

Blocking Optimisation

The following general blocking reagents were tested: human AB serum (obtained from healthy AB blood group donor), and FcR blocking reagent (Miltenyi Biotechnology, Cologne, Germany). Better staining pattern was observed using Fc-receptor blocking compared to the human AB serum (Figure VI).

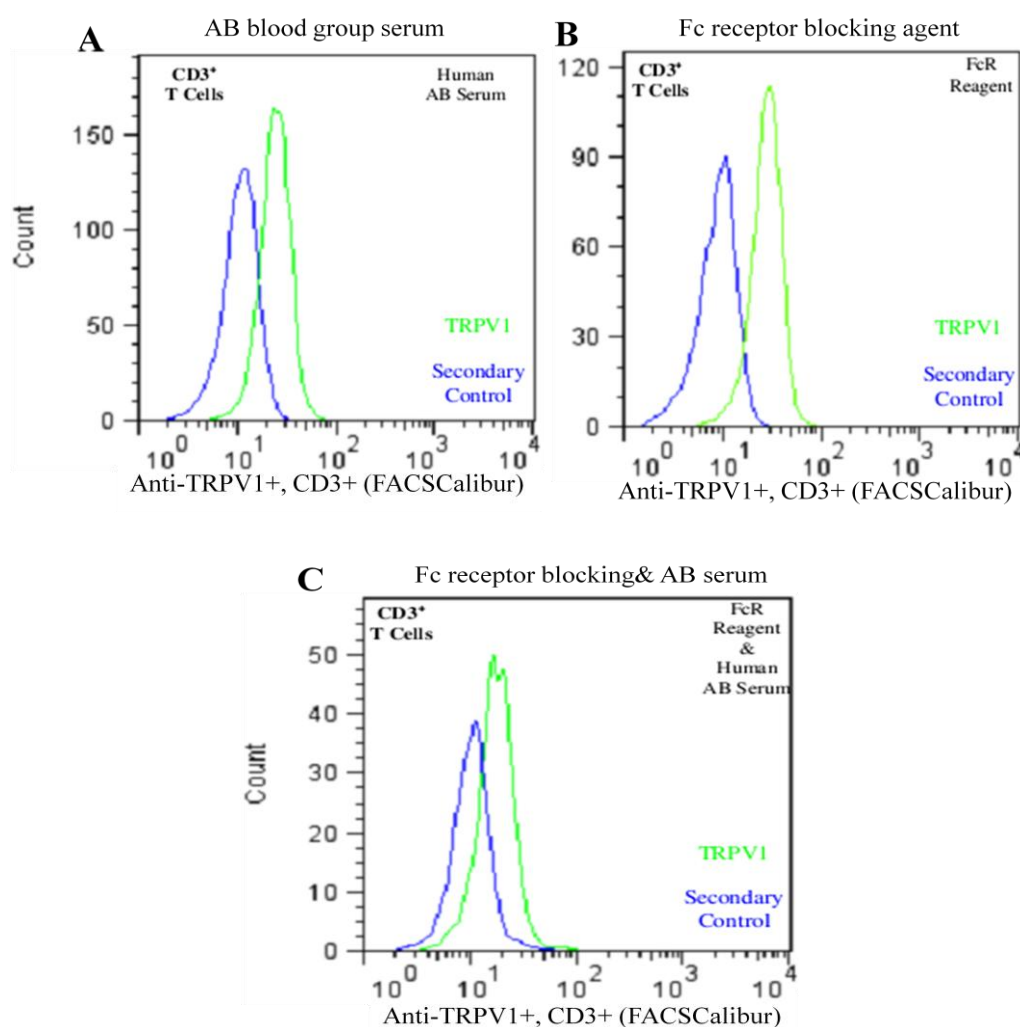


Figure VI: Blocking optimisation using FACSCalibur™ cytometer on anti-TRPV1 antibody (Santa Cruz Biotechnologies). Results demonstrate better staining pattern for (B) Fc-receptor blocking compared to (A) human AB serum. (C) a significant decrease in signal was observed when using a combination of both.

Primary antibody dilution

A dilution study for Santa Cruz Biotechnology anti-TRPV1 antibody was performed to optimise the TRPV1 signal, by incubating one million cells with 0.1 to 2 μg of the primary antibody diluted 1:20 (Santa Cruz Biotechnologies, CA, USA). There was a direct correlation between the amount of primary antibody added and the TRPV1 signal, with optimum TRPV1 antibody quantity of 1 μg (Figure VII). Still, no separation between isotype control and this anti-TRPV1 antibody; as similar or weaker signal than the isotype control was detected (Figure VIII).

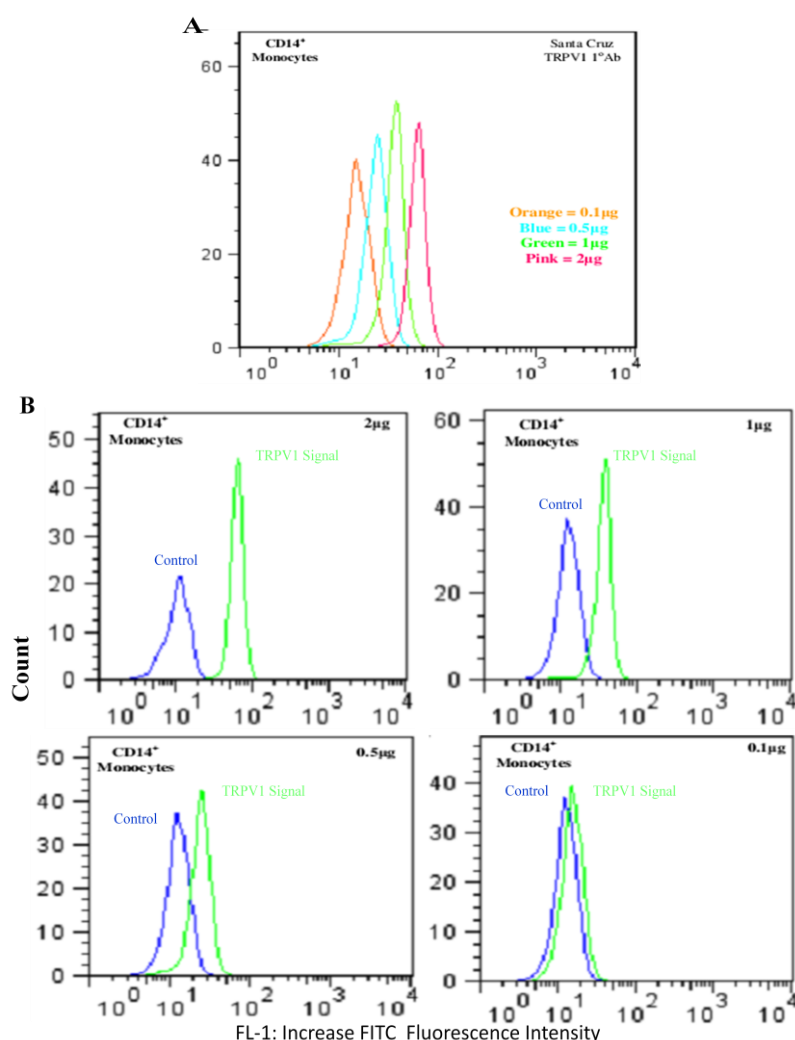


Figure VII: TRPV1 primary antibody optimisation (Santa Cruz Biotechnologies, CA, USA). (A) Results demonstrate increase in TRPV1 signal with increasing quantity of TRPV1 primary antibody, (B) TRPV1 signal comparing to noise.

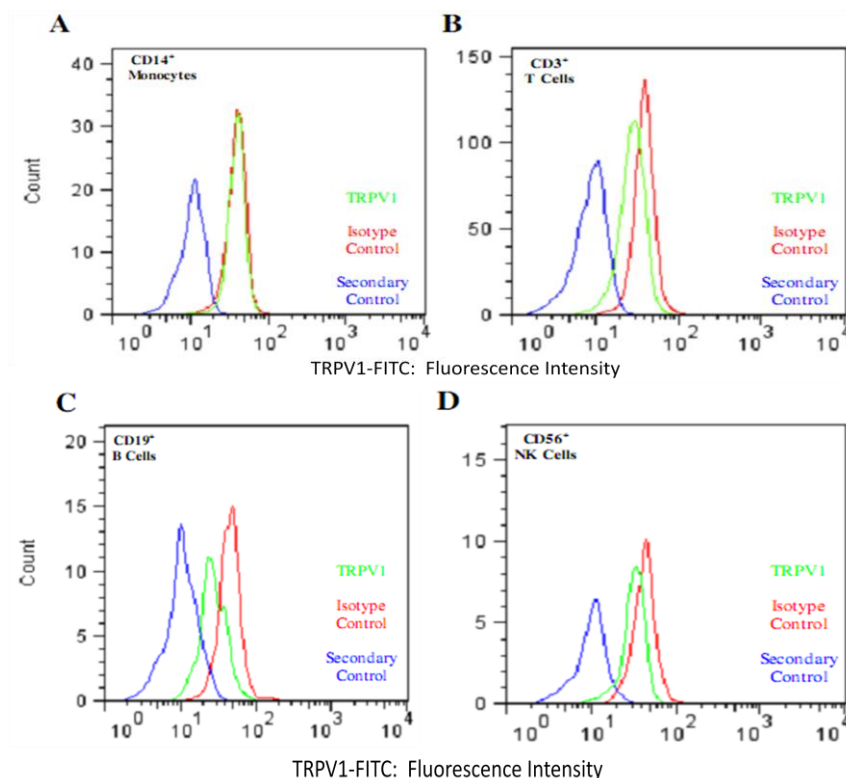


Figure VIII: TRPV1 signal compared to isotype control in human leukocyte subpopulations. Data demonstrate staining pattern for isotype control (Santa Cruz biotechnology, USA) compared with TRPV1 signal (Santa Cruz biotechnology, USA) and secondary antibody only control (goat anti-rabbit-FITC, Santa Cruz biotechnology, USA) for (A) Monocytes, (B) T cells, (C) B Cells and (D) natural killer cells using FACSCalibur™.

Data Acquisition & Analysis

Data acquisition and analysis were completed using the FACSCalibur™ flow cytometer and Cell QuestPro software (version 5.1) (Becton Dickinson Biosciences, San Jose, USA). 10,000 events were acquired for each sample. Fluorescence intensity is expressed in arbitrary units on a logarithmic scale. Final analysis was performed using FlowJo (version 7/9, Oregon, U.S.A.).

Appendix II: Patients and Control Subjects Consent Forms

1. Patient consent form

School of Human Life Sciences, University of Tasmania, Launceston TASMANIA



PATIENT INFORMATION AND CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Study Title:

Transient Receptor Vanilloid 1 (TRPV1) in Haematological Malignancies.

Chief Investigators:

Dr Murray Adams, School of Human Life Sciences, University of Tasmania.

A/Prof Dominic Geraghty, School of Human Life Sciences, University of Tasmania.

A/Prof Alhossain Khalafallah, Launceston General Hospital.

Mrs Sofia Omari, School of Human Life Sciences, University of Tasmania (PhD Student).

Dear Patient

This consent form may contain words that you do not understand. Please ask the study doctor or the study staff to explain any words or information that you do not clearly understand.

INTRODUCTION AND PURPOSE

It has been shown in previous research studies that the transient receptor potential vanilloid 1 (TRPV1, pronounced “TRiP-V-one”) is important in a number of diseases. Evidence suggests that in some diseases the number of sensory nerves increase and more TRPV1 is produced.

The purpose of the present study is to determine whether the expression of TRPV1 in human white blood cells is altered in patients with haematological malignancies (blood cancers), e.g., leukaemia, plasma cell myeloma (or, multiple myeloma), lymphoma, compared to people who do not have a haematological malignancy. Data will be compared with a comparable number of control participants (people without haematological malignancy).

PROCEDURE

If you choose to participate in this study you will be required to attend the Pathology Department at your local hospital/pathology laboratory or the School of Human Life Sciences Clinical Laboratory for a blood test.

Samples:

You will be required to give 30 millilitres of blood, which will be taken at Pathology Department at your local hospital/pathology laboratory or at the School of Human Life Sciences Clinical Laboratory. Some patients maybe asked to provide a bone marrow aspirate sample or lymph node biopsy if the blood cells of interest are not detected in the blood sample. Collection of these specimens is performed using standard procedures by A/Prof Alhossain Khalafallah (Consultant Haematologist). If you have specific questions related to collection procedures please contact A/Prof Khalafallah (alhossain@yahoo.com or 6348 7140) for further information.

RISKS AND DISCOMFORTS

Blood sampling is a low risk activity but participants should be aware that there are a number of minor complications that can result from the procedure. In order for subjects to give informed consent to blood sampling and/or collection of bone marrow or lymph node material, they should read and understand the following:

Complications of **blood sampling**:

1. Syncope (fainting): This is not common in healthy volunteers, but can more commonly occur if subjects are unwell or suffering from a viral infection such as a cold or flu. Subjects who are extremely apprehensive about the procedure or the sight of blood are also prone to fainting. If this does occur, the subject will be laid down and adequate ventilation with fresh air is provided. A glass of cold water often helps to alleviate the symptoms.
2. Nausea and Vomiting: Whilst a feeling of nausea is a relatively common response (especially in first time subjects), vomiting is quite uncommon in adults as a response to **blood sampling**. Subjects who are apprehensive may experience feelings of nausea and are encouraged to return when they feel more confident with the procedure, if that is suitable.
3. Bruising and Haematoma Formation: Bruising is the most common post-procedure complication. The likelihood of bruising can be greatly diminished by applying pressure to the puncture site for 5 minutes after the completion of the procedure and maintaining a straight arm. Patients taking anticoagulants or any other medication that hinders the body's ability to form a clot (e.g., aspirin) should apply pressure for an additional 5 minute period and then reassess whether any more pressure should be applied. Haematoma formation (bleeding under the skin to form a raised swelling) can also occasionally occur and can be minimised with prolonged application of pressure to the site.

4. Convulsions: Usually only seen in patients who faint. These are usually minor in nature and last less than a minute. The procedures for this are the same as for fainting although, of course, care is taken to ensure that the patient does not hurt themselves.
5. Nerve Injury: Rare reports exist of patients who have experienced a partial loss of arm movement following **blood sampling**. The incidence of this is less than 1 in 10,000 and as such it is considered extremely unlikely.

Complications of collecting **bone marrow** and **lymph node** material.

As with **blood sampling**, there is a small risk of minor complications with the collection of **bone marrow** and **lymph node** material.

Bone marrow is collected from the pelvic hip bone using a needle and syringe. This is performed under local anaesthesia. This procedure generally takes about one hour. The chance of a serious complication in a healthy person is very low but some people may experience nausea, bruising and/or local pain and discomfort. The two most common complications that may occur from the procedure are bleeding and infection at the puncture site. The bleeding at the puncture site is usually controlled with external compression and gauze. Local infection at the puncture site is rare, but can require oral antibiotics. The procedure is performed under sterile conditions to prevent infections.

Lymph node material is collected using a fine needle biopsy. A fine needle biopsy uses a thin, hollow needle to obtain fluid and tissue samples. This is performed under local anaesthesia. This procedure takes between 30-60 min to complete. There are generally few complications after a lymph node biopsy but some people may experience nausea, bruising and/or local pain and discomfort. There is a small risk of bleeding or infection. The bleeding at the puncture site is usually controlled with external compression and gauze. Local infection at the puncture site is rare, but can require oral antibiotics. The procedure is performed under sterile conditions to prevent infections.

BENEFITS

We hope that studies such as this one will lead to the development of treatments that have less severe side effects for people with blood cancers. However, there will be no direct benefit to you as a result of the research performed with the material obtained from your blood sample. If you have specific questions related to blood cancers please contact A/Prof Alhossain Khalafallah (alhossain@yahoo.com or 6348 7140) for further information.

COST

Participation in this trial will not result in any costs for you and there is no payment for participation in this study.

CONFIDENTIALITY

All data will be treated in the strictest of confidence. The information that will be collected will only be used for the purpose of this project. Records identifying your identity will not be made publicly available. If the results of the trial are published, your identity will remain confidential. If reference to you is made, this will only be done via code numbers.

PARTICIPATION

Your participation is voluntary and you may withdraw at any time without prejudice.

INCLUSION AND EXCLUSION

To enter this study as a control participant there must be no prior history of haematological disease (blood cancer) and over the age of 18.

WITHDRAWAL

If you choose to withdraw at any stage, you may request that any, or all, of your data collected for the purpose of this project be destroyed. The researchers will act in accordance with your wishes.

RESULTS

The results of this study will be analysed and presented as group data only. A summary of results will be available at the end of the study and these results will be mailed to you on request.

CONTACTS

If you have a question about this study please contact the chief investigators;

- Dr Murray Adams (Murray.Adams@utas.edu.au or 6324 5483)
- A/Prof Dominic Geraghty (D.Geraghty@utas.edu.au or 6324 5488)
- A/Prof Alhossain Khalafallah (alhossain@yahoo.com or 6348 7140)

This study has been cleared by the Tasmanian Health and Medical Human Research Ethics Committee in accordance with the National Health and Medical Research Council's guidelines.

If you have any concerns of an ethical nature or complaints about the manner in which the project is conducted, you may contact the Executive Officer of the Human Research Ethics Committee (Tasmania) Network. The Executive officer can direct participants to the relevant Chair that reviewed the research. Executive Officer: Jen Bruyn Schmidt (02 6226 7479).

Thankyou for your time,

INFORMED CONSENT FORM

If you decide to take part in this study, you will not receive results of these tests. We do not offer you your personal results at this time as the study is entering a new field of research and results will not be interpreted until the study is complete. (You will be asked if you wish to receive a summary the results of this research once it has been completed).

1. I, _____ have read the above information and I agree to take part in the study investigating TRPV1 in blood cells in patients with haematological malignancy and normal controls.

2. I was given a copy of this signed and dated Informed Consent Form and the corresponding Subject Information Sheet. I have received an explanation of the nature, purpose, duration and foreseeable effects of the study and what I will be expected to do. The possible risks and benefits of the study have been explained to me. I was given time and opportunity to inquire about the trial and all my questions were answered to my satisfaction.

3. I am aware that an Independent Ethics Committee (University of Tasmania) has subjected this study for review and approval.

4. I am free to withdraw from the study at any time, without the need to justify my decision.

5. I agree that the results of the study may be published or presented, however my name and contact details will be kept confidential.

6. I understand that the research will be conducted in accordance with the Declaration of Helsinki, NH&MRC Guidelines and applicable privacy laws.

7. I voluntarily consent to participate in this study.

Subject's Signature

Date

Investigator Statement

I, _____ have explained this study and the implications of participating in it to this volunteer and I believe that the consent is informed and that he/she understands the implications of participating in the study. The subject consented to participate by his/her personally dated signature

Investigator's Signature

Date

Witness

Date

2. Control subject consent form

School of Human Life Sciences, University of Tasmania, Launceston TASMANIA



CONTROL PARTICIPANT INFORMATION AND CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Study Title:

Transient Receptor Vanilloid 1 (TRPV1) in Haematological Malignancies.

Chief Investigators:

Dr Murray Adams, School of Human Life Sciences, University of Tasmania.

A/Prof Dominic Geraghty, School of Human Life Sciences, University of Tasmania.

A/Prof Alhossain Khalafallah, Launceston General Hospital.

Mrs Sofia Omari, School of Human Life Sciences, University of Tasmania (PhD Student).

This consent form may contain words that you do not understand. Please ask the study doctor or the study staff to explain any words or information that you do not clearly understand.

INTRODUCTION AND PURPOSE

It has been shown in previous research studies that the transient receptor potential vanilloid 1 (TRPV1, pronounced “TRiP-V-one”) is important in a number of diseases. Evidence suggests that in some diseases the number of sensory nerves increase and more TRPV1 is produced.

The purpose of the present study is to determine whether the expression of TRPV1 in human white blood cells is altered in patients with haematological malignancies (blood cancers), e.g., leukaemia, multiple myeloma, lymphoma, compared to people who do not have a haematological malignancy. **You are being asked to act as a control (i.e., no history of haematological malignancy).**

PROCEDURE

If you choose to participate in this study you will be required to attend the Pathology Department at your local hospital/pathology laboratory or the School of Human Life Sciences Clinical Laboratory for a blood test.

Blood Samples:

You will be required to give 30 millilitres of blood, which will be taken at Pathology Department at your local hospital/pathology laboratory or at the School of Human Life Sciences Clinical Laboratory.

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CONFIDENTIALITY

All data will be treated in the strictest of confidence. The information that will be collected will only be used for the purpose of this project. Records identifying your identity will not be made publicly available. If the results of the trial are published, your identity will remain confidential. If reference to you is made, this will only be done via code numbers.

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7. I voluntarily consent to participate in this study.

Subject's Signature

Date

Investigator Statement

I, _____ have explained this study and the implications of participating in it to this volunteer and I believe that the consent is informed and that he/she understands the implications of participating in the study. The subject consented to participate by his/her personally dated signature

Investigator's Signature

Date

Witness

Date

Appendix III: Experiments Sheets and Protocols

1. Cell metabolic activity assay in the presence of CAP (Alamar Blue method):

Alamar blue[®] reagent is used to assess cell metabolic activity. Resazurin, the active ingredient of alamarblue[®] reagent, is a non-toxic, cell permeable compound that is blue and non-fluorescent. Upon entering the cells, resazurin is reduced to resorufin, a red compound which is highly fluorescent. Viable cells will continuously convert resazurin to resorufin, increasing the overall fluorescence and colour of the media surrounding the cells.

Resazurin (blue, non fluorescence) \longrightarrow Resorufin (red, fluorescence)

THP-1 cells metabolic activity in the presence of CAP:

1. Count the cell number using haemocytometer or Invitrogen Countess.
2. Calculate the total volume to get 5×10^5 cells/ 100 μ L/ well. Take the desired cell suspension, centrifuge, resuspend in the calculated volume.
3. In a 96 wells microplate, add 100 μ L of cell suspension to each well and incubate.
4. Prepare serial dilution of CAP: 2000, 1000, 500, 250, 125, 63, 32 and 16 μ M. Each well will contain 100 μ L of CAP. A vehicle should be used as a negative control

Note: vehicle should contain the same amount of ethanol used to reconstitute CAP. In our experiments, CAP contains 20 μ L of ethanol for every 980 μ L of CAP solution.

CAP Serial Dilution:

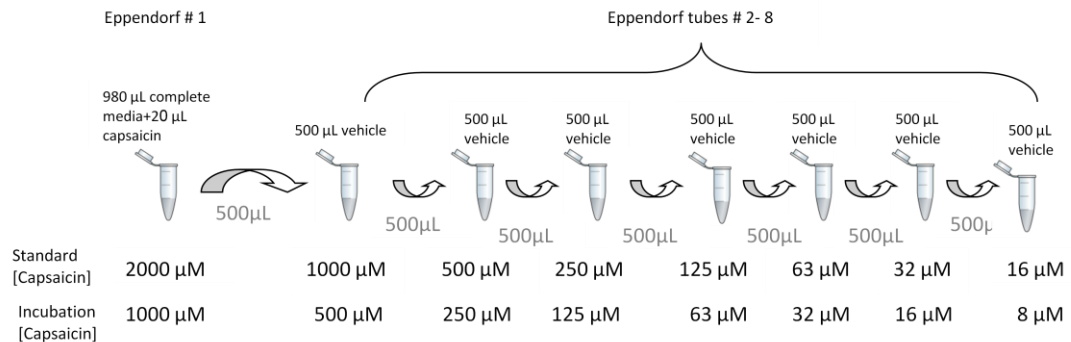
- 8 Eppendorf tubes, write the concentration on the lid. Start with 2000 μ M.
- To prepare a vehicle: in a 15 ml centrifuge tube, add 5 ml complete media, and for each 980 μ L of media, add 20 μ L absolute ethanol and mix.
- Starting with 2000 μ M (2mM) concentration, CAP stock concentration is 100 mM. To calculate the required initial CAP volume, use the formula:

$$C_i \times V_i = C_f \times V_f$$

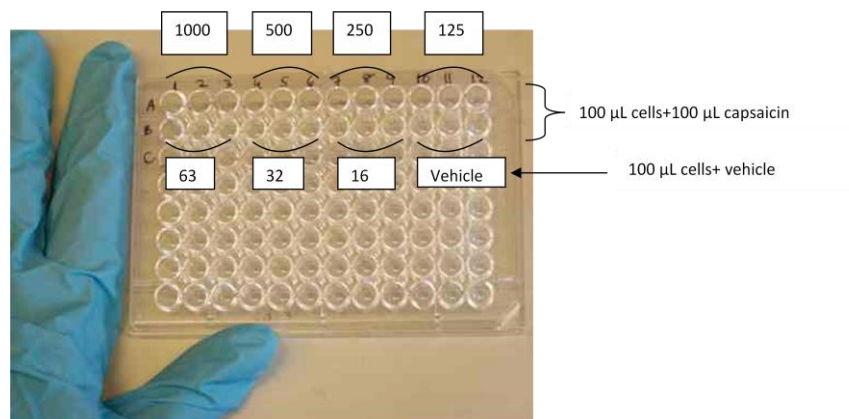
$$100\text{mM} \times V = 2\text{mM} \times 1000$$

$$V_i = 20 \mu\text{L}.$$

- For Eppendorf #1 (2000 μM): add 980 μL of complete media+ 20 μL of CAP (100 mM) (the mixture will be turbid).
- To the rest of the tubes, add 500 μL of vehicle. Then start serial dilution by taking 500 μL from tube #1 to #2, mix then take 500 μL from #2 to #3....etc (dilution factor= $\frac{1}{2}$).



5. Bring the microplate from the incubator and add 100 μL of each CAP concentration to 3 wells. In the last 3 wells add vehicle only as follows (incubation concentration is shown below), then incubate at 37°C, 5% CO_2 :



6. After 22hr of incubation, take 0.1 of the total volume of alamarblue[®] reagent in an Eppendorf tube and add it to each well, then incubate for another 2hr.

7. After 2hr, the fluorescence reading will be performed using GENios, TECAN reader at excitation wavelength of 540-570 nm and emission at 580-610 nm.

GENios, TECAN reading procedure:

1. Turn on the fluorescence plate reader (GENios, TECAN)
2. On the desktop, open XFluor4.xls excel data sheet.
3. Press Add Ins button; select XFluor4 → Connect → Edit Measurement Parameters (optimal gain, fluorescence intensity, no temperature...).

4. Load your plate → Start Measurements.
5. Save the readings.
6. Calculate the mean for each CAP concentration readings (3 wells each).
7. Calculate the mean for the vehicle readings.
8. $\% \text{ metabolic activity} = \frac{\text{mean (CAP concentration readings)}}{\text{mean(vehicle readings)}} \times 100\%$.
9. By GraphPad Prism Program, create the diagram.

Tips:

1. Use reverse pipetting to avoid air bubbles in the microplate. Few bubbles are not going to affect the result, as the fluorescence reader read the bottom of the well only.
2. Use wide end tips for cell suspension to avoid stressing the cells.
3. Alamarblue[®]-treated cells plate could be incubated for 1-4 hr in cell culture incubator. Discard after 4 hr.
4. The assay plate can be wrapped in foil, stored at 4°C, and read within 1-3 days without affecting the fluorescence or absorbance value.

2. Cell metabolic activity of malignant haematological cell lines malignancies exposed to CAP in the presence of TRPV1, CB1 and CB2 antagonists (SB452533, AM251 and AM630)

- ❖ Total volume in each well= 250µL:
100µL cell suspension + 50µL antagonist/vehicle+ 100µL CAP.
CAP concentration used= 125 µM.

❖ Solutions:

- ❖ SB free vehicle: 30µL ethanol/ 1 ml complete media (500µL needed).
- ❖ AMs free vehicle: 3µL DMSO/ 1 mL complete media (500µL needed).
- ❖ CAP free solution: 3.13µL ethanol/ 1 ml complete media. To prepare 5 ml of this solution: 15.7µL ethanol + 4984.4µL complete media.

❖ **CAP**= 100mM. Prepared dilutions: 312.5µM to end up with 125µM CAP concentration¹.

➤ CAP Volume= 2500 µL/ 15 wells.

$$C_i V_i = C_f V_f \text{ (i= initial, f= final)}$$

$$100000\mu\text{M} * V_i = 312.5\mu\text{M} * 2500$$

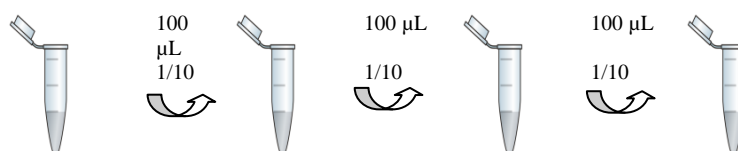
$V_i = 7.8 \mu\text{L}$ of stock CAP into 2492 µL of CAP free solution.

❖ **SB 452533**= stock concentration 10 mM. Required concentration: 3.0 µM to end up with 1.0 µM².

❖ **SB 452533 experiment preparation:**

1. SB diluent: 30µL alcohol³/ 1 ml complete media (used in the SB dilutions only). Required volume= 3 ml (90µL alcohol+ 2910µL complete media).
2. CAP free solution: see below.

❖ **SB452533 dilution (1000 µL):** added to all SB wells (including SB vehicle wells)



	970µL complete media+ 30µL SB	900µL SB diluent	900µL SB diluent	900µL SB diluent
Preparation concentration	300µM	30µM	3.0µM	0.3µM
Incubation concentration	100µM	10µM	1.0µM	0.1µM

¹ CAP= solute/t.volume= 100/ 250= 2.5x. We want incubation [CAP] =125 µM, so 125* 2.5= 312.5 µM.

² We will add 50 µL antagonist+ 100 µL cells first, so 50/150= 1:3= 3 x. So, 3 * 100 µM= 300 µM, 30,...etc.

³ Because we add 30 µL of SB in 1 ml media to get 300 µM final concentration. Stock [SB]= 10mM.

❖ **AM251 (CB1 antagonists) and AM630 (CB2 antagonist):** stock concentration

100 mM. Required dilutions: 300, 30, 3.0 and 0.3 μ M to end up with 100, 10, 1.0 and 0.1 μ M incubation concentrations.

❖ **AM251 and 630 dilutions:**

$$C_i V_i = C_f V_f \quad (i = \text{initial}, f = \text{final})$$

$$100000 \mu\text{M} * V_i = 300 \mu\text{M} * 1000$$

$$V_i = 3 \mu\text{L} \text{ of stock AM251 or AM630 into } 997 \mu\text{L complete media.}$$

❖ **To start serial dilution:**

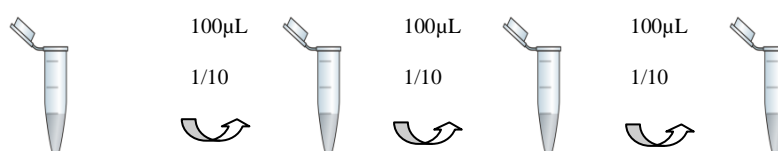
$$\text{Dilution} = \text{solute} / \text{total volume}$$

$$1/10 = s/1000 \rightarrow S = 100 \mu\text{L.}$$

❖ **Vehicle:** Both of AM251 and AM630 (AMs) have the same vehicle:

1. AM dilution solution: 3 μ L DMSO/ 1 ml complete media (for AMs dilution only). Required vehicle volume= 6 ml (5991 μ L complete media + 9 μ L DMSO).
2. CAP free solution: see below.

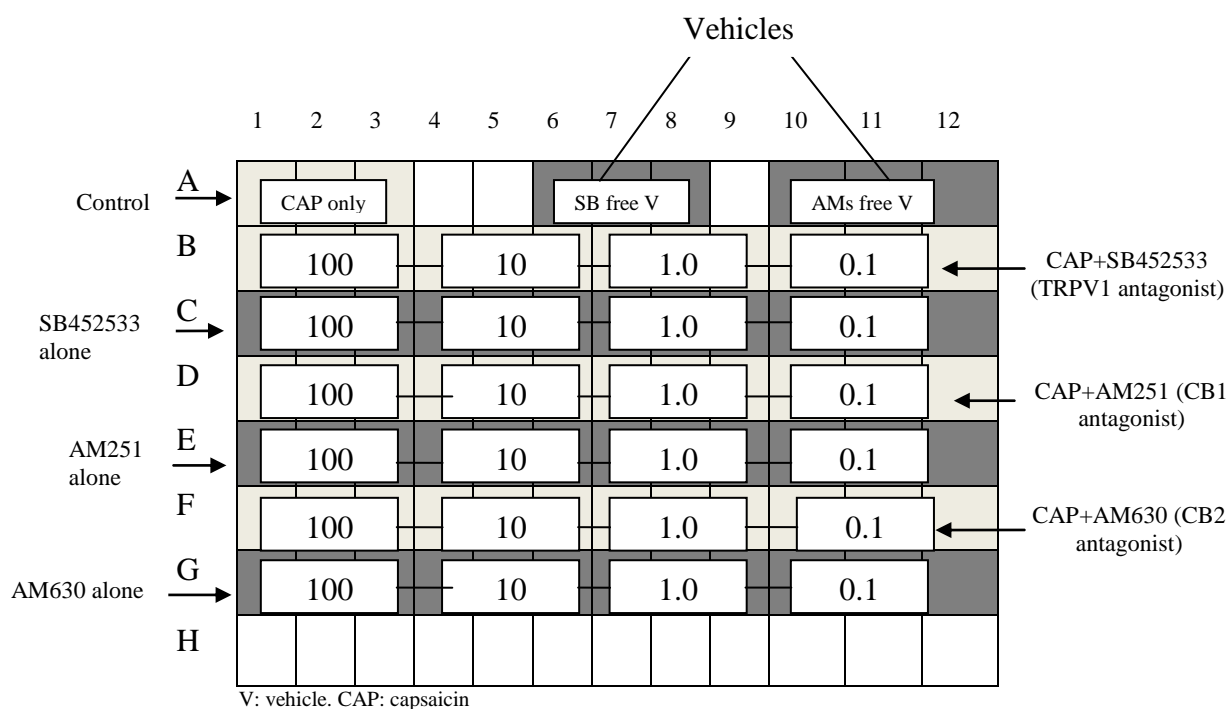
❖ **AM 251 and AM630 dilutions (1000 μ L):**



	997 μ L complete media+ 3.0 μ L AM251 or AM630	900 μ L AM dilution solution	900 μ L AM dilution solution	900 μ L AM dilution solution
Preparation concentration	300 μ M	30 μ M	3.0 μ M	0.3 μ M
Incubation concentration	100 μ M	10 μ M	1.0 μ M	0.1 μ M

Experiment procedures:

- In a 96 well microplate, add 100 μ L cell suspension + 50 μ L antagonist (all rows except row A).
 - For row A:
 - 1-3: add 50 μ L of complete media.
 - 6-8: add 50 μ L of SB free vehicle.
 - 10-12: add 50 μ L of AMs free vehicle.
- Incubate 30 min at 5% CO₂, 37°C.
- After the incubation, add 100 μ L of CAP to (B, D and F) wells. Add CAP free vehicle to the rest of wells → mix well and incubate for 22hr.
- Add 0.1 of the total volume of prewarmed Alamar blue reagent (25 μ L/250 μ L total volume) → incubate for another 2 hr → test at 540 excitation wavelength and 595 emission wavelengths.



2. Cell Cryopreservation Protocol

Freezing medium:

Cryopreservation freezing media composes of complete media + 5% (v/v) DMSO. After calculating the required quantity of DMSO, aspire by syringe and filter using syringe filter into a tube then add the media to the desired total volume. Use the formula:

$$(v/v)\% = [(volume\ of\ solute)/(volume\ of\ total\ solution)] \times 100\%$$

(Solution is not the solvent)

Cryopreservation Protocol:

1. Prepare a freezing medium consisting of a complete growth medium and 5% DMSO. Store between 2-8°C until use.
2. Measure total number of cells and viability percentage using a haemocytometer, cell counter and Trypan Blue exclusion, or the Countess[®] Automated Cell Counter. Calculate the required freezing medium volume depending on the viable cell density.
3. Collect cells by gentle centrifugation (10 min at 125 x g). Aseptically decant supernatant without disturbing the cell pellet and resuspend them in the freezing medium at a concentration of 1×10^6 to 5×10^6 viable cells per 1 ml.
4. Label the appropriate number of vials, then add 1 to 1.8 ml of the cell suspension to each vial (depending on the volume of the vial) and seal. As you aliquot the cell suspension, frequently and gently mix the cells to maintain a homogeneous cell suspension.
5. Allow cells to equilibrate in the freezing medium at room temperature for a minimum of 15 min and no longer than 60 min, as metabolic activity may decline by the DMSO.
6. Place the vials into a controlled-rate freeze chamber and place the chamber in a -70°C (or colder) mechanical freezer for at least 24 hr. Alternatively, use a programmable freezer unit set to cool the cryovials at -1°C per min until a temperature below -70°C is achieved.
7. Quickly transfer the vials to a liquid nitrogen or -130°C freezer. Frozen material will warm up at a rate of 10°C per min and cells will deteriorate rapidly if warmed above -50°C.
8. Record the location and details of the freezer.
9. After 24 hr, remove one cryovials from the liquid nitrogen; restore the cells in culture, and measure metabolic activity and sterility.

Notes:

1. Do not add undiluted DMSO to a cell suspension as dissolution of DMSO in aqueous solutions releases heat (exothermic).

2. Freeze cultured cells at a high concentration and low passage number as possible. Make sure the cells are at least 90% viable before freezing. Note that the optimal freezing conditions depend on the type of the cell line in use.
3. Always use sterile cryovials for storing frozen cells. Cryovials containing the frozen cells may be stored immersed in liquid nitrogen or in the gas phase above the liquid nitrogen (see **Safety Note** below).
4. All solutions and equipment that come in contact with the cells must be sterile. Always use proper sterile technique and work in a laminar flow hood.

Safety Note: Biohazardous materials **must** be stored in the gas phase above the liquid nitrogen. Storing the sealed cryovials in the gas phase eliminates the risk of explosion. If you are using liquid-phase storage, be aware of the explosion hazard with both glass and plastic cryovials and always wear a face shield or goggles.

References: <http://www.invitrogen.com/site/us/en/home/References/gibco-cell-culture-basics/cell-culture-protocols/freezing-cells.html>

www.atcc.org

3. Reagents used for Western blotting protocol :

Washing Solution: phosphate buffered saline-Tween20 (PBST) (1000mL):

0.5 mL of Tween 20+ 50 mL of PBS, then fill the water up to 1000 mL.

Blocking Solution: (3%BSA+5% NFM in PBST) (50 mL):

2.5g of NFM+ 1.5g of BSA in 50 mL of PBST.

Running Buffer: Biorad 10X Tris mix

Transfer Buffer (1 X): (25 mM Tris, 0.2 M glycine and 5% methanol)

100 mL 10 X Transfer solution+ 50 mL methanol+850 mL water.

Transfer Buffer (10X):

Tris: 25 mM, M.W: 121.14

Glycine: 0.2 M, M.W: 75.07 (Check the bottle). To prepare 1000mL of 10X of the transferring buffer:

30.3 g Tris + 150.1 g Glycine + water to 1000mL.

4X Laemli Buffer:

0.5 M Tris-Cl (2.5 mL)

SDS (0.4g)

Glycerol: 2 mL

Bromophenol Blue: 20mg

B-mercaptoethanol: 400uL

Water: to 10 mL. If the solution become yellow, so the PH< 6.8, add drops of 1.5 M Tris-HCL to return the colour to the blue.

Notes:

- Solutions should be fresh and cold, especially the transferring buffer.
- Blocking reagent should be sterile.
- Adjust the pH using HCl to increase acidity of Tris (pH=10).
- Rinse the wells with the running buffer using syringe and needle or pipette to remove excess acrylamide.
- When transferring overnight, set the 'amplification to constant' for even band transferring.
- More transferring time, bigger protein transferring will be successful.
- If using HRP, don't use sodium azide in any solution, because azide is an inhibitor for the HRP enzyme.
- When preparing the gel, ammonium persulfate (APS, oxidizing agent) should be fresh, because it only lasts for few days after reconstitution.

4. Flow cytometry Protocol:

Step	Details
Step 1	Label the tube according to the experiment design.
Step 2	Add the required volumes of the antibodies (according to package insert) carefully to the bottom of the tube.
Step 3	Add 1×10^6 cells to the each tube.
Step 4	Gently vortex to mix. Incubate the tubes in the dark, RT, 10 min.
Step 5	Add 2 ml of the RBCs lysing solution to the whole blood tubes only, vortex gently to mix. Incubate in the dark at room temperature for 5 min.

	Note: This incubation must NOT exceed 10 minutes.
Step 6	Centrifuge for 5 min on high (300 -350g), then remove supernatant. Wash with PBSA (3 ml/tube), centrifuge 3 min at 350g.
Step 8	Add 250µL/ tube of BD cytofix/cytoperm, 20 min at 4C.
Step9	Wash 2 x in saponin-based wash (1ml/tube/wash).
Step 10	Add 50µL of the blocking reagent. Incubate 15min, dark, RT.
Step 11	Centrifuge all Abs at 10,000 x g for 5 min, use ONLY the supernatant.
Step 12	Add primary Ab or isotype control, Mix gently. Incubate : 45 min, dark, 4° C.
Step 13	Wash 2x: add saponin-based wash (1ml/tube/wash), centrifuge for 3 min at 350 g on high. Remove supernatant, resuspend cells
Step 14	Add 50 µL of secondary Ab, (1/25, diluted in the saponin-wash, mix gently. Incubate 20 min, 4° C in the dark.
Step 15	Wash 2x: add saponin-based wash (1ml/tube/wash), centrifuge for 3 min at 350 g on high. Remove supernatant, resuspend cells
Step 16	Before RUN , add 2.5 ml PBS (Ca ²⁺ / Mg ²⁺ free).

Blocking preparation (500uL):

10% AB serum or goat serum: 10/100 * 500= 50uL.	0.05% sodium azide: <i>0.05/100* 5 ml= 0.0025 g= 2.5 mg.</i>
0.1% BSA: 0.1/100 * 5 ml= 0.005 g= 5mg.	<i>Then made up to 4.5 mL wash. Then add 50 µL of serum to 450 µL (0.1%BSA+0.05% sodium azide solution).</i>

Table 1: Attune cytometer channels and the associated fluorochromes

Channel	Detected CD-fluorochrome
BL1	2° TRPV1-FITC
BL2	CD19-PE, CD7-PE, CD33-PE, CD56-PE
BL3	CD45-PerCP, CD45-PE-Cys5
VL1	CD14-V450, CD3-BV421, CD13-BV421, CD13-BV421, CD20-BV421.
VL2	CD14-V500
VL3	CD38-BV605

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