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**An investigation of the potential anti-inflammatory  
properties of novel compounds with respect to LFA-1  
mediated T cell motility and cytotoxicity profiling.**

By

Jennifer P Coppins. MSc

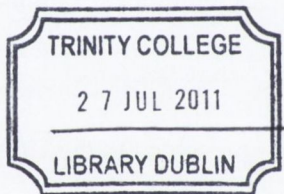
A thesis presented to the University of Dublin  
for the degree of Doctor of Philosophy

School of Pharmacy and Pharmaceutical Sciences

Trinity College

University of Dublin

2009.



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## **To my parents**

Romance fails us and so do friendships, but the relationship of parent and child, less noisy than all the others, remains indelible and indestructible, the strongest relationship on earth.

*Theodore Reik*



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## Abbreviations

AIF	Apoptosis inducing factor
ALPS	Autoimmune lymphoproliferative syndrome
ANOVA	Analysis of variance
APC	Antigen presenting cell
Bcl-2	B cell lymphoma 2 family
Ca <sup>2+</sup>	Calcium
CAD	Caspase activated deoxyribonuclease
Cdna	complement DNA
CPT	Camptothecin
DD	Death domain
DISC	Death inducing signalling complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DSS	Dextran sulfate sodium
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
Endog G	Endonuclease G
ERM	Ezrin, Radixin, Moesin proteins
FAC	Fluorescent activated cell sorting
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FLICA	Fluorochrome inhibitor of caspase

HC	Heavy chain
HEPES	N-2-hydroxyethylpiperazine-N'-2 ethanesulphonic acid
IAP	Inhibitors of apoptosis proteins
IBD	Inflammatory bowel disease
ICAMs	Intracellular adhesion molecules
IDDM	Insulin dependent diabetes mellitus
IFN-	Interferon gamma
IgSF	Immunoglobulin superfamily
IL	Interleukin
LDH	Lactate dehydrogenase
LFA-1	Leukocyte function associated antigen-1
Mg <sup>2+</sup>	Magnesium
Mg <sup>2+</sup>	milligram
MHC	Major histocompatibility complex
MPT	Mitochondrial permeability transition
Mrna	Messenger RNA
MS	Multiple sclerosis
MTOC	Microtubule organising centre
MTT	((3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide)
NF-Kb	Nuclear factor kappa B
NFQ	Non-fluorescent quencher
NK	Natural killer cell
NKT	Natural killer T cell
nm	nanometers
P/S	Penicillin / Streptomycin

p53	phosphoprotein
PCR	Polymer chain reaction
PI	Propidium iodide
PKC	Protein kinase C
PML	Progressive multifocal leukoencephalopathy
PMN	Polymorphonuclear phagocytes
PS	Phosphatidylserine
RFU	Relative fluorescent unit
RNA	Ribonucleic acid
ROS	Radical oxygen species
RPMI	Roswell Park Memorial Institute
SEM	Standard error mean
TCR	T cell receptor
TNBS	Trinitrobenzene sulphonic acid
TNFR	Tumor necrosis factor receptor
Tregs	Regulatory T cells
UNT	Untreated
UV	Ultra violet
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen

## **Declaration**

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

  
Jennifer Coppins

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## Summary

The aim of this thesis was to investigate the effects *in vitro* of novel compounds on LFA-1 mediated T cell motility, and, to develop a profile of the compounds with respect to cytotoxicity and apoptosis. The compounds evaluated in this work were categorised as those that are synthetic derivatives of the naturally occurring pharmacological products from the fern *Pteridium aquilinum*, which had previously been evaluated in animal models of inflammation, denoted with a prefix PH. Following a pilot study, the following were chosen for further investigation; 6C6, 6C6-OH, 7C9 and 7C17 based on activity demonstrated and to examine the potential with varying structures. PH 6 (6C6) had demonstrated significant efficacy in animal models of inflammation. A second group of compounds were isolated in house, known as quinones, and are denoted with a prefix HIH. This group of compounds were also assessed for their inhibitory properties in LFA-1 mediated T cell motility. The cytotoxicity of these compounds was restricted to investigation in MTT and LDH assays owing to limited quantities.

This thesis opens with an introduction that describes inflammation and autoimmunity with the emphasis on T cell involvement. The process of, and molecules involved in LFA-1 mediated T cell motility are also described. Cytotoxicity and apoptosis are introduced, and the involvement of apoptosis in the regulation of the immune system is also described.

A pilot study was performed and determined the utility of the LFA-1 mediated T cell motility assay as an appropriate model to evaluate drugs in an anti-inflammatory context with respect to T cell function. The pilot study demonstrated the significant effect of PH 11 and PH 12 as inhibitors of LFA-1 mediated T cell motility. The effect of the four main PH compounds studied in this work was determined firstly, in the LFA-1 mediated T cell motility assay 4 h post treatment, and the findings indicated PH 6 to be a significantly potent inhibitor. Furthermore, its effect was found to be dose dependent. Cytotoxicity experiments in HuT-78 and Jurkat T lymphocytes demonstrated PH 6 again to be the most significant drug. The cytotoxic effect was also found to be dose dependent, and more pronounced in Jurkat T lymphocytes. The toxicity of the test compounds was further investigated

with determination of Annexin V and PI (Propidium iodide) binding. PH 6 was found to induce apoptosis significantly in Jurkat T lymphocytes, and also HuT-78 T lymphocytes, which are resistant to apoptosis as a result of constitutively activated NF- $\kappa$ B. This fact indicated that PH 6 was mediating the apoptotic effect in an NF- $\kappa$ B independent manner. Caspase activity following treatment with PH 6 was then determined in a poly caspase FLICA assay and PH 6 was found to cause significant caspase activation. The expression of TNF- $\alpha$  mRNA was then determined and PH 6 was found to upregulate its expression. These results are discussed, in the context of inflammation and autoimmunity.

Based on the findings of this work, PH 6, HIH 6 and HIH 7 could be further investigated with respect to cytokine regulation and also mRNA expression of LFA-1 and ICAM-1 to determine the means by which these compounds mediate their effects.

The appendices include supplemental images acquired during the pilot study of LFA-1 mediated T cell motility, supplemental preliminary cytotoxicity experiments which were carried out in HMC-1 mast cells and A549 epithelial cells, and structures of the compounds used in the pilot study.



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# 1. Introduction

## 1.1 The immune system

The immune system mediates a resistance to infection and the invasion of pathogens through an assortment of cells, tissues and molecules which in a concerted manner provide defence mechanisms against foreign invaders (Abbas, Lohr et al. 2004). These defence mechanisms include the skin, tears, saliva, mucous layers, and pH controlled surfaces, as well as cellular responses. In the event of the protective surface defence mechanism failing to destroy the invading pathogen, the cellular response is mounted (Janeway et al., 2005).

### Innate immunity

The responses of the immune system can be classified as innate and adaptive, the former also being referred to as natural. They are always present in the healthy individual. The innate response is the first line of defence. Epithelia, phagocytes and natural killer (NK) cells, cytokines and plasma proteins, including those of the complement system, make up the components of the innate immune system. A physical barrier against microbes is provided by the epithelial cells. Neutrophils and monocytes are the main phagocytes recruited to the site of infection. They ingest microbes and secrete cytokines. NK cells function by killing cells infected by intracellular microbes and aid in the activation of macrophages by secreting IFN- $\gamma$ . The proteins of the complement system stimulate inflammation, lyse microbes, and opsonise microbes for phagocytosis (Abbas, Lohr et al. 2004).

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## Adaptive immunity

The adaptive response of the immune system, also known as specific or acquired immunity, is a defence mechanism that is mediated by the presence of microbial invaders. With each successive encounter with a microbe the adaptive response is more effective. The two types of adaptive immunity are humoral immunity and cell mediated immunity. The former is mediated by antibodies produced by B cells, and the latter by T cells. The role of the antibodies in humoral immunity is to neutralise extracellular toxins, while the T lymphocytes in cell mediated immunity function to eliminate intracellular microbes. The purpose of the adaptive immune response is long lived memory against immune insult. This is achieved by a series of phases. They are lymphocyte recognition of antigen, lymphocyte proliferation and differentiation induced by activation, and elimination of microbes followed by a decline in immune response (Abbas et al., 2006).

### **1.2 Inflammation**

Inflammation is a complex reaction of the innate immune system, involving the cells of the immune system. It is a local response to injury, at a local site (Watkins, Maier et al. 1995) the cardinal signs of which are redness, heat, swelling and pain. Distinguishable by duration and cells involved, inflammation is classified as acute or chronic (Lydyard et al., 2004), with acute inflammation being short in duration and resulting from the initial response by polymorphonuclear phagocytes (PMN). Chronic inflammation, on the other hand, is longer in duration and is the result of lymphocytes, macrophages and plasma cells responding to a persistent microbe. Both acute and chronic inflammation, result in some tissue damage. The main causes of

---

acute inflammation include microbial infection, trauma, allergens and autoimmunity (Lydyard et al., 2004).

Regulation of host responses to infection, immune responses and inflammation occurs by way of cytokine mediation. Those cytokines that function to make disease worse are referred to as pro-inflammatory. The cytokines that work to reduce inflammation and promote healing are known as anti-inflammatory (Dinarello 2000). Pro-inflammatory cytokines include IL-6, TNF- $\alpha$ , IL-12, IFN- $\gamma$ . Anti-inflammatory cytokines include IL-4, IL-10, and TGF- $\beta$  (Janeway et al., 2005).

### **1.3 Autoimmunity**

As mentioned above, the immune system is designed to react to, and prevent against foreign microbes. Distinction between self and non self antigens, by the immune system, is essential in the response to an invading pathogen. Self tolerance or immunologic tolerance occurs in the normal immune system, remains unresponsive to self antigens or autoantigens when lymphocytes are exposed to these autoantigens. This is known as *self tolerance* or *immunologic tolerance*. Autoimmunity occurs when there is a breakdown or failure of self tolerance (Sinha, Lopez et al. 1990; Kamradt and Mitchison 2001). Mechanisms are in place to maintain this tolerance of, or lack of immune response to, self antigens. It is when these mechanisms fail that the immune system responds by mounting an attack on the individual's own cells and tissues (Abbas et al., 2006).

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### **1.3.1 Mechanisms of self tolerance**

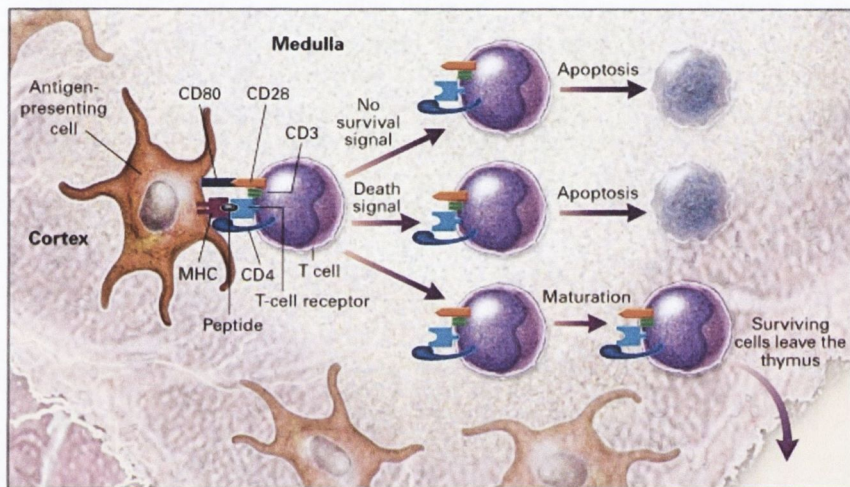
Various mechanisms have been proposed to describe how self antigens are tolerated by the immune system while maintaining an effective response to foreign pathogens (Chen, Benoist et al. 2005). The mechanisms of self tolerance can be categorised into central tolerance and peripheral tolerance. Central tolerance is the primary mechanism that leads to self tolerance and is maintained by clonal deletion of immature T cells in the thymus and immature B cells in the bone marrow. Peripheral tolerance involves several mechanisms that inhibit autoimmune diseases (Kamradt and Mitchison 2001; Abbas, Lohr et al. 2004).

Self tolerance involves T cells and B cells. T cells do not recognise T-independent antigens such as self polysaccharides, lipids and nucleic acids. The role of B cells in self tolerance comes onto play at this point to prevent the production of autoantibodies (Abbas et al., 2006). T cells have a prevailing role over B cells in self tolerance and autoimmunity.

#### **1.3.1.1 Central T lymphocyte tolerance**

Central tolerance is also known as thymocyte negative selection and entails the deletion by apoptosis of self reactive immature T cells in the thymus. When the immature T cells have relocated to the thymus from the bone marrow, they encounter self antigens. T cells can express receptors that recognise the self antigen with high or low affinity. Deletion occurs by apoptosis. Those T cells whose receptors have a low affinity for the antigen do not receive the signal to inhibit spontaneous apoptosis and therefore die in the thymus. T cells whose receptors, on the other hand, have a high affinity

for the antigen are also deleted by undergoing apoptosis. Maturation of T cells in the thymus with no deletion by apoptosis allows the residual T cells to migrate to the periphery. This is achieved when the T cells have receptors with neither high nor low, but an intermediate affinity for the antigen. This process is known as positive selection (Kamradt and Mitchison 2001; Chen, Benoist et al. 2005).



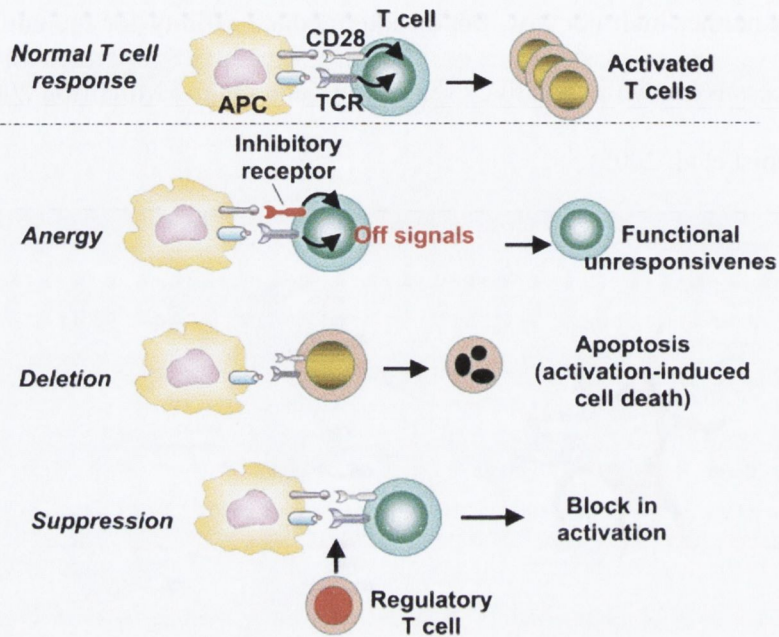
*Figure 1.1: (Kamradt and Mitchison 2001)*

The mechanism of central tolerance described above is inadequate in acting alone to prevent autoimmune events. The existence of self antigens and potentially pathogenic lymphocytes in the periphery lends itself to the suggestion of mechanisms of peripheral self tolerance (Kamradt and Mitchison 2001; Ballotti, Chiarelli et al. 2006).

### **1.3.1.2 Peripheral T lymphocyte tolerance**

Peripheral tolerance is the second line of protection against self recognition. Several mechanisms function to maintain tolerance in the periphery. Not just deletion, but also functional anergy and suppression by regulatory T cells

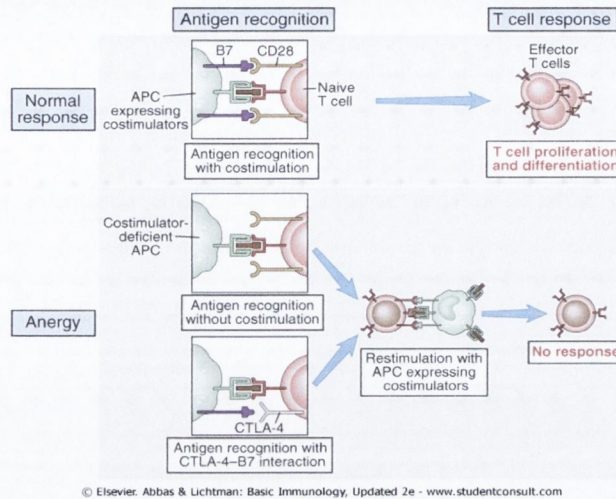
(Tregs), prevent the initiation of autoimmune diseases (Abbas, Lohr et al. 2004).



**Figure 1.2 : Mechanisms of peripheral T cell tolerance.**

The mechanisms that maintain tolerance in CD4 T cells are illustrated with antigen presenting cells (APC), T cells and activated T cells, and compared with a normal immune response (Abbas, Lohr et al. 2004)

T cells require the engagement of the T cell receptor (TCR) and co-stimulatory molecules for full activation. The functional inactivation of a lymphocyte, when it encounters an antigen in the absence of sufficient levels of co-stimulators required for full lymphocyte activation, is known anergy. In the anergic state, the T cells do not transcribe the gene that encodes interleukin 2 (IL-2), when stimulated again with appropriate amounts of antigen. This failure to produce IL-2 prevents T cells from proliferating and differentiating (Davis and Neriah 2004).



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**Figure 1.3:** An antigen presented by costimulator-expressing antigen presenting cells (APCs) induces a normal T cell response. If the T cell recognises antigen without costimulation, or in the presence of CTLA-4-B7 interactions, the T cell fails to respond and is rendered unresponsive even if the antigen is subsequently presented by costimulator-expressing APCs.

Abbas et al., 2006

Immune suppression involves different subsets of regulatory cells including NKT (natural killer T cells),  $CD8^+CD28^-$ , and  $\gamma\delta$  T cells, which prevent the activation of other autoreactive lymphocytes. Suppression can be exerted by the production of cytokines that can block activation or by mechanisms of cell to cell interactions (Abbas, Lohr et al. 2004).

There are numerous types of regulatory T (Tregs) cells. The subsets of Tregs that prevent activation are nTregs and iTregs. Of these two subsets, the prevailing importance lies with the nTregs, or  $CD4^+CD25^+$  T cells. This

naturally occurring subpopulation of T cells develops in the thymus and suppression by these cells is proposed to be mediated through cell to cell contact. iTregs, the CD4<sup>+</sup>CD25<sup>+</sup> counterpart of nTregs, occur extrathymically and the induction of these regulatory T cells occurs in the presence of certain cytokines or cells(Lan, Ansari et al. 2005; Ballotti, Chiarelli et al. 2006).

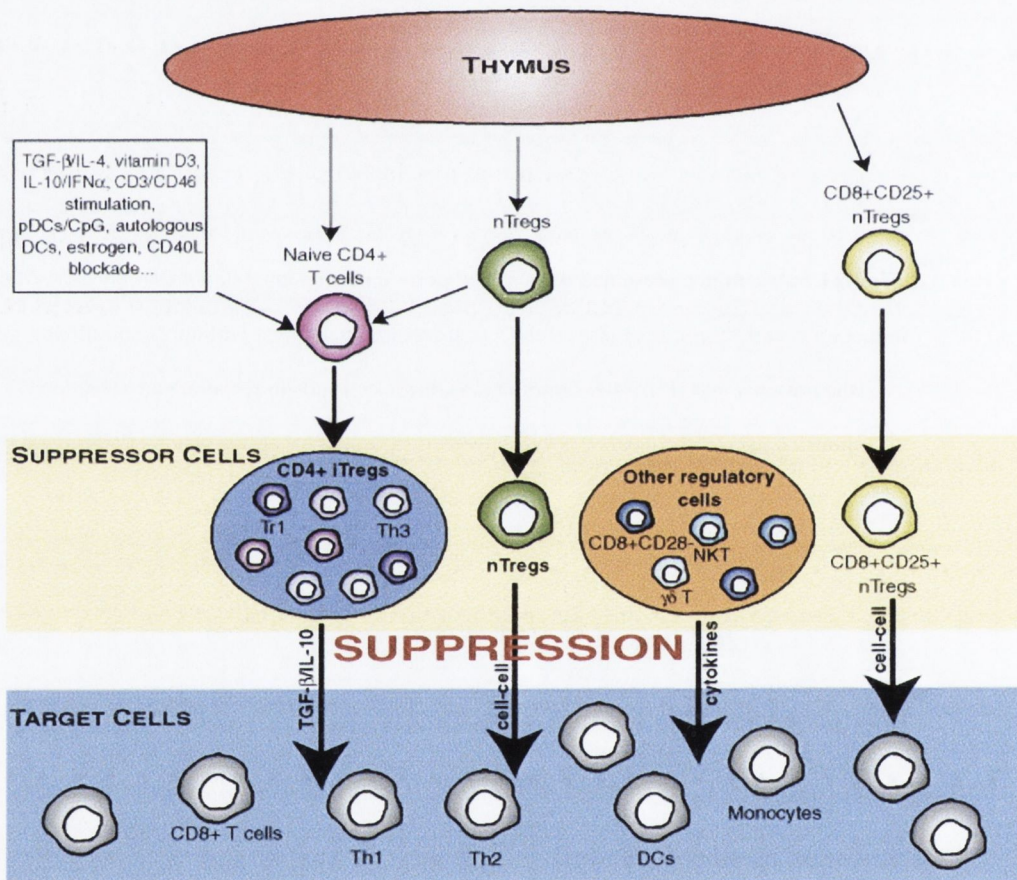


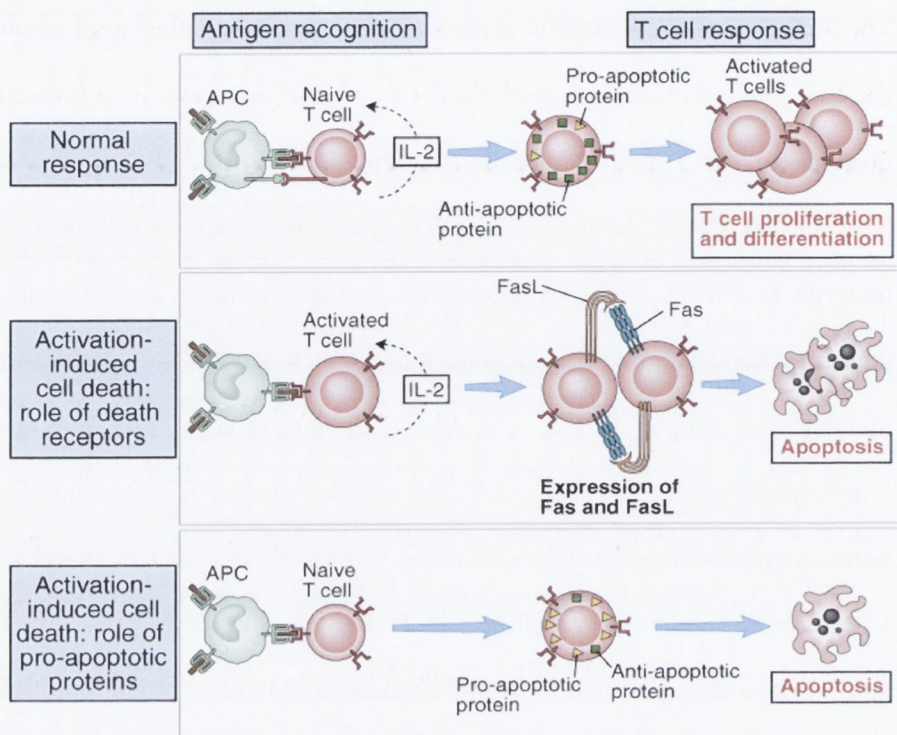
Figure 1.4: R.Y. Lan et al., Autoimmunity Reviews 4 351-363,2005

Deletion is the elimination of self reactive lymphocytes. As with the process of negative selection described earlier, deletion in the periphery also occurs through apoptotic pathways.



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The initiation of the apoptotic pathways is mediated by either engagement of the Fas receptor with its ligand (FasL) or by the induction of pro-apoptotic proteins in the T cells(Kamradt and Mitchison 2001). When interaction between Fas - FasL occurs, a sequence of events ensues, causing the cell to undergo apoptosis. These events include the activation of caspase cascades and mitochondrial alterations(Kamradt and Mitchison 2001). A failure in this mechanism to maintain self tolerance is demonstrated by the autoimmune lymphoproliferative syndrome (ALPS). In this instance, alterations in Fas induced apoptosis result from mutations in Fas sequences. The alterations in Fas induced apoptosis resulting in ALPS are also the cause of other autoimmune diseases such as multiple sclerosis (MS)(Ballotti, Chiarelli et al. 2006).



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**Figure 1.5: Activation-induced death of T lymphocytes.** T cells respond to antigen presented by normal APCs by secreting IL-2, expressing anti-apoptotic proteins, and undergoing proliferation and differentiation. In one form of activation-induced cell death, restimulation of recently activated T cells by antigen leads to coexpression of Fas and Fas ligand (FasL), engagement of Fas, and apoptotic death of the T cells. Note that FasL on one T cell may engage Fas either on a neighboring cell (as shown) or on the same cell. Fas-independent activation-induced cell death may also occur when antigen recognition by T cells without costimulation or innate immunity leads to expression of intracellular pro-apoptotic proteins. Abbas et al., 2006

### 1.3.1.3 B lymphocyte tolerance

Tolerance breakdown can occur as a result of the activation of either T or B cells. Self tolerance involves T cells and B cells. T cells do not recognise T-independent antigens such as self polysaccharides, lipids and nucleic acids. The role of B cells in self tolerance comes onto play at this point to prevent

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the production of autoantibodies (Abbas et al., 2006). As with T cell tolerance there are central and peripheral B cell tolerance mechanisms to filter autoreactive B cells out of the B cell repertoire (Kamradt and Mitchison 2001).

#### **1.3.1.4 Central B lymphocyte tolerance**

The interaction of immature B lymphocytes in the bone marrow with self or autoantigens results in the clonal deletion of the B cell, or with receptor editing. The process of deletion occurs by apoptosis as in the T cell repertoire (Kamradt and Mitchison 2001). Receptor editing is a change in receptor specificity. Rearrangements and expression of the genes of the heavy chain (HC) of the B cell receptor changes the specificity of the receptor for antigen. Receptor editing allows the cell to interact with the antigen which in turn allows for the events of negative (deletion by apoptosis) or positive selection. Positive selection occurs in B cells that express an intermediate level of signals. Cells escaping negative selection and undergoing positive selection, enter the periphery (Ballotti, Chiarelli et al. 2006).

#### **1.3.1.5 Peripheral B lymphocyte tolerance**

Again, as with the T cell repertoire, B cell tolerance is maintained in the periphery by the mechanism of anergy. Having escaped the fate of deletion in the bone marrow, B cells that have migrated to the periphery with a lower affinity to membrane – bound self antigens undergo transition to an anergic or indifferent state. This transition is dependent on the availability of T cell help ((Miller and Basten 1996). In the presence of T cell help the B cells become activated. In their absence the B cells become either anergic or

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activated by T independent antigens that trigger strong signals in B cells. Anergic autoreactive B cells are excluded from lymphoid follicles and as such are more likely to undergo apoptosis as a result of a lack of survival stimuli (Ballotti, Chiarelli et al. 2006).

#### **1.4 LFA-1 mediated T cell motility**

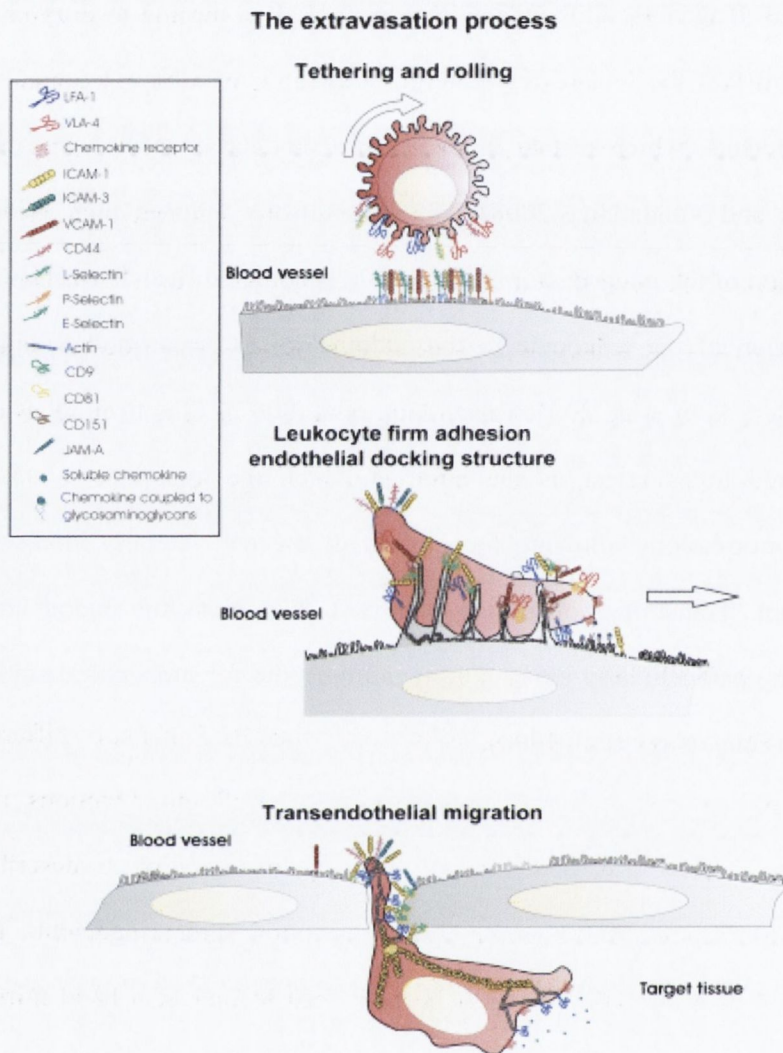
The migration of cells from the blood into peripheral tissues acts as an essential part of the immune response by facilitating the participation of cells in both immune surveillance and host defence (Rose, Alon et al. 2007). This “trafficking” of cells across the vascular endothelium comprises a multistep process that is highly controlled and is mediated by the collective effects of adhesion and signalling molecules (Butcher 1991; Springer 1994).

Three main molecular families of adhesion proteins coordinate to form an integrated adhesion network to facilitate leukocyte adhesion. The three families are namely the intracellular adhesion molecules (ICAMs), the integrins (CD11/CD18) and the carbohydrate binding L-, E- and P-selectins (Kotovuori, Tontti et al. 1993).

Also known as extravasation, this multistep adhesion cascade is initiated when leukocytes circulating the blood interact with the endothelium, or platelets attached to the endothelium. (**Figure 1.6**) With the transitory contact between the leukocyte selectins and their vascular ligands, or subsets of leukocyte integrins and their ligands of the immunoglobulin superfamily, in the presence of shear flow, the cells begin to roll along the endothelium (Barreiro, Fuente et al. 2007; Rose, Alon et al. 2007). As the leukocyte

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begins rolling along the endothelium, the contact time between the selectins and integrins lengthens, allowing tethering of the cell to the endothelium. It is at this point that the leukocytes undergo activation, typically triggered by chemoattractants, which results in the *in situ* modulation of the integrins (Woodside and Vanderslice 2008). The subsequently induced high avidity conformation of the integrins for their specific endothelial ligands establishes firm adhesion of the leukocyte to the endothelium (Carman and Springer 2003; Luster, Alon et al. 2005; Rose, Alon et al. 2007). This firm adhesion, also referred to as arrest, is the point at which the leukocyte adopts a polarised morphology allowing locomotion of the cell laterally along the endothelium. Transmigration of the polarised cell across the endothelium then occurs, paracellularly being the prevailing route for most extravasation processes (Feng, Nagy et al. 1998).



**Figure 1.6 : Key migratory steps of immune cells at sites of inflammation**

Tethering and rolling of the cell along the endothelium. Activation by chemokine signal. Integrin and ligand binding causing docking of the cell and arrest. Polarisation of the leukocyte followed by diapedesis (Barreiro, Fuente et al. 2007).

Networks of intracellular signalling co-ordinate the repeated cycles of cytoskeletal rearrangement, that is characteristic of actively migrating cells, thereby facilitating the net translocation of the cell contents (Volkov, Long et al. 2001). These intracellular signalling networks are based largely on phosphorylation - dependent cascades integrated by GTPases of small

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molecular weight, including protein kinase C (PKC) (Volkov, Long et al. 1998). The PKC family of isoforms is subdivided, according to enzymatic properties, into the classical  $\alpha$ ,  $\beta$  and  $\gamma$ , the novel  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$  and  $\mu$  and the atypical  $\zeta$  and  $\lambda/\iota$  (Nishizika 1992). With respect to T cells, which express the isoforms  $\alpha$ ,  $\beta$  (I),  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$  and  $\theta$ , PKC plays an important functional role in motility, through the cytoskeleton (Thorp, Verschueren et al. 1996). During LFA-1 induced T cell locomotion, PKC  $\beta$  (I) and  $\delta$  translocate, to the centrosome and microtubule-rich tail of the polarised cell, and close to the organising centre (MTOC), respectively (Fanning, Volkov et al. 2005).

#### **1.4.1 The actin cytoskeleton**

The reorganisation of the actin cytoskeleton is involved at many different points during signaling, migration, and execution of effector functions, and controls the shape of T cells. During the key steps of migration, as described earlier, the actin cytoskeleton undergoes substantial rearrangement. The morphology of a migrating cell has been likened to that of a hand mirror, with a broad leading edge that is enriched in chemokine receptors such as CCR2 and CCR5, a large cell body and narrow trailing uropods (Nieto, Frade et al. 1997; Wei, Tromberg et al. 1999). Contained in this trailing uropod are different cytoskeletal elements including the microtubule organising centre (MTOC), actin filaments, ERM (ezrin, radixin, moesin) proteins, along with the cytoskeletal linker protein known as plectin (Brown, Hallam et al. 2001), that work in concert to produce a movement that is, in essence, amoeboid-like (Jacobelli, Chmura et al. 2004).

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The leading edge of the migrating cell also contains the activated integrins that are involved in the attachment to the substratum of the leading pseudopodal projections. The rear of the moving cell, the trailing uropod, holds the MTOC, as mentioned above, and it is here that de-adhesion occurs to facilitate the translocation of the cell and retraction of the trailing uropod, a process indicated to be the result of de-activation of integrins (Vicente-Manzanares, Sancho et al. 2002).

To elaborate on the above introduction to cell migration, and in the context of the work carried out for this thesis, the adhesion molecules involved in the migratory process will be reviewed in more detail, with the emphasis being on those molecules involved specifically in the assay performed, that is, LFA-1 and ICAM-1.

#### **1.4.2 Adhesion molecules**

The molecules that participate in the extravasation process are collectively known as adhesion molecules. Expressed on all cell surfaces, adhesion molecules are glycoproteins that are involved at each point of the extravasation process by mediating the contact between two cells, homotypically or heterotypically, and the contact between cells and the extracellular matrix (Carlos and Harlan 1994; Gumbiner 1996). These adhesion molecules are categorised, according to their structural characteristics and functions, into families namely cadherins, integrins, selectins and the immunoglobulin superfamily (Bochner 1997; Henricks and Nijkamp 1998; Petruzzelli, Takami et al. 1999).



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#### **1.4.2.1 Cadherins**

Cadherins are a family of cell adhesion receptors,  $\text{Ca}^{2+}$ -dependent in nature, which bind cells together by haemophilic interactions. They fall into groups of classic cadherins and subfamilies of structurally related proteins. Classic cadherins contain a single protein chain, spanning the cell membrane. The external domain of this protein chain contains motifs bridged by  $\text{Ca}^{2+}$  (Petruzzelli, Takami et al. 1999).

#### **1.4.2.2 Selectins**

Selectins are protein receptors that are divided into three families based on the cell type on which they are expressed. L-selectin is expressed by leukocytes, P-selectin on both platelets and endothelial cells, while expression of E-selectin occurs on just endothelial cells (Stefanelli, Malesci et al. 2008). Selectins are involved in the early stages of cell adhesion, facilitating the attachment of leukocytes to cells of the endothelium, by interacting with their sialyl-Lewis X-like carbohydrate ligands, which are presented by sialomucin-like surface molecules (Luster, Alon et al. 2005). Regulation of selectin and selectin ligand adhesion is affected by their topographical distribution, shedding, and also associations with the actin cytoskeleton (Hafezi-Moghadam, Thomas et al. 2001). L-selectin is expressed on all leukocytes and is upregulated by the pro-inflammatory cytokine  $\text{TNF-}\alpha$  (Stefanelli, Malesci et al. 2008).

#### **1.4.2.3 Integrins**

Representing a large family of transmembrane glycoproteins, integrins consist of two noncovalently associated heterodimers known as  $\alpha$  and  $\beta$  subunits. It is the association of these two subunits in the presence of divalent

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cations that is vital for ligand binding. Subdivision of this family of adhesion molecules, on the basis of the type of  $\beta$  subunit expressed yields various integrin subfamilies. Those subfamilies with  $\beta_1$ ,  $\beta_2$  and  $\beta_7$  chains and those with  $\alpha_4$  chains are of particular relevance to leukocyte migration, especially  $\beta_2$  integrins (Panés 1999; Luster, Alon et al. 2005).

From the repertoire of more than 20 integrins that have been identified to date, leukocytes are known to express 13, and just five of these are major contributors to leukocyte-endothelial adhesion (Luster, Alon et al. 2005).

Integrins of the  $\beta_1$  (CD29) subfamily, which result from the binding of the  $\beta_1$  chain to variable  $\alpha$  chains, participate in tissue organisation (Henricks and Nijkamp 1998), and the  $\alpha_4\beta_1$  (very late antigen-4 /VLA-4) heterodimer is one of the five main modulators in adhesion. VLA-4 mediates the homing of a subset of lymphocytes that reside in the gut, to Peyer's patches, and also participates in the adhesion of lymphocytes, monocytes, eosinophils and natural killer cells to endothelial cells stimulated by cytokines. VLA-4 is not present on neutrophils (Henricks and Nijkamp 1998; Ben-Horin and Bank 2004).

Members of the  $\beta_2$  subfamily are also referred to as leukocyte integrins due to their expression being a phenomenon solely associated with this cell type. The common  $\beta_2$  subunit (CD18) of these integrins binds to one of three  $\alpha$  subunits CD11a, CD11b, or CD11c yielding CD11a/CD18 (LFA-1; lymphocyte function associated antigen-1), CD11b/CD18 (myeloid-specific integrins Mac-1) and CD11c/CD18 (gp150, 95). The contact between leukocytes expressing  $\beta_2$  integrins and various target cells is mediated through intracellular adhesion molecules (ICAMs) expressed on the target

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cells, namely ICAM-1, 2 or 3 (Stefanelli, Malesci et al. 2008). The interaction of CD11a/CD18 (LFA-1) with the intracellular adhesion molecule-1 and 2, and CD11b/CD18 with ICAM-1 on endothelial cells places them as two more of the main modulators of adhesion previously mentioned. (Henricks and Nijkamp 1998; Barreiro, Fuente et al. 2007; Stefanelli, Malesci et al. 2008). Given the specific involvement of the integrins LFA-1 in the study performed for this thesis, it is reviewed in more detail below.

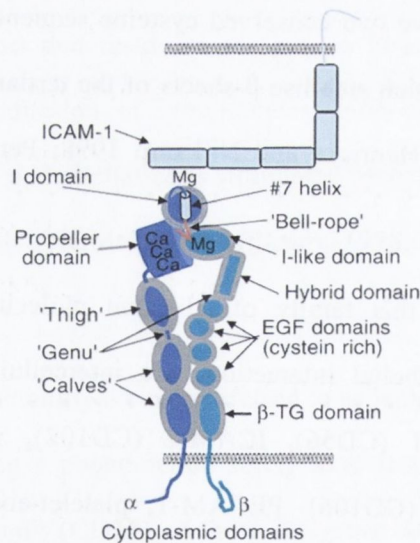
#### **1.4.2.4 Immunoglobulin superfamily**

Exhibiting variety both structurally and functionally, members of the immunoglobulin (Ig) superfamily (IgSF), act as ligands for the leukocyte integrins (Petruzzelli, Takami et al. 1999; Anderson and Siahaan 2003). These cell surface proteins are characterised by multiple extracellular Ig-like domains, which have two conserved cysteine sequences, separated by 55 to 75 amino acids, which stabilise  $\beta$ -sheets of the tertiary structure by forming disulphide bonds (Henricks and Nijkamp 1998; Petruzzelli, Takami et al. 1999).

The members of this family of adhesion molecules that participate in leukocyte – endothelial interactions are intercellular adhesion molecules (ICAMs), ICAM-1 (CD56), ICAM-2 (CD102), vascular cell adhesion molecule VCAM (CD106), PECAM-1; platelet-endothelial cell adhesion molecule-1 (CD31) and MAdCAM-1 which is the mucosal addressin -1 (Panés 1999). The adhesive interactions of these proteins with their integrins are dependent on both the leukocyte and endothelial cell involved.

### 1.4.3 Structural interaction of LFA-1 / ICAM-1

As mentioned previously, LFA-1 is a cell surface receptor which belongs to the  $\beta_2$  family of integrins, comprised of  $\alpha$  and  $\beta$  subunits. The I (inserted)-domain contained in the  $\alpha_L$  - subunit of LFA-1, is fundamental for the binding of this integrin to its ligand ICAM-1 (Lu, Shimaoka et al. 2001). The I-domain x-ray structure has shown a di-nucleotide binding fold of a central  $\beta$ -sheet, with 7  $\alpha$ -helices surrounding it. The N-terminus of the  $\alpha$ -subunit, consisting of a  $\beta$ -propeller structure made up of 7  $\beta$ -sheets, sits below the I-domain, and is connected to the I-domain by a hinge sequence which facilitates movement (McDowall, Leitinger et al. 1998; Ma, Shimaoka et al. 2002). Also present on the  $\beta_2$  subunit of LFA-1, near the N-terminus, is a  $\beta$ I-like domain, which is structurally homologous to the  $\alpha_L$ -subunit.



**Figure 1.7: Structural model of LFA-1.** The position of the C-terminal  $\alpha$ -helix, shown above as the blue cylinder of the I domain, controls ligand binding affinity. The red tail (peptide) associated with the helix connects the seventh helix of the I domain to the propeller domain. Conformational changes in the integrin are transmitted to the ligand-binding site by the peptide. Ligand-binding status and overall ligand-binding affinity may be controlled by the angle between the hybrid domain and I-like domain. (Dustin, Bivona et al. 2004)

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#### **1.4.3.1 Activation of LFA-1**

For activation of LFA-1 to happen both conformational changes of the integrin and receptor clustering occur (Hynes 2002; Carman and Springer 2003; Dustin, Bivona et al. 2004). One of two mechanisms can activate LFA-1; inside out signaling and outside in signaling. The former process is produced upon induction of secondary messengers by activated cell surface molecules such as TCR or chemokine receptors (Hogg, Laschinger et al. 2003), and is also known as priming.

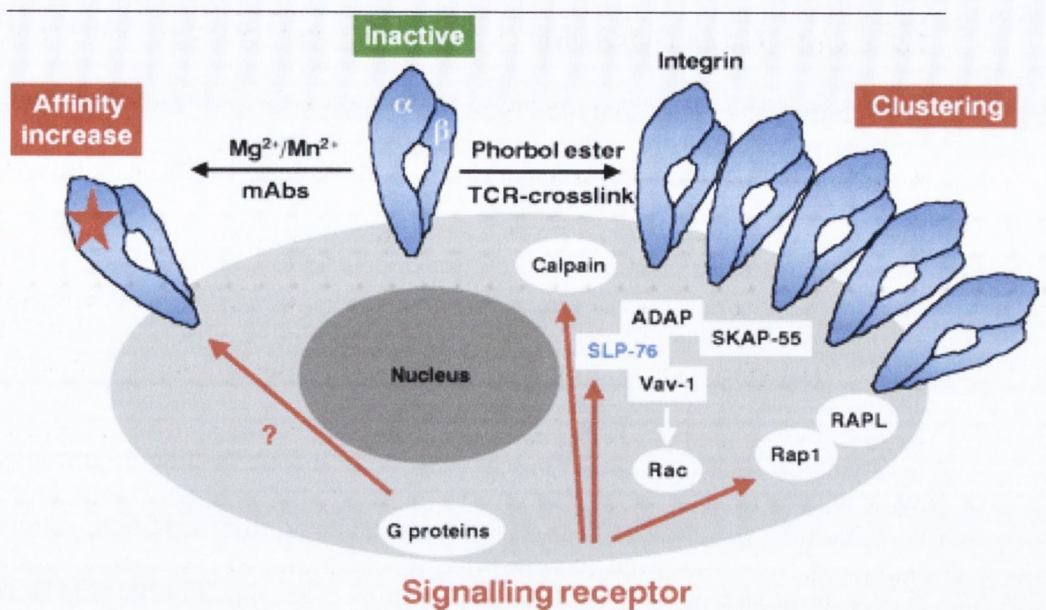
The latter process of outside-in signaling occurs in response to external or “outside” stimuli, such as ICAM-1 or activating antibodies, which results in a signalling cascade in the cytoskeletal region of the cell which results in activation of the tyrosine kinase  $\zeta$ - associated protein-70 (Zap-70), that in turn causes the formation of clusters resulting from the activation of other LFA-1 molecules (Soede, Driessens et al. 1999). *In vitro*, as in the work carried out for this thesis using monoclonal antibodies, outside-in activation occurs and increased affinity of LFA-1 for its ligand ICAM-1 is observed.

Integrin activation occurs from either the clustering, also known as avidity, of the cell surface integrins or a conformational change which results in high affinity for the ligand (van Kooyk and Figdor 2000). When the structure of LFA-1 undergoes a conformational change, from a low affinity state, where adhesive contacts are not formed by cells in circulation, to a high affinity state, whereby the I-domain of the integrin goes from a closed to an open form, ligand binding is possible (Ma, Shimaoka et al. 2002).

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There are two suggested divalent cation binding sites present on both the  $\alpha_L$ - and  $\beta_2$ - subunits of LFA-1, namely binding sites for  $Ca^{2+}$  and  $Mg^{2+}$  ions which work together to allow a change in affinity of LFA-1 (Binnerts and van Kooyk 1999). Low affinity LFA-1, or closed conformation, is a result of the displacement of  $Mg^{2+}$  from its site in the presence of high concentrations of  $Ca^{2+}$ . The high affinity form of LFA-1 is the result of  $Mg^{2+}$  stimulation extracellularly, and a conformational change in the integrin then occurs (Labadia, Jeanfavre et al. 1998). Fittingly, for the rapid formation of adhesions and then de-adhesion during migration, LFA-1 maintains a relatively low affinity for its ligand ICAM-1, even when activated (Woska, Morelock et al. 1996).

Following the conformational change, binding of LFA-1 to ICAM-1 occurs via the I-domain of LFA-1 and the D1 domain of ICAM-1, D1 referring to the immunoglobulin-like domain number on the transmembrane glycoprotein, ICAM-1.



**Figure 1.8 :Integrin activation.** Integrins are activated by either clustering of the integrins on the cell surface, or increased affinity for the ligand resulting from conformational changes. Experimentally, the above states are achieved by the use of activating mAbs, binding of  $Mg^{2+}$  and  $Mn^{2+}$ , or by treatment with phorbol ester or cross linking of TCR. (Hogg, Laschinger et al. 2003).

#### 1.4.4 Therapeutic implication of inhibiting motility

The blockade or modulation of the expression of adhesion molecules provides a potential strategy for the treatment of inflammatory conditions by reducing the number of cells that migrate to the site of injury.

The inhibition of the LFA-1 / ICAM-1 interaction provides a therapeutic target in terms of inflammation and autoimmunity as when this interaction is interrupted or modulated, the activated T cell can no longer participate in the immune response (Anderson and Siahaan 2003).

Various methods of modulating or inhibiting the LFA-1 / ICAM-1 interaction have been developed including antibodies, peptides and small molecules

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## 1.5 Apoptosis and Cell death

It was during the 1970s that the discovery of new patterns of cell death led to the arrival of apoptosis as a concept (Kerr, Whyllie et al. 1972).

Kerr et al were the first to use the term apoptosis to describe this essential physiological process of cell death. As a programmed form of cell death, apoptosis, which comes from Greek with *apo* = for, *ptosis* = falling, is a tightly controlled intracellular process involving the eradication of unwanted cells, thereby fulfilling critical roles in development, differentiation, cellular homeostasis and pathophysiological processes (Sun and Shi 2001; O'Brien and Kirby 2008).

Cell death can occur by either necrosis or apoptosis. Cells that are damaged by external injury die by the process of necrosis and those cells that undergo the programmed cell death of apoptosis can be induced to do so by either external and internal stimuli (Ghobrial, Witzig et al. 2005).

Necrosis, on the other hand, is characterised by rapid swelling of the cell, disruption of the plasma membrane and its organelles, resulting in cell lysis. An inflammatory reaction can subsequently be observed, caused by the release of the cell contents into the surrounding tissue (Proskuryakov, Konoplyannikov et al. 2003; Vanden Berghe, Kalai et al. 2006). However, in the event of the apoptotic bodies escaping phagocytosis *in vivo*, or as is the case *in vitro*, where there are no cells to mop up the apoptotic debris, a process known as secondary necrosis is observed with the apoptotic bodies swelling, subsequent loss of density leading to rupture of the membrane with the content of organelles being released, as in necrosis (Power, Fanning et al. 2002).



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The morphological features of apoptosis, at the early stages, include cell shrinkage with the organelles becoming more closely packed and the cytoplasm becoming more dense. Chromatin condensation also occurs, which is one of the most characteristic features of apoptosis. Membrane blebbing also occurs and is followed by the formation of apoptotic bodies during “budding” where the cell disintegrates into tightly packed organelles. These apoptotic bodies made up of cytoplasm and compacted organelles can occur either with or without nuclear fragments. Lack of adhesion and rounding are also associated with apoptosis (Elmore 2007). Biochemical alterations indicative of apoptosis are DNA fragmentation, protein cleavage at specific locations, the mitochondrial membrane potential is increased, and phosphatidylserine (PS) appears on the cell membrane (Hengartner 2000).

### **1.5.1 Pathways of apoptosis**

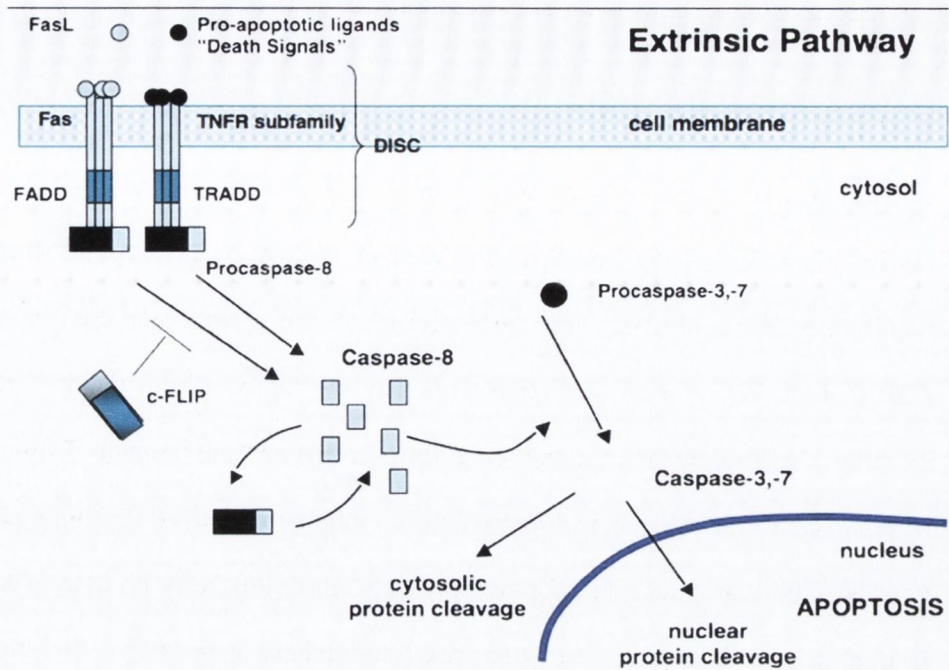
The sequences of energy dependent molecular events that result in apoptosis are highly complex and sophisticated. Two independent pathways have been identified as the main signalling pathways by which apoptosis occurs (Strasser, O'Connor et al. 2000). The extrinsic pathway involves the activation of death receptors and is therefore also known as the death receptor pathway and the intrinsic or mitochondrial pathway involves the release of the apoptogenic factors cytochrome c and AIF (apoptosis inducing factor) from the mitochondria into the cytoplasm. When these pathways are activated the end point results with their convergence on the activation of caspases which execute the final stages of apoptosis (Strasser, O'Connor et al. 2000; Elmore 2007).

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### **1.5.1.1 Extrinsic pathway**

Known also as the death receptor pathway and cytoplasmic pathway, the extrinsic pathway is initiated through the ligation of the Fas (Apo-1) death receptor. As a member of the tumour necrosis factor receptor (TNFR) gene superfamily, the Fas receptor contains cyteine rich extracellular domains. The cytoplasmic or intracellular domain is referred to as the “death domain” (DD) of this receptor and consists of approximately 80 amino acids (Wajant 2002; Ghobrial, Witzig et al. 2005; Elmore 2007). The role of the death domain is to transmit the death signal to the intracellular signalling pathways from the cell surface and when this pathway is triggered (Elmore 2007).

Formation of a death inducing signalling complex (DISC) results from the interaction of the FasL with inactive Fas complexes (Ghobrial, Witzig et al. 2005). The Fas complex comes about when the receptors cluster together and bind with the homologous trimeric ligands. Cytoplasmic adaptor proteins subsequently recruited exhibit the corresponding death domains to which the receptors bind (Elmore 2007).



**Figure 1.9: The extrinsic pathway.** Also known as the death receptor pathway. Apoptosis is initiated by the effector caspases 3 and 7 which are activated when initiator caspase 8 releases active caspase enzyme into the cytosol. (O'Brien and Kirby 2008).

### 1.5.1.2 Intrinsic pathway

The intrinsic or mitochondrial pathway occurs when the cell is exposed to stresses or injury such as DNA damage, ROS (radical oxygen species), radiation, cytokines, chemotherapeutic agents, hormone or growth factor deprivation and glucocorticoids, which act independently of the receptors. Mitochondrial events are initiated when targets within the cell are acted on by intracellular signals resulting from these non-receptor-mediated stimuli (Zimmermann, Bonzon et al. 2001; Elmore 2007). The effect of these stimuli is seen with the alteration of the inner mitochondrial membrane which causes the mitochondrial permeability transition (MPT) pore to open. Subsequently, a loss of mitochondrial transmembrane potential is observed that facilitates

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the release of pro-apoptotic proteins into the cytosol from the mitochondria (Saelens, Festjens et al. 2004).

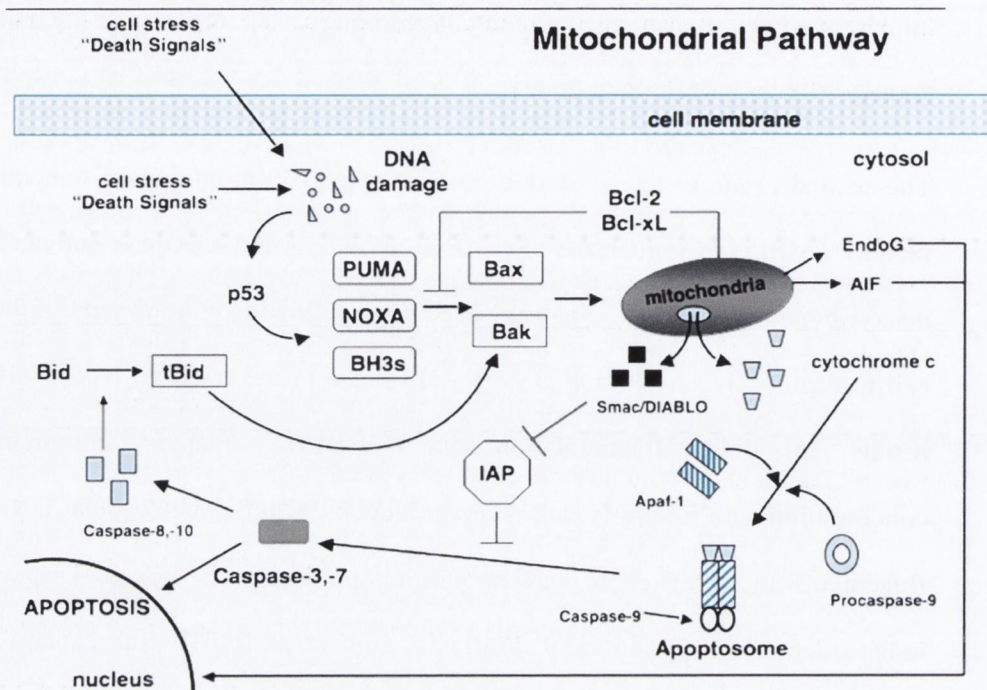
Membrane permeability is controlled by the B cell lymphoma-2 (Bcl-2) family members of proteins that are present on the outer mitochondrial membrane. By controlling the permeability of the membrane, the Bcl-2 family members in turn determine whether the apoptogenic factors are released into the cytosol through creation of pores or ion channels (Minn, Velez et al. 1997; O'Brien and Kirby 2008). Included in the Bcl-2 family are both pro-apoptotic and anti-apoptotic proteins. Bax, Bak, Bcl-Xs, Bid, Bik, Bim, and Hrk are some of the pro-apoptotic members, while Bcl-2, Bcl-X<sub>L</sub>, Bcl-W, Bfl-1 and Mcl-1 are some of the anti-apoptotic members. It is the fine balance between pro and anti-apoptotic Bcl-2 proteins that regulates whether the cell commits to apoptosis, and places the Bcl-2 family of proteins of high importance in the regulation of this pathway (Ghobrial, Witzig et al. 2005; Elmore 2007).

Of the apoptogenic factors released from the mitochondria there are two of the main groups of proteins. Firstly, cytochrome c, Smac /DIABLO, and the serine protease HtrA2, also known as Omi, (van Loo, van Gurp et al. 2002; Ghobrial, Witzig et al. 2005; O'Brien and Kirby 2008). The effect of the cytochrome c release is seen with the activation of pro-caspase 9, which occurs following interaction of Apaf-1 with cytochrome-c which combines with pro-caspase 9, forming an apoptosome. The apoptosome then in turn activates caspase-3, followed by the activation of the caspase cascade that results in apoptosis. The pro-apoptotic effect of the Smac/DIABLO and HtrA2/Omi proteins is reported to be a result of the inhibition of the

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inhibitors of apoptosis proteins (IAP) (Schimmer 2004; O'Brien and Kirby 2008).

The second group of pro-apoptotic proteins released are apoptosis inducing factor (AIF), endonuclease G (Endog G), and caspase-activated deoxyribonuclease (CAD). Their release occurs following commitment of the cell to death. AIF and Endog G both translocate to the nucleus, where AIF results in DNA fragmentation and peripheral nuclear chromatin condensation, and Endog G causes the production of oligonucleosomal DNA fragments by cleavage of nuclear chromatin. Their effects are caspase independent (Joza, Susin et al. 2001; Li, Luo et al. 2001). Subsequently, mitochondrial release of CAD and translocation to the nucleus allows cleavage of caspase-3. Oligonucleosomal DNA fragmentation then occurs with a more pronounced chromatin condensation than that caused by AIF(Enari, Sakahira et al. 1998).



**Figure 1.10: The intrinsic pathway.** Also known as the mitochondrial pathway, as apoptosis is initiated intracellularly. Cytochrome c released from the mitochondria forms an apoptosome with Apaf-1 and pro-caspase 9. Activation of caspases 3 and 7 follows activation of caspase 9 and apoptosis occurs. Caspase independent apoptosis occurs via stimulation by EndoG and AIF. (O'Brien and Kirby 2008).

### 1.5.1.3 p53

As mentioned above it is the Bcl-2 family of proteins that control the permeability of the membrane. Regulation of this family is by the tumor suppressor phosphoprotein p53, whose function as a transcription factor is to regulate downstream genes that are not only important in apoptosis, but cell cycle arrest and DNA repair as well (Ghobrial, Witzig et al. 2005). p53 also functions as a stress sensor that is capable of inducing the intrinsic pathway (O'Brien and Kirby 2008). Promotion of apoptosis by p53 occurs by way of the suppression of the transcription of the anti-apoptotic factors such as Bcl-

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2. Alternatively, it can also promote apoptosis by up regulating the Fas receptor (Bauer and Helfand 2006).

### **1.5.2 Caspases**

As cysteine-dependent aspartate-specific proteases, caspases have a critical role in the process of apoptosis, through their participation in both the signal transduction and execution of the process (Yi and Yuan 2009).

It is as inactive latent pro-forms as precursor zymogens, that caspases are present in the cell. Expressed as these proenzymes, caspases are composed of three domains; an N-terminal prodomain, a large subunit containing the active site, and a small C-terminal subunit, also known as the catalytic subunit (Zimmermann, Bonzon et al. 2001; Chowdhury, Tharakan et al. 2008).

Based on their preference for substrate, the extent of sequence identity and the structural similarities, caspases are divided into subfamilies. These subgroups are the initiator caspases, the executioner caspases and those caspases involved in cytokine activation (Zhang, Wu et al. 2004). The initiator caspases include caspase-2, 8, 9 and 10, while caspase-3, 6 and 7 are the executioner caspases, also known as effector caspases. Those involved in cytokine regulation are caspase-1, 4, 5, 11, 12, 13 and 14. As their name suggests, initiator caspases are involved in initiating the apoptotic process (Hengartner 2000) and can also be molecularly defined on the basis of the of a long N-terminal domain, the presence of which allows the formation of the protein complexes that provide molecular platform for caspase activation and inhibition (Degterev, Boyce et al.).

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Caspase activation, from latent zymogens to fully functional proteases, involves two cleavage events at specific aspartic acid residues thereby forming the active heterotetrameric protease subunit.

While in the context of apoptosis, the activation of caspases results in the eradication of the cell, the involvement of these proteolytic enzymes in functions other than cell death is demonstrated by their participation in immune function, cellular proliferation and differentiation (Chowdhury, Tharakan et al. 2008).

### **1.5.3 Necrosis**

While apoptosis is a tightly regulated and highly controlled process, necrosis serves to demonstrate the opposite. Necrosis is the passive and degenerative side of cell death and is characterised by the rapid and extensive lysis of the cell, the contents of which are released into the surrounding tissue and may induce an inflammatory reaction (Formigli, A. et al. 2000). Also, necrosis does not display the same stimulus-specific and cell – type specific behaviour associated with apoptosis. In the context of therapeutic strategies, and while induction of apoptosis is discussed as a desirable approach, where once apoptosis and necrosis were considered two distinct modes of cell death, it is now recognised that necrosis and apoptosis are not entirely separate processes and have been demonstrated to occur separately, sequentially and at the same time.



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### 1.5.4 Camptothecin (CPT)

The positive control of apoptosis used in the work carried out for this thesis was Camptothecin, a modified monoterpene indole alkaloid, produced by *Camptotheca acuminata* (Nyssaceae), *Nothapodytes foetida*, *Pyrenacantha klaineana*, *Merrilliodendron megacarpum* (Icacinaceae), *Ophiorrhiza pumila* (Rubiaceae), *Ervatamia heyneana* (Apocynaceae) and *Mosteua brunonis* (Gelsemiaceae), species of unrelated orders of angiosperms (Lorence and Nessler 2004)(Lorence and Nessler, 2004)(Lorence and Nessler, 2004)(Lorence and Nessler, 2004)(Lorence and Nessler, 2004)(Lorence and Nessler, 2004)(Lorence and Nessler, 2004). Camptothecin was first isolated in 1958 and has been used widely in Chinese medicine. Camptothecin is a 5-ring heterocyclic alkaloid that contains an  $\alpha$ -hydroxylactone (Figure xxxxx).

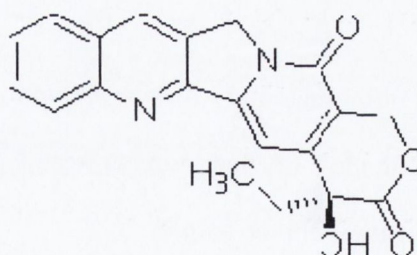


Figure 1.11: Camptothecin

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The mechanism of action of Camptothecin involves the poisoning of the ubiquitous enzyme topoisomerase, which participates in DNA replication, transcription, recombination, repair, chromatin assembly, and chromosome segregation (Avemann, Knippers et al. 1988). The use of CPT as an apoptotic inducing agent is widely referred to in the literature where many cell lines have been studied and topoisomerase inhibitors such as Camptothecin have been shown to either inhibit proliferation or induce apoptosis, depending on the cell line used (Hannun 1997). Treatment of both HuT-78 and Jurkat T cells with Camptothecin for the induction of apoptosis has been reported by many groups (Zisterer, Campiani et al. 2000; Sané, Cantin et al. 2004), with HuT-78 reported to demonstrate resistance to apoptosis but not immunity, following treatment (Giri and Aggarwal 1998). Furthermore, CPT has shown success by way of anticancer properties as demonstrated by its use in preclinical studies in tumours of both colonic and gastric origin and the CPT derivatives, irinotecan and topotecan, are widely used treatments for various cancers. (Lorence and Nessler 2004).

### **1.5.5 Apoptosis and the immune system- inflammation / autoimmunity**

Apoptosis plays a central regulatory role in the immune system and as such dysregulation or extremes of this process result in numerous diseases, including those of an autoimmune nature.

This central regulatory role is mediated at many stages of the immune response, including T cell development. While T cells originate in the bone marrow, they emigrate to the thymus and it is here that they undergo either positive or negative selection, which is dependent on the affinity of the T cell

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antigen receptors (TCRs) for major histocompatibility antigens (Krammer 2000). Autoimmunity can be avoided at this point, as tolerance of the T cells with a high affinity for self MHC molecules and peptide, is induced by their elimination, by apoptosis, which is negative selection. Those that avoid cell death because of recognition for self MHC molecules and peptide with low affinity, are referred to as positively selected and this low affinity binding to the MHC allows the specificity of the adaptive immune response (Palmer 2003).

Resolution of inflammation involves the return to normal, with respect to overall cell numbers, and the proportion of different cells that make up this population (Akbar and Salmon 1997), and it is at this point that the role of apoptosis demonstrates its importance.

Autoimmune and allergic reactions can result from the excess of lymphocytes, and this surplus can be the result of impaired apoptosis. Therefore, cell death processes are necessary for the removal of unwanted cells and in this context of autoimmunity, the induction of apoptosis provides a strategy for disease treatment.

Defects in the Fas pathway are associated with autoimmune diseases as Fas signaling plays an important part in both the removal of self-reactive lymphocytes and in immune surveillance of transformed or virus infected lymphocytes (Lamy, Liu et al. 1998; Landowski, Moscinski et al. 2001).

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### **1.5.6 Cytokines and Inflammation**

As previously mentioned, the immune response and inflammatory process are highly regulated, and cytokines are involved in the initiation, mediation and resolution of inflammation (Williams, Paleolog et al. 2007).

Stanley Cohen first introduced the term cytokine in 1974 to describe soluble proteins which are hormone like, and their fundamental role is that of communication between cells and the microenvironment. The secretion of cytokines is a response to inducing stimuli, and they are constitutively expressed (Cohen, Biggazzi et al. 1974). The involvement of cytokines in immunoregulation and inflammation has been well documented

In basic terms, cytokines can fall under the heading of pro-inflammatory or anti-inflammatory. The concept of the role of pro-inflammatory cytokines, being solely that of an up-regulatory nature, while anti-inflammatory cytokines act by way of suppression, is too simple. It is now known that many cytokines demonstrate paradoxical effects as a result of their basic pleiotropy, redundancy and multifunctional properties (Tayal and Kalra 2008). This is exhibited by the pro-inflammatory cytokines TNF- $\alpha$  and interleukin-1 $\beta$  (IL-1 $\beta$ ). A positive feedback loop between the two results in the up regulation of both cytokines.

As with apoptosis, the role that cytokines play in the immune system is critical to the development, and regulation of immunity, inflammation and hematopoiesis (Dinarello 2000).

In terms of the production of cytokines, T helper cells are the group of cells that are the major producers of cytokines (Tayal and Kalra 2008).

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With emphasis of this work on the apoptosis inducing potential of the compounds, the role of TNF- $\alpha$  in mediating apoptosis is introduced below.

#### **1.5.6.1 TNF- $\alpha$**

TNF- $\alpha$  is a pro-inflammatory cytokine, a 17-kDa protein composed of three identical subunits, the production of which is predominantly from activated mononuclear phagocytic cells, but it is also a product of NK (natural killer), B cells, and activated T cells. As a pro-inflammatory cytokine, it causes the activation of cells, the expression of adhesion molecules, chemokines and other cytokines (Issazadeh, Ljungdahl et al. 1995; Issazadeh, Lorentzen et al. 1996).

#### **1.5.7 Current anti-inflammatory treatments in autoimmunity**

Glatiramer acetate, a mixture of synthetic polypeptides made up of four amino acids similar to myelin basic protein (MBP). It is used for the immunomodulatory treatment of multiple sclerosis (MS). The exact mechanism of action is not yet fully understood but *in vitro* investigations have shown that glatiramer acetate binds with MHC molecules in a competitive manner and inhibition of T cell response to several myelin antigens (Fridkis-Hareli, Teitelbaum et al. 1994).

Immunological effects observed following treatment included decreased T cell proliferation both *in vitro* and *in vivo*, reduced T cell migration, and a shift from Th1 to Th2 cells was recorded (Thibault 2009).

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Natalizumab is a selective adhesion molecule  $\alpha_4$  antagonist. By binding to the  $\alpha_4$  subunit of the  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$  integrins, binding of the integrins to their endothelial receptors is blocked and so attenuates inflammation (Polman, O'Connor et al. 2006). Natalizumab was first administered in a randomised, double blind placebo controlled study to patients with Crohn's disease, demonstrating efficacy and subsequently resulted in significant clinical remission was observed. This drug has also been reported to be effective in a study of 10 patients with Ulcerative Colitis (Gordon, Hamilton et al. 2002)

Infliximab, an anti- TNF- $\alpha$  monoclonal antibody with murine variable regions and human IgG1 constant regions has been shown to be effective against rheumatoid arthritis (RA). (Knight, Trinh et al. 1993) It was first approved in 1999 for treatment of patients with RA, in conjunction with methotrexate (MTX), in cases where MTX treatment alone was inadequate. Since then it has been prescribed for the treatment of other inflammatory diseases such as Crohn's disease, ulcerative colitis, psoriatic arthritis and psoriasis. The proposed mechanisms of action include down regulation of pro-inflammatory cytokines, reduced leukocyte recruitment as a result of the reduced expression of adhesion molecules and chemokines into the joint and a reduction in angiogenesis by way of reduced VEGF production (Feldmann and Steinman 2005). Etanercept, a TNF receptor/immunoglobulin fusion protein is another anti- TNF- $\alpha$  agent used in the treatment of autoimmune diseases. The proposed mechanisms of action is similar to that of Infliximab, however, one notable difference is the lack of activity of Etanercept in Crohn's disease (Hochberg, Tracy et al. 2003). Adalimumab is a fully humanised anti-TNF monoclonal IgG1 antibody that binds only to TNF- $\alpha$

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and is approved for the treatment of moderate to severe RA, psoriatic arthritis, ankylosing spondylitis and Crohn's disease (Tayal and Kalra 2008).

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## 1.6 Background of test compounds

### PH compounds

The first group of test compounds evaluated in the work for this thesis stemmed from the naturally occurring pharmacological products from the fern *Pteridium aquilinum*, Pterosin Z and acetylpterodin. Their activity has been demonstrated with the inhibition of intestinal contractions to histamine and 5-hydroxy tryptamine (5-HT), along with inhibition of calcium-induced contractions in  $K^+$  - depolarised smooth muscle guinea-pig ileum (Sheridan H, Frankish N et al. 1999).

Work carried out to develop a synthetic route revealed Pterosin Z to have potent inhibitory capacity. Subsequently, the development of over 200 compounds, after research of the derivatives of these natural products, led to large scale testing in smooth muscle relaxant and mast cell stabilisation studies (Sheridan, Frankish et al. 1999). Any compound showing efficacy in these studies could provide a potential anti-inflammatory compound. The compounds selected for this work belonged to the following groups of compounds; 3C8, 6C6, 7C9 and 7C17.

Numerous in vivo models of inflammation were then employed to evaluate the potential of the compounds as possible drugs for autoimmune diseases such as multiple sclerosis and ulcerative colitis. Specifically, PH 6 (6C6) was shown to have a substantial effect in the animal model of multiple sclerosis, EAE, experimental autoimmune encephalomyelitis. However, behavioural



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indications of toxicity were also observed. Other previous work included IL2 secretion, and oxazalone mouse ear studies.

The indication that these compounds could inhibit inflammation, in *in vivo* models, warranted further investigation of their effects *in vitro*. Therefore, the selected compounds were evaluated in various systems representative of lymphocyte function. The areas investigated were T cell motility, toxicity profiling and regulation of TNF- $\alpha$ .

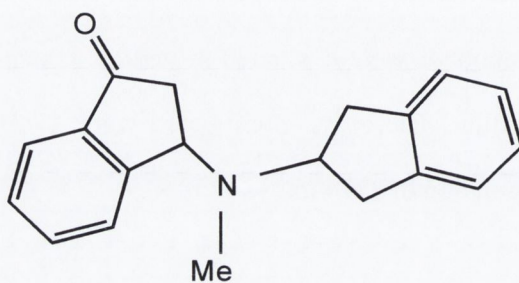
### **Quinones**

As part of an ongoing study into the use of natural products as anti-inflammatory agents, the effects of a series of quinones on T-cell motility has been investigated. Quinones are an important group of pharmacologically active pigments widely distributed in nature; they are well known to demonstrate various physiological effects such as antimicrobial, anti-inflammatory and anti-cancer activity. Many efficient antineoplastic drugs are either quinones (anthracycline derivatives, mitoxantrone, actinomycin), quinonoid derivatives (quinolones, genistein, bactracyclin), or drugs such as etoposide that can easily be converted to quinones by *in vivo* oxidation (Hsu, Cho et al. 2006). The compounds evaluated in the work carried out for this thesis were isolated in house by

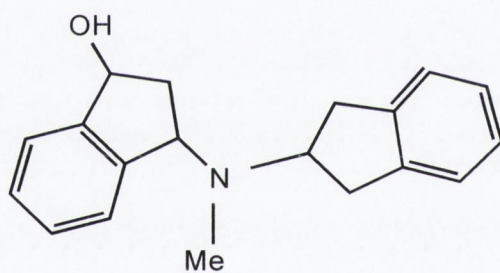
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## Compound structures

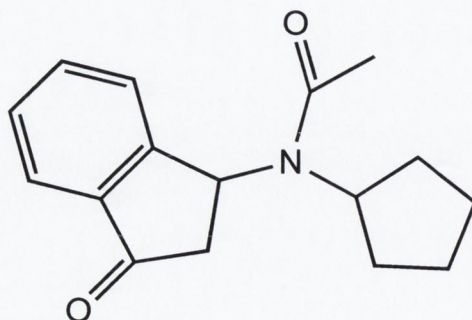
PH 6 (6C6)



PH 7 (6C6 OH)

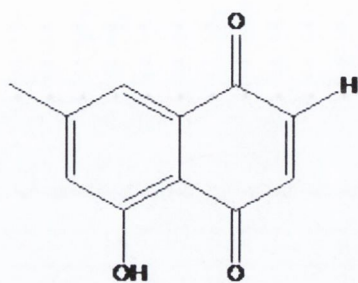


PH8 (7C9)



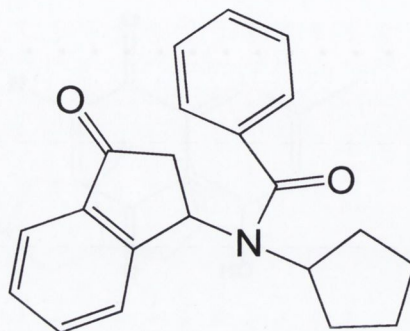
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HIH 7

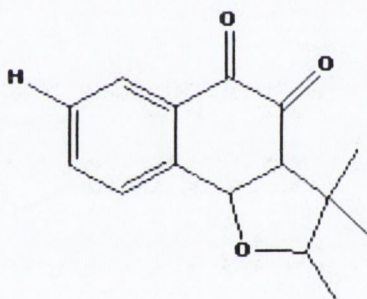


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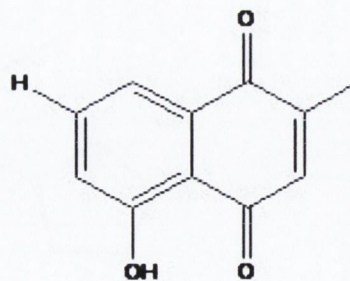
PH 34 (7C17)



HIH 4



HIH 6



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## 1.7 Aims

The aims of the work performed for this thesis were

To determine use of the LFA-1 mediated T cell motility assay as an indicator to the potential anti-inflammatory properties of novel compounds in T cell function.

To investigate the potential inhibitory properties of novel compounds *in vitro* on LFA-1 T cell mediated motility

To develop a profile of the compounds with respect to cytotoxicity by evaluating the effect of the compounds on mitochondrial viability (MTT) and membrane integrity (LDH), Annexin V and Propidium Iodide binding and poly caspase activity.

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## 2. Materials and Methods

### 2.1 Materials

#### 2.1.1 General cell culture

Isopropanol	TCD solvent stores
Biozidal ZF™	Wak-Chemie
Virkon	DuPont
RPMI 1640 media	Gibco
Dulbecco's Modified Eagle Medium (DMEM)	Gibco
Iscove's Modified Dulbecco's Medium (IMDM)	Invitrogen
DPBS	Gibco
$\alpha$ -Thioglycerol	Invitrogen
Sodium pyruvate (100 mM)	Sigma
Sodium bicarbonate (7.5 %)	Sigma
Trypsin/EDTA	Sigma
Sterile-filtered DMSO	Sigma
HEPES	Invitrogen
Sterile 25 mL serological pipettes	Greiner
Sterile 10 mL serological pipettes	Greiner
Sterile 5 mL serological pipettes	Greiner

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Sterile eppendorf 1.5 mL tubes	Sarstedt
Sterile eppendorf 0.5 mL tubes	Sarstedt
Sterile transfer pipettes	Sarstedt
10 – 300 $\mu$ L pipette tips	Greiner
200 – 1000 $\mu$ L pipette tips	Greiner
Sterile 24 well tissue culture plates	Nunc
Sterile 96 well tissue culture plates	Greiner
Sterile 15 mL tubes	Greiner
Sterile 50 mL tubes	Greiner
Sterile 0.22 $\mu$ M filters	Spark
Penicillin / Streptomycin (P/S)	Gibco
Fetal bovine serum (FBS)	Biowest
Iron supplemented fetal bovine serum	Sigma
Trypan blue	Sigma
Sigma clean water bath treatment	Sigma
T75 cm <sup>3</sup> tissue culture flasks	Greiner
T25 cm <sup>3</sup> tissue culture flasks	Greiner

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### **2.1.2 Motility assay**

Rabbit anti-mouse IgG	Dako
CD11a, LFA-1 antibody clone SPV-L7	Monosan
Go6976	Calbiochem UK

### **2.1.3 Cytotoxicity and Apoptosis**

MTT assay	Roche Applied Science
LDH assay	Roche Applied Science
Triton-X	Sigma
Poly caspase assay	AbD Serotec
Camptothecin	Sigma
Annexin V	BD Biosciences
Propidium iodide	BD Biosciences
Wash buffer for FACs	BD Biosciences

### **2.1.3 Gene expression profiling**

Total RNA isolation and purification kit	Qiagen
cDNA reverse transcription kit	Applied Biosystems
Taqman master mix	Applied Biosystems



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### 2.1.5 Equipment

CO <sub>2</sub> Incubator	NuAire
Microscope	Olympus
Image Proplus software	Media Cybernetics
FLUOStar Optima plate reader	BMG Labtech
Flow cytometer (FACS)	BD Biosciences
Pipette-aid	Drummond Scientific Co.
Micro-balance	Mettler, Toledo, mt5

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## **2.2 Methods**

### **2.2.1 *In vitro* cell culture**

#### **2.2.1.1 Aseptic technique**

Aseptic techniques were utilised during all cell culture work and also in the preparation of cell culture reagents. This is necessary to maintain a sterile environment free from fungal, bacterial and viral infections that can alter normal cellular functions. Aseptic techniques utilised, include the use of sterile disposable plastics, and sterilisation of glassware, plastics and H<sub>2</sub>O by autoclaving at 121°C for 30-60 minutes. All cell culture work was carried out in a laminar flow hood (Hera Safe, category 2). This allows only filtered air to come into contact with cells, thus preventing contamination with airborne pathogens. The interior of the hood was wiped down with 70% isopropanol before and after use. The hood surface was also exposed to ultraviolet (UV) light for 15-30 minutes after use. Any items taken into the flow hood were lightly sprayed with 70% isopropanol to prevent introduction of any pathogens to the hood work area. Disposable latex gloves were worn and sprayed with isopropanol before use. Gloves were changed regularly during cell culture work. Cells were maintained in a sterile Nuaire incubator (95% air, 5% CO<sup>2</sup> at 37°C) and any items placed in the incubator were lightly sprayed with isopropanol to prevent contamination with any pathogens. Both the incubator and laminar flow hood were regularly cleaned with Biocidal ZF™ to maintain a sterile environment.

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### 2.2.1.2 Cell culture

Hut-78 cells.

HuT 78, a cutaneous T cell lymphoma derived from peripheral blood of a Caucasian patient with Sezary syndrome, with the properties of a mature T cell line of inducer/helper phenotype, was purchased from the ATCC, and cells were cultured in RPMI 1640 supplemented with 1% penicillin / streptomycin (25 U / mL, 25 $\mu$ G /mL), 1 % L-glutamine, 40 mL FBS, 5 mL HEPES at 10  $\mu$ M, 5 mL glucose at 4.5 g / L, 5 mL sodium pyruvate at 1  $\mu$ M and 2 mL sodium bicarbonate at 1.5 g / L in a humidified atmosphere at 37 ° C and 5% CO<sub>2</sub>. Cells were seeded at a density of 0.5 x 10<sup>6</sup> cells per mL in T-75 cm<sup>2</sup> flasks with filter lid and maintained at a density of 0.5-0.9 x 10<sup>6</sup> cells per mL. Cells were passaged every 3 to 4 days.

Jurkat cells.

Jurkat cells, a human lymphoblastoid T-cell line, were purchased from the ATCC, and cells were maintained in RPMI-1640 supplemented with with 1% penicillin / streptomycin (25 U / mL, 25 $\mu$ G /mL), 1 % L-glutamine, 40 mL FBS, 5 mL HEPES at 10  $\mu$ M, 5 mL glucose at 4.5 g / L, 5 mL sodium pyruvate at 1  $\mu$ M and 2 mL sodium bicarbonate at 1.5 g / L in a humidified atmosphere at 37 ° C and 5% CO<sub>2</sub>. Cells were seeded at a density of 0.5 x 10<sup>6</sup> cells per mL in T-75 cm<sup>2</sup> flasks with filter lid and maintained at a density of 0.5-0.9 x 10<sup>6</sup> cells per mL. Cells were passaged every 3 to 4 days.

A549 and HMC-1 (Appendix 3)

A549, human type II-like epithelial lung carcinoma cells, were purchased from the ATCC, and were cultured in Dulbecco's Modified Eagles Medium

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(DMEM) supplemented with 10% FBS, 1% penicillin / streptomycin and 1% L-glutamine. Cells were seeded at a density of  $0.2 \times 10^6$  cells per mL in T-75cm<sup>2</sup> flasks with filter lid and maintained at a density of  $0.5\text{-}0.9 \times 10^6$  cells per cm<sup>2</sup>. Cells were passaged every 4 to 5 days.

The human mast cell line HMC-1 was kindly provided by Dr J. H. Butterfield of the Mayo Clinic and Mayo Foundation, Rochester, USA. HMC-1 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 25 mM HEPES, sodium bicarbonate and without  $\alpha$ -thioglycerol supplemented with 10% iron supplemented FBS, 1.2 mM  $\alpha$ -thioglycerol and 1% penicillin / streptomycin. Cells were seeded in T-75cm<sup>2</sup> flasks with filter lid at a density of  $0.5 \times 10^6$  cells per mL and maintained at a density of  $0.6 - 1 \times 10^6$  cells per mL. Cells were passaged every 5 to 6 days.

Cell culture media was stored at 4 °C. Aliquots of FBS and P/S were stored at -18 °C.

### **2.2.2 LFA-1 mediated T cell motility**

This assay was based on a model developed by Kelleher et al where T cell migration is induced by the presence of antibodies, in this case anti-LFA-1.(Fanning, Volkov et al. 2005) This study differed in that cells were not fixed for subsequent morphology examination, nor was immunofluorescence carried out. The deformation index was calculated similarly to previously published, with greater emphasis on the extent of deformation being an indicator of the inhibition of LFA-1 T cell motility, when calculated with respect to the overall number of cells. See appendix for sample calculation.

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A 24 well sterile flat-bottomed tissue culture plate was pre-coated with 250  $\mu$ L per well of a 1 / 100 dilution of rabbit anti-mouse IgG in sterile PBS. The plate was sealed with parafilm and stored at 4 °C overnight. The antibody coated plate was then washed twice with sterile 1x PBS and subsequently coated with 250  $\mu$ L per well of a 1 / 100 dilution of mAbs to  $\alpha$ -chain of LFA-1, clone SPV-L7, in sterile PBS (Kelleher, Murphy et al. 1995). The plate was covered and incubated in a humidified atmosphere at 37 °C and 5% CO<sub>2</sub> for at least 1 hour.

Drugs and controls were prepared on a second plate. The model used for this investigation was the HuT-78 T lymphocyte cell line. 10<sup>4</sup> cells / well were required. Cell density was determined by suspension of 50  $\mu$ L of cells in 50  $\mu$ L Trypan blue with 400  $\mu$ L PBS and a haemocytometer was used to count the cells. Adjustments to the cell density were made as appropriate.

Cells were dispensed into each well, and the plate was incubated at 37 °C, 5% CO<sub>2</sub> for 10 minutes. The density was then checked under the microscope and adjusted accordingly if necessary.

Cells were treated with the appropriate volumes of stock concentrations, of test compounds in DMSO, to give the desired final concentration. Go6976, a selective PKC- $\alpha$  and  $\beta$  inhibitor, was used as a positive control of inhibition of T cell motility. The cell / drug mixture was incubated in a humidified atmosphere at 37 °C and 5% CO<sub>2</sub> for 30 minutes.

The antibody plates were washed as before. The cell / drug mixture was added and incubated at 37 °C again, until sufficient cytoskeletal rearrangement in control untreated cells had been observed.

Image Pro Plus (Media Cybernetics), was used to analyse the cells. Triplicate photos of each well were recorded. Motility was then determined by calculation of the deformation index, based on the following equations. A deformation index greater than 3 was considered to indicate motility.

Equation 1: Elongation index = major ellipse diameter / minor ellipse diameter

Equation 2: Circularity index =  $4 \times \pi \times (\text{area} / (\text{perimeter})^2)$ .

Equation 3: Deformation index = elongation index / circularity index

### **2.2.3 Cytotoxicity and cell death**

#### **2.2.3.1 FACS Apoptosis – Annexin V / PI staining**

The analysis of Annexin V and propidium iodide (PI) binding was carried out with Annexin V and propidium iodide (BD Pharmingen) according to the manufacturer's instructions. The human T lymphoma cell line, HuT-78, was firstly used in this study to investigate the apoptotic potential of the test compounds. The initial incubation period of 4 h was not sufficient to yield statistically significant different results between the control samples and therefore the incubation period was increased to 24 h. This incubation period was also applied to the study of the test compounds on the Jurkat, human T cell line.

7 mL of 500,000 cells / mL were incubated in a humidified atmosphere at 37 °C and 5% CO<sub>2</sub> in T25 cm<sup>3</sup> tissue culture flasks with test compounds at the indicated concentration for 24 h. Positive control samples were treated with

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Camptothecin (6  $\mu$ M). Following incubation with test compounds, cells were transferred to 15 mL sterile tubes, washed twice with cold PBS and centrifuged at 300 X g for 5 min. The cell pellet was then re-suspended in 1 X Binding Buffer at a concentration of  $1 \times 10^6$  cells / mL. Of this solution, 100  $\mu$ L ( $10^5$  cells) was transferred to a 1.5 mL eppendorf to which 5  $\mu$ L Annexin V and 5  $\mu$ L PI were added. The solution was mixed by gently pipetting up and down and then incubated for 15 min, protected from light at room temperature. A further 400  $\mu$ L of 1 X Binding Buffer was subsequently added. Samples were analysed by FACSArray (BD Biosciences). For each sample 10,000 ungated events were acquired. Annexin V<sup>+</sup> PI<sup>-</sup> cells represented the early apoptotic populations, while Annexin V<sup>+</sup> PI<sup>+</sup> cells represented late apoptotic or secondary necrotic populations.

#### **2.2.3.2 Caspase activity (Fluorochrome inhibitor of caspase, FLICA)**

The potential caspase activity of PH 6 in Jurkat T cells was determined with Carboxyfluorescein FLICA™ Apoptosis detection kit, Caspase Assay (AbD Serotec). The polycaspase kit contains a caspase inhibitor which is fluorescently labelled. This inhibitor, FAM-VAD-FMK, is a carboxyfluorescein derivative of valylalanylaspatic acid fluoromethyl ketone, and once inside the cell, covalently binds to the cysteine residue of the active caspase. When bound to the active caspase FAM-VAD-FMK inhibits any subsequent enzyme activity.

To determine if caspase activity was associated with the apoptotic effect of PH 6 as demonstrated in the FACS study, 7 mL of 500,000 cells / mL were treated with PH 6 (10  $\mu$ M) in a T25 cm<sup>3</sup> flask and incubated for 12 h in a

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humidified atmosphere at 37 °C and 5% CO<sub>2</sub>. The incubation period of 12 h, as opposed to 24 h for Annexin V and PI binding, was chosen as the morphological changes associated with apoptosis as determined by Annexin V and PI binding are the result of a cell that has fully committed to the process of cell death. Apoptosis of control cells was induced by treatment with camptothecin (2 µg / mL) for 12 h.

Following treatment of the Jurkat cells with PH 6 and induction of apoptosis in the control samples, the cells were labelled with FLICA. The cells were transferred to 15 mL sterile tubes and centrifuged at 300 X g for 5 min. The cell pellet was resuspended at a density of 10<sup>7</sup> cells / mL. To 290 µL of this cell suspension, 10 µL of 30X FLICA label was added. The 30X FLICA was prepared by a 1 / 5 dilution of 150X FLICA stock in PBS. The tubes were flicked gently to ensure uniform mixing of the cells with the FLICA label and covered with aluminium foil to protect from light. The tubes were then incubated for 1 h in a humidified atmosphere at 37 °C and 5% CO<sub>2</sub>.

After incubation with the FLICA label, the cells were washed three times. Firstly, 2 mL of wash buffer was added to each tube and the cells were centrifuged at 300 X g for 5 min. The supernatant was gently decanted and the cell pellet was resuspended with another 1 mL of wash buffer. Again, the cells were centrifuged at 300 X g for 5 min and the supernatant gently decanted. The last wash was repeated once more, but before centrifugation, the cell density of each sample was determined and adjusted so that all tubes contained a similar quantity of cells to that of the control untreated sample. The cells were again centrifuged at 300 X g for 5 min, the supernatant decanted and the cell pellet resuspended in 400 µL of sterile PBS.



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100  $\mu$ L of each sample was then transferred to a black 96 well plate and the fluorescence measured with an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

### **2.2.3.3 Lactate dehydrogenase (LDH) release.**

The cytotoxicity of the test compounds was determined by the measurement of the lactate dehydrogenase (LDH) enzyme activity as released in the supernatant of the treated cells. This is a colorimetric assay where the extent of lactate dehydrogenase released by the damaged cells is proportional to the amount of formazan formed. The enzymatic reaction whereby the formazan is formed is a two step reaction. Upon incubation of the reaction mixture with the cell free supernatant the first step of the reaction occurs when the LDH in the supernatant catalyzes the conversion of lactate to pyruvate by reduction. In the second step, the diaphorase catalyst of the reaction mixture reduces the pale yellow tetrazolium salt to the red formazan salt. (Roche MTT manual). The assay was carried out according to the manufacturer's instructions.

Prior to the experiment it was necessary to prepare assay media. Media for each cell line was prepared with 1% FBS as opposed to the standard 10% for culture media, as FBS has inherent LDH activity, and as such interferes with the enzyme reaction. Also for the HuT-78 assay media both sodium pyruvate and sodium bicarbonate were omitted during media preparation, as they would participate in the enzyme reaction.

100  $\mu$ L of LDH assay media were dispensed into the wells of a 96 well plate. HuT-78 or Jurkat cells were counted and the density was adjusted to 500,000 cells / mL. 100  $\mu$ L of this cell suspension was then added to the wells containing the LDH assay media. The cells were then treated with the desired

concentration of test compound, as indicated in the results. Untreated cells provided both the spontaneous release of LDH for the cytotoxicity calculation, and therefore the negative control of cytotoxicity, where the LDH release observed was that of the cells in their resting state. DMSO (0.01%) was added to multiple wells as vehicle control and cells treated with Triton X-100 (2 % in assay media) provided the positive control of cytotoxicity resulting in maximal release of LDH. The cell and drug mixture was then incubated in a humidified atmosphere at 37 °C and 5% CO<sub>2</sub> for either 4 h or 24 h.

Following the incubation period, the 96 well plate was centrifuged at 300 X g for 10 min. 100 µL of the supernatant was then transferred to the corresponding wells of another 96 well plate. During this time the reaction mixture was prepared by addition of 250 µL catalyst (diaphorase / NAD<sup>+</sup> mixture) to 11.25 mL of the dye solution (both supplied in kit). 100 µL of this reaction mixture was then added to each well containing 100 µL supernatant and the plate was incubated at room temperature for 15 min and protected from light. Absorbance was then read on an OPTIMA plate reader at 490 nm. Using the absorbance values obtained, the percentage of cytotoxicity was determined by the following calculation.

% Cytotoxicity =

$$\frac{((\text{sample value} - \text{untreated}) / (\text{Triton-X} - \text{untreated}))}{\text{Triton-X} - \text{untreated}} \times 100$$

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#### **2.2.3.4 Cell proliferation Cytotoxicity ((3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide) MTT)**

As with the above mentioned LDH assay, the determination of cytotoxicity and cellular proliferation by the MTT assay is colorimetric. However, while the LDH assay is a determination of cell membrane damage, the MTT assay is a direct measure of those cells in the population that are metabolically active. The extent of colour produced by the cleavage of the yellow tetrazolium salt MTT by enzymes of the endoplasmic reticulum, to purple formazan crystals by metabolically active cells is directly proportional to the number of viable cells.

This assay was carried out according to the manufacturer's instructions. HuT-78 or Jurkat cells were counted and the density was adjusted to 500,000 cells / mL. 100  $\mu$ L of cell suspension was dispensed into a 96-well plate. The cells were then treated with the desired concentration of test compound, as indicated in the results. Multiple wells were left untreated as a negative control of cytotoxicity. DMSO (0.01%) was added to multiple wells as vehicle control and cells treated with Triton X-100 (2 % in assay media) provided the positive control of cytotoxicity. The cell and drug mixture was then incubated in a humidified atmosphere at 37 °C and 5% CO<sub>2</sub> for either 4 h or 24 h.

Following the incubation period, 10  $\mu$ L of yellow MTT labelling reagent was added to each well and the plate was incubated for a further 4 h in a humidified atmosphere at 37 °C and 5% CO<sub>2</sub>. To solubilise the water insoluble formazan dye formed during this incubation period, 100  $\mu$ L per well of solubilisation solution (10% SDS in 0.01 M HCl, supplied in the kit)

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was added and the plate was incubated overnight in a humidified atmosphere at 37 °C and 5% CO<sub>2</sub>. The absorbance was then recorded at 550 nm.

## **2.2.4 Cell preparation for QPCR**

### **2.2.4.1 RNA isolation**

Purified total RNA was isolated from treated cells according to the Qiagen RNeasy mini kit manual. To harvest cells, the total cell suspension was placed in a 2 mL sterile tube and centrifuged for 5 min at 300 x g. The supernatant was gently aspirated. The cell pellet was then loosened by vigorously flicking the tube. To disrupt and lyse the cells, 350 µL of buffer RTL was then added to the pellet and the tube was vortexed to mix the buffer RTL and cell pellet. 350 µL of 70 % ethanol was then added to each sample of homogenised lysate and pipetted up and down to mix. This total volume of 700 µL for each sample was then transferred to RNeasy spin columns which were placed in supplied 2 mL collection tubes and centrifuged for 15 s at 8000 x g to bind the RNA to the columns. The flow-through was then discarded and the collection tube re-used for the following wash steps. 700 µL of Buffer RW1 was then added to each spin column and centrifuged for 15 s at 8000 x g to wash the spin column membranes. Again the flow-through was discarded carefully to ensure no contact of the spin columns with the flow-through. 500 µL of buffer RPE was then added to each spin column and centrifuged for 2 min at 8000 x g. A further 500 µL buffer RPE was then added to wash each spin column, and centrifuged for 2 min at 8000 x g. Each RNeasy spin column was then transferred to new 1.5 mL supplied collection

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tube. 50  $\mu$ L of RNase-free water was then added directly to each spin column membrane and centrifuged for 1 minute at 8000 x g to elute the RNA. The eluated RNA was then aliquoted and stored at -80° C.

#### **2.2.4.2 cDNA synthesis**

*RNA quantification:* The optical density (OD) of RNA was measured using a spectrophotometer (UV/vis Beckman Coulter Du730) to determine RNA concentration and purity. The concentration of RNA can be measured due to its ability to absorb light at 260nm. As an OD reading of 1.0 at 260nm is equivalent to an RNA concentration of 40 $\mu$ g/ml, sample RNA concentrations can be quantified using the following equation: RNA = OD<sub>260</sub> X dilution factor X 40 $\mu$ g/ml. The purity of RNA may also be established by measuring absorbance at 280nm. A ratio of OD<sub>260</sub>/OD<sub>280</sub> of approx. 1.8-2.1 is indicative of pure RNA. All RNA samples used had ratios of >1.5. RNA concentrations were then equalised so that equal concentrations of RNA could be used as template for cDNA transcription. Samples were aliquoted in equal volumes until reverse- transcribed.

#### **2.2.4.3 cDNA synthesis by Reverse Transcription**

RNA samples were reverse transcribed into cDNA using a high capacity cDNA archive kit (Applied Biosystems) according to the manufactures protocol. Briefly, 20 $\mu$ l of 0.5-2.5 $\mu$ g of RNA was mixed in a PCR mini-tube with an equal volume of 2X master mix that was made up as follows: 1:5 dilution of 10X Reverse Transcription Buffer, 1:12.5 dilution of 25X dNTPs, 1:5 dilution of Random Primers, 1:10 dilution of MultiScribe Reverse

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Transcriptase and 1:2.381 dilution of H<sub>2</sub>O. Samples were then placed in thermal cycler and incubated at 25°C for 10 minutes followed by a 2 hour incubation at 37°C. Resultant cDNA was frozen at -20°C until ready for real time polymerase chain reaction (PCR) analysis.

#### **2.2.4.4 Real-time PCR**

Assesment of target genes was performed using Taqman gene expression assays containing specific target primers, and FAM-labelled MGB target probes (Applied biosystems).  $\beta$ -actin gene expression was used to normalize gene expression between samples, and was quantified using a GAPDH endogenous control gene expression assay containing specific primers, and a VIC-labelled MGB probe for human GAPD (GAPDH). No requirement for optimisation of the primer sequences was necessary as all primers used were pre-optimised by Applied Biosystems.

Amplification of a gene of interest and endogenous control ( $\beta$ -actin) was carried out for each cDNA sample. Briefly, cDNA was diluted 1:4 and 10 $\mu$ l of diluted cDNA was pipetted onto a PCR plate, to which 1.25 $\mu$ l of primer/probe, 1.25 of  $\mu$ l of  $\beta$ -actin/probe and 12.5 $\mu$ l of Taqman master mix was added. Samples were run in duplicate, and electronic pipettes (EDP3 20-200 $\mu$ l, 2-20 $\mu$ l and 10-100 $\mu$ l) were used to ensure pipetting accuracy. Samples were placed in the real-time PCR thermocycler (Applied Biosciences 7300) using the following programme; step 1: 95°C for 10 minutes, step 2: 95°C for 15 seconds followed by 1 minute at 60°C. Step two was repeated 40 times,

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and fluorescence read when the cycle was at 60°C for the duration of the programme.

During step two of the PCR reaction, the double stranded cDNA is denatured at 95°C for 15seconds. As the temperature begins to fall to 60°C (annealing and extension) the target probe is first to anneal to the single-stranded cDNA as it has a higher melting temperature than the target primers (Applied Biosystems). This probe contains a FAM/VIC dye and a proprietary non-fluorescent quencher (NFQ) dye, this quencher prevents the dye from emitting a fluorescent signal by fluorescence resonance energy transfer (FRET) technology (Applied Biosystems). At 60°C the primers anneal and the strand is extended by 5' nuclease activity of the Taq polymerase. This displaces the FAM/VIC-labeled probe causing the FRET between the dye and quencher to be broken, and the generation of a fluorescent signal. Due to the specificity of the probe and primers for the cDNA sequence, one fluorescent signal is generated for each new cDNA copy and measured during the annealing stage of the PCR cycle (60°C).

### **2.2.5 Statistical analysis**

All data was analysed using the Prism statistical software package. Statistical comparisons were initially performed using a one or two-way analysis of variance (ANOVA) as indicated in the experimental sections. Data was deemed significant when  $P < 0.05$ . Results are expressed as means and standard error of the mean (SEM).

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### **3. Results**

#### **3.1 LFA-1 mediated T cell motility**

The inhibitory property of the test compounds on LFA-1 T cell motility was assessed in this assay. Calculation of the deformation index determined whether a cell was considered to be motile or not. A cell, upon activation by LFA-1, developed a motile phenotype illustrated by leading edges, lamellae and a deformation index greater than three.

As a pilot study, a range of compounds were evaluated in the LFA-1 mediated T cell motility assay to determine its expediency as a model of T cell function in the context of immune function (**Figure 3.1**). This pilot study was followed by evaluation of the four main test compounds of interest; PH 6, PH 7, PH 8 and PH 34 (**Figure 3.2**).



Pilot study with PH compounds.

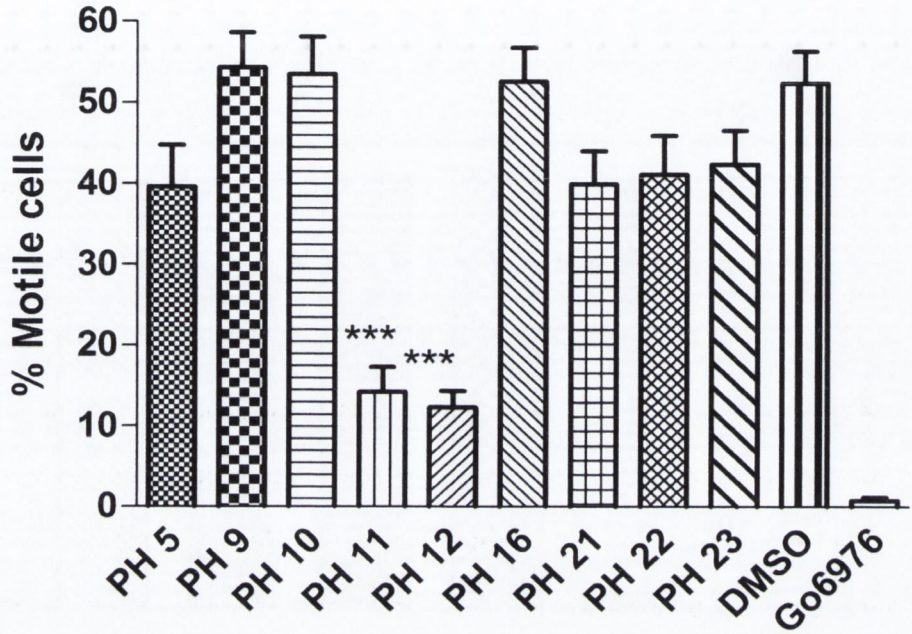


Figure 3.1: % Motile HuT-78 cells 4 h post incubation with test compounds (10  $\mu$ M).

PH 11 and PH 12 significantly inhibited LFA-1 mediated T cell motility. Data are expressed as mean  $\pm$  SEM where \*\*\* indicates statistical significance ( $P < 0.001$ ) compared to DMSO (0.5%). Go6976 (5  $\mu$ M).

Determination of inhibitory effects of PH 6 in Hut-78 cells

PH 6 significantly inhibits LFA-1 mediated T cell motility

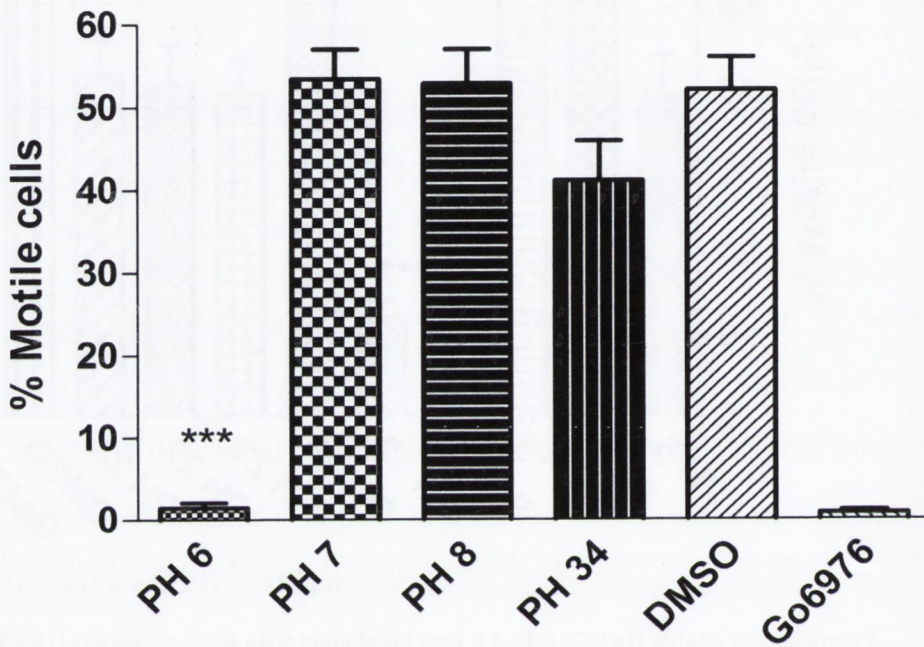
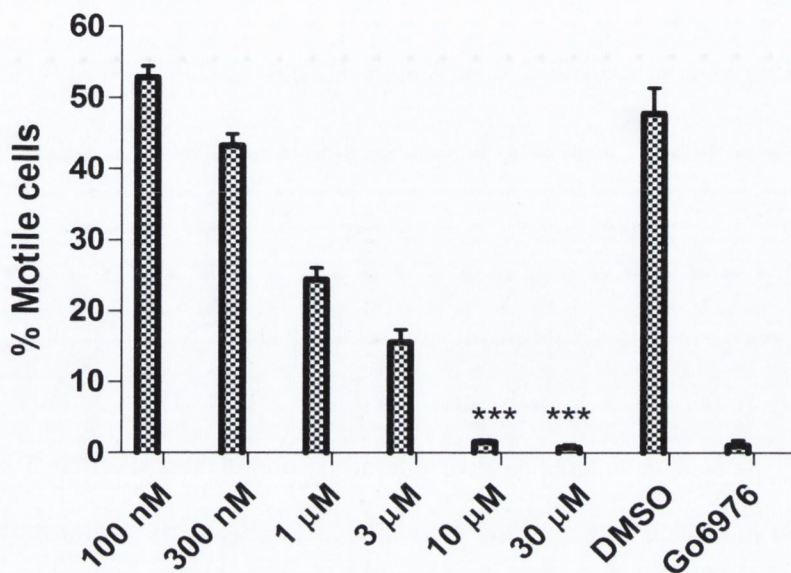


Figure 3.2: % Motile HuT-78 cells 4 h post incubation with test compounds (10  $\mu$ M).

PH 6 (10  $\mu$ M) significantly inhibited T cell motility. Data are expressed as mean  $\pm$  SEM (N=24) from three experiments carried out independently, where \*\*\* indicated statistical significance with  $P < 0.001$  compared to DMSO (0.5%). Go6976 (5 $\mu$ M).

*PH 6 inhibits T cell motility in a dose dependant manner.*



**Figure 3.3: PH 6 Dose response. % Motile HuT-78 cells 4 h post incubation with 0.01, 0.03, 0.1, 0.3, 10 and 30  $\mu$ M PH 6.**

PH 6 (10 $\mu$ M) significantly inhibited T cell motility. Data are expressed as mean  $\pm$  SEM (N=52) from three experiments carried out independently, where \*\*\* indicated statistical significance with  $P < 0.001$  compared to DMSO.

Compared to Go6976 (5 $\mu$ M), the only test compounds to inhibit motility significantly were PH 6, PH 11, and PH 12 ( $P < 0.001$ ). Cells treated with the PKC selective inhibitor did not undergo cytoskeletal rearrangement, as observed in the untreated and DMSO controls, where cells remained spherical in shape and no spreading of the cytoskeleton was recorded. Untreated and DMSO treated HuT 78 cells, originally non-adherent, were observed to spread, and, over the 4 hour incubation period, showed extensive

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rearrangement of the cytoskeleton culminating in long cytoplasmic projections.

Treatment of cells with PH 11 and PH 12 significantly inhibited the locomotory behaviour of the HuT-78 cells. Treatment with these compounds, at 10  $\mu$ M, resulted in  $15.45 \pm 0.79\%$  and  $11.78 \pm 1.36\%$  motile cells respectively.

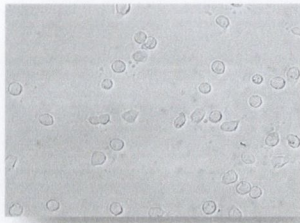
PH 6 showed the most significant inhibition of HuT-78 motility at 10  $\mu$ M, with only  $1.40 \pm 0.02\%$  of cells determined to be motile. The remaining PH 6 treated cells were calculated to have a deformation index of less than 3 and as such had not developed a motile phenotype. This is clearly demonstrated in the images as the cells displayed a predominantly spherical or oval shape, with little or no evidence of cytoskeletal rearrangement, compared to that seen in DMSO treated cells.

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Table 1: Representative images of PH test compound treated HuT-78 cells acquired for analysis and calculation of deformation index.

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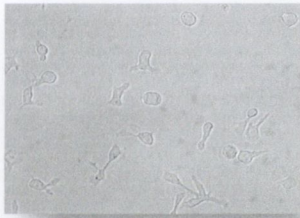
**PH 6**



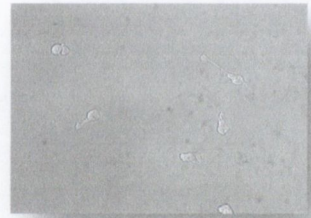
**PH 34**



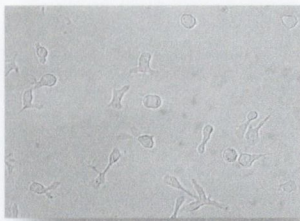
**PH 7**



**DMSO**



**PH 8**



**Go6976**

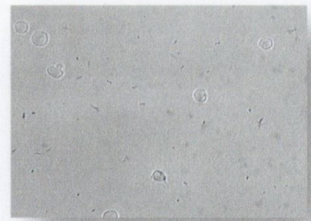
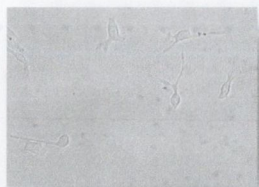
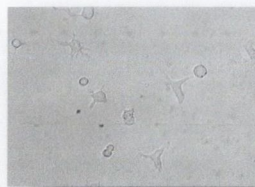


Table 2: Representative images of HIH test compound treated HuT-78 cells acquired for analysis and calculation of deformation index.

**HIH 1**



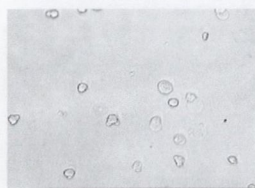
**HIH 5**



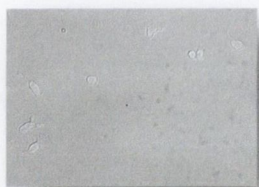
**HIH 2**



**HIH 6**



**HIH 3**



**HIH 7**



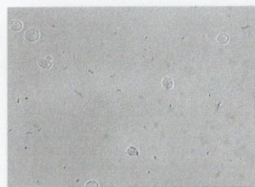
**HIH 4**



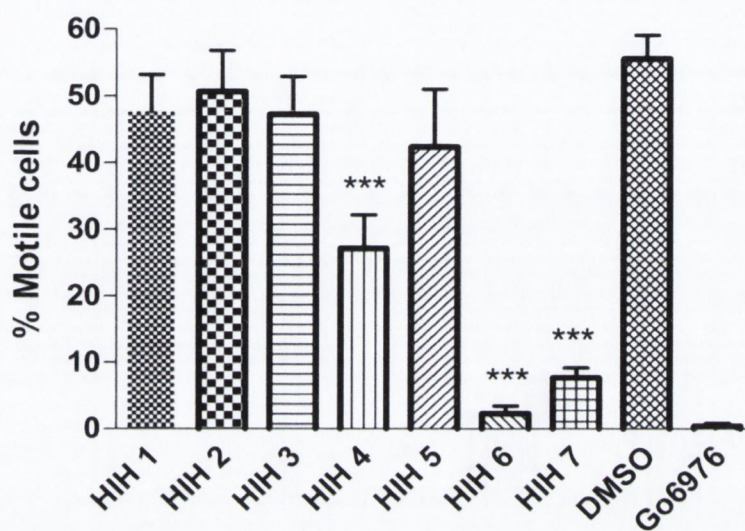
**DMSO**



**Go6976**



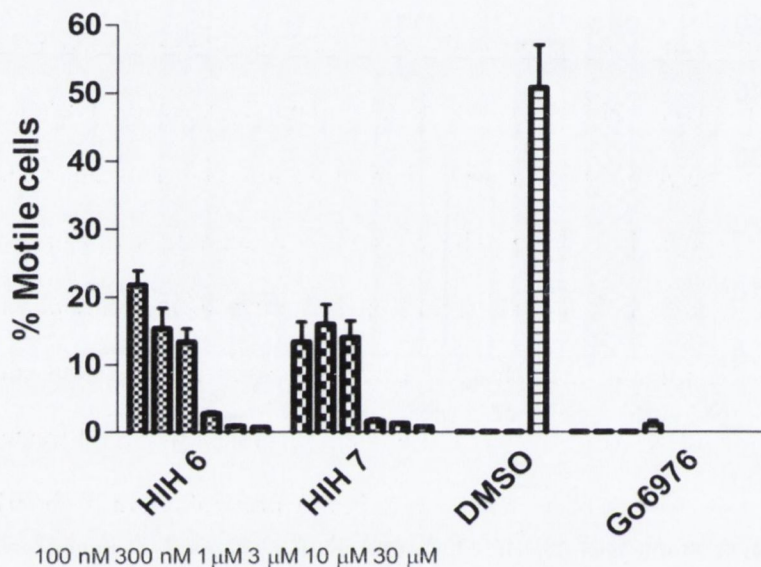
*HIH 4, HIH 6 and HIH 7 significantly inhibit LFA-1 T cell motility.*



**Figure 3.4: % Motile HuT-78 cells 4 h post incubation with naphtoquinone compounds (10  $\mu$ M).**

HIH 4, HIH 6 and HIH 7 all significantly inhibited LFA-1 mediated T cell motility. Data are expressed as mean  $\pm$  SEM, where \*\*\* indicates statistical significance ( $P < 0.001$ ) compared to DMSO.

*HIH 6 and HIH 7 significantly inhibit LFA-1 T cell motility in a dose dependent manner.*



**Figure 3.5: Dose Response % Motile HuT-78 cells 4 h post incubation with HIH 6 and HIH 7.**

HIH 6 and HIH 7 (10  $\mu$ M) inhibited T cell motility dose dependent manner. Data are expressed as mean  $\pm$  SEM (N=24) from three experiments carried out independently, where \*\*\* indicated statistical significance with  $P < 0.001$  compared to DMSO (0.5%). Go6976 (5 $\mu$ M).

All compounds were tested at 10  $\mu$ M initially. The PKC selective inhibitor, Go6976, was again used as a control of motility inhibition. Of the seven HIH compounds tested, HIH 6 and HIH 7 were the only compounds that had a



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significant effect on LFA-1 mediated T cell motility. Treatment for 4 hours with these compounds (Figure 3.4), resulted in negligible rearrangement of the cytoskeleton. No leading lamella or trailing uropods were observed, which in turn determined a consistent deformation index of less than three. Hence, cell motility for HIH 6 and HIH 7 was observed at  $1.75 \pm 0.21\%$  and  $5.8 \pm 0.58\%$  respectively. HIH 1, HIH 2, and HIH 3 treatment of the Hut 78 cells resulted in  $47.6 \pm 0.39\%$ ,  $47.09 \pm 6.25\%$  and  $44.68 \pm 5.25\%$  motile cells, respectively. In all three of these compounds extensive rearrangement of the cytoskeleton was observed. There were low cell numbers resembling the original spherical shape. The cells were observed as having adhered, spread and undergone similar rearrangement to that of the untreated DMSO control. HIH 4 treatment of the HuT 78 cells was more effective in inhibiting motility, than HIH 1, HIH 2 or HIH 3. Cell adherence in HIH 4 treated cells was observed, however, migration was significantly inhibited ( $P < 0.001$ ) with the result that  $30.05 \pm 6.1\%$  of cells were motile.

The significant inhibition of HuT-78 motility in response to treatment with HIH 6 and HIH 7 at  $10 \mu\text{M}$ , necessitated a further study, to examine the effects on T cell locomotory behaviour, with respect to a varying doses of the compounds. The assay was performed as previously described, with cells treated with compounds, at final concentrations of  $100 \text{ nM}$ ,  $300 \text{ nM}$ ,  $1 \mu\text{M}$ ,  $3 \mu\text{M}$ ,  $10 \mu\text{M}$  and  $30 \mu\text{M}$ .

Data of cells treated in the dose response study demonstrated the concentration dependent effect of HIH 6 and HIH 7 on T cell motility. Treatment at  $30 \mu\text{M}$  resulted in almost complete inhibition of cell motility,

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where only  $0.7 \pm 0.0006\%$  of cells were determined to be motile, for HIH 6 and  $0.79 \pm 0.03\%$  for HIH 7. The very low numbers of cells assigned a deformation index greater than three, from which the percentage of motile cells was calculated, confirmed the visual observations where limited rearrangement of the cytoskeleton, resulting in cytoplasmic projections, had occurred. The cells had not developed a motile phenotype. Furthermore, even at the lowest concentration of 100 nM, the motility of cells remained low at  $21.7 \pm 2.16\%$  and  $13.34 \pm 2.96\%$  respectively for HIH 6 and HIH 7. This suggests that both HIH 6 and HIH 7 are potent inhibitors of LFA-1 mediated T cell locomotory behaviour.

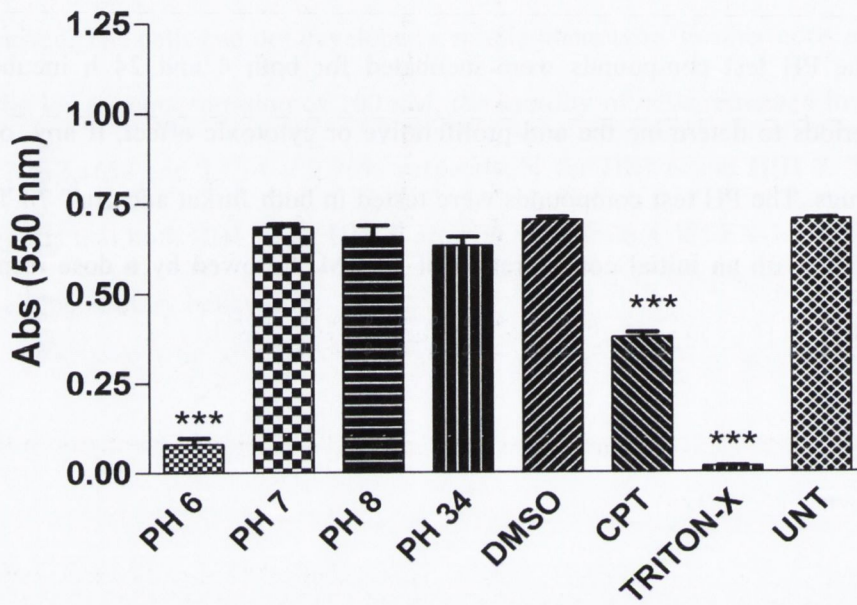
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### **3.2 Cytotoxicity and Apoptosis**

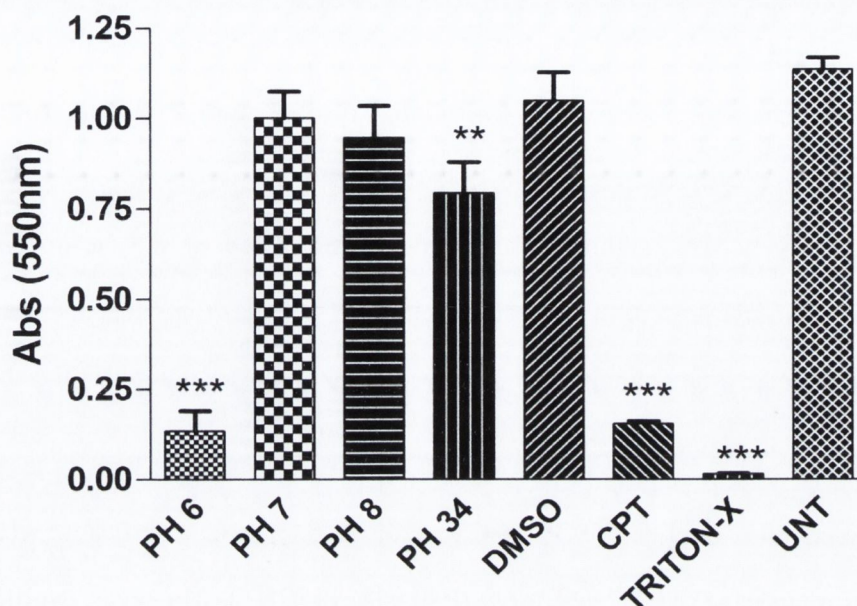
The premise of this MTT assay is that those cells with viable and live mitochondria will cleave the yellow tetrazolium salt to form purple formazan crystals in the reaction, and as such the live cells are directly proportional to absorbance observed.

The PH test compounds were incubated for both 4 and 24 h incubation periods to determine the anti-proliferative or cytotoxic effect, if any, of the drugs. The PH test compounds were tested in both Jurkat and HuT 78 T cell lines, with an initial concentration of 10  $\mu$ M, followed by a dose response study.

PH 6 significantly induces cell death at both 4 and 24 h.



**Figure 3.6: MTT Cytotoxicity of Jurkat T cells 4 h post treatment with PH compounds at 10  $\mu$ M.** The cytotoxic effect of the compounds as determined by MTT assay. Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT.



**Figure 3.7: MTT Cytotoxicity of Jurkat T cells 24 h post treatment with PH compounds at 10 µM.**

The cytotoxic effect of the compounds as determined by MTT assay. Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT.

Following 4 h incubation with the test compounds, PH 6 was the only drug to have a significant effect, compared to the untreated control, on the Jurkat cells with an absorbance value of  $0.135 \pm 0.0548$ , which was also more potent than the apoptosis inducing Camptothecin, as illustrated in **Figure 3.6**. Absorbance values for PH 7, PH 8 and PH 34 were all substantially higher and did not differ significantly from the vehicle and untreated control, clearly indicating that these drugs do not have any cytotoxic or anti-proliferative effect on Jurkat T cells.

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From data of the 24 h incubation with the test compounds, PH 6 was revealed to have the most substantial effect on Jurkat cell viability with a mean absorbance value of  $0.135 \pm 0.0007$  compared to the untreated cells,  $1.138 \pm 0.343$ . The absorbance values for both CPT and Triton-X were indicative of maximum cytotoxicity with  $0.154 \pm 0.0536$  and  $0.015 \pm 0.0036$  values respectively. This significant difference is explained by the lack of viable mitochondria present in the test sample. This effect is time dependent and is illustrated by data obtained for Jurkat cells following treatment for the shorter incubation period of 4 h.

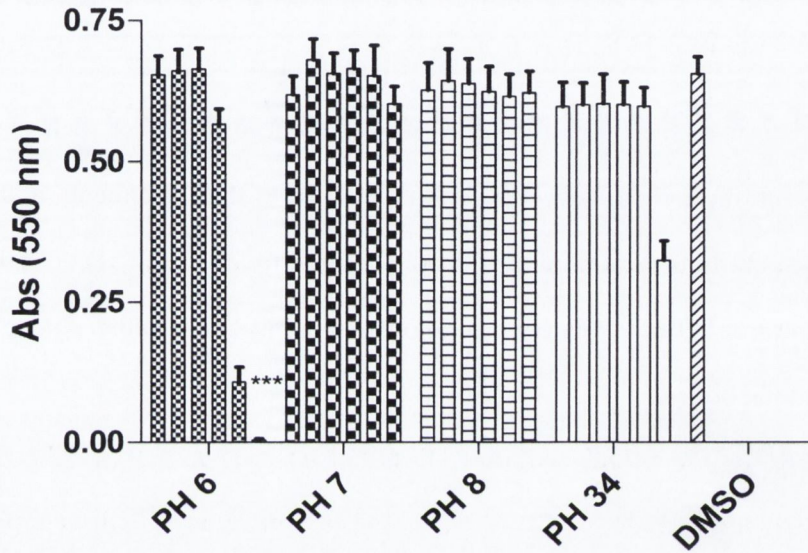
The cytotoxic effect of PH 34 was observed to be significant, with an absorbance of  $0.794 \pm 0.1464$  ( $P < 0.001$  vs UNT). However, worthy of mention is the lack of significant difference for the same drug when compared to the vehicle control, DMSO ( $1.052 \pm 0.2830$ ). Furthermore, given the length of the incubation period of 24 h, coupled with the significant difference compared to the positive controls of cytotoxicity, PH 34 is not considered to have a cytotoxic effect.

Neither PH 7, nor PH 8 demonstrated a significant effect on the Jurkat cells with absorbance values of  $1.001 \pm 0.1328$  and  $0.948 \pm 0.1504$  respectively.

A subsequent dose response study was performed to determine if a relationship between the concentration of test compounds used and the extent of cell death, existed. As PH 6 had in the initial MTT study demonstrated, at  $10 \mu\text{M}$ , significant anti-proliferative capacity and PH 7, PH 8 and PH 34 showed negligible effect at the same concentration, it was necessary to use a series of doses that would incorporate both higher and lower concentrations

than the initial 10  $\mu\text{M}$ . Therefore, Jurkat cells were incubated with 1, 3, 10, 30, 10 and 30  $\mu\text{M}$  of each of the test compounds for 4 hours.

*PH 6 induced cytotoxicity occurs in a dose dependent manner.*



**Figure 3.8: MTT Cytotoxicity of Jurkat T cells 4 h post treatment with PH compounds at 0.01, 0.03, 0.1, 0.3, 10 and 30  $\mu\text{M}$ .**

The cytotoxic effect of the compounds as determined by MTT assay. Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT.

As illustrated in Figure 3.7, a dose dependent relationship did not exist between PH 7, PH 8, PH 34 and cell death in Jurkat cells 4 h post treatment. Only at the highest dose of 30  $\mu\text{M}$  did PH 34 have a significant impact on Jurkat cell viability, compared to the untreated and DMSO vehicle control cells, with an absorbance value of  $0.325 \pm 0.0345$  recorded. Camptothecin

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treatment was observed to have a similar effect on the cells with an absorbance value of  $0.323 \pm 0.017$  recorded.

The most significant effect observed was that of the dose dependent relationship following treatment with PH 6. Results following incubation with the lower concentrations of 0.1, 0.3 and 1  $\mu\text{M}$  revealed no difference between drug treated cells and untreated or DMSO vehicle treated cells. Those cells treated with 10  $\mu\text{M}$  gave the expected result, as per the initial study. However the highest dose of 30  $\mu\text{M}$  induced an effect such that no significant difference between Jurkat cells with this treatment, and the positive control of maximum cell death, Triton-x treated cells, was observed. Furthermore, PH 6 at this concentration was significantly more potent than Camptothecin.

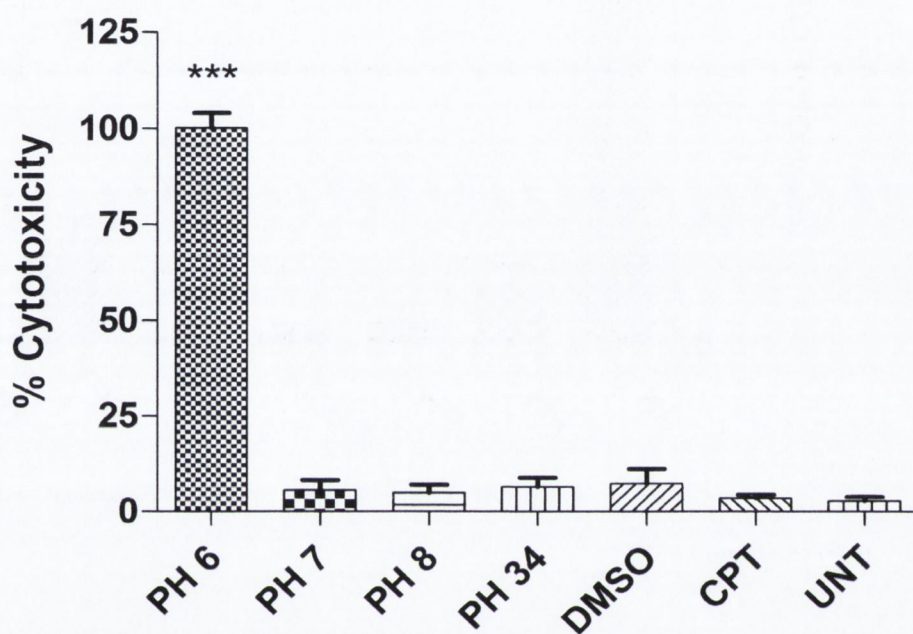
#### *LDH*

#### *Determination of cytotoxic effects of PH test compounds in Jurkat T cells*

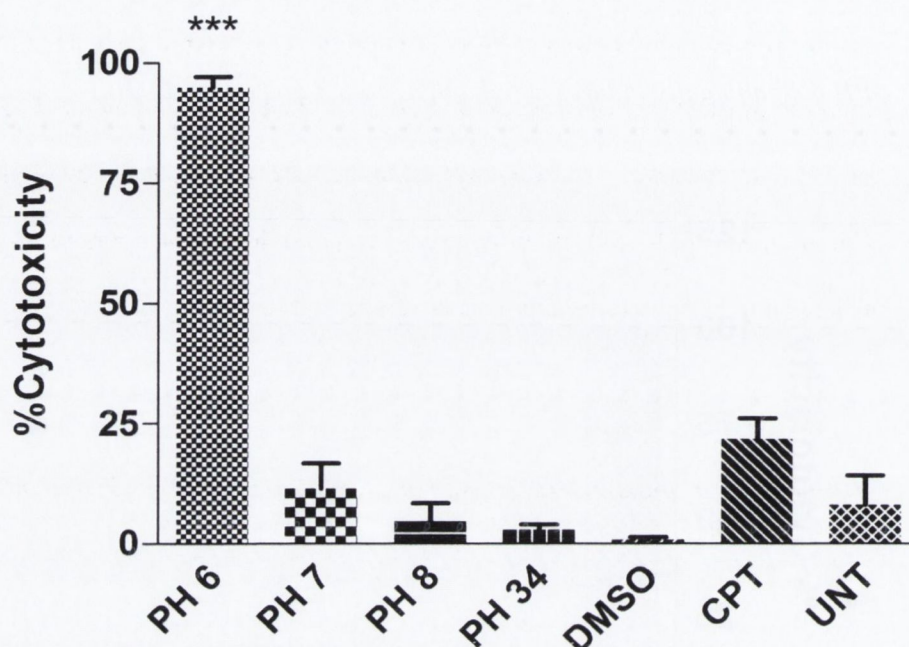
Further investigation of the potential toxicity of PH6, PH7, PH 8 and PH 34 was performed by evaluating the lactate dehydrogenase (LDH) activity. Again, the test compounds were incubated for 4 and 24 hours respectively, in both Jurkat and HuT 78 T cells.



*PH 6 significantly induces cell death at both 4 and 24 h. (LDH)*



**Figure 3.9: LDH Cytotoxicity of Jurkat T cells 4 h post treatment with PH compounds at 10 μM.** The cytotoxic effect of the compounds is determined by LDH assay. Data are expressed as mean ± SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT.



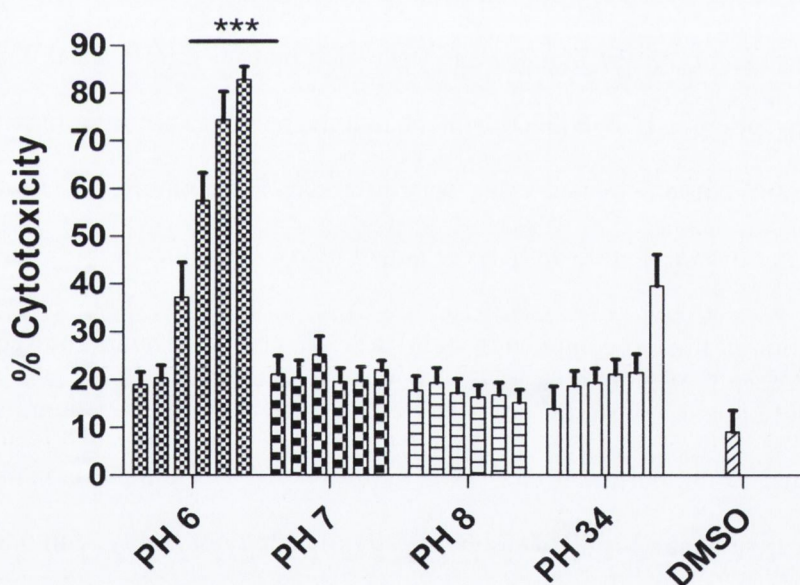
**Figure 3.10: LDH Cytotoxicity of Jurkat T cells 24 h post treatment with PH compounds at 10  $\mu$ M.**

The cytotoxic effect of the compounds is determined by LDH assay. Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT.

Following the initial incubation of 4 hours (**Figure 3.8**), only PH 6 was observed to have any significant effect on the Jurkat cells. Treatment with this compound induced an almost equipotent release of LDH to the positive control Triton-X, where maximum cell death of Jurkat cells was observed. PH 7, PH 8 and PH 34 did not result in LDH release indicative of cell death when compared to the vehicle or untreated control. Nor did Camptothecin induce cell death with just  $1.590 \pm 0.749$  % cytotoxicity calculated based on LDH released. This is explained by the fact that the mode of action of Camptothecin is to induce apoptosis, and as such the cell membrane remains

intact with no release of the cell contents. As expected, 24 hours post treatment with the test compounds, PH 6 and the positive control Triton-X, again resulted in maximum cytotoxicity. Of the other treatments, a significant increase in LDH release was not observed for the test compounds PH 7, PH 8 or PH 34. However, cytotoxicity following the extended incubation with Camptothecin did result in an increase in cytotoxicity at 4 hours to 24 hours where significant cytotoxicity was observed.

*PH 6 induces cytotoxicity (LDH) in a dose dependent manner in Jurkat T cells*



**Figure 3.11: LDH Cytotoxicity of Jurkat T cells 4 h post treatment with PH compounds at 0.1, 0.3, 1, 3, 10 and 30 µM.**

The cytotoxic effect of the compounds as determined by LDH assay. Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT.

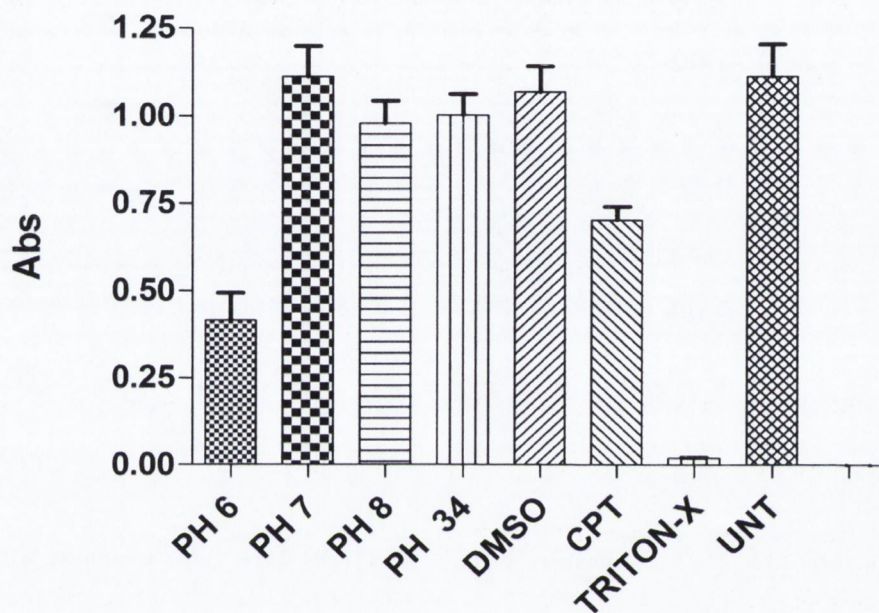
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As observed in the MTT dose response study, a dose dependent relationship did not exist between PH 7, PH 8, PH 34 and cell death as determined by lactate dehydrogenase (LDH) release in Jurkat cells 4 h post treatment (**Figure 3.10**). Only at the highest dose of 30  $\mu$ M did PH 34 have a notable, but not significant, impact on Jurkat membrane integrity, compared to the untreated and DMSO vehicle control cells, with  $39.51 \pm 6.62$  % cytotoxicity observed. PH 6 treatment was again demonstrated to have the most significant effect on the cell population with a dose dependant relationship clearly illustrated (**Figure 3.10**). Results following incubation with the lower concentrations of 0.01, 0.03 and 0.1  $\mu$ M revealed no difference between drug treated cells and untreated or DMSO vehicle treated cells. Those cells treated with 10  $\mu$ M gave the expected result, as per the initial study. However the highest dose of 30  $\mu$ M induced an effect such that no significant difference between Jurkat cells with this treatment, and the positive control of maximum cell death, Triton-x treated cells, was observed. Furthermore, PH 6 at this concentration was significantly more potent than Camptothecin.

Incubation of the test compounds with HuT 78 cells for 24 hours showed that PH 7, PH 8 and PH 34 did not affect cell proliferation after 24 hours when compared to the untreated and DMSO control. As with the aforementioned results, treatment with PH 6 resulted in significant cytotoxicity with  $0.827 \pm 0.042$  absorbance recorded.

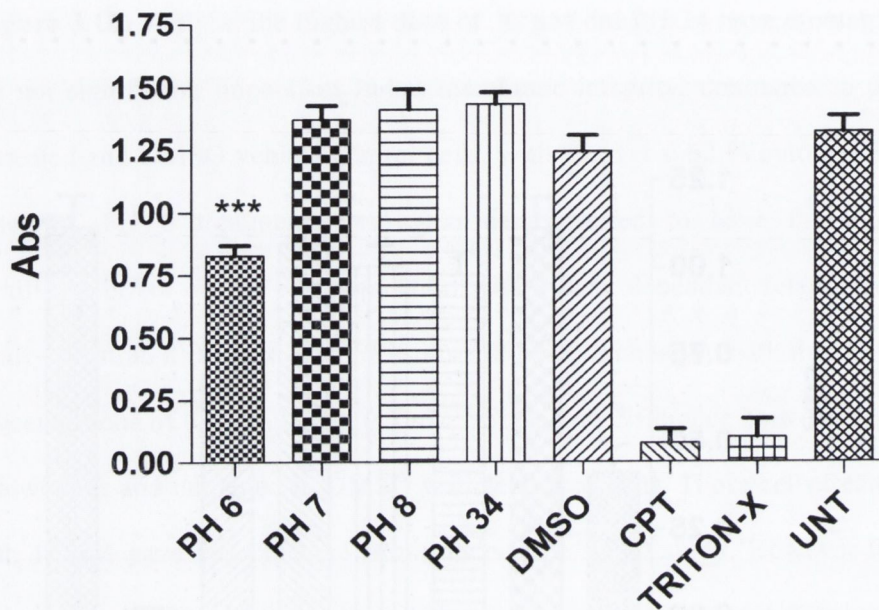
*Determination of the cytotoxic effects of test compounds in Hut -78*

*PH 6 significantly induces cell death at both 4 and 24 h.*



**Figure 3.12: MTT Cytotoxicity of HuT 78 T cells 4 h post treatment with PH compounds at 10 μM.**

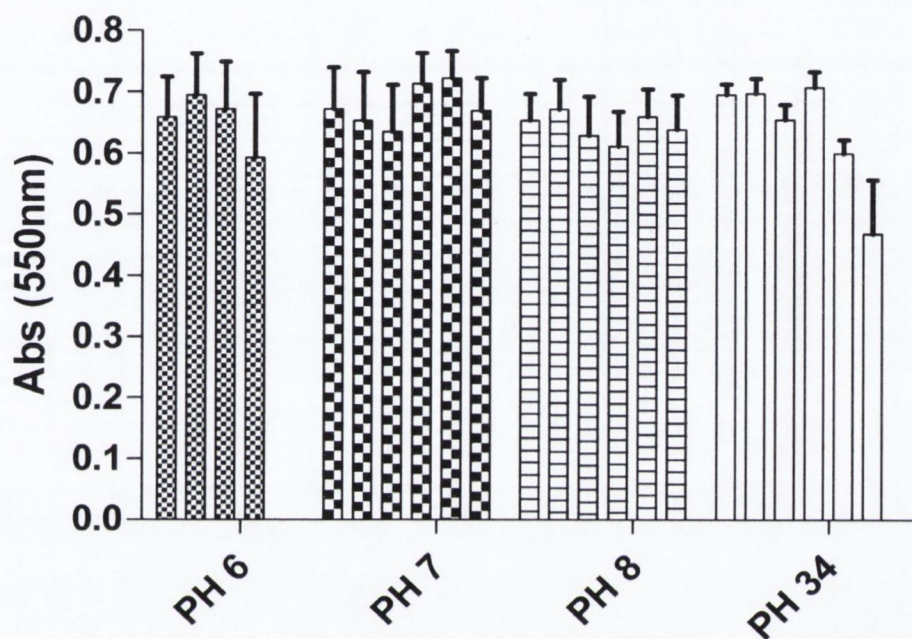
The cytotoxic effect of the compounds as determined by MTT assay. Data are expressed as mean ± SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT, DMSO, CPT and TRITON-X.



**Figure 3.13: MTT Cytotoxicity of HuT 78 T cells 24 h post treatment with PH compounds at 10 μM.**

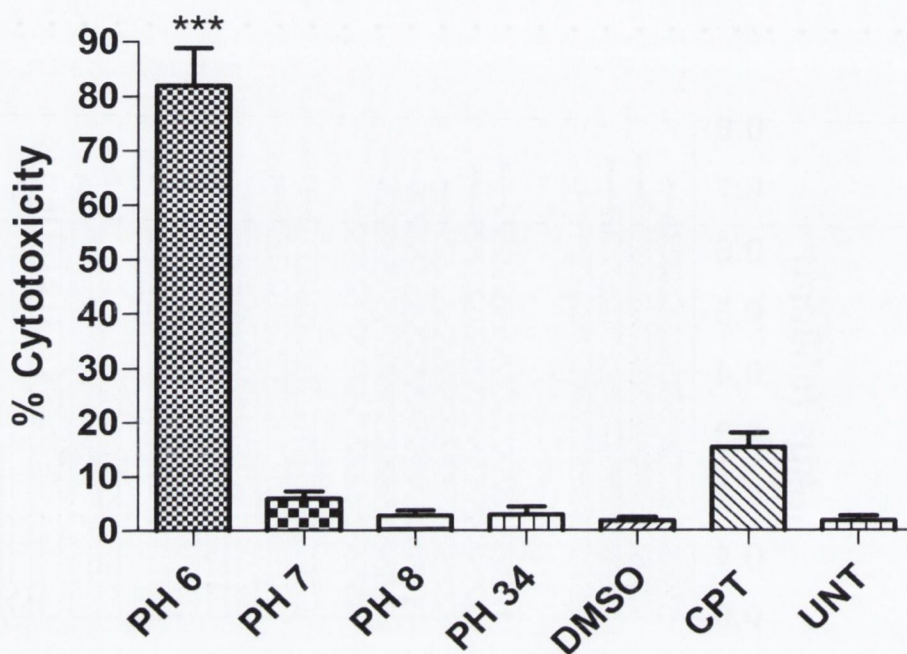
The cytotoxic effect of the compounds as determined by MTT assay. Data are expressed as mean ± SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT, DMSO, CPT and TRITON-X.

*HuT 78 MTT Dose Response (4HRS)*



**Figure 3.14: MTT Cytotoxicity of HuT 78 T cells 4 h post treatment with PH compounds at 0.01, 0.03, 0.1, 0.3, 10 and 30  $\mu$ M.**

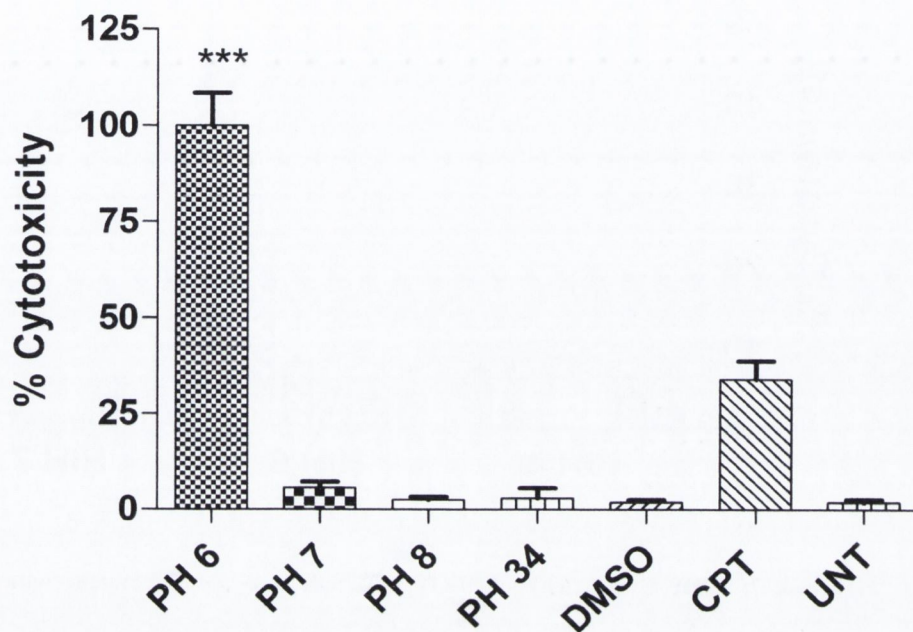
The cytotoxic effect of the compounds as determined by MTT assay. Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT.



**Figure 3.15: LDH Cytotoxicity of HuT 78 T cells 4 h post treatment with PH compounds at 10  $\mu$ M.**

The cytotoxic effect of the compounds as determined by LDH assay. Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT, DMSO, CPT and TRITON-X.

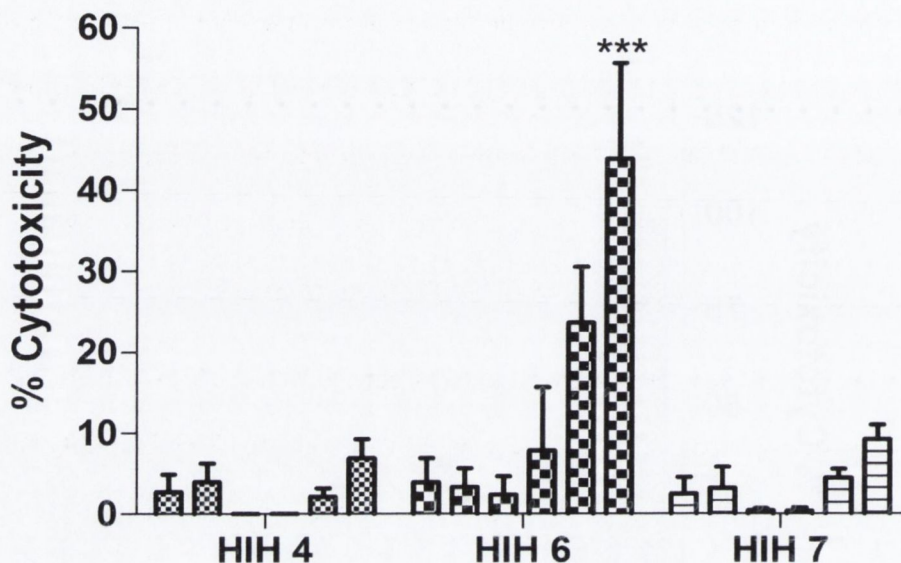




**Figure 3.16: LDH Cytotoxicity of HuT 78 T cells 24 h post treatment with PH compounds at 10  $\mu$ M.**

The cytotoxic effect of the compounds as determined by LDH assay. Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT, DMSO, CPT and TRITON-X.

*HIH 6 induces cytotoxicity in a dose dependent manner.*



**Figure 3.17: LDH Cytotoxicity of HuT-78 T cells 4 h post treatment with HIH compounds at 1, 3, 10, 30, 100 and 300 µM.**

The cytotoxic effect of the compounds as determined by LDH assay. Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to UNT.

Treatment of HuT-78 cells with HIH 6 was the only compound to induce cytotoxicity in a dose dependant manner. Even at the highest concentrations, HIH 4 and HIH 7 did not affect the viability of the cell population with the % cytotoxicity for these drugs close to just 10 %. HIH 6 caused a significant increase from 0.1 µM to 30 µM with cytotoxicity observed to be higher than 50 %.(Sheridan, Hook et al. 2008)

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### **3.3 Apoptosis - FACs Annexin V /PI binding**

Further investigation of any cytotoxic effect associated with treatment with the test compounds was warranted to determine the nature of the type of cell death observed. This was especially true for PH 6 which had, in both the MTT and LDH studies, shown significant anti-proliferative capacity. Annexin V and Propidium Iodide binding was analysed by flow cytometry to determine the apoptotic potential of the test compounds.

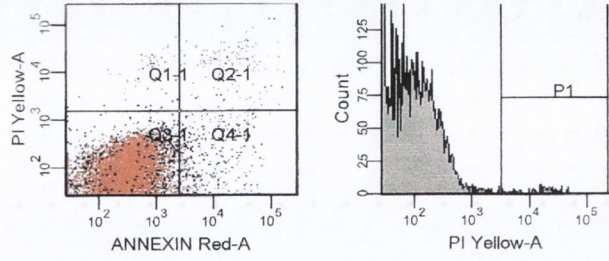
The initial flow cytometry study was carried out in HuT-78 cells, as these cells were used as the model for the LFA-1 mediated T cell motility study. It followed that, given the results of the test compounds in the cytotoxicity study, most notably the impact of PH 6 on cell viability, in both HuT-78 and Jurkat cells, coupled to the significant inhibition of LFA-1 mediated T cell motility observed, HuT-78 cells were primary initial interest.

HuT-78 cells were initially incubated for 4 h, as per LFA-1 mediated T cell motility protocol, with the positive control of apoptosis, Camptothecin (2 µg / mL). No significant difference between Camptothecin and the untreated cells was observed, and a time course study was performed to optimise experimental conditions, that is, the optimum incubation period to obtain significant difference between controls. This revealed the optimum incubation period to be 24 h. The lengthy incubation period results from the constitutively activated NF-κB which promotes cell survival and as such renders HuT-78 cells resistant, but not immune, to apoptosis. The alternative T lymphocyte Jurkat line was then incubated with the test compounds to determine any apoptotic effect. PH 6 demonstrated significant capacity to induce apoptosis in this cell line. The next question to be answered was

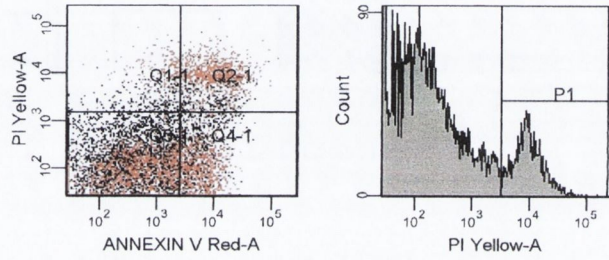
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whether PH 6 could induce apoptosis in the resistant HuT-78 cell line. Results demonstrated the potency of PH 6 in HuT-78 cells to be similar to that observed in Jurkat cells, and as such were deemed to do so independently of NF- $\kappa$ B.

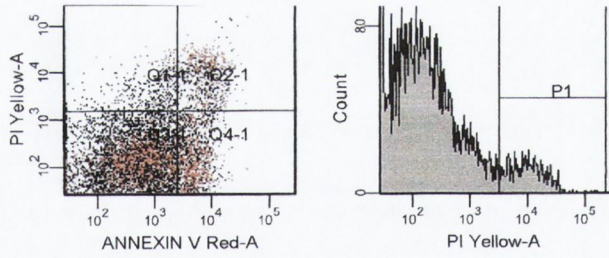
HuT-78 CPT 4hrs



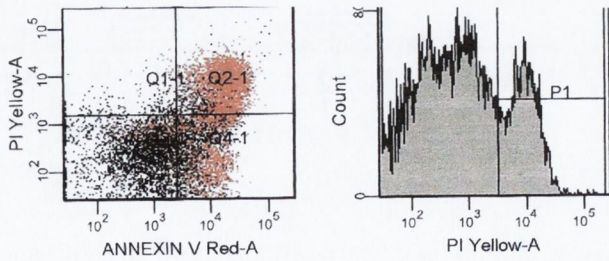
HuT-78 CPT 12 hrs



HuT-78 CPT 18 hrs

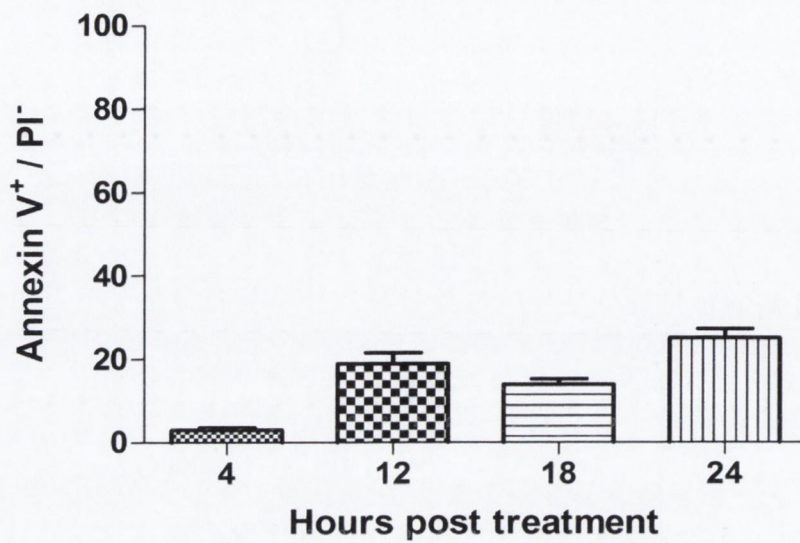


HuT-78 CPT 24 hrs



**Figure 3.18: FACS. Camptothecin induction of apoptosis is significant 24 h post treatment.**

(a)



(b)

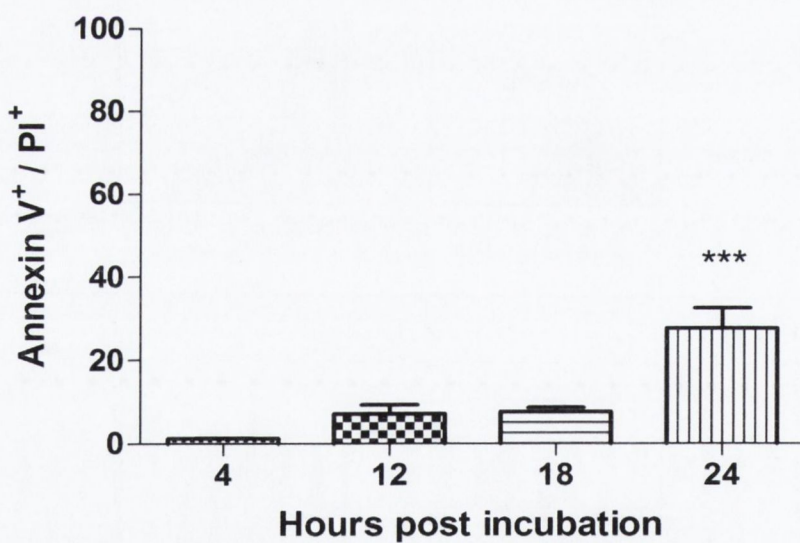
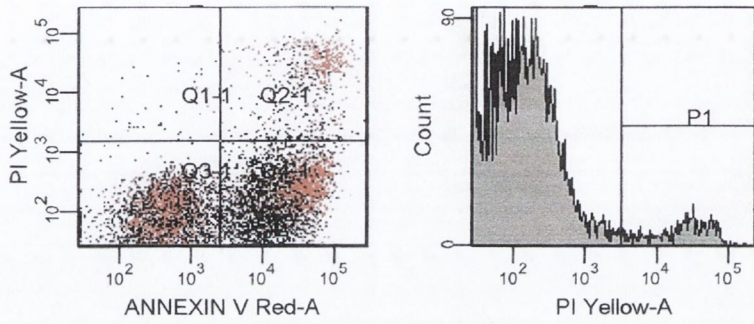


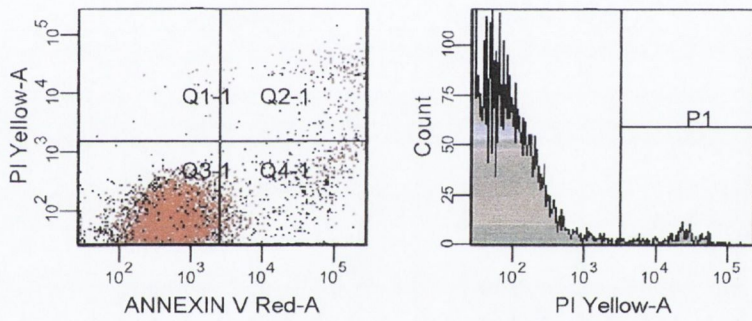
Figure 3.19 : The % of Annexin V<sup>+</sup> / PI<sup>-</sup> (early apoptosis) (a) and Annexin V<sup>+</sup> / PI<sup>+</sup> (late apoptotic) (b) HuT-78 cells following treatment with CPT at 2 $\mu$ g /mL at 4, 12,18 and 24 hours incubation as determined by FACs with Annexin V and PI binding.

*PH 6 significantly induces apoptosis in Jurkat T cells as determined by FACs analysis 24 h post incubation.*

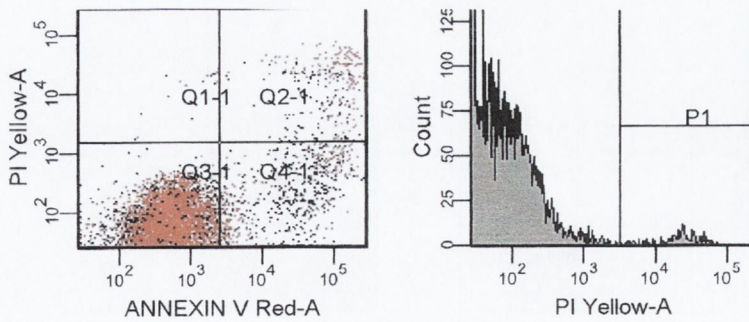
PH 6 Jurkat 24hrs



PH 7 Jurkat 24hrs

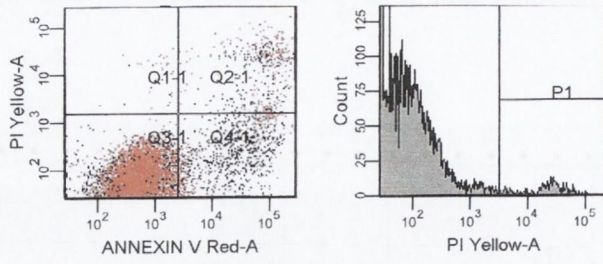


PH 8 Jurkat 24 hrs

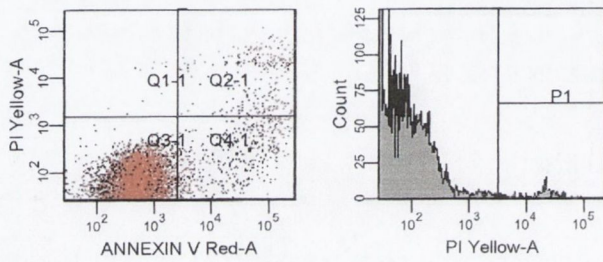


**Figure 3.20(a): FACs. PH 6 significantly induces apoptosis in Jurkat T cells as determined by FACs analysis 24 h post incubation.**

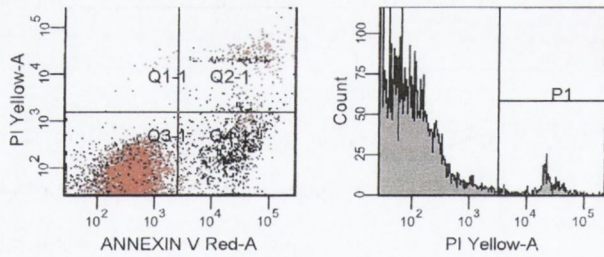
PH 34 Jurkat 24 hrs



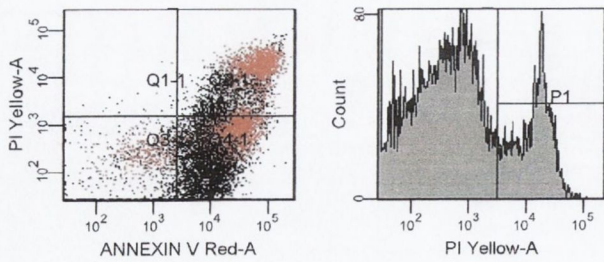
DMSO Jurkat



UNT Jurkat

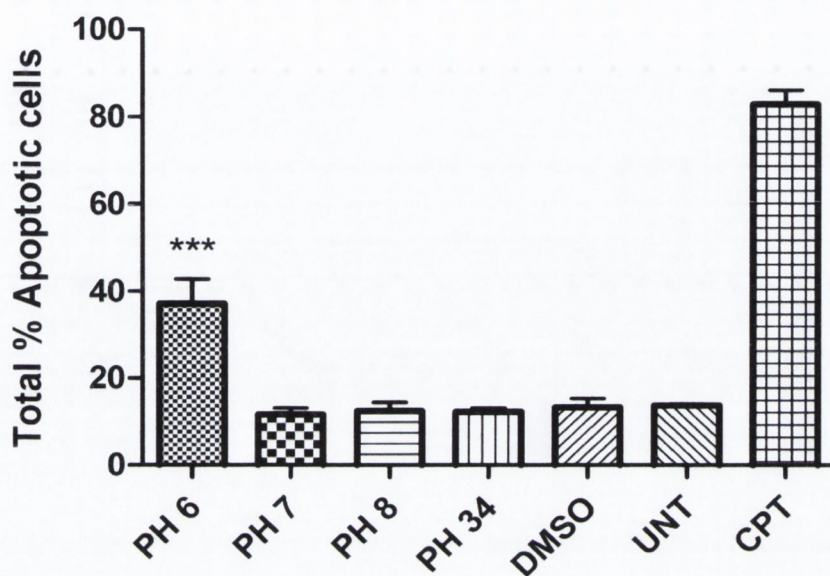


CPT Jurkat



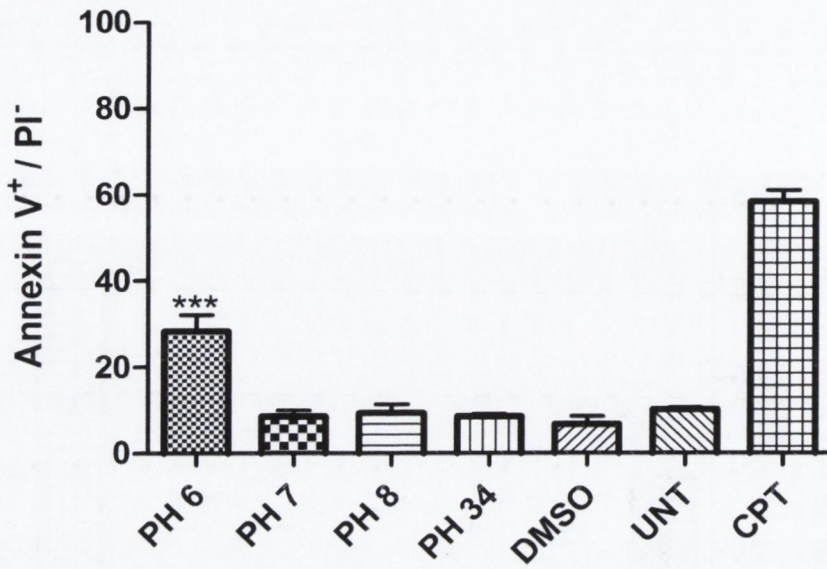
**Figure 3.20(b): FACs. PH 6 significantly induces apoptosis in Jurkat T cells as determined by FACs analysis 24 h post incubation.**





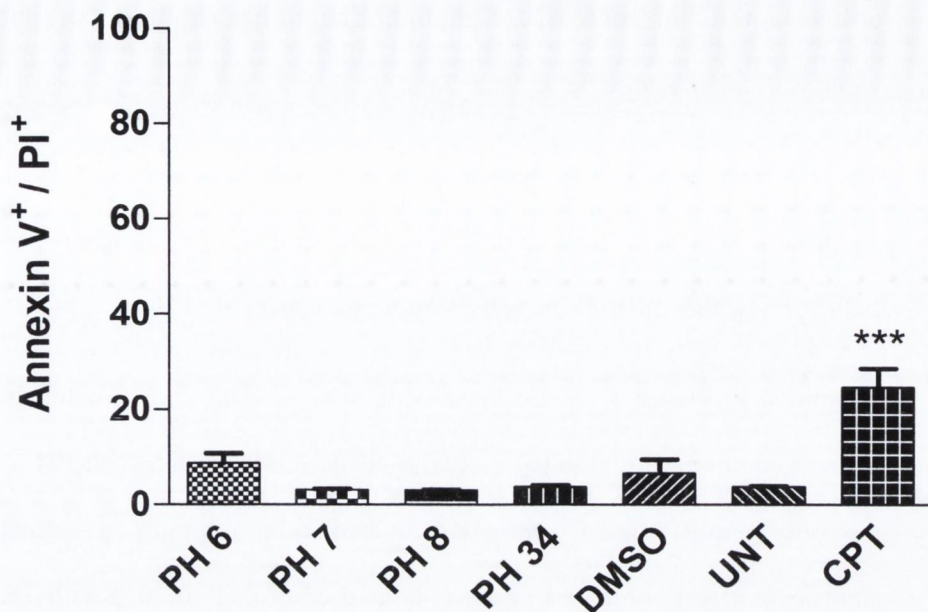
**Figure 3.21: Total % apoptotic Jurkat cells following treatment with test compounds at 10 μM for 24 hours.**

Data of cells positive for Annexin V binding and negative for PI binding (early apoptotic) and positive for both Annexin V and PI binding (late apoptotic) were combined to give the total percentage of cells that demonstrated apoptotic properties following treatment with compounds at 10 μM. Data are expressed as mean ± SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to DMSO, UNT, CPT and all other compounds.



**Figure 3.22: % Jurkat cells Annexin V<sup>+</sup> / PI<sup>-</sup> (early apoptotic) following treatment with test compounds at 10 μM for 24 hours.**

Data of cells positive for Annexin V binding and negative for PI binding (early apoptotic) following treatment with compounds at 10 μM for 24 hours. Data are expressed as mean ± SEM (N=3) where \*\*\* indicates statistical significance with P<0.001 compared to DMSO, UNT, CPT and all other compounds.



**Figure 3.23: The percentage of Jurkat cells positive for both Annexin V and PI (late apoptotic) following treatment with test compounds at 10  $\mu$ M for 24 hours.**

Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to all other samples.

The total percentage of cells undergoing apoptosis was calculated by addition of the values observed for Annexin V<sup>+</sup> / PI<sup>-</sup> (early apoptotic) cells and Annexin V<sup>+</sup> / PI<sup>+</sup> (late apoptotic) cells. Following treatment with the test compounds, all at 10  $\mu$ M for 24 hours, PH 6 was revealed to be the only compound to have a significant apoptotic effect, where the total percentage was calculated to be  $37.1 \pm 10$ . While this value indicated the potential of PH 6 to induce apoptosis when compared to the untreated control, it also demonstrated that the effect of PH 6 was not comparable to that of the

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positive control of apoptosis, Camptothecin, for which the total apoptotic value was calculated to be  $82.96 \pm 5.4$ .

The separate analysis of the data obtained for Annexin V<sup>+</sup> / PI<sup>-</sup> (early apoptotic) cells and Annexin V<sup>+</sup> / PI<sup>+</sup> (late apoptotic) cells gave a clearer indication of the effect of the test compounds, especially PH 6.

Illustrated in Figure 3.15, are those cells that, 24 hours post treatment with the test compounds, exhibited early apoptosis. Cells treated with PH 6 were the only population of compound treated cells to show a statistically significant effect compared to the untreated control ( $P < 0.001$ ) with  $28.36 \pm 6.5$  % of cells binding Annexin V<sup>+</sup> / PI<sup>-</sup>. As with the total percentage of apoptotic cells, PH 6 was also significantly different to the positive control Camptothecin. PH 7, PH 8 and PH 34 showed negligible difference compared to the vehicle control DMSO and the untreated cells. They all displayed statistically significant difference to Camptothecin ( $P < 0.001$ ).

The percentage of cells positive for both Annexin V and Propidium Iodide binding indicates those cells that are late apoptotic or dead. Following 24 hours incubation none of the test compounds showed a statistically significant effect on the Jurkat cells compared to the untreated control. All test compounds, DMSO and untreated samples were significantly different to the positive control of apoptosis, Camptothecin ( $P < 0.001$ ).

These results clearly demonstrate that PH 6 mediates its effect by way of apoptosis. Given that the majority of the total apoptotic population, 24 hours post treatment with PH 6, consists of cells in early apoptosis, coupled with the lack of statistical significance for PH 6 treated cells displaying Annexin V and PI binding characteristic of dead cells or those in late apoptosis, the

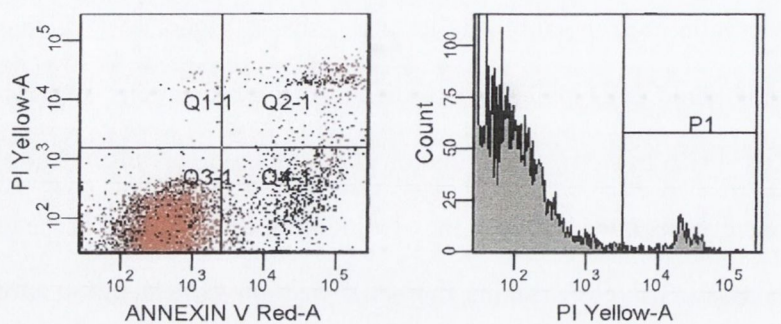
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conclusion that the cytotoxic effect observed in LDH and MTT results was not one of necrosis but apoptosis, required further investigation.

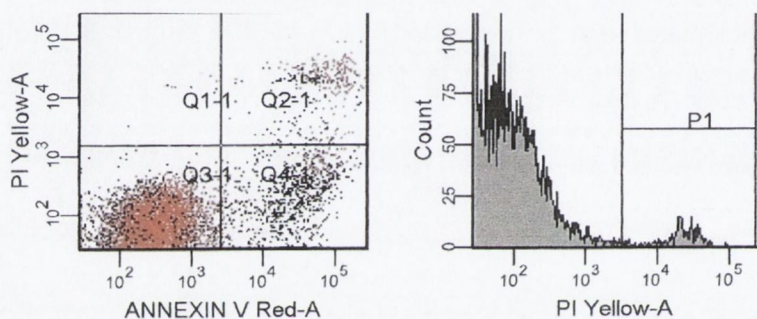
A visual observation of a notably smaller pellet size 24 hours post treatment with PH 6, compared to the pellet size of the untreated Jurkat cells, suggested that part of the PH 6 treated cell population had undergone apoptosis and the dead cells had disintegrated. As such they would not be identifiable at the end point of this assay. Therefore, taking the pellet size into consideration, along with the statistical significance of treatment with PH 6 at 10  $\mu$ M for 24 hours ( $P < 0.001$ ) as illustrated in Figure 3.14, which resulted in the decreased pellet size, it was postulated that a lower dose may yield a higher apoptotic population of cells. A dose response study was subsequently performed to determine if there was a dose dependent relationship.

*PH 6 does not induce apoptosis in a dose dependent manner.*

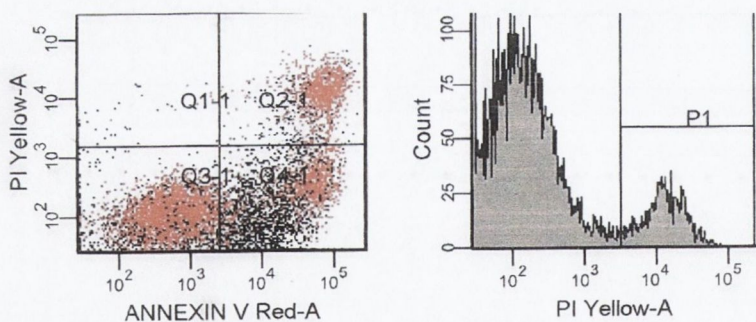
PH 6 0.1  $\mu$ M Jurkat 24 hrs



PH 6 1  $\mu$ M Jurkat 24hrs

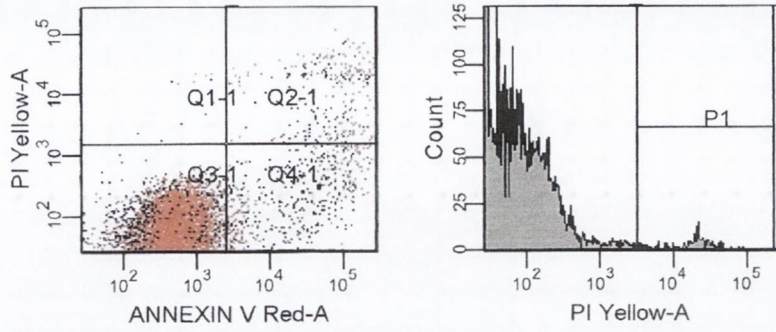


PH 6 10  $\mu$ M Jurkat 24hrs

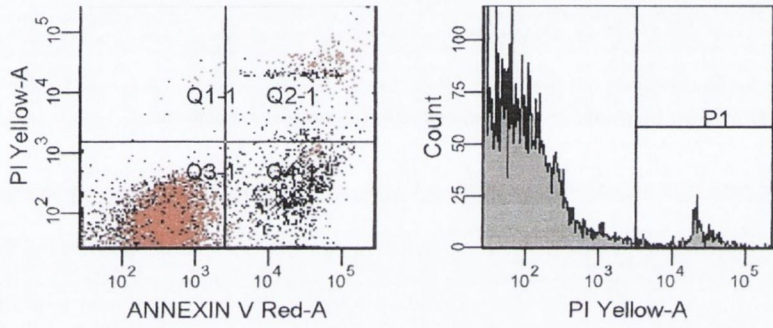


**Figure 3.24: FACS. PH 6 does not induce apoptosis in a dose dependent manner.**

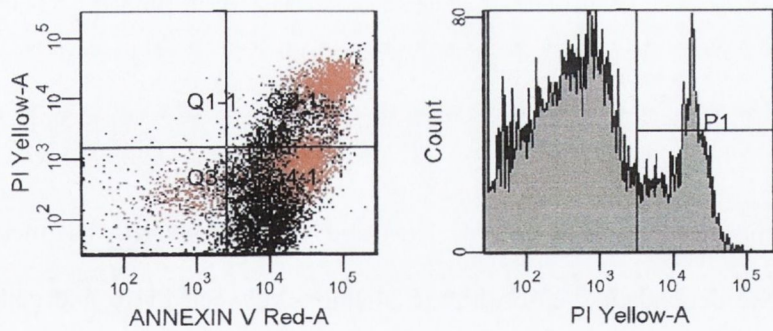
DMSO



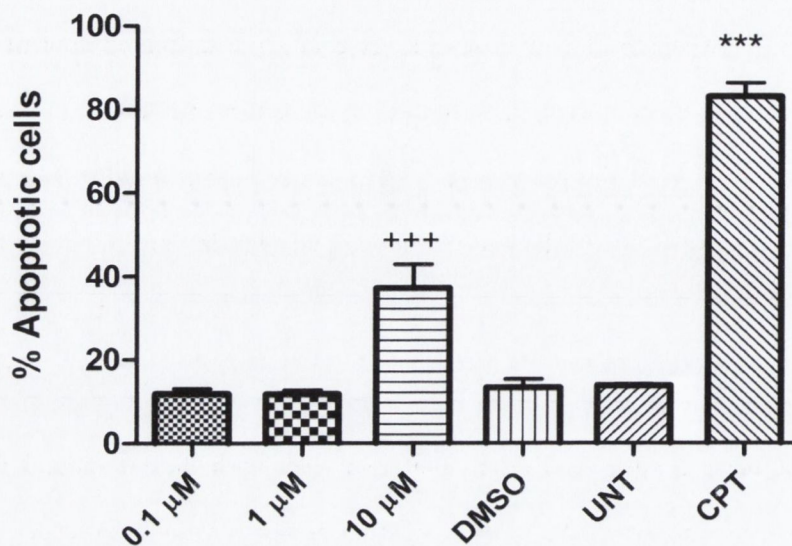
UNT



CPT



**Figure 3.25: FACS Vehicle control (DMSO), Untreated (UNT) control and positive control of apoptosis, CPT.**



**Figure 3 .26: PH 6 Dose response in Jurkat cells 24 hours post treatment.**

Data of cells positive for Annexin V binding and negative for PI binding (early apoptotic) and positive for both Annexin V and PI binding (late apoptotic) were combined to give the total percentage of cells that demonstrated apoptotic properties following treatment with PH 6 at 0.1, 1 and 10  $\mu\text{M}$ . Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to DMSO, UNT, CPT and all concentrations of PH 6, and where +++ indicates statistical significance compared to DMSO, UNT and CPT.

The dose response study with 0.1 $\mu\text{M}$ , 1 $\mu\text{M}$  and 10  $\mu\text{M}$  revealed that there was not a dose dependent relationship at 24 hours between PH 6 and cells binding Annexin V and PI. There was negligible difference between 0.1 and 1 $\mu\text{M}$  compared to the untreated controls. As expected, Camptothecin was statistically significantly different from all samples and again PH 6 at 10  $\mu\text{M}$  was significantly different to the untreated controls.

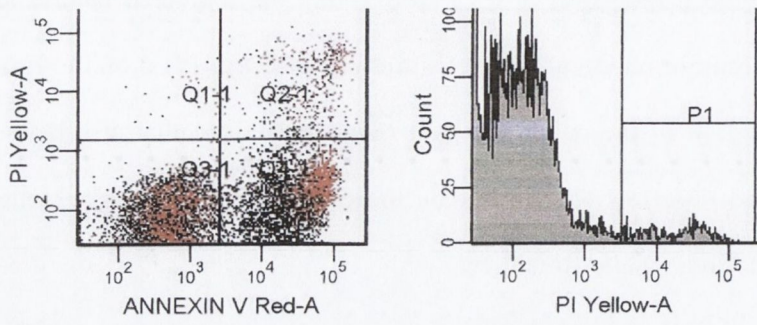


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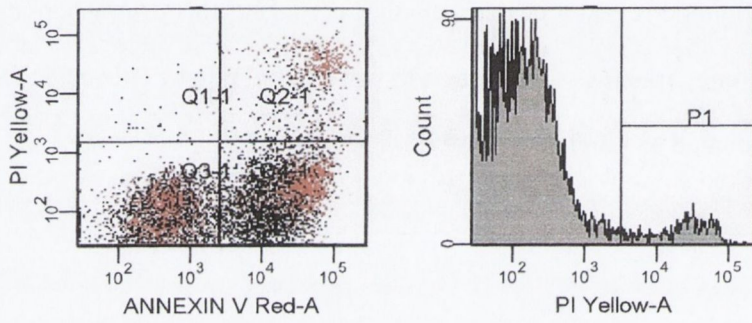
Following the dose response study which demonstrated no significant difference between the lower doses of PH 6 and the untreated control and with no further information gained regarding the effect of PH 6 on the Jurkat cells, the question of the pellet size still remained. Given that at 0.1 and 1  $\mu\text{M}$ , PH 6 had no effect whatsoever on the cell population after a 24 hour incubation period, while treatment with 10  $\mu\text{M}$  induced a significant response, the most evident avenue to pursue was a time course study of PH 6.

The hypothesis for this study was that treatment with the compound for a shorter period could reveal a peak incubation period for this drug where cells were undergoing apoptosis but had not yet disintegrated. The cells were therefore incubated at 4 and 12 hours with PH 6(10 $\mu\text{M}$ ).

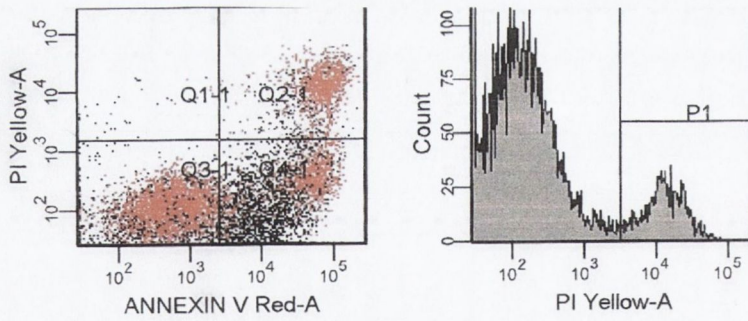
PH 6 Jurkat 4 hrs



PH 6 Jurkat 12 hrs

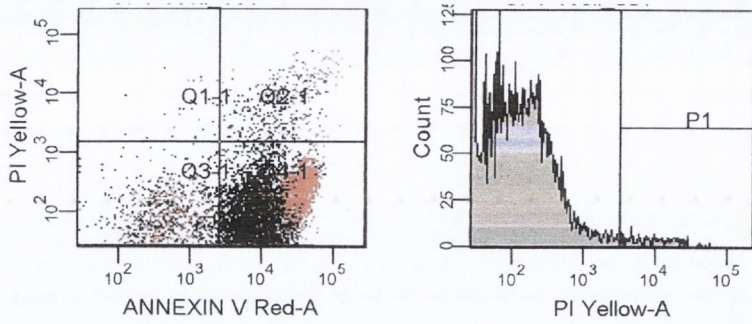


PH 6 Jurkat 24 hrs

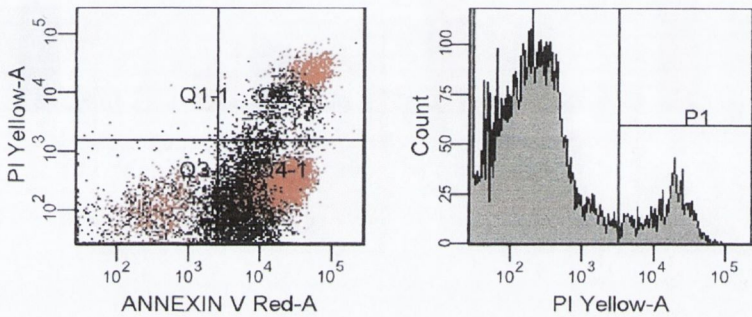


**Figure 3.27:** FACS Representative FACS plots of Jurkat cells obtained following incubation for 4, 12 and 24 h with PH 6 (10 $\mu$ M).

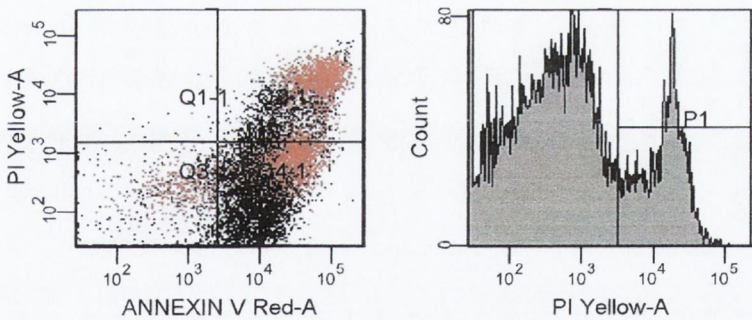
CPT Jurkat 4 hrs



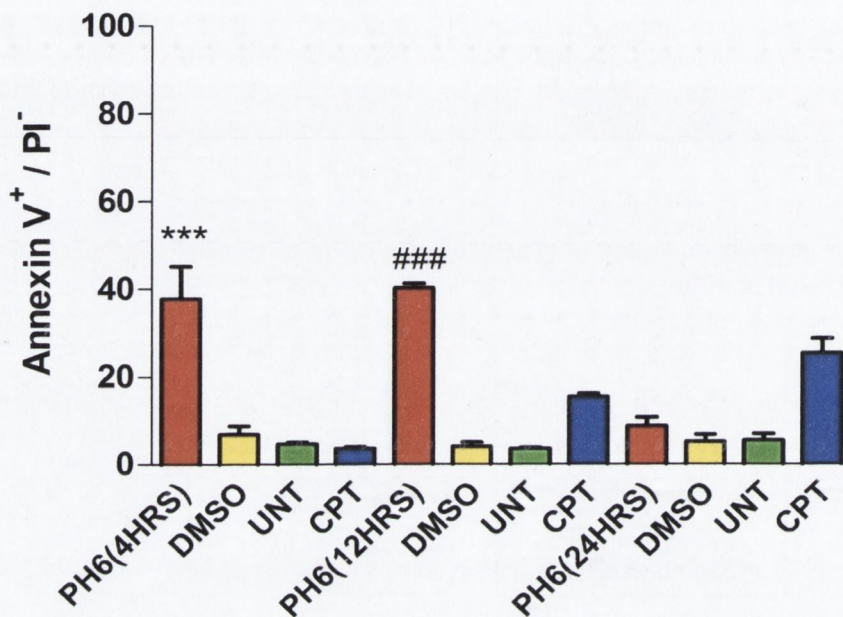
CPT Jurkat 12hrs



CPT Jurkat 24 hrs

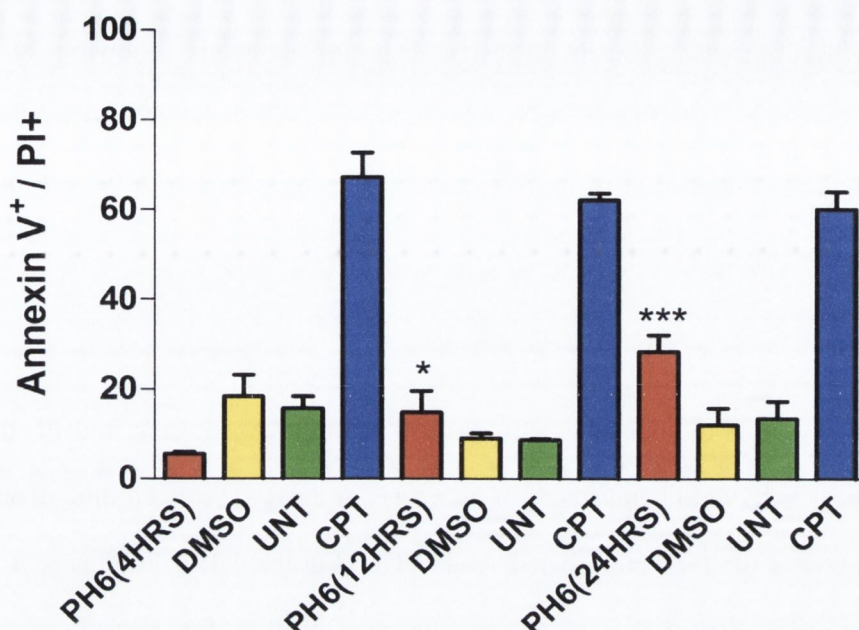


**Figure 3.27:** FACS Representative FACS plots of Jurkat cells obtained following incubation for 4, 12 and 24 h with CPT (2 $\mu$ g/mL).



**Figure 3.28: The % of early apoptotic Jurkat cells (Annexin V<sup>+</sup> / PI<sup>-</sup>) following treatment with PH 6 at 10 $\mu$ M at 4, 12 and 24 hours incubation.**

Data of cells positive for Annexin V binding and negative for PI binding (early apoptotic) following treatment with PH 6 (10  $\mu$ M). Data is expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance compared to DMSO, UNT and CPT (at all time points) and compared to PH 6 (24 hours), and where ### indicates statistical significance compared to DMSO, UNT and CPT (at all time points) and compared to PH 6 (24 hours).



**Figure 3.29: The % of late apoptotic Jurkat cells (Annexin V<sup>+</sup> / PI<sup>+</sup>) following treatment with PH 6 at 10µM at 4, 12 and 24 hours incubation.**

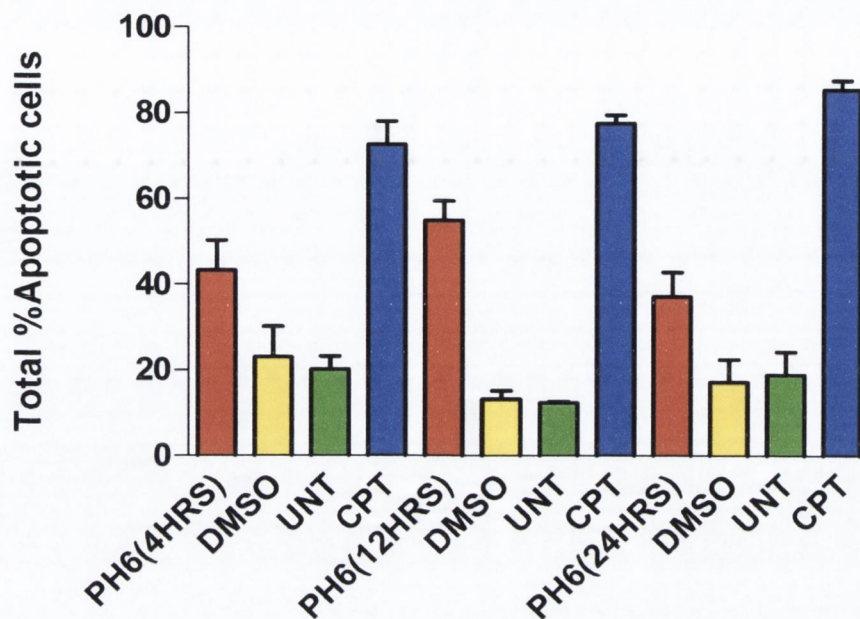
Data of cells positive for both Annexin V and PI binding (late apoptotic) following treatment with PH 6 (10 µM). Data is expressed as mean ± SEM (N=3) where \*\*\* indicates statistical significance (P<0.001) compared to PH 6 (4 hours) and where \* indicates statistical significance (P<0.05) compared to PH 6 (24 hours).

Incubation of Jurkat cells with PH 6 (10 µM) for a shorter period had a clearly significant effect on the percentage of cells undergoing early apoptosis. At 4 hours a significant percentage, 37.83 ± 12.7 %, of cells were determined to be in the early stages of apoptosis, compared to not only the untreated control, but also Camptothecin, the positive control of apoptosis, for which just 3.63 ± 0.5 % displayed Annexin V<sup>+</sup> / PI<sup>+</sup> binding. At 12 hours there was not a significant increase for PH 6 where 40.2 ± 1.7 % of cells were observed as Annexin V<sup>+</sup> / PI<sup>+</sup>. Again, the test compound was statistically different to Camptothecin, even though a statistically significant time

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dependent increase was observed for Camptothecin. The sharp decrease of cells observed to be in the early stages of apoptosis following 24 hours incubation with PH 6, from 12 hours, to  $8.73 \pm 3.4$  % proves that the optimum incubation period for induction for apoptosis with PH 6 should be shorter than 24 hours.

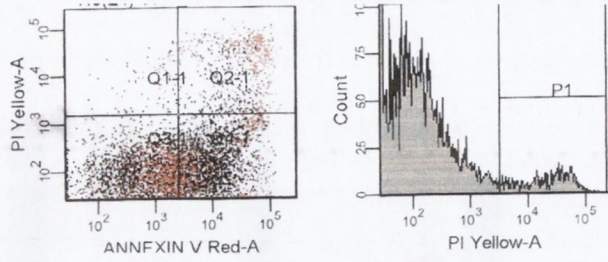
Analysis of the data for cells positive for both Annexin V and PI, that is, those cells in the late stages of apoptosis or dead, revealed a time dependent increase for the cells treated with PH 6 (**Figure 3.19**). Following 4 hours treatment, just  $5.5 \pm 0.7$  % of cells were positive for Annexin V and PI binding. At 12 hours this increased to  $14.76 \pm 8.2$  %, ( $P > 0.05$  versus PH 6 at 4 hours) and was subsequently observed to be significantly higher ( $P < 0.05$ )  $28.36 \pm 6.5$  % at 24 hours. This value fell sharply at 24 hours to  $8.7 \pm 3.4$  %. The increase in dead cells, over time, suggests an apoptotic effect as opposed to necrotic, as with necrosis a more rapid cell death would be observed. Furthermore, the proportion of cells Annexin V<sup>+</sup> and PI<sup>-</sup> (early apoptotic) again demonstrate this point where treatment with PH 6 induced a significant level of early apoptosis at just 4 hours (**Figure 3.18**). The biochemical alterations associated with early stage apoptosis, which facilitate the binding of Annexin V by the movement of phosphatidylserine to the external position on the lipid bilayer, but do not allow for Propidium iodide binding, as is the case with necrosis. This indicates that cell death associated with PH 6 occurred via an apoptotic pathway as opposed to its effect being that of necrosis.



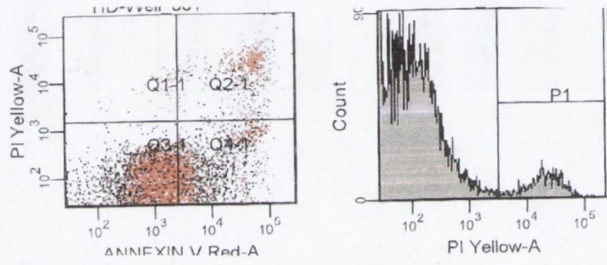
**Figure 3.30 : The total % of apoptotic Jurkat cells following treatment with PH 6 at 10 $\mu$ M at 4, 12 and 24 hours incubation as determined by FACs with Annexin V and PI binding.**

Data of cells positive for Annexin V binding and negative for PI binding (early apoptotic) and positive for both Annexin V and PI binding (late apoptotic) were combined to give the total percentage of cells that demonstrated apoptotic properties following treatment with PH6 at 10  $\mu$ M. Data is expressed as mean  $\pm$  SEM (N=3).

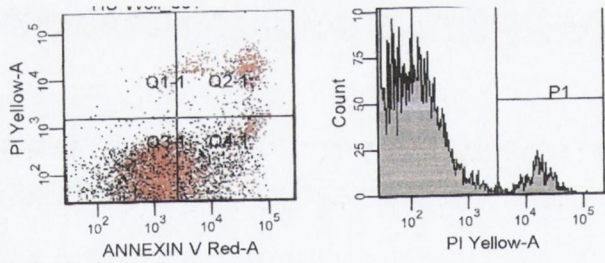
PH 6 (10 $\mu$ M) HuT-78 24 hrs



HuT-78 DMSO 24hrs



HuT-78 Untreated 24 hrs



HuT-78 CPT (2 $\mu$ g / mL) 24 hrs

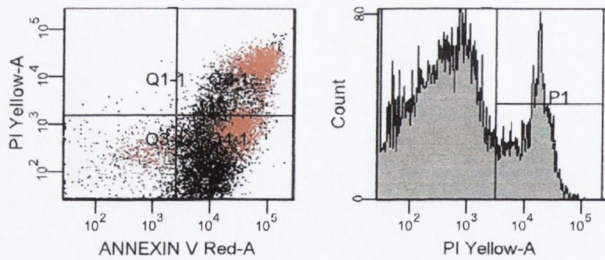
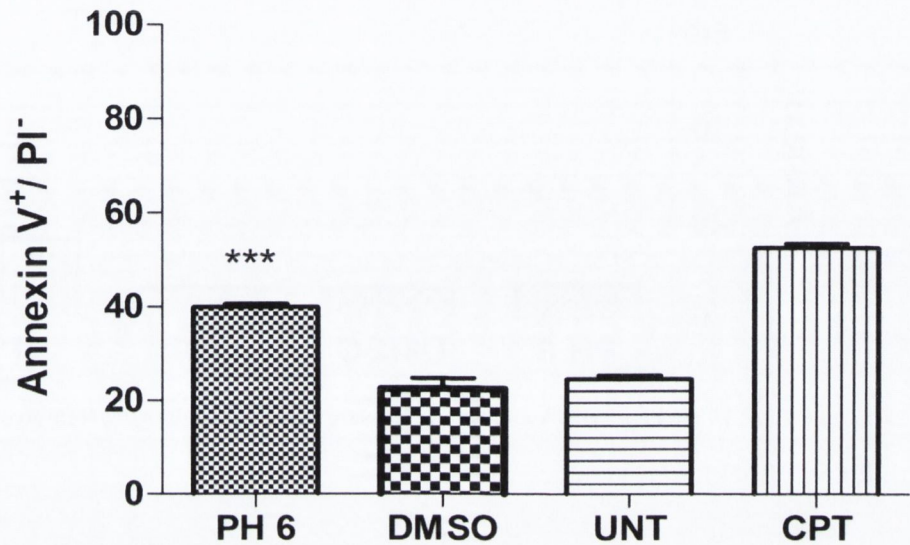


Figure 3.31. FACS Representative FACS plots of HuT-78 cells obtained following



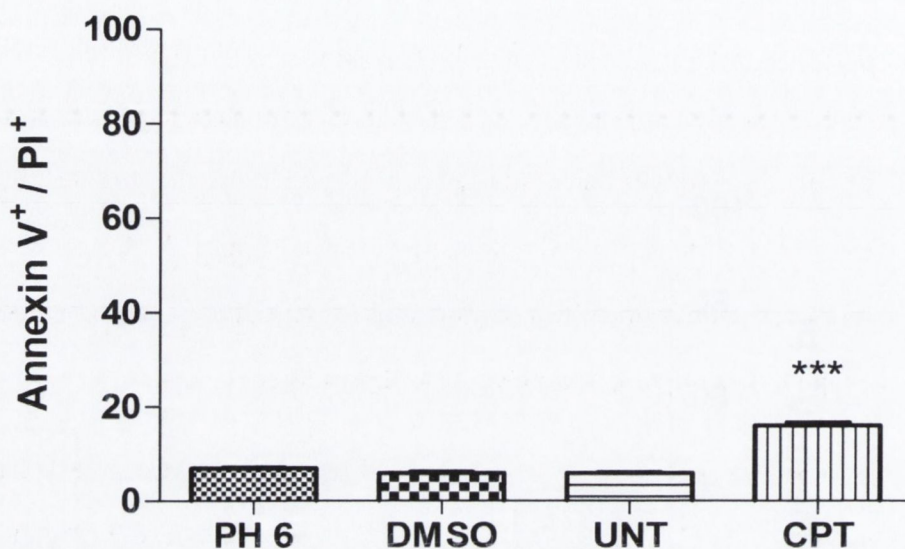
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*PH 6 significantly induces apoptosis in HuT-78 cells.*



**Figure 3.32: % HuT-78 cells Annexin V<sup>+</sup> / PI<sup>-</sup> (early apoptotic) following treatment with PH 6 at 10  $\mu$ M for 24 hours.**

Data of cells positive for Annexin V binding and negative for PI binding (early apoptotic) following treatment with PH 6 at 10  $\mu$ M for 24 hours. Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to DMSO, UNT, CPT.



**Figure 3.33: % HuT-78 cells Annexin V<sup>+</sup> / PI<sup>+</sup> (late apoptotic) following treatment with PH 6 at 10  $\mu$ M for 24 hours.**

Data of cells positive for Annexin V binding and negative for PI binding (early apoptotic) following treatment with PH 6 at 10  $\mu$ M for 24 hours. Data are expressed as mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance with  $P < 0.001$  compared to DMSO, UNT, PH 6.

As illustrated by both Figure 3.21 and Figure 3.22 x: % HuT-78 cells Annexin V<sup>+</sup> / PI<sup>-</sup> (early apoptotic) following treatment with PH 6 at 10  $\mu$ M for 24 hours, PH 6 significantly induced apoptosis in HuT-78 cells 24 h post treatment, with  $39.96 \pm 0.351$  % of cells binding Annexin V<sup>+</sup> / PI<sup>-</sup> and  $6.93 \pm 0.34$  % binding Annexin V<sup>+</sup> / PI<sup>+</sup>. This was compared to the untreated population of cells, for which  $24.3 \pm 0.98$  % of cells binding Annexin V<sup>+</sup> / PI<sup>-</sup>, and  $5.83 \pm 0.31$  % were found to bind Annexin V<sup>+</sup> / PI<sup>+</sup>. Camptothecin significantly induced apoptosis following 24 h with a total population of apoptotic cells  $69.33 \pm 1.42$  %.

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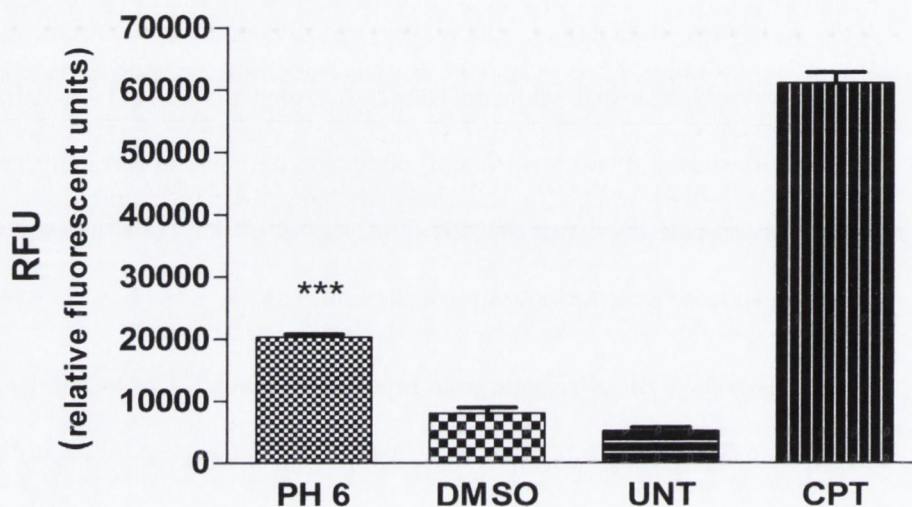
### **3.4 Poly-Caspase Activity (FLICA)**

The observation of morphological changes associated with apoptosis following treatment with PH 6 demonstrated in the Annexin V and PI binding study were clear indicators of the activity of this compound. Further to this, was the observation that while resistance to apoptosis in HuT-78 cells had been demonstrated, PH 6 significantly induced apoptosis in this cell line. To further investigate the effect that PH 6 had on the Jurkat cells, in the context of apoptosis, poly caspase activity was determined.

In this assay the FLICA reagent used binds to Caspase 1, Caspase 3, Caspase 4, Caspase 5, Caspase 6, Caspase 7, Caspase 8 and Caspase 9. Therefore, in this instance it was used as a generic method of detection of the total caspase activity, as an adjunct evaluation of the apoptotic inducing capacity of PH 6. In this way a greater understanding of the cell death associated with PH 6 treatment, in Jurkat cells, was developed.

The rationale for investigating Jurkat cells was based on the resistance demonstrated by HuT-78 cells. Given that this poly caspase assay was the initial examination with respect to caspase activity, the Jurkat T cell line was preferable given its susceptibility to apoptosis.

*PH 6 significantly induces caspase activity*



**Figure 3.34: Poly Caspase activity of PH 6 (10 $\mu$ M) treated Jurkat cells.**

Data represents the relative fluorescent units (RFU) measured with excitation wavelength of 490 nm and emission wavelength of 520 nm, of Jurkat treated cells with PH 6 (10 $\mu$ M) 12 hours post treatment. Data is expressed at mean  $\pm$  SEM (N=3) where \*\*\* indicates statistical significance compared to the untreated control, (P<0.001).

Caspase activity of Jurkat cells 12 hours post treatment with PH 6 at 10  $\mu$ M further demonstrated the apoptotic potential of this compound. The RFU value obtained for PH 6 treated cells was 20,269  $\pm$  1293, which was statistically significantly different to both untreated control and vehicle control DMSO. However, PH 6 did not induce caspase activity to the same extent as the Camptothecin control, where statistical significance was again observed.

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Given that caspase activity is an effect that occurs downstream of the morphological changes associated with apoptosis and those identified in the Annexin V and PI binding study, the indication is that PH 6 is inducing apoptosis in these cells. Also as the optimum effect observed in the Annexin V and PI binding study followed 12 hours incubation, and as caspase activity occurs downstream of the morphological changes, the caspase inducing activity of PH 6 should be investigated at a shorter incubation period.

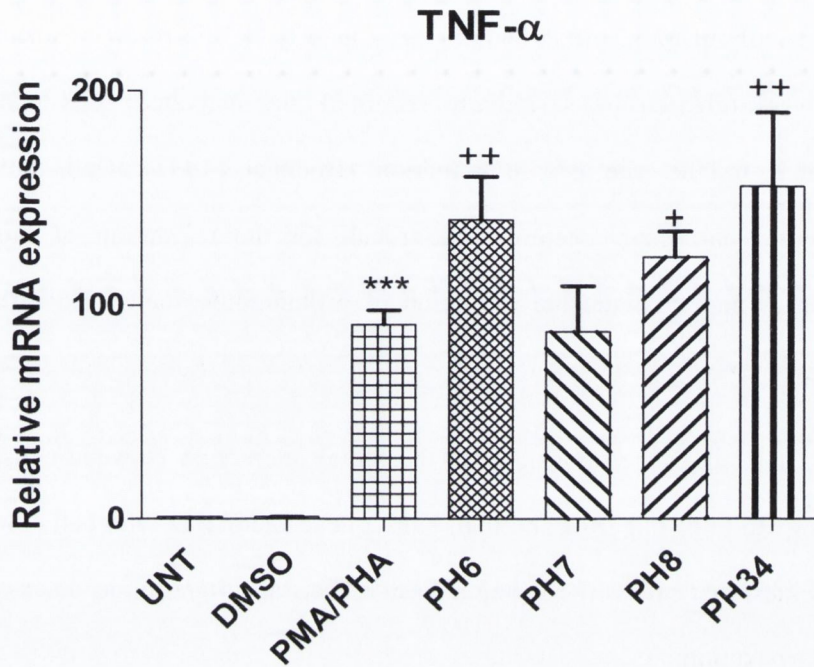
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### **3.5 Pro-inflammatory cytokine regulation**

The expression of TNF- $\alpha$  following treatment with the PH test compounds was carried out to determine if the drugs had any effect on the expression of the pro-inflammatory cytokine TNF- $\alpha$ . This cytokine was chosen due to its involvement with apoptosis, but also even though widely reported and demonstrated to behave in a pro-inflammatory context, it has been shown in TNF- $\alpha$  deficient mice that the absence of this cytokine resulted in the development of a more severe form of the animal model of multiple sclerosis, EAE (experimental autoimmune encephalomyelitis). (Imitola, Chitnis et al. 2005)

With respect to apoptosis, PH 6 had demonstrated significant efficacy in the induction of apoptosis as discussed previously. In determining whether PH 6 was mediating this effect by the up regulation of TNF- $\alpha$  a greater understanding of the action of this drug could be achieved.

*PH6, PH8 and PH 34 significantly upregulate TNF- $\alpha$  mRNA expression*



**Figure 3.35: Relative mRNA expression of TNF- $\alpha$  4 h post treatment**

Data of Jurkat cells treated with PH compounds at 10  $\mu$ M. Data is expressed as mean  $\pm$  SEM (N=3). PH 6 significantly upregulates expression of TNF- $\alpha$

Following treatment with the PH test compounds a significant upregulation of TNF- $\alpha$  was observed with PH 6 causing  $140 \pm 28.2$  fold change with respect to the untreated control ( $P < 0.001$ ) and ( $P < 0.01$ ) compared to PMA/PHA treatment.

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## **4. Discussion**

### **4.1 LFA-1 mediated T cell motility**

Defined more than a century ago as a localised swelling with redness, pain and loss of function, inflammation was found to be the result of chemicals that were released by selectively recruited and activated cells that had migrated to the site of inflammation (Bochner, 1997). Cell adhesion molecules and their receptors are crucial for the regulation of immune responses and facilitate the migration of inflammatory cells into inflamed tissues.

While selectins are responsible for the initial interaction between cells, it is the activation of integrins and their subsequent interaction with cell adhesion molecules, that causes the strong adhesion and transmigration as discussed in the introduction.

These adhesion molecules present numerous potential targets for the treatment of inflammation and autoimmunity, and given the focus of this thesis on LFA-1 mediated T cell motility, the results are discussed in that context.

The involvement of activated leukocytes, especially T lymphocytes, in mediating the immune response constitutes a pivotal role in both mounting and amplifying the immune response. The adaptive defence against pathogens is composed of both the cellular and humoral immune response. The humoral response is also referred to as B-lymphocyte immune response and involves antibody production, whereas the T lymphocyte or cellular response centres on cell-cell interactions (Anderson and Siahaan 2003).



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Autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis and insulin-dependent diabetes mellitus (IDDM) all entail autoreactive lymphocyte infiltration to a particular organ or site (Helena Yusuf-Makagiansar 2002). Adhesion molecules as a target for immunomodulation, therefore provide the means of reducing the infiltration of inflammatory cells to sites of inflammation.

Anti-adhesion therapy, as a strategy for the treatment of autoimmune conditions, stems from the essential participation of adhesion molecules in the pathophysiology of autoimmune diseases such as IBD. The clinical success of the use of Natalizumab in the treatment of both multiple sclerosis and Crohn disease substantiates this idea. Natalizumab is a monoclonal antibody to the  $\alpha_4$  integrin chain that works by blockade of binding, of  $\alpha_4\beta_7$  (VLA-4) to VCAM-1 on  $T_H1$  cells infiltrating the brain, and blockade of binding of  $\alpha_4\beta_7$  to MadCAM-1 on  $T_H1$  cells infiltrating the gut (Luster, Alon et al. 2005).

Also, elevated levels of LFA-1 and ICAM-1 expression have been shown in the CNS of MS patients. High levels of LFA-1 and VLA-4 expression, in memory T cells, were observed in intestinal inflammation, along with high levels of ICAM-1 and VCAM-1 (Li, Jevnikar et al. 1997; Brandtzaeg, Farstad et al. 1999).

Further demonstrating the potential of inhibiting cell recruitment to the site of inflammation as a target, is asthma, an inflammatory lung disease that is characterised by the infiltration of  $CD4^+$  lymphocytes and eosinophils (David

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Broide 2001). Prevention of allergen induced recruitment of eosinophils into the airways has been shown by the blockade of  $\alpha_4$ -integrin (W R Henderson, E Y Chi et al. 1997).

In terms of modelling blockade of adhesion molecules, extensive preclinical work has demonstrated the efficacious blockade of adhesion molecules in diverse disease models. One of the earliest investigations examined the administration of anti- $\beta_7$  integrin and anti-MAdCAM-1 antibodies to colitic SCID mice, where a significant reduction in the number of infiltrating leukocytes was observed in the lamina propria and mesenteric lymph nodes of inflamed colons (Picarella, Hurlbut et al. 1997). Further to this, the attenuation of colitis that was induced by CD4<sup>+</sup> T cells, which were isolated from LFA-1 (CD11a/CD18) - deficient mice, and a similar attenuation of disease that was observed in CD18 and CD11a null mice when DSS was administered (Abdelbaqi, Chidlow et al. 2006; Pavlick, Ostanin et al. 2006) clearly demonstrate the impact of modulating adhesion molecules. The effect of altering expression of ICAM-1 is also demonstrated in many animal models. While increased ICAM-1 and VCAM-1 mRNA levels have been observed in TNBS-colitic mice, administration of an anti-ICAM-1 antibody resulted in attenuation of disease in rats (Sans, Salas et al. 2001; Yoshida, Yamaguchi et al. 2001).

Antibodies against several adhesion molecules in experimental autoimmune encephalomyelitis (EAE) have also shown the critical role for adhesion molecules in the pathogenesis of EAE.

Noteworthy, regarding the use of antibodies in the treatment of autoimmune conditions, are issues such as physicochemical properties, rendering oral

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administration difficult, the cost associated with production, and stability issues that are problematic with respect to formulation (Anderson and Siahhaan 2003). While literature regarding monoclonal antibody use is substantial, the use of a small chemical entity may prove more favourable in pharmaceutical terms.

The use of monoclonal antibodies provides a blockade of adhesion receptors. However, alternative strategies include inhibition of the expression of adhesion receptors, and the inhibition of the activation of integrins. Anti-inflammatory drugs have been shown to mediate their effects through both the former and latter of these strategies (Gonzalez-Amaro, Portales-Perez et al. 1998).

Further to the above, the cytoskeletal machinery involved in driving the T cell migratory events, present further potential targets for the action of the compounds, including Rho, Rac, cdc-42, ROCK, and MLCK.

In this work, the potential of the test compounds to inhibit LFA-1 mediated T cell motility was determined by measuring the major and minor axis, and the perimeter of each activated cell. The extent of cytoskeletal rearrangement and deformation was subsequently calculated as the deformation index. A pilot study demonstrated the use of this assay in evaluating the compounds for their potential anti-inflammatory properties, where significant inhibition of LFA-1 mediated T cell motility was observed following treatment with PH 11 and PH 12.

The extent of inhibition was initially indicated by visual observation whereby cells had maintained a non-adherent property, a possible result of constitutive expression of LFA-1 and VLA-4 integrins with low binding capacity which

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facilitates the function of cells in a non-adherent or circulating mode (Helena Yusuf-Makagiansar 2002). In comparison to the control DMSO treated cells, where cells had undergone cytoskeletal rearrangement, PH 11 and PH 12 treated cells displayed morphology similar to that of the negative control, Go6976, where cells maintained a spherical shape. This observation is in keeping with the archetypal events of migration, whereby the formation of a leading lamella is followed by nucleokinesis, and extension of the uropod and eventual retraction of the trailing uropod (Volkov et al., 1998). The inhibitory effect of these two compounds was further demonstrated by the fact that the deformation index was less than three.

A subsequent study to ascertain the potential of the main test compounds for this thesis revealed PH 11, PH 12, to be less potent than PH 6, at inhibiting T cell motility. This comparison is evident, again in the images, where cells were observed to be more oval in shape. Slight spreading of the cytoskeleton was also seen and appears as very light grey projections from the front and rear of the cells. These projections were more clearly defined in the images of the DMSO treated cells, and also, the images of cells treated with compounds that did not significantly inhibit migration, such as PH 9, PH 10 or PH 16.

These results demonstrate the potential of PH 6 as a pharmacological inhibitor of LFA-1 mediated T cell motility and as such, upon further investigation, could provide an alternative to mAbs therapy, where there are concerns with respect to the immunogenic potential and systemic administration as a result of challenging relative efficacy / safety ratio (Yusuf-Makagiansar, Anderson et al. 2002).

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With respect to the naphthoquinone compounds assessed, all compounds were tested at 10  $\mu$ M initially. Experimental and control conditions were the exact same as for the PH compounds. Of the seven HIH compounds tested, HIH 6 and HIH 7 were the only compounds that had a significant effect on LFA-1 mediated T cell motility following 4 h incubation. Upon visual inspection, limited rearrangement of the cytoskeleton was observed. No leading lamella or trailing uropods were observed, which in turn determined a consistent deformation index of less than three. Hence, cell motility for HIH 6 and HIH 7 was observed at  $1.75 \pm 0.21\%$  and  $5.8 \pm 0.58\%$  respectively. HIH 1, HIH 2, and HIH 3 treatment of the Hut-78 cells resulted in  $47.6 \pm 0.39\%$ ,  $47.09 \pm 6.25\%$  and  $44.68 \pm 5.25\%$  motile cells, respectively with extensive rearrangement of the cytoskeleton observed. Limited cells resembling the original spherical shape were observed. The cells were observed as having adhered, spread and undergone similar rearrangement to that of the untreated DMSO control. HIH 4 treatment of the HuT-78 cells was more effective in inhibiting motility, than HIH 1, HIH 2 or HIH 3. While leading lamella and cytoplasmic projections were observed, some of the cells had retained a slightly spherical shape. They had adhered, however, and migration was inhibited with the result that  $30.05 \pm 6.1\%$  of cells were motile.

The significant inhibition of HuT-78 motility in response to treatment with HIH 6 and HIH 7 at 10  $\mu$ M necessitated a further dose response study. The assay was performed as previously described, with varying concentration.

A dose dependent relationship was observed. Treatment at 30  $\mu$ M resulted in almost complete inhibition of cell motility, where only  $0.7 \pm 0.0006\%$  of

cells were determined to be motile, for HIH 6 and  $0.79 \pm 0.03\%$  for HIH 7. The very low numbers of cells assigned a deformation index greater than three, from which the percentage of motile cells was calculated, confirmed the visual observations where no rearrangement of the cytoskeleton, resulting in cytoplasmic projections, had occurred. The cells retained their initial shape. Furthermore, even at the lowest concentration of 100 nM, the motility of cells remained low at  $21.7 \pm 2.16\%$  and  $13.34 \pm 2.96\%$  respectively for HIH6 and HIH7. This suggests that both HIH6 and HIH7 are potent inhibitors of LFA-1 mediated T cell locomotory behaviour.

As discussed with respect to the PH compounds, the significant inhibition of LFA-1 mediated T cell motility following treatment with HIH 6 and HIH 7 provides potential alternatives to monoclonal antibody treatment and as such warrants further investigation to ascertain the mechanism of this action.

#### **4.2 Cytotoxicity and Apoptosis**

For any novel compound, determination of its cytotoxic properties is imperative in assessing the overall potential of the drug, be it intended as a toxic compound or not. For this work all test compounds were initially evaluated for their cytotoxic properties in MTT and LDH assays.

Results from these experiments showed significant cell death associated with PH 6 in both HuT-78 and Jurkat T lymphocytes. The significant effect of PH 6 on cell viability, as determined by both MTT and LDH assays, was further exemplified by the fact that from 4 h to 24 h incubation, the cell death observed in Jurkat T lymphocytes, altered only marginally, clearly indicating

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that PH 6 was most potent at a shorter incubation period. For all other drug treatments no substantial increase in cell death was observed in either the MTT or LDH assays. Keeping in line with the reported mechanism of action of Camptothecin, which mediates its effect by topoisomerase I inhibition (Leroy F. Liu 2000), Jurkat cells incubated with this treatment did not show significant cytotoxicity as membrane integrity was not compromised.

Notable, however, was the variation of results observed for HuT-78 and Jurkat treated T lymphocytes. The effect of treatment with PH 6 on cell viability in the MTT assay was more pronounced in Jurkat cells, with absorbance at 24 h recorded to be less than 0.25. HuT-78 cells showed significantly higher values following 4 h incubation with PH 6, indicating a higher survival population, compared to Jurkat cells. **Figure 3.10.**

Furthermore, at 24 h post treatment with PH 6, absorbance values recorded for HuT-78 cells illustrated the less potent effect of PH 6 on these cells, where absorbance rose substantially, to over 0.75, compared to treatment at 4 h. This larger margin in cell viability between incubation periods, and cell type, is attributable to the fact that in HuT-78 cells, at 4 h post treatment a larger population of cells were deemed viable. Therefore, there was a greater starting population to proliferate in the following 20 h. This is due to the issue of resistance in HuT-78 cells as a result constitutively activated NF- $\kappa$ B. This effect is demonstrated by the difference in absorbance recorded in both cell lines at 4 and 24 h post incubation. (**Figure 3.6, Figure 3.12**). The presence of activated NF- $\kappa$ B promotes survival in the cell and therefore the Jurkat cells showed a higher level of cytotoxicity.

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While both assays are widely utilised in the cytotoxic evaluation of novel compounds, the results of each are not entirely comparable. On the one hand, MTT is a direct measure of those cells that, following treatment, retain viable mitochondria. LDH, on the other hand, assesses cell viability with respect to membrane integrity, as measured by the release of lactate dehydrogenase. Therefore, while both are considered valid in terms of determining cytotoxicity, they are fundamentally different.

Given the extent of toxicity associated with treatment with PH 6, determining the means by which this effect was mediated necessitated evaluation with respect to the apoptotic inducing potential of these drugs.

As mentioned in the introduction, the process of programmed cell death, or apoptosis, is essential in T cell development, shaping the immune repertoire, and in the initiation and resolution of immune responses (Krammer 2000). Further to this, and in the context of the work carried out for this thesis, the dysregulation of apoptosis is implicated in many autoimmune conditions. Given the role of apoptosis in balancing T cell numbers and in the development of the T cell repertoire, pharmacological induction of apoptosis poses a promising target to control those activated T cells that have escaped apoptosis in autoimmune diseases (Rüdiger Arnold 2006). This is demonstrated by the inherited disorder of apoptosis (ALPS) where an inability to maintain normal lymphocyte homeostasis is the result of failed apoptotic mechanisms (Giovannetti, Pierdominici et al. 2008). As with all organs, balancing cell proliferation with cell death is critical to the local physiology and pathology. This is illustrated by immune-mediated diseases in the gastrointestinal tract, where either an increase or decrease in apoptosis



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can induce autoimmune conditions, namely Ulcerative colitis and Crohn's disease, which occur in the event of abnormally decreased apoptosis. Sulfasalazine has been shown to induce apoptosis, and while the mechanism of its therapeutic effect is yet to be fully elucidated, the interference of this drug with NF- $\kappa$ B/Rel activation may play a part (Liptay, Fulda et al. 2002).

It is the survival of a subset of T cells, which upon recognition of "self" peptide as "non-self peptide" or antigenic peptide, become activated and proliferate, that result in autoimmune disease pathology (Anderson and Siahaan 2003). In rheumatoid arthritis, defects observed in apoptosis activation cause the dysregulation of cell proliferation and the inflamed synovium becomes infiltrated with inflammatory cells, proliferating fibroblasts, lymphocytes and endothelial cells (Buckley, Pilling et al. 2001). FLIP blocks the Fas dependent apoptotic pathways in monocytes / macrophages of rheumatoid synovium which results in inhibition of activation of caspase-8. NF- $\kappa$ B and PI3K/AKT-1 activation occurs, causing the upregulation of Bcl-2 which is involved with mitochondrial stabilisation and apoptosis inhibition (Choy and Panayi 2001; Liu and Pope 2003). All of this evidence demonstrates the magnitude of the involvement of apoptosis in autoimmune disease and as such, implicates the potential of modulating apoptosis as a pharmacological target.

Initially, to determine the apoptotic effect, if any, of the test compounds, HuT-78 T lymphocytes were incubated with the test compounds for 4 h, and assessed flow cytometrically for Annexin V and Propidium Iodide binding. The results from this experiment were inconclusive as no significant difference between the untreated population of cells and those treated with

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the positive control for apoptosis, Camptothecin, was observed. A subsequent attempt to determine the apoptotic effect of the compounds, with respect to caspase activity, yielded a similar lack of disparity between controls. A time course study yielded only a significant difference at 24 h in assessment of Annexin V and PI binding. This was explained by the presence of constitutively activated NF- $\kappa$ B in HuT-78 cells, which causes resistance to apoptosis (Giri and Aggarwal 1998).

The transcription factor NF- $\kappa$ B mediates its participation in immune and inflammatory responses by regulating the expression of numerous genes, including those involved in apoptosis (Shishodia and Aggarwal 2001; Ashikawa, Majumdar et al. 2002). Anti-apoptotic proteins such as Bcl-2, Bcl-xl, c-FLIP, Bfl-1/A1 and c-IAP are NF- $\kappa$ B dependent. Down regulation of these proteins increases sensitivity to apoptotic stimuli by altering the apoptotic threshold in favour of commitment to cell death. Activation and constitutive activation of NF- $\kappa$ B however, can repress the apoptotic activity normally associated with TNF- $\alpha$  treatment, cytotoxic drugs or radiation (Burstein and Duckett 2003). The relationship between NF- $\kappa$ B and anti-apoptotic genes has been demonstrated by various experimental techniques, such as cell death caused by TNF (Wallach, Varfolomeev et al. 1999). Exemplifying the effect of NF- $\kappa$ B, in terms of resistance to apoptosis induced by TNF, were mice lacking in Rel A. NF- $\kappa$ B signaling was impaired as a result and the animals were observed to die *in utero* resulting from hepatocyte cell death. When fibroblasts derived from the embryos of these mice were cultured *in vitro*, sensitivity to TNF-mediated apoptosis increased significantly (Beg and Baltimore 1996). The same was found to be true for mice deficient in IKK $\beta$ , where again, *in utero* death was observed, and

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fibroblast sensitivity to TNF, of the embryos of these mice, was also significantly augmented (Li, Chu et al. 1999; Tanaka, Fuentes et al. 1999).

Further evidence of the anti-apoptotic effect of NF- $\kappa$ B is demonstrated by relationship between the expression of anti-apoptotic proteins and NF- $\kappa$ B. The levels of these anti-apoptotic proteins and subsequent sensitivity to apoptotic stimuli can be affected by the presence  $\kappa$ B sites on the gene promoters (Burstein and Duckett 2003).

As mentioned in the introduction, the release of mitochondrial pro-apoptotic factors such as cytochrome c and Smac/DIABLO, is a process regulated by the Bcl-2 gene family. It has been shown that induction of the anti-apoptotic proteins in this gene family, is mediated by NF- $\kappa$ B (Chen, Edelstein et al. 2000).

Given the resistance to apoptosis in HuT-78 cells, assessment of the effect of treatment with PH compounds on the alternative T lymphocyte cell line, Jurkat was carried out. Following induction of apoptosis by Camptothecin at 24 h, a significant difference between positive and untreated controls was observed. The test compounds were subsequently incubated with Jurkat T lymphocytes for 24 h and PH 6 was shown to be the only compound to induce apoptosis significantly. **Figure 3.14.**

Further to this, was the visual observation of the experimental endpoint pellet size of PH 6 treated cells, which was markedly smaller than that of the untreated cells, and to a lesser extent, the positive control cells. This factor was not observed in HuT-78 treated cells, where negligible variation in pellet size was observed between controls, except 24 h post treatment.

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While indicative of the apoptotic effect of PH 6, this data related to the total population of cells, and as such included both those cells that were deemed to be in early apoptosis and those that were either in late apoptosis or dead. Analysis of the data for cells Annexin V<sup>+</sup> /PI<sup>-</sup> and Annexin V<sup>+</sup> /PI<sup>+</sup>, separately, further exemplified that the effect of treatment with PH 6 was indeed that of apoptosis, as the majority of cells making up the total percentage fell into the category of those that were Annexin V<sup>+</sup> /PI<sup>-</sup>.

The disparity of results observed for the controls, between the HuT-78 and the Jurkat cell lines, in the initial apoptosis study, was in keeping with the results observed in both the MTT and LDH assays. While both were demonstrative of the cytotoxic capabilities of PH 6, the more potent effect on Jurkat cells reiterated the issue of resistance of HuT-78 cells as seen in the FACs and caspase study. This was clear in the MTT assay with HuT-78 treated cells, where a significantly higher population survived following treatment with PH 6 compared to that of the Jurkat treated cells. Figure 3.6 and Figure 3.10

Furthermore, while the intracellular contents of apoptotic cells are not released into the surrounding tissue, as membrane integrity is maintained, the observed results following both 4 h and 24 h incubation where Jurkat cells showed significant release of LDH release can be explained by the fact that as the cell disintegrates into apoptotic bodies, they maintain membrane integrity. In the event of these apoptotic cells not being removed quickly, a process known as secondary necrosis occurs, whereby, subsequent loss of membrane integrity causes the release of the contents of the apoptotic bodies, including LDH into the surrounding tissues (Sun and Shi 2001). Given that

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the work carried out for this thesis was *in vitro* in nature, phagocytosis of the apoptotic bodies did not occur. *In vivo*, rapid disposal of these bodies by phagocytosis occurs, and no inflammation is observed (Power, Fanning et al. 2002).

Caspase activity was also investigated in a poly caspase FLICA assay. In this assay the active caspases bind to the fluorophore. The extent of fluorescence is proportional to the presence of active caspases.

The activation of caspases is fundamental to the initiation and execution of programmed cell death. Assessment of Annexin V and PI binding determines whether the morphological changes associated with apoptosis are present. Positive binding for these indicates that the cell has already committed to and initiated the process of cell suicide. By assessing the effect of treatment with PH 6 on Jurkat cells with respect to caspase activity a more comprehensive understanding of the means by which PH 6 was inducing cell death was achieved.

Following incubation with PH 6 for 12 h, significant activation of caspases was observed, further indicating the apoptotic effect of PH 6. **Figure 3.23.**

Given the survival of a larger population in the constitutively activated NF- $\kappa$ B HuT-78 cells, and the data for Jurkat treated cells in both FACs and caspase activity studies, which demonstrated the apoptotic properties of PH 6, the overall conclusion was that PH 6 associated cell death was mediated by apoptosis.

The next question posed was whether or not the apoptotic affect of treatment with PH 6 was dependent on NF- $\kappa$ B. To determine this, the constitutively

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activated NF- $\kappa$ B HuT-78 cell line was incubated with PH 6 for 24 h. The subsequent observation of the equipotent effect of PH 6 in this cell line compared to that of treatment in the Jurkat cell line, coupled to the fact that the results obtained following FACs analysis and caspase activity argue against general toxicity, indicated the possibility that apoptosis was mediated in an NF- $\kappa$ B independent manner. Further investigation would be required to confirm this, such as gene expression profiling.

Following determination of the apoptotic inducing properties of PH 6, it was investigated whether this compound altered regulation of the pro-inflammatory cytokine, TNF- $\alpha$ . The rationale for this study was two-fold. Firstly, given the pro-inflammatory nature of this cytokine, whether or not the test compounds showed the ability to down regulate expression of TNF- $\alpha$  was of interest and secondly, the role of TNF- $\alpha$  in mediating apoptosis posed the question of whether the PH 6 was inducing apoptosis by regulation of TNF- $\alpha$ .

TNF- $\alpha$  is a common treatment for induction of apoptosis, however, it is also known to activate NF- $\kappa$ B, which as discussed above, is involved with the regulation of anti-apoptotic proteins. Therefore, the observed effect of PH 6 in upregulation of the mRNA expression of TNF- $\alpha$ , posed some questions. On the one hand, PH 6 has significantly induced apoptosis and results obtained in the Hut-78 cell line were indicative of this effect being independent of NF- $\kappa$ B. With that evidence, the conclusion could be that PH 6 was mediating its cytotoxic effects through apoptosis induced by upregulation of TNF- $\alpha$ . However, TNF- $\alpha$  does not always induce apoptosis.

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Moreover, and by way of explanation of this, TNF- $\alpha$  is known to activate NF- $\kappa$ B.

### **4.3 Concluding remarks**

The aims of this work were to determine the use of the LFA-1 mediated T cell motility assay as an indicator to the potential anti-inflammatory properties of novel compounds in T cell function and subsequently to investigate the potential inhibitory properties of novel compounds *in vitro* in this model of T cell motility. Further to this, the aim was to develop of a profile of the compounds with respect to cytotoxicity, by evaluating the effect of the compounds on mitochondrial viability (MTT) and membrane integrity (LDH), Annexin V and Propidium Iodide binding and poly caspase activity.

From the results discussed above, it has been shown that the aims of this work were met. Of the PH compounds tested, PH 6 consistently demonstrated potency in the experiments. Similarly, HIH 6 and HIH 7 were the most potent of their group, with HIH 6 demonstrating a more potent effect in cytotoxicity.

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#### 4.4 Future work

Based on the results presented in this thesis for PH 6 and, HIH 6 and HIH 7, which demonstrate their potency, future work to determine the modes of action of the compounds could be carried out. To further elucidate the effect of the drugs in the LFA-1 mediated T cell motility assay, gene expression profiling of the effect of the drugs on mRNA expression, of the molecules involved in the assay, such as ICAM-1 and LFA-1 could be carried out. With respect to the significant effect seen with PH 6 in terms of inducing apoptosis, further work is needed to determine the apoptotic pathway by which PH 6 is mediating this effect. Also given the ability of PH 6 to induce apoptosis in an NF- $\kappa$ B independent manner as observed in the HuT-78 cells, investigation into the effect of PH 6 on NF- $\kappa$ B could further reveal the mechanism of the effect of this drug.

With respect to the overall investigation of the PH compounds, and as PH 6 was observed to be the most active, further work could also incorporate the synthesis of analogues of this compound to determine if a structure activity relationship exists.

Regarding the natural product compounds, they too could be investigated further to determine the mode by which the inhibition they exerted on LFA-1 T cell motility, again this could involve gene expression profiling to evaluate the effect on mRNA expression. Furthermore, given the significant effect of HIH 6 in the cytotoxicity study, this compound could be further evaluated with respect to apoptotic potential.



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## References

- Abbas, A. K., J. Lohr, et al. (2004). "T cell tolerance and autoimmunity." Autoimmunity Reviews **3**(7-8): 471-475.
- Abdelbaqi, M., J. H. Chidlow, et al. (2006). "Regulation of dextran sodium sulfate induced colitis by leukocyte beta 2 integrins." Lab Invest **86**(4): 380-390.
- Akbar, A. N. and M. Salmon (1997). "Cellular environments and apoptosis: tissue microenvironments control activated T-cell death." Immunology Today **18**(2): 72-76.
- Anderson, M. E. and T. J. Siahaan (2003). "Targeting ICAM-1/LFA-1 interaction for controlling autoimmune diseases: designing peptide and small molecule inhibitors." Peptides **24**(3): 487-501.
- Ashikawa, K., S. Majumdar, et al. (2002). "Piceatannol Inhibits TNF-Induced NF- $\kappa$ B Activation and NF- $\kappa$ B-Mediated Gene Expression Through Suppression of I $\kappa$ B $\alpha$  Kinase and p65 Phosphorylation." J Immunol **169**(11): 6490-6497.
- Avemann, K., R. Knippers, et al. (1988). "Camptothecin, a specific inhibitor of type I DNA topoisomerase, induces DNA breakage at replication forks." Mol. Cell. Biol. **8**(8): 3026-3034.
- Ballotti, S., F. Chiarelli, et al. (2006). "Autoimmunity: Basic Mechanisms and Implications in Endocrine Diseases." Hormone research **66**(3): 132-141.
- Barreiro, O., H. d. I. Fuente, et al. (2007). "Functional insights on the polarized redistribution of leukocyte integrins and their ligands during leukocyte migration and immune interactions." Immunological Reviews **218**(1): 147-164.

- 
- Bauer, J. H. and S. L. Helfand (2006). New tricks of an old molecule: lifespan regulation by p53. Aging Cell, Blackwell Publishing Limited. **5**: 437-440.
- Beg, A. A. and D. Baltimore (1996). "An Essential Role for NF-kappa B in Preventing TNF-alpha -Induced Cell Death." Science **274**(5288): 782-784.
- Ben-Horin, S. and I. Bank (2004). "The role of very late antigen-1 in immune-mediated inflammation." Clinical Immunology **113**(2): 119-129.
- Binnerts, M. E. and Y. van Kooyk (1999). "How LFA-1 binds to different ligands." Immunology Today **20**(5): 240-245.
- Bochner, B. S. (1997). "Cellular adhesion and its antagonism." Journal of Allergy and Clinical Immunology **100**(5): 581-585.
- Brandtzaeg, P., I. N. Farstad, et al. (1999). "Regional specialization in the mucosal immune system: primed cells do not always home along the same track." Immunology Today **20**(6): 267-277.
- Brown, M. J., J. A. Hallam, et al. (2001). "Cutting Edge: Integration of Human T Lymphocyte Cytoskeleton by the Cytolinker Plectin." J Immunol **167**(2): 641-645.
- Buckley, C. D., D. Pilling, et al. (2001). "Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation." Trends in Immunology **22**(4): 199-204.
- Burstein, E. and C. S. Duckett (2003). "Dying for NF-[kappa]B? Control of cell death by transcriptional regulation of the apoptotic machinery." Current Opinion in Cell Biology **15**(6): 732-737.

- 
- Butcher, E. C. (1991). "Leukocyte-endothelial cell recognition: Three (or more) steps to specificity and diversity." Cell **67**(6): 1033-1036.
- Carlos, T. M. and J. M. Harlan (1994). "Leukocyte-endothelial adhesion molecules." Blood **84**(7): 2068-2101.
- Carman, C. V. and T. A. Springer (2003). "Integrin avidity regulation: are changes in affinity and conformation underemphasized?" Current Opinion in Cell Biology **15**(5): 547-556.
- Chen, C., L. C. Edelstein, et al. (2000). "The Rel/NF-kappa B Family Directly Activates Expression of the Apoptosis Inhibitor Bcl-xL." Mol. Cell. Biol. **20**(8): 2687-2695.
- Chen, Z., C. Benoist, et al. (2005). "How defects in central tolerance impinge on a deficiency in regulatory T cells." Proc Natl Acad Sci U S A. **102**(41): 14735-40.
- Chowdhury, I., B. Tharakan, et al. (2008). "Caspases -- An update." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **151**(1): 10-27.
- Choy, E. H. S. and G. S. Panayi (2001). "Cytokine Pathways and Joint Inflammation in Rheumatoid Arthritis." N Engl J Med **344**(12): 907-916.
- Cohen, S., P. E. Biggazzi, et al. (1974). "Similarities of T cell function in cell-mediated immunity and antibody production." Cellular Immunology **2**: 150-159.
- David Broide, P. S. (2001). "Eosinophil trafficking to sites of allergic inflammation." Immunological Reviews **179**(1): 163-172.

- 
- Davis, M. and Y. Neriah (2004). "Behind the scenes of anergy: a tale of three E3s." Nat Immunol **5**: 238-240.
- Degterev, A., M. Boyce, et al. (2003). "A decade of caspases." Oncogene **22**(53): 8543-8567.
- Dinarello, C. A. (2000). "Proinflammatory cytokines." Chest **118**(2): 503-508.
- Dustin, M. L., T. G. Bivona, et al. (2004). "Membranes as messengers in T cell adhesion signaling." Nat Immunol **5**(4): 363-372.
- Elmore, S. (2007). "Apoptosis: A Review of Programmed Cell Death." Toxicologic Pathology **35**(4): 495-516.
- Enari, M., H. Sakahira, et al. (1998). "A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD." Nature **391**(6662): 43-50.
- Fanning, A., Y. Volkov, et al. (2005). "CD44 cross-linking induces protein kinase C-regulated migration of human T lymphocytes." Int. Immunol. **17**(4): 449-458.
- Feldmann, M. and L. Steinman (2005). "Design of effective immunotherapy for human autoimmunity." Nature **435**(7042): 612-619.
- Feng, D., J. A. Nagy, et al. (1998). "Neutrophils Emigrate from Venules by a Transendothelial Cell Pathway in Response to FMLP." J. Exp. Med. **187**(6): 903-915.
- Formigli, L., P. A., et al. (2000). "Aponecrosis: Morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis." Journal of Cellular Physiology **182**(1): 41-49.

- 
- Fridkis-Hareli, M., D. Teitelbaum, et al. (1994). "Direct binding of myelin basic protein and synthetic copolymer 1 to class II major histocompatibility complex molecules on living antigen-presenting cells--specificity and promiscuity." Proceedings of the National Academy of Sciences of the United States of America **91**(11): 4872-4876.
- Ghobrial, I. M., T. E. Witzig, et al. (2005). "Targeting Apoptosis Pathways in Cancer Therapy." CA Cancer J Clin **55**(3): 178-194.
- Giovannetti, A., M. Pierdominici, et al. (2008). "Apoptosis in the Homeostasis of the Immune System and in Human Immune Mediated Diseases." Current Pharmaceutical Design **14**(3): 253-268.
- Giri, D. K. and B. B. Aggarwal (1998). "Constitutive Activation of NF-kappa B Causes Resistance to Apoptosis in Human Cutaneous T Cell Lymphoma HuT-78 Cells. AUTOCRINE ROLE OF TUMOR NECROSIS FACTOR AND REACTIVE OXYGEN INTERMEDIATES." J. Biol. Chem. **273**(22): 14008-14014.
- Gonzalez-Amaro, R., D. Portales-Perez, et al. (1998). "Pentoxifylline Inhibits Adhesion and Activation of Human T Lymphocytes." J Immunol **161**(1): 65-72.
- Gordon, F. H., M. I. Hamilton, et al. (2002). "A pilot study of treatment of active ulcerative colitis with natalizumab, a humanized monoclonal antibody to alpha-4 integrin." Alimentary Pharmacology & Therapeutics **16**(4): 699-705.
- Gumbiner, B. M. (1996). "Cell Adhesion: The Molecular Basis of Tissue Architecture and Morphogenesis." Cell **84**(3): 345-357.

- 
- Hafezi-Moghadam, A., K. L. Thomas, et al. (2001). "L-Selectin Shedding Regulates Leukocyte Recruitment." J. Exp. Med. **193**(7): 863-872.
- Hannun, Y. A. (1997). "Apoptosis and the Dilemma of Cancer Chemotherapy." Blood **89**(6): 1845-1853.
- Hengartner, M. O. (2000). "The biochemistry of apoptosis." Nature **407**(6805): 770-776.
- Henricks, P. A. J. and F. P. Nijkamp (1998). "Pharmacological modulation of cell adhesion molecules." European Journal of Pharmacology **344**(1): 1-13.
- Hochberg, M. C., J. K. Tracy, et al. (2003). "Comparison of the efficacy of the tumour necrosis factor  $\hat{\pm}$  blocking agents adalimumab, etanercept, and infliximab when added to methotrexate in patients with active rheumatoid arthritis." Annals of the Rheumatic Diseases **62**(suppl 2): ii13-ii16.
- Hogg, N., M. Laschinger, et al. (2003). "T-cell integrins: more than just sticking points." J Cell Sci **116**(23): 4695-4705.
- Hsu, Y.-L., C.-Y. Cho, et al. (2006). "Plumbagin (5-Hydroxy-2-methyl-1,4-naphthoquinone) Induces Apoptosis and Cell Cycle Arrest in A549 Cells through p53 Accumulation via c-Jun NH2-Terminal Kinase-Mediated Phosphorylation at Serine 15 in Vitro and in Vivo." J Pharmacol Exp Ther **318**(2): 484-494.

- 
- Hynes, R. O. (2002). "Integrins: Bidirectional, Allosteric Signaling Machines." Cell **110**(6): 673-687.
- Imitola, J., T. Chitnis, et al. (2005). "Cytokines in multiple sclerosis: from bench to bedside." Pharmacology & Therapeutics **106**(2): 163-177.
- Issazadeh, S., A. Ljungdahl, et al. (1995). "Cytokine production in the central nervous system of Lewis rats with experimental autoimmune encephalomyelitis: dynamics of mRNA expression for interleukin-10, interleukin-12, cytolysin, tumor necrosis factor [alpha] and tumor necrosis factor [beta]." Journal of Neuroimmunology **61**(2): 205-212.
- Issazadeh, S., J. C. Lorentzen, et al. (1996). "Cytokines in relapsing experimental autoimmune encephalomyelitis in DA rats: persistent mRNA expression of proinflammatory cytokines and absent expression of interleukin-10 and transforming growth factor-[beta]." Journal of Neuroimmunology **69**(1-2): 103-115.
- Jacobelli, J., S. A. Chmura, et al. (2004). "A single class II myosin modulates T cell motility and stopping, but not synapse formation." Nat Immunol **5**(5): 531-538.
- Joza, N., S. A. Susin, et al. (2001). "Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death." Nature **410**(6828): 549-554.
- Kamradt, T. and N. A. Mitchison (2001). "Tolerance and Autoimmunity." N Engl J Med **344**(9): 655-664.

- 
- Kelleher, D., A. Murphy, et al. (1995). "Leukocyte function-associated antigen 1 (LFA-1) and CD44 are signalling molecules for cytoskeleton-dependent morphological changes in activated T cells." J Leukoc Biol **58**(5): 539-546.
- Kerr, J. F., A. H. Whyllie, et al. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**: 239-257.
- Knight, D. M., H. Trinh, et al. (1993). "Construction and initial characterization of a mouse-human chimeric anti-TNF antibody." Molecular Immunology **30**: 1443-1453.
- Kotovuori, P., E. Tontti, et al. (1993). "The vascular E-selectin binds to the leukocyte integrins CD11/CD18." Glycobiology **3**(2): 131-136.
- Krammer, P. H. (2000). "CD95's deadly mission in the immune system." Nature **407**(6805): 789-795.
- Labadia, M. E., D. D. Jeanfavre, et al. (1998). "Molecular Regulation of the Interaction Between Leukocyte Function-Associated Antigen-1 and Soluble ICAM-1 by Divalent Metal Cations." J Immunol **161**(2): 836-842.
- Lamy, T., J. H. Liu, et al. (1998). "Dysregulation of CD95/CD95 Ligand-Apoptotic Pathway in CD3+ Large Granular Lymphocyte Leukemia." Blood **92**(12): 4771-4777.
- Lan, R. Y., A. A. Ansari, et al. (2005). "Regulatory T cells: Development, function and role in autoimmunity." Autoimmunity Reviews **4**(6): 351-363.



- 
- Landowski, T. H., L. Moscinski, et al. (2001). "CD95 Antigen Mutations in Hematopoietic Malignancies." Leukemia & Lymphoma **42**(5): 835.
- Leroy F. Liu, S. D. D. T.-K. L. Y. M. M. S. S.-P. S. (2000). "Mechanism of Action of Camptothecin." Annals of the New York Academy of Sciences **922**(THE CAMPTOTHECINS: UNFOLDING THEIR ANTICANCER POTENTIAL): 1-10.
- Li, L. Y., X. Luo, et al. (2001). "Endonuclease G is an apoptotic DNase when released from mitochondria." Nature **412**(6842): 95-99.
- Li, X. C., A. M. Jevnikar, et al. (1997). "Expression of Functional ICAM-1 and VCAM-1 Adhesion Molecules by an Immortalized Epithelial Cell Clone Derived from the Small Intestine." Cellular Immunology **175**(1): 58-66.
- Li, Z.-W., W. Chu, et al. (1999). "The IKKbeta Subunit of I kappa B Kinase (IKK) is Essential for Nuclear Factor kappa B Activation and Prevention of Apoptosis." J. Exp. Med. **189**(11): 1839-1845.
- Liptay, S., S. Fulda, et al. (2002). "Molecular mechanisms of sulfasalazine-induced T-cell apoptosis." British Journal of Pharmacology **137**(5): 608-620.
- Liu, H. and R. M. Pope (2003). "The role of apoptosis in rheumatoid arthritis." Current Opinion in Pharmacology **3**(3): 317-322.
- Lorence, A. and C. L. Nessler (2004). "Camptothecin, over four decades of surprising findings." Phytochemistry **65**(20): 2735-2749.

- 
- Lu, C., M. Shimaoka, et al. (2001). "An isolated, surface-expressed I domain of the integrin  $\alpha_5\beta_1$  is sufficient for strong adhesive function when locked in the open conformation with a disulfide bond." Proceedings of the National Academy of Sciences of the United States of America **98**(5): 2387-2392.
- Luster, A. D., R. Alon, et al. (2005). "Immune cell migration in inflammation: present and future therapeutic targets." Nat Immunol **6**(12): 1182-1190.
- Ma, Q., M. Shimaoka, et al. (2002). "Activation-induced Conformational Changes in the I Domain Region of Lymphocyte Function-associated Antigen 1." J. Biol. Chem. **277**(12): 10638-10641.
- McDowall, A., B. Leitinger, et al. (1998). "The I Domain of Integrin Leukocyte Function-associated Antigen-1 Is Involved in a Conformational Change Leading to High Affinity Binding to Ligand Intercellular Adhesion Molecule 1 (ICAM-1)." J. Biol. Chem. **273**(42): 27396-27403.
- Miller, J. F. A. P. and A. Basten (1996). "Mechanisms of tolerance to self." Current Opinion in Immunology **8**(6): 815-821.
- Minn, A. J., P. Velez, et al. (1997). "Bcl-xL forms an ion channel in synthetic lipid membranes." Nature **385**(6614): 353-357.
- Nieto, M., J. M. R. Frade, et al. (1997). "Polarization of Chemokine Receptors to the Leading Edge during Lymphocyte Chemotaxis." J. Exp. Med. **186**(1): 153-158.
- Nishizika, Y. (1992). "Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C." Science **258**(5082): 607-14.

- 
- O'Brien, M. A. and R. Kirby (2008). "Apoptosis: A review of pro-apoptotic and anti-apoptotic pathways and dysregulation in disease." Journal of Veterinary Emergency & Critical Care **18**(6): 572-585.
- Palmer, E. (2003). "Negative selection [mdash] clearing out the bad apples from the T-cell repertoire." Nat Rev Immunol **3**(5): 383-391.
- Panés, J. (1999). "Adhesion molecules in inflammatory bowel disease." Pathophysiology **5**(4): 271-282.
- Pavlick, K. P., D. V. Ostanin, et al. (2006). "Role of T-cell-associated lymphocyte function-associated antigen-1 in the pathogenesis of experimental colitis." Int. Immunol. **18**(2): 389-398.
- Petruzzelli, L., M. Takami, et al. (1999). "Structure and function of cell adhesion molecules." The American Journal of Medicine **106**(4): 467-476.
- Picarella, D., P. Hurlbut, et al. (1997). "Monoclonal antibodies specific for beta 7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) reduce inflammation in the colon of scid mice reconstituted with CD45RB<sup>high</sup> CD4<sup>+</sup> T cells." J Immunol **158**(5): 2099-2106.
- Polman, C. H., P. W. O'Connor, et al. (2006). "A Randomized, Placebo-Controlled Trial of Natalizumab for Relapsing Multiple Sclerosis." N Engl J Med **354**(9): 899-910.
- Power, C., N. Fanning, et al. (2002). "CELLULAR APOPTOSIS AND ORGAN INJURY IN SEPSIS: A REVIEW." SHOCK **18**(3): 197-211.

- 
- Proskuryakov, S. Y. a., A. G. Konoplyannikov, et al. (2003). "Necrosis: a specific form of programmed cell death?" Experimental Cell Research **283**(1): 1-16.
- Rose, D. M., R. Alon, et al. (2007). "Integrin modulation and signaling in leukocyte adhesion and migration." Immunological Reviews **218**(1): 126-134.
- Rüdiger Arnold, D. B. M. B. Christian R. F. Peter H. K. (2006). "How T lymphocytes switch between life and death." European Journal of Immunology **36**(7): 1654-1658.
- Saelens, X., N. Festjens, et al. (2004). "Toxic proteins released from mitochondria in cell death." Oncogene **23**(16): 2861-2874.
- Sané, A.-T., A. M. Cantin, et al. (2004). "Ascorbate modulation of H<sub>2</sub>O<sub>2</sub> and camptothecin-induced cell death in Jurkat cells." Cancer Chemotherapy and Pharmacology **54**(4): 315-321.
- Sans, M., A. Salas, et al. (2001). "Differential role of selectins in experimental colitis." Gastroenterology **120**(5): 1162-1172.
- Schimmer, A. D. (2004). "Inhibitor of Apoptosis Proteins: Translating Basic Knowledge into Clinical Practice." Cancer Res **64**(20): 7183-7190.
- Sheridan H, Frankish N, et al. (1999). "Smooth muscle relaxant activity of pterosin Z and related compounds." Planta Medica **65**(3): 271-272.
- Sheridan, H., N. Frankish, et al. (1999). "Synthesis and antispasmodic activity of analogues of natural pterosins." European Journal of Medicinal Chemistry **34**(11): 953-966.
- Sheridan, H., I. Hook, et al. (2008). "Inhibition of LFA-1 Mediated T-Cell Motility by Naphthoquinones." Planta Med **74**(11): 1383-1387.

- 
- Shishodia, S. and B. B. Aggarwal (2001). "Nuclear Factor-kB: A Question of Life or Death." Journal of Biochemistry and Molecular Biology **35**(1).
- Sinha, A. A., M. T. Lopez, et al. (1990). "Autoimmune diseases: the failure of self tolerance." Science **248**(4961): 1380-1388.
- Soede, R. D. M., M. H. E. Driessens, et al. (1999). "LFA-1 to LFA-1 Signals Involve {zeta}-Associated Protein-70 (ZAP-70) Tyrosine Kinase: Relevance for Invasion and Migration of a T Cell Hybridoma." J Immunol **163**(8): 4253-4261.
- Springer, T. A. (1994). "Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm." Cell **76**(2): 301-314.
- Stefanelli, T., A. Malesci, et al. (2008). "Anti-adhesion molecule therapies in inflammatory bowel disease: Touch and go." Autoimmunity Reviews **7**(5): 364-369.
- Strasser, A., L. O'Connor, et al. (2000). "APOPTOSIS SIGNALING." Annual Review of Biochemistry **69**(1): 217.
- Sun, E. W. and Y. F. Shi (2001). "Apoptosis: the quiet death silences the immune system." Pharmacology & Therapeutics **92**(2-3): 135-145.
- Tanaka, M., M. E. Fuentes, et al. (1999). "Embryonic Lethality, Liver Degeneration, and Impaired NF-kB Activation in IKK-B-Deficient mice." Immunity **10**: 421-429.
- Tayal, V. and B. S. Kalra (2008). "Cytokines and anti-cytokines as therapeutics -- An update." European Journal of Pharmacology **579**(1-3): 1-12.

- 
- Thibault, M. (2009). "Link of the mechanisms of action of glatiramer acetate to its long-term clinical data." Journal of the neurological sciences **277**: S12-S15.
- Thorp, K. M., H. Verschueren, et al. (1996). "Protein kinase C isotype expression and regulation of lymphoid cell motility." Immunology **87**(3): 434-8.
- van Kooyk, Y. and C. G. Figdor (2000). "Avidity regulation of integrins: the driving force in leukocyte adhesion." Current Opinion in Cell Biology **12**(5): 542-547.
- van Loo, G., M. van Gorp, et al. (2002). "The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity." Cell Death & Differentiation **9**(1): 20.
- Vanden Berghe, T., M. Kalai, et al. (2006). "Necrosis is associated with IL-6 production but apoptosis is not." Cellular Signalling **18**(3): 328-335.
- Vicente-Manzanares, M., D. Sancho, et al. (2002). "The leukocyte cytoskeleton in cell migration and immune interactions." International Review of Cytology **216**(233-289).
- Volkov, Y., A. Long, et al. (1998). "Inside the Crawling T Cell: Leukocyte Function-Associated Antigen-1 Cross-Linking Is Associated with Microtubule-Directed Translocation of Protein Kinase C Isoenzymes {beta}(I) and {delta}." J Immunol **161**(12): 6487-6495.
- Volkov, Y., A. Long, et al. (2001). "Crucial importance of PKC-[beta](I) in LFA-1-mediated locomotion of activated T cells." Nat Immunol **2**(6): 508-514.

- 
- W R Henderson, J., E Y Chi, et al. (1997). "Blockade of CD49d (alpha4 integrin) on intrapulmonary but not circulating leukocytes inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma." J Clin Invest **100**(12): 3083-92.
- Wajant, H. (2002). "The Fas Signaling Pathway: More Than a Paradigm." Science **296**(5573): 1635-1636.
- Wallach, D., E. E. Varfolomeev, et al. (1999). "TUMOR NECROSIS FACTOR RECEPTOR AND Fas SIGNALING MECHANISMS." Annual Review of Immunology **17**(1): 331-367.
- Watkins, L. R., S. F. Maier, et al. (1995). "Immune activation: the role of pro-inflammatory cytokines in inflammation, illness responses and pathological pain states." Pain **63**(3): 289-302.
- Wei, X., B. J. Tromberg, et al. (1999). "Mapping the sensitivity of T cells with an optical trap: Polarity and minimal number of receptors for Ca<sup>2+</sup> signaling." Proceedings of the National Academy of Sciences of the United States of America **96**(15): 8471-8476.
- Williams, R. O., E. Paleolog, et al. (2007). "Cytokine inhibitors in rheumatoid arthritis and other autoimmune diseases." Current Opinion in Pharmacology **7**(4): 412-417.
- Woodside, D. G. and P. Vanderslice (2008). "Cell Adhesion Antagonists: Therapeutic Potential in Asthma and Chronic Obstructive Pulmonary Disease." BioDrugs **22**(2): 85.

- 
- Woska, J. R., Jr., M. M. Morelock, et al. (1996). "Characterization of molecular interactions between intercellular adhesion molecule-1 and leukocyte function- associated antigen-1." J Immunol **156**(12): 4680-4685.
- Yi, C. H. and J. Yuan (2009). "The Jekyll and Hyde Functions of Caspases." Developmental Cell **16**(1): 21-34.
- Yoshida, N., T. Yamaguchi, et al. (2001). "Role of P-Selectin and Intercellular Adhesion Molecule-1 in TNB-Induced Colitis in Rats." Digestion **63**(Suppl 1): 81-86.
- Yusuf-Makagiansar, H., M. E. Anderson, et al. (2002). "Inhibition of LFA-1/ICAM-1 and VLA-4/VCAM-1 as a therapeutic approach to inflammation and autoimmune diseases." Medicinal Research Reviews **22**(2): 146-167.
- Zhang, A., Y. Wu, et al. (2004). "Apoptosis-A Brief Review." Neuroembryology(3): 47-59.
- Zimmermann, K. C., C. Bonzon, et al. (2001). "The machinery of programmed cell death." Pharmacology & Therapeutics **92**(1): 57-70.
- Zisterer, D. M., G. Campiani, et al. (2000). "Pyrrolo-1,5-benzoxazepines Induce Apoptosis in HL-60, Jurkat, and Hut-78 Cells: A New Class of Apoptotic Agents." Journal of Pharmacology and Experimental Therapeutics **293**(1): 48-59.

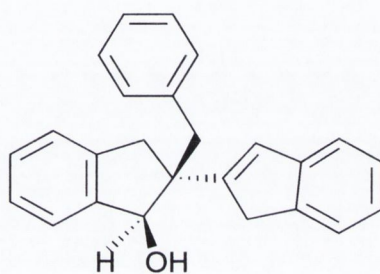


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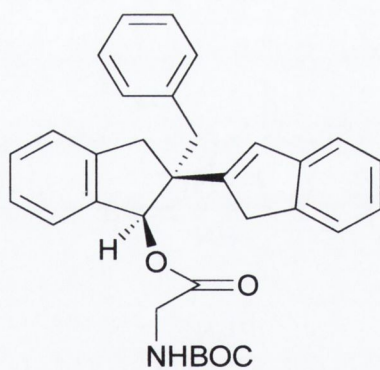
## Appendix 1

### Structures of compounds used in pilot study

PH 5

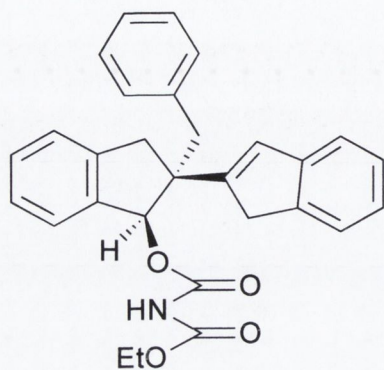


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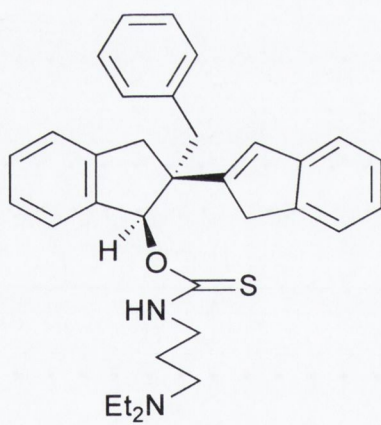


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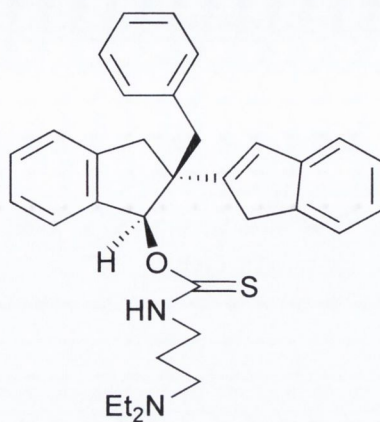
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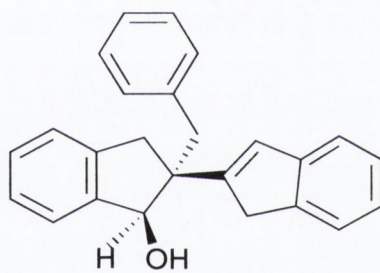
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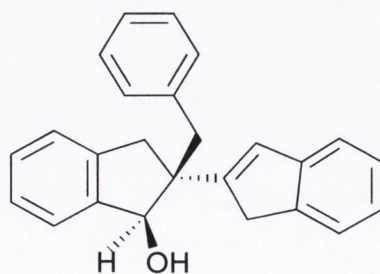
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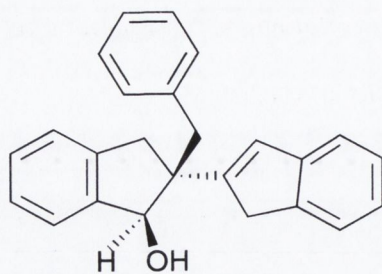
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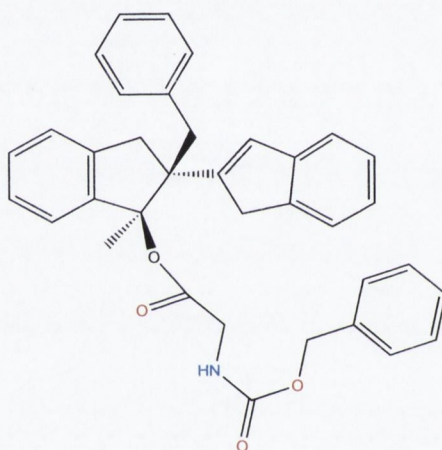
PH 21



PH 22



PH 23

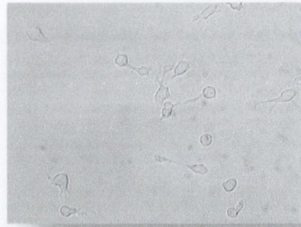


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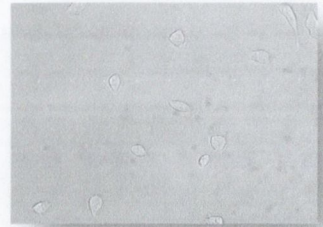
**Appendix 2**

**Table 3: Representative images of compounds tested in the pilot LFA-1 mediated T cell motility assay.**

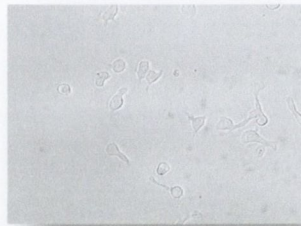
**PH 5**



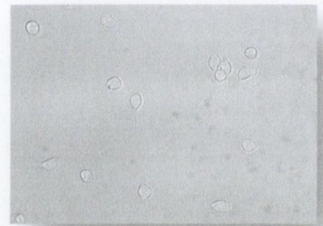
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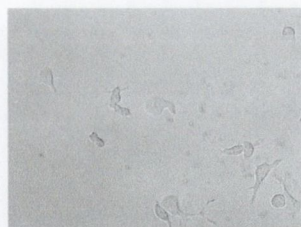
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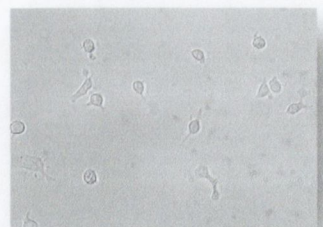
**PH 12**



**PH 10**



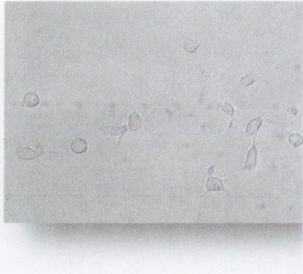
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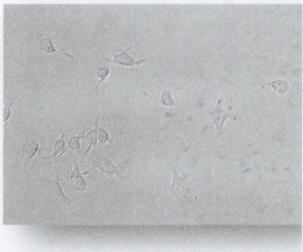
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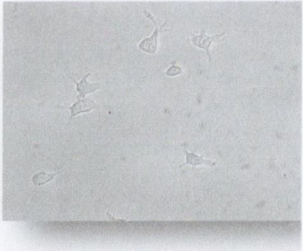
**PH 21**



**PH 22**



**PH 23**

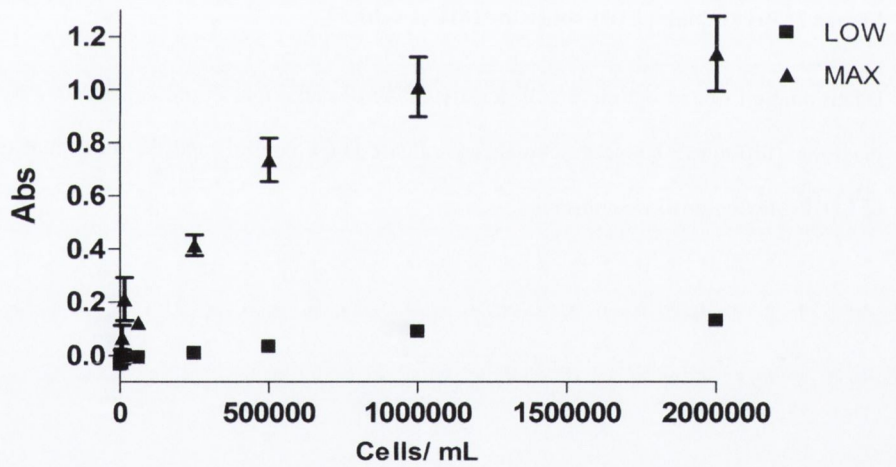


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### Appendix 3

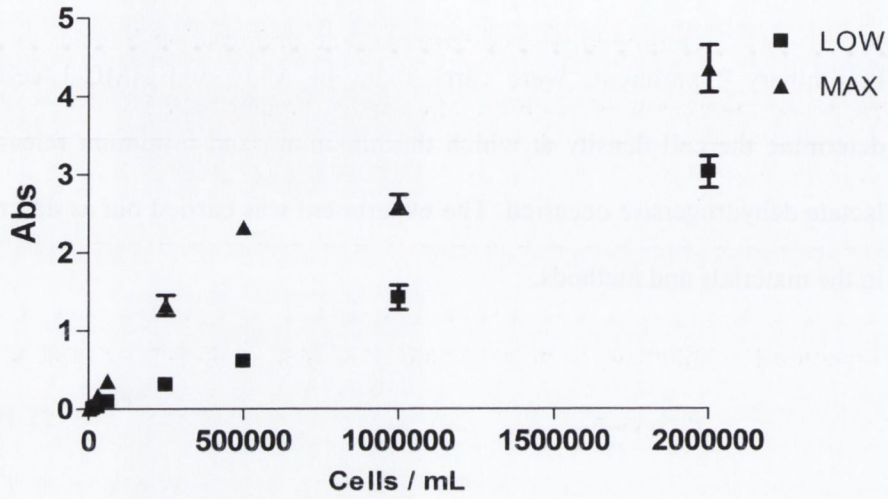
Supplemental cytotoxicity in A549 lung epithelial cells and HMC-1 mast cells.

Preliminary experiments were carried out in A549 and HMC-1 cells to determine the cell density at which the maximum and minimum release of lactate dehydrogenase occurred. The experiment was carried out as described in the materials and methods.



**Figure 1: Preliminary LDH study in A549 cells.**

Determination of the optimum cell density of A549 cells, that is the density at which the maximum difference between spontaneous release (Low control) and the maximum release of LDH (Max control) was observed.



**Figure 2: Preliminary LDH study in HMC-1 cells.**

Determination of the optimum cell density of A549 cells, that is the density at which the maximum difference between spontaneous release (Low control) and the maximum release of LDH (Max control) was observed.



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#### **Appendix 4: Motility calculation**

Sample calculation of the determination of % Motile cells.

Motility was determined by calculation of the deformation index, based on the following equations. A deformation index greater than 3 was considered to indicate motility.

Equation 1: Elongation index = major ellipse diameter / minor ellipse diameter

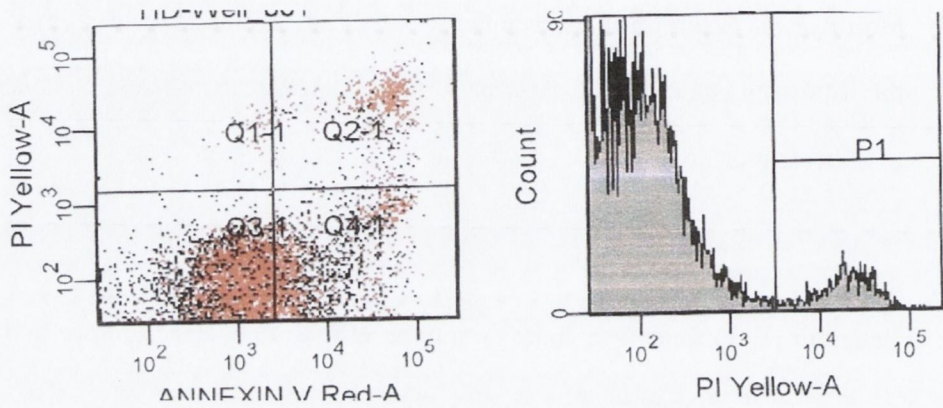
Equation 2: Circularity index =  $4 \times \pi \times (\text{area} / (\text{perimeter})^2)$ .

Equation 3: Deformation index = elongation index / circularity index

The percentage of motile cells was then calculated by:

$(\text{Number of motile cells} / \text{Total number of cells}) \times 100$

## Appendix 5: FACs calculation



The four quadrants shown above represent the stages of apoptosis;

Q1-1 cellular debris

Q2-1 Annexin V<sup>+</sup> / PI<sup>+</sup> (Late apoptotic / dead cells)

Q3-1 Annexin V<sup>-</sup> /PI<sup>-</sup> (Live cells)

Q4-1 Annexin V<sup>+</sup>/ PI<sup>-</sup> (Early apoptotic)

The total percentage of apoptotic cells was calculated by adding the results obtained for Q4-1 and Q2-1.

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## **Appendix 6: Paper**

## Inhibition of LFA-1 Mediated T-Cell Motility by Naphthoquinones

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### Abstract

An *in vitro* T-cell migration assay has been established that can be used to study the effects of compounds on the development of T-cell polarisation with HuT-78 T lymphocytes. This assay indicates the ability of compounds tested to inhibit the inflammatory response by decreasing LFA-1-mediated T-cell motility. The effect of a series of naturally occurring quinone isolates on motility has been evaluated in this assay. Distinct differences have been observed between naphthoquinones, dihydrofuranonaphthoquinones and anthraquinones

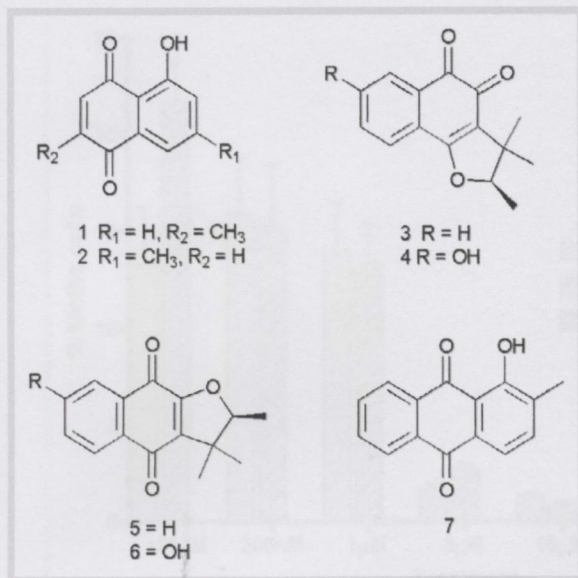
### Key words

T cell polarisation · HuT-78 T lymphocytes · naphthoquinone · plumbagin · dunnione · 7-hydroxydunnione

The migration of T lymphocytes from the blood stream into surrounding tissues and lymph nodes is critical to ensure that an effective immune response is generated. This process also contributes to the mechanism of inflammation in the body. Migration of the cells toward antigenic challenge is permitted by cytoskeletal-based rearrangements, generated in response to integrin activation on the surface of the T lymphocyte. One of the best characterised integrin adhesion molecules on T lymphocytes is lymphocyte functional antigen-1 (LFA-1) [1], [2].

Activation of LFA-1 on T cells by stimulatory monoclonal antibodies to the adhesion molecule (anti-LFA-1) induces the same migratory phenotype as that acquired upon activation of LFA-1 by its cognate physiological ligand (ICAM-1). This *in vitro* induced migratory phenotype mimics the ability of lymphocytes to migrate to sites of inflammation in the body, as the interaction between LFA-1 and its physiological ligand (ICAM-1) are crucial for cell migration and inflammation *in vivo*. Weitz-Schmidt and co-workers [3] demonstrated that the anti-inflammatory-immunosuppressive properties exhibited by statins (cholesterol-lowering drugs) are mediated by their binding to and inhibition of the LFA-1 adhesion molecule.

As part of an ongoing study into the use of natural products as anti-inflammatory agents, the effects of a series of quinones on T cell motility has been investigated. Quinones are an important group of pharmacologically active pigments widely distributed in nature. They demonstrate a range of physiological effects including antimicrobial, anti-inflammatory and anticancer activity [4]. The mechanism of action of naphthoquinones has received much attention and is still under investigation. To date the most important biochemical reaction of quinones is their reversible reduction to the corresponding hydroquinone [5]. The theory of free radical generation may be consistent as a mechanism for cytotoxicity and enzyme inhibition; however, it does



not fully explain the observed anti-inflammatory activity of many quinones.

A range of quinones 1–7 isolated from a series of plant cell cultures currently under investigation in our laboratories [6], [7] have been evaluated in the LFA-1 mediated T-cell motility assay. Three structural classes of quinones were investigated in this study including naphthoquinones (1, 2), dihydro-furanonaphthoquinones (3–6) and an anthraquinone (7). All compounds were tested at  $10 \mu\text{M}$  initially (● Fig. 1) and those compounds (1 and 2) that substantially inhibited motility were then tested in a dose-response study (● Fig. 2). The PKC inhibitor Go6976 ( $1 \mu\text{M}$ ) was used as a positive control for migration inhibition [1]. Untreated cells were used as a control of the extent of deformation; LFA-1 in the absence of any test compound induced motility (deformation index > 3) in every cell observed. While all compounds ( $10 \mu\text{M}$ ) significantly ( $P < 0.05$ ) reduced motility compared to untreated controls, a relatively high dose was em-

ployed to achieve a minimal effect. Compared to the positive control Go6976 ( $1 \mu\text{M}$ ), the only test compounds (at  $10 \mu\text{M}$ ) to substantially inhibit motility were the structurally related naphthoquinones 7-methyljuglone (1) and plumbagin (2) where cell motility dropped to  $7.7 \pm 3.8\%$  and  $2.3 \pm 1.6\%$ , respectively. A subsequent dose-response study showed a concentration dependent relationship (● Fig. 2). The percentage of motile cells ranged from  $13.3 \pm 2.9\%$  and  $21.6 \pm 2.1\%$  for (1) and (2), respectively, at  $100 \text{ nM}$ , down to  $0.8 \pm 0.3\%$  and  $0.6 \pm 0.1\%$  at  $30 \mu\text{M}$ . Of the furanonaphthoquinones, 7-hydroxydunnione (4) decreased cell motility markedly but not significantly. Apoptotic bodies were also identified in cells incubated with this compound (● Fig. 3). The anthraquinone (7) had no effect on motility.

A cytotoxicity study using a range of concentrations was carried out to investigate the effect on lactate dehydrogenase (LDH) release (● Fig. 4). Negligible toxicity was observed below  $100 \mu\text{M}$  for tested compounds. At  $100 \mu\text{M}$  and  $300 \mu\text{M}$ , toxicity increased to  $16.6 \pm 17.1\%$  and  $55.1 \pm 3.0\%$  for plumbagin (2). At the same concentrations, toxicity from 7-methyljuglone (1) remained low at  $5.0 \pm 2.9\%$  and  $7.6 \pm 3.0\%$ , respectively [6]. The inhibitory effect of 1 and 2 on LFA-1-mediated polarity of HuT 78 cells is dose-dependent and is indicative of an inhibitory effect by naphthoquinones on elements involved in the process of T-cell chemoattraction and movement towards areas of inflammation. The inhibition in the number of motile cells caused by either the furanonaphthoquinones or the anthraquinone is much less pronounced and may indicate a non-specific activity. The inhibitory effects observed with 7-methyljuglone (1) and plumbagin (2) are not mediated by or associated with cell toxicity, which is only evidenced at a concentration of  $100 \mu\text{M}$ . While the mechanism of action is unclear, further investigation might focus on the potential inhibition of 12(S)-HETE and the deactivation of protein kinase C, which has been reported for some naphthoquinones [8].

#### Materials and Methods

The naphthoquinones 7-methyljuglone (1) and plumbagin (2) were obtained from suspension cultures of *Dionaea muscipula*

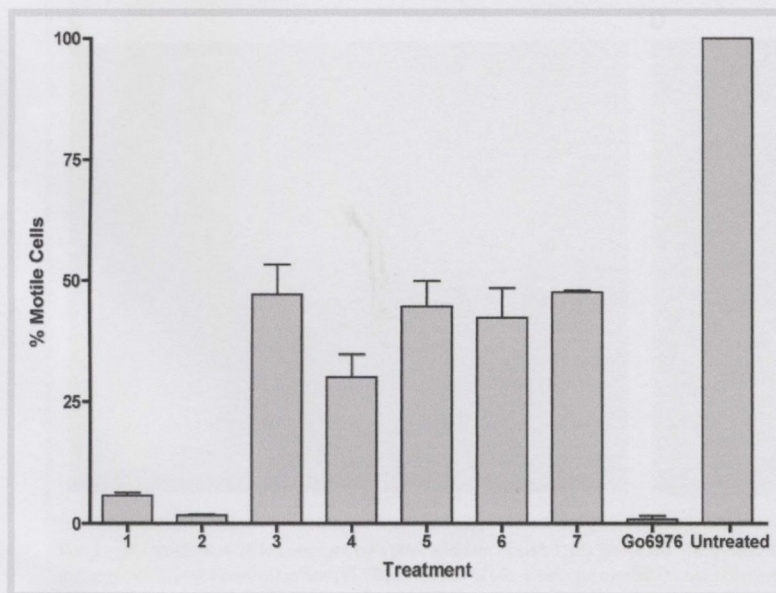
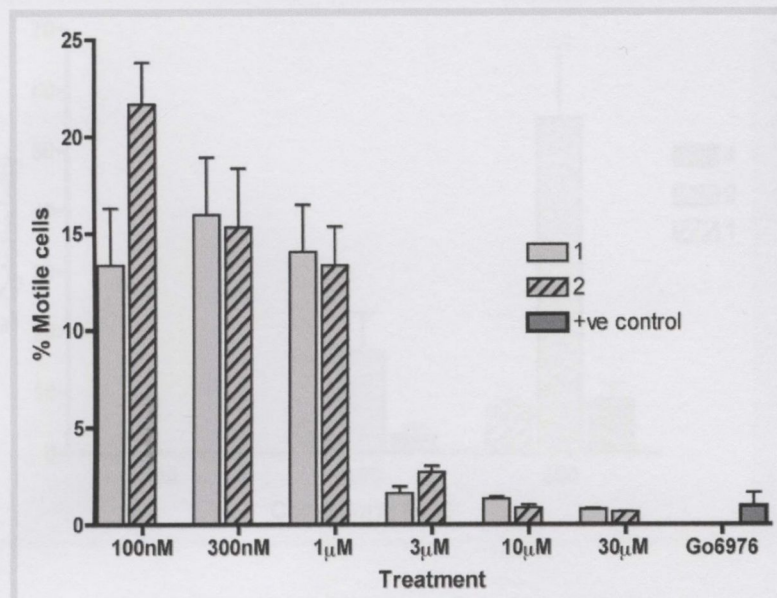
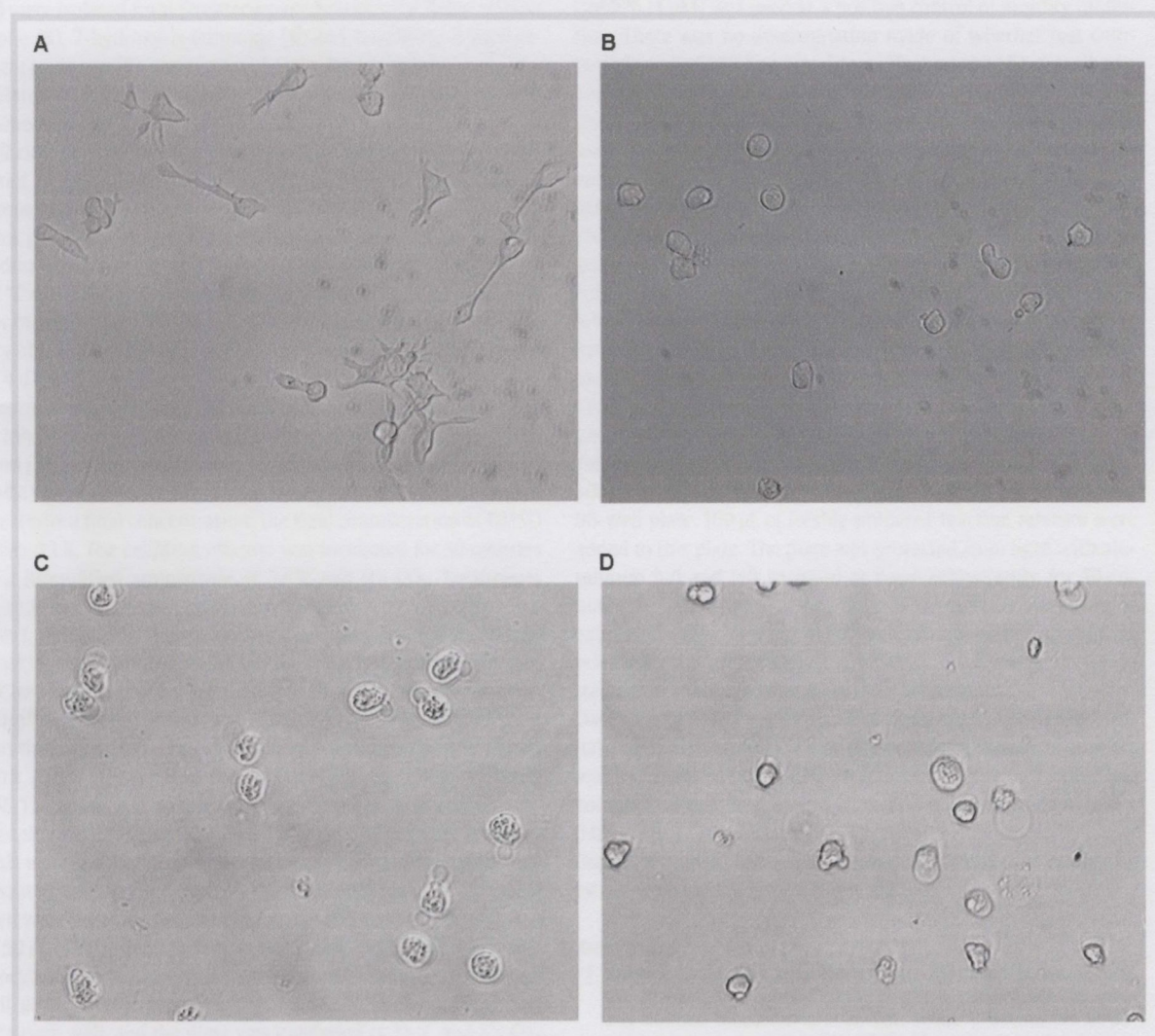


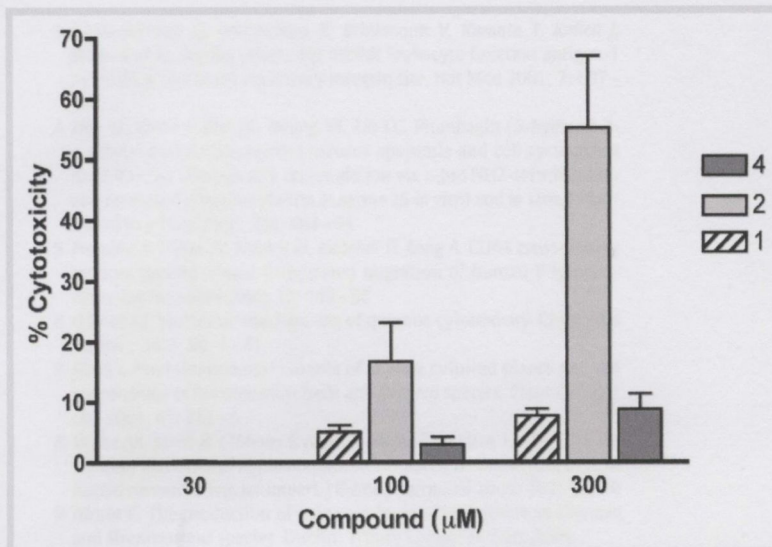
Fig. 1 Percentage motile Hut-78 cells treated with naphthoquinones ( $10 \mu\text{M}$ ) and Go6976 ( $1 \mu\text{M}$ ). Data are expressed as mean  $\pm$  S.E.M. ( $n = 8$ ).



**Fig. 2** Dose-response study. Percentage of motile Hut-78 cells treated with a range of concentrations of 7-methyljuglone (1) and plumbagin (2) together with Go6976 (1  $\mu$ M). Data are expressed as mean  $\pm$  S.E.M. (n = 8).



**Fig. 3** (A) Motile HuT 78 lymphocyte cells after addition of LHA-1. (B) Motile HuT 78 lymphocyte cells after addition of LHA-1. (C) Inhibition of LFA-1 induced motility by 7-methyljuglone (1). (D) Inhibition of LFA-1 induced motility by plumbagin (2).



**Fig. 4** LDH cytotoxicity: Hut-78 cells treated with 30–300  $\mu\text{M}$  of 7-methyljugulone (1), plum-bagin (2) and 7-hydroxydunnione (4). Cytotoxicity is expressed as the amount of LDH released as percentage of LDH release by the positive control, Triton-X 100 (2 mM), after subtraction of spontaneous release from both values. Data are expressed as mean  $\pm$  S.E.M. ( $n = 7$ ).

and *Drosera* species [7]. Dunnione (3) and 7-hydroxydunnione (4) were isolated from *Streptocarpus hybridus* var Ruby.  $\alpha$  Dunnione (5), 7-hydroxy- $\alpha$ -dunnione (6) and 1-hydroxy-2-methyl-anthraquinone (7) were isolated from *Streptocarpus dunnii* [9]. Isolates were characterised by their physical and spectroscopic properties.

**Cell culture:** HuT 78 T lymphocytes (LGC Promochem) were cultured in RPMI 1640 (Gibco) supplemented with 1% penicillin/streptomycin, 1% L-glutamine, 40 mL FBS, 5 mL HEPES at 10  $\mu\text{M}$ , 5 mL glucose at 4.5 g/L, 5 mL sodium pyruvate at 1  $\mu\text{M}$  and 2 mL sodium bicarbonate at 1.5 g/L in a humidified atmosphere at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Cells were seeded at a density of  $0.5 \times 10^6$  cells per mL in T-75  $\text{cm}^2$  flasks with filter lid and maintained at a density of  $0.5$ – $0.9 \times 10^6$  cells per mL. Cells were subcultured every 3 to 4 days.

**Treatments:** Go6976 at 1  $\mu\text{M}$  (Calbiochem), a selective PKC- $\alpha$  and - $\beta$  inhibitor [10], was used as a positive control of motility inhibition [2]. Stock concentrations of test compounds and Go6976 in DMSO were added to the cells at the appropriate volume to give the desired final concentration; the final concentration of DMSO being 0.1%. The cell/drug mixture was incubated for 30 minutes in a humidified atmosphere at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Treatments were prepared on the day of experiment.

**LFA-1 bioassay:** A 24-well sterile flat-bottomed tissue culture plate was coated with 250  $\mu\text{L}$  per well of a 1/100 dilution of rabbit anti-mouse IgG in sterile PBS. The plate was sealed with Parafilm<sup>®</sup> and stored at 4  $^{\circ}\text{C}$  overnight. The antibody coated plate was then washed twice with sterile PBS and subsequently coated with 250  $\mu\text{L}$  per well of a 1/100 dilution of LFA-1 in sterile PBS. The plate was covered and incubated in a humidified atmosphere at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for at least 1 hour. Drugs and controls were incubated on a second plate. The model used for this investigation was the HuT 78 T lymphocyte cell line.  $10^4$  cells/well were required. Cell density was determined by a suspension of 50  $\mu\text{L}$  of cells in 50  $\mu\text{L}$  Trypan blue with 400  $\mu\text{L}$  PBS and a haemocytometer was used to count the cells. Adjustments to the cell density were made as appropriate. Cells were dispensed into each well, and the plate was incubated at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 10 minutes. The density was then checked under the microscope and adjusted accordingly if necessary. Cells were treated with the appropriate volumes of stock concentrations of test

compounds in DMSO, to give the desired final concentration. Go6976 (1  $\mu\text{M}$ ) was used as a positive control of motility inhibition. There was no determination made of whether test compounds or Go6976 had any direct effect on the LFA-1 antibody. The cell/drug mixture was incubated in a humidified atmosphere at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 30 minutes. The antibody plates were washed as before. The cell/drug mixture was added and incubated at 37  $^{\circ}\text{C}$  again, until sufficient cytoskeletal rearrangement in control untreated cells had been observed. Image Pro Plus (Media Cybernetics) was used to analyse the cells. Photographs (in triplicate) were taken of each well; each photograph containing between 8 and 21 cells. Motility was then determined by calculation of the deformation index. A deformation index greater than 3 was considered to indicate motility. Deformation Index = elongation index/circularity index [1], [2].

**Cytotoxicity assay:** Cytotoxicity was assessed using a LDH cytotoxicity assay (Roche). Briefly, 50,000 HuT 78 cells/well were incubated with the test compounds for 4 h at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . 100  $\mu\text{L}$  of supernatant were then removed, and added to a new 96-well plate. 100  $\mu\text{L}$  of freshly prepared reaction mixture were added to this plate. The plate was protected from light with aluminium foil and left to stand at room temperature for 10 minutes. The absorbance at 492 nm was measured. Cytotoxicity is expressed as the amount of LDH released as percentage of LDH release by the positive control, Triton-X 100 (2 mM), after subtraction of spontaneous release from both values.

Data are expressed as mean  $\pm$  S.E.M. of  $n$  wells from three individual experiments conducted on different days. Statistical analysis was by means of 1-way ANOVA followed by Dunnett's multiple comparison test as a post test, with significance taken as  $P < 0.05$ .

Copies of original spectra and biological activity data can be obtained from the corresponding author.

#### References

- 1 Volkov Y, Long A, Kelleher D. Inside the crawling T cell: leukocyte function-associated antigen-1 cross-linking is associated with microtubule-directed translocation of protein kinase C isoenzymes beta(1) and delta. *J Immunol* 1998; 161: 6487–95
- 2 Volkov Y, Long A, McGrath S, Ni Eidhin D, Kelleher D. Crucial importance of PKC-beta(1) in LFA-1-mediated locomotion of activated T cells. *Nat Immunol* 2001; 2: 508–14

- 3 Weitz-Schmidt G, Welzenbach K, Brinkmann V, Kamata T, Kallen J, Bruns C et al. Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. *Nat Med* 2001; 7: 687–92
- 4 Hsu YL, Cho CY, Kuo PL, Huang YT, Lin CC. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) induces apoptosis and cell cycle arrest in A549 cells through p53 accumulation via c-Jun NH2-terminal kinase-mediated phosphorylation at serine 15 *in vitro* and *in vivo*. *J Pharmacol Exp Ther* 2006; 318: 484–94
- 5 Fanning A, Volkov Y, Freeley M, Kelleher D, Long A. CD44 cross-linking induces protein kinase C-regulated migration of human T lymphocytes. *Int Immunol* 2005; 17: 449–58
- 6 O'Brien PJ. Molecular mechanisms of quinone cytotoxicity. *Chem Biol Interact* 1991; 80: 1–41
- 7 Hook I. Naphthoquinone contents of *in vitro* cultured plants and cell suspensions of *Dionaea muscipula* and *Drosera* species. *Plant Cell Tiss Org* 2001; 67: 281–5
- 8 Wube AA, Streit B, Gibbons S, Asres K, Bucar F. *In vitro* 12(S)-HETE inhibitory activities of naphthoquinones isolated from the root bark of *Euclea racemosa* ssp. *schimperi*. *J Ethnopharmacol* 2005; 102: 191–6
- 9 Nestor C. The production of quinones by *in vitro* cultures of *Dionaea* and *Streptocarpus* species. Dublin: Trinity College Dublin; 2006
- 10 Martiny-Baron MG, Kazanietz H, Mischak PM, Blumberg G, Kochs H, Hug D et al. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J Biol Chem* 1993; 268: 9194–7

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#### Bibliography

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