SYNTHESIS OF QUINOLINE-FATTY ACID CONJUGATES AND CHARACTERIZATION OF THEIR IMMUNOMODULATORY PROPERTIES

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ABSTRACT

The anti-malarial chloroquine (CQ) and derivatives are useful anti-inflammatory agents which have been useful in treating patients with rheumatoid arthritis and systemic lupus erythromytosus. Nevertheless derivatives of CQ, such as hydroxychloroquine (HCQ) have been made to reduce the toxicity and increase the potency of the drug. We have approached this challenge by synthesizing 13 quinoline based compounds bearing a saturated or un-saturated fatty acid side chain (carbon chain length of 3 to 20).

Examination of their immunomodulatory properties *in vitro* at concentrations up to 50μM showed these compounds to have no effect on phytohaemagglutinin- and antigen, tetanus toxoid- induced human lymphocyte proliferation and interferon-γ, interleukin(IL)-2, IL-10 and lymphotoxin production. Similarly, there was no effect on the responses induced by the B cell mitogen, *Staphylococcus aureus.* In contrast, at the same concentrations, CQ significantly inhibited lymphoproliferation and cytokine production induced by these agents. Using bacterial lipopolyssacharide (LPS) as a stimulus for monocytes in the peripheral blood mononuclear fraction, we found that cytokine production, tumour necrosis factor alpha (TNFα), IL-1β and IL-6 was inhibited by CQ but not the NT compounds.

In contrast the data demonstrated that a number of the NT compounds were effective in inhibiting human neutrophil adherence induced by TNF. However, this activity was not related to either the carbon chain length or their saturated versus un-saturated state of the fatty acid side chain. In comparison, both CQ and HCQ had no effect on this response. The compound NT8 which had lauric acid on the side chain of the quinoline structure was the

most active. Further studies with this compound revealed that it was particularly effective in inhibiting TNF-induced neutrophil cytokine (IL-1β and IL-8) production but not TNF-induced neutrophil migration inhibition or TNF-induced respiratory burst. CQ and HCQ had no effect on all of these responses. At the lower concentrations of NT8 which significantly inhibited neutrophil adherence and cytokine production, the compound had no effect on adherence induced by phorbol 12-myristate 13-acetate (PMA) and calcium ionophore (A23187). These agents are known to by-pass the surface receptors and act on protein kinase C and cause an increase in intracellular calcium levels respectively, suggesting that the effects of NT8 are upstream in the signalling cascade. Interestingly we found that NT8 caused a significant decrease in surface expression of TNF receptor II (TNFRII) but not TNFRI. This selective effect of NT8 on neutrophil functions such as adherence was lost with an increase in concentration, where the neutrophil responses to the agonists, complement fragment C5a, IL-8, granulocyte macrophage-colony stimulating factor, formyl-Met-Leu-Phe, PMA, A23187 and arachidonic acid were also inhibited. A similar selectivity was seen for neutrophil responses to leukotriene B_4. The anti-inflammatory effects of NT8 were confirmed *in vivo* in a mouse model of LPS-induced peritonitis, where both TNF and neutrophils play an important role.

The research in this thesis has enabled the development and characterization of a new class of quinoline based compounds with anti-inflammation properties distinguishable from those of CQ and HCQ, thus, providing a new avenue for the development of anti-inflammatory agents.

DECLARATION

Mei-Chun Yeh — Doctor of Philosophy

Name — Program

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ABBREVIATION

A23187	calcium ionophore
AA	arachidonic acid
APC	antigen presenting cells
ASK1	apoptosis signal-regulating kinase
ATF	activation transcription factors
ATP	adenosine triphosphate
BSA	bovine serum albumin
C5a	complement 5a
COX	cyclo-oxygenases
CPLA2α	Ca^{2+} dependent cytosolic phospholipase A2α
CQ	chloroquine
CR	complement receptor
CTLA	cytotoxic T lymphocyte-associated antigen
DAG	diacylglycerol
DC	dendritic cells
DHA	docosahexaenoic acid
DTT	1,4-dithiothreitol
E.coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
ELAM	endothelial leucocyte adhesion molecule
ERK	extracellular-signal-regulated kinases
EPA	eicosapentaenoic acid

FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FK	fractalkine
fMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
Foxp3	forkhead box P3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocytes colony-stimulating factor
GM-CSF	granulocytes macrophage colony stimulating factor
GTP	guanosine-5'-triphosphate
HBSS	Hanks' Balanced salt solution
HCQ	hydroxychloroquine
HPLC	high performance liquid chromatography
ICAM	intracellular-adhesion-molecule
IFN-γ	interferon gamma
IgG	immunoglobulins
IL	interleukin
IMPDH	inosine monophosphate dehydrogenase
IP	intraperitoneal
IP3	inositol trisphosphate
ITAMS	immunoreceptor tyrosine-based activation motifs
IV	intravenous
JNK	c-Jun NH_2-terminal kinase
LA	lauric acid
LFA	leukocyte functional antigen
LPS	lipopolysaccharide

LT	lymphotoxin
LTB_4	leukotriene B_4
MAPK	mitogen activated protein kinase
MBL	mannose-binding lectin
MCP	monocyte chemotactic protein
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatability complex
MIP	macrophage inflammatory protein
MPO	myeloperoxidase
MPS	mononuclear phagocyte system
NADPH	nicotinamide adenine nucleotide phosphate
NBPP	bis-nitrophenyl phosphate
NK	natural killer
NMR	nuclear magnetic resonance
NOD	nucleotide-binding oligomerization domain
NOS	nitric oxide synthase
NOX	NADPH oxidase
ODRS	oxygen derivatives reactive species
OA	oleic acid
PAF	platelet activating factor
PAMP	pathogen-associated microbial patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate based saline
PCoA	palmitoyl CoA
PE	phycoerythrin

PECAM-1	platelet endothelial adhesion molecule-1
PGE2	prostaglandin E2
PHA	phytohaemagglutinin
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PLA_2	phospholipase A_2
PLAD	pre-ligand assembly domain
PLC-γ	phospholipase C-gamma
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocytes
PPAR	peroxisome-proliferation-activated receptor γ
PPD	paraphenylenediamine
PPR	pattern recognition receptors
PR3	proteinase 3
PRS	pathogen recognition system
PSGL-1	P-selectin glycoprotein ligand 1
PyrP	pyridoxal phosphate
RANTES	regulated on activation normal T cell expressed and secreted
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
S.aureus	Staphylococcus aureus
SLE	systemic lupus erythematosus
SPT	serine palmitoyl transferase
TCR	T cell receptor
TGF-β	transforming growth factor beta

Th	T helper cells
THF	tetrahydrofuran
TLC	thin layer chromatography
TLR	toll-like receptors
TNF	tumour necrosis factor
TNFRI	TNF receptor I
TNFRII	TNF receptor II
TNF-RM	tumour necrosis factor-rich medium
T regs	regulatory T cells
TT	tetanus toxoid
VCAMS	vascular cell adhesion molecules
VLA	very late antigen
UV	ultraviolet

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1.0 CHAPTER 1 INTRODUCTION

1.1 General introduction

The immune system through its innate and acquired characteristics protects the body against microbial pathogens (Kaufmann and Steward, 2005). These responses involve a network of different leukocyte types and mediators such as cytokines as well as cell surface receptors. In this manner, the immune system is able to mount an effector action which leads to the elimination of bacteria or other foreign matter in tissues and organs. However, these characteristics of the immune system can also induce allergic and autoimmune reactions in the body, which are responsible for many debilitating diseases in our community, namely the immune system can either act as a friend or a foe to the human body. The very nature of the foreign structure, whether of microbial or auto-antigen origin, dictates the characteristics of the inflammatory response displayed (Ferrante *et al.*, 1992). The onset of the inflammatory reaction and its resolution is guided by a balance of positive and negative regulators of both the cellular and molecular elements of the immune system. In simplistic terms, the inflammatory response can be classified into the acute or chronic type.

Cells of the immune system can participate in innate immunity. Some of these cells are more specialised for this function than others. In the absence of antibodies and T cells, which can recognise antigen specifically, the host defence system is dependent on the recognition of patterns of distinct differences of defined molecular structures on the surface of microbial pathogens, tumor cells and altered self structures (Chaplin, 2006; Goldsby *et al.*, 2003a). These are known as pathogen recognition systems (PRS). Pattern recognition receptors (PRR) on leukocytes such as toll like receptors (TLR) have a spectrum of recognition functions. Engagement of these receptors leads to cellular activation and production of cytokines, phagocytosis of particles and killing of tumor cells (Chaplin, 2006).

For example, killing of tumor cells via pattern recognition is carried out by natural killer (NK) cells (large granular lymphocytes) (Goldsby *et al.*, 2003b). Both macrophages and neutrophils have TLRs which recognise microbial structures (Goldsby *et al.*, 2003b; Ferrante, 2005). This is a first line recognition system, which when initated activates the leukocytes to release a weak range of mediators to either directly deal with microbial pathogens and/or promote the infiltration and activation of cells which assist in defence against infection.

The classification of inflammatory responses can also be based on the types of cells infiltrating infected area; mononuclear infiltrate (T lymphocytes, macrophages and B cells) or predominantly polymorphonuclear cells (PMN) (neutrophil, eosinophils and basophils) (Goldsby *et al.*, 2003c). Regulating the characteristic of the cellular infiltration is now recognised to occur via different T cell subpopulations which mediate their effect through the release of specific cytokine patterns. Apart from T cells existing as T helper/ inducer cells (Th) and T suppressor/cytotoxic (Ts) cell, the Th cells have now been further characterised into Th1, Th2 and Th17 (Chaplin, 2006). The cytokines they release influence the type of inflammatory response observed. Other T cells that are designated to play a key role in regulating the immune response are the regulatory T cells (Treg) (Sakaguchi, 2008). Again, these have a distinct cytokine pattern from the Th cells. The T cell arm of the immune system is essential to the adaptive or acquired immune response. The diversity of the TCR repertoire is generated by combinational rearrangement of a variety of germ-line immunoglobulin (Ig) or TCR gene segments (Goldsby *et al.*, 2003c). T cells respond to antigen, presented in association with major histocompatibility complex (MHC) molecules by the antigen presenting cells (APC) (Goldsby *et al.*, 2003a; Chaplin, 2006).

The other component of the adaptive immune response is the B cell, which can be triggered by antigen to develop into plasma cells to produce antibodies complimentary to the stimulating antigens. In order for B cells to be activated and mature into antibody-producing cells, T cells are required to release several cytokines (Goldsby *et al.*, 2003c). This T cell assistance is also necessary for downstream immunoglobulin class switching. The anti-microbial resistance mediated by adaptive immunity occurs through the production of antibodies which neutralise the microbial toxin or promote the phagocytosis of bacteria. This represents the humoral immunity of the immune system which is principally a function of B cells.

Cell-mediated immunity occurs as a result of the generation of cytotoxic T lymphocytes which attack virus infected target cells or by T cells recruiting and activating macrophages for microbial killing (Kumaratilake *et al.*, 1992). Th1 cells play important roles, by releasing cytokines such as interferon-γ (IFN-γ) which activate macrophages to promote the killing of intracellular bacterial parasites or virus infected cells. In comparison, protection against helminths requires eosinophils, a response best promoted by Th2 cytokines such as interleukin 4 (IL-4) and IL-5(Coffman *et al.*, 1989; Shin *et al.*, 2009). However, the interplay between the Th1 and Th2 arm of the immunity is quite complex and requires further direction.

Inflammatory reactions may be initiated by autoantigens or allergens. Autoantigens can elicit autoimmune responses involving the cellular and humoral arms of the immune system. Antibodies and sensitised T cells promote target destruction through antibody-mediated cellular cytotoxicity or cytotoxic T cells which destroy the host cells. Type 1 diabetes, multiple sclerosis, rheumatoid arthritis and systemic erythematosus are all examples in which the Th1 response plays a critical role in tissue damage (Kowanko and Ferrante, 1996; Ware

and Matthay, 2000).

Although there have been several developments in providing anti-inflammatory therapeutics, the search for newer approaches continues. Present therapies range from those which target the immune system non-specifically, such as steroidal and non-steroidal anti-inflammatory agents, to those which target a specific cytokine or its receptor e.g. the use of anti-TNF antibodies or TNF soluble receptors (Chu *et al.*, 1991; Feldmann and Maini, 2001; Gabay *et al.*, 1997). Other targets include the intracellular signalling pathways such as the calcium/calmodulin pathway and the p38 mitogen activated protein (MAP) kinase (Cole and Kohn, 1994; Mayer and Callahan, 2006). In some cases, antimicrobial agents have shown immunosuppressive properties are used as anti-inflammatory agents. This includes chloroquine (CQ) and its derivatives. The interest extends to various attempts to produce derivatives of CQ which may improve the drug action of the compound, such as hydroxychloroquine (HCQ). This literature review will describe the nature and function of cells of the innate and acquired immune system, the intercellular signalling mediators and also review what is currently known about the use of anti-malarials as anti-inflammatories. It will also cover areas of possible therapies and target sites will also be explored.

1.2 Mononuclear phagocytes

In the bone marrow, through haematopoiesis, myloid cells mature into monocytes which are released into the blood stream and become a source of tissue resident macrophages and tissue infiltrating mononuclear cells during the inflammatory reaction (Figure 1.1) (Goldsby *et al.*, 2003c).

Macrophages are distributed in tissues throughout the body, mainly seen in the skin, liver, peritoneum, kidney, heart, blood vessels and brain. These cells can display functional characteristics which assist both innate and adaptive immunity. Macrophages mainly achieve their objectives by possessing marked functional heterogeneity in accordance with their role in the tissues in which these reside (Fujiwara and Kobayashi, 2005). Resident tissue macrophages display characteristics and specialized function relevant to the tissues in which they are found, e.g. the kupffer cells of the liver line the sinusoids and play a major role in filtering blood to remove foreign matter (Fujiwara and Kobayashi, 2005) whereas the macrophages of the lung phagocytose airborne particles and bacteria which lodge in the lungs.

In the initial stages of an infection, macrophages depend on PRRs to distinguish self from non-self. They recognise conserved regions on microbial pathogen structures termed pathogen-associated microbial patterns (PAMP) which include bacterial lipopolysaccharide (LPS) (Brown and Gordon, 2005). The PRR are found in serum, on the surface membrane or intracellularly. Table 1.1 shows selected molecules of the different types and their microbial ligands. Those free in serum, such as complements, can bind to microbial pathogens and promote their phagocytosis (Brown and Gordon, 2005). Interaction with PRR in plasma membrane (such as TLRs) or intracellular (such as nucleotide-binding oligomerization domains-NOD) to PAMPs will elicit key responses in macrophages leading to phagocytosis, microbicidal activity and the release of cytokines (Brown and Gordon, 2005). The nature of this macrophage-microbial pathogen interaction is likely to dictate the effectiveness of the adaptive immune response which is important for the destruction of infection.

The role of macrophages in adaptive immune response is to prime naïve T cells as well as to present antigens to primed T cells, like dendritic cells (DCs) (Porcheray *et al.*, 2005). Antigen-presentation by macrophages and their DC relatives leads to the generation of functional T cells with different properties relevant to the elimination of microbial pathogens (Goldsby *et al.*, 2003a). The main subsets, $CD4^+$ and $CD8^+$ T cells play quite different roles in immunity. $CD4^+$ T cells are considered helper T cells and are instrumental in producing cytokines which enable the development of effector cytotoxic T cells ($CD8^+$ T cells) that target and kill cells infected with cytosolic pathogens e.g. viruses (Goldsby *et al.*, 2003c). Depending on the environmental stimulus, $CD4^+$ T cells may differentiate into Th1 or Th2 cell types and can also be influenced by cytokines released from macrophages. These T cells types produce different cytokine patterns which mediate immunity to different type of pathogens.

The anti-microbial activity of macrophages has been well characterized. Usually extracellular microbial pathogens are killed following opsonisation with antibody which not only promotes their phagocytosis, but stimulates signals through the Fcγ receptors. This process stimulates a combination of biochemical pathways that generates several microbicidal substances delivered to the intracellular site where the pathogen is confined; the phagocytic vacuole (Goldsby *et al.*, 2003d). These substances include enzymes, peptides and oxygen derived reactive species. Many of these overlap with those used by the neutrophil and are extensively described in section 1.4. However, the most striking characteristic of the macrophage which distinguishes it from the neutrophil is the process of macrophage activation. This is classically described for the killing of the intracellular bacteria, *Listeria monocytogenes* (Sonoda *et al.*, 1997). These bacteria can live and multiply within macrophages but when macrophages are transformed into an activated state the bacteria are

killed. The activation is brought about by Th1 cytokines such as IFN-γ (Sonoda *et al.*, 1997), whereas Th2 cytokines including IL-10, IL-12 and TGF- β are considered to deactivate macrophage (Fujiwara and Kobayashi, 2005; Kumaratilake and Ferrante, 1992).

Macrophages activate the immune response by secreting a wide range of mediators. These include cytokines, pro-and anti-angiogenic factors, a variety of enzymes, complement components and metabolites of arachidonic acid. An area of intensive investigation is the cytokines released by macrophages and their role in the cytokine network. Macrophages are major producers of TNF, IL-1, IL-10, IL-12, IFN-α and IL-18 (Brown and Gordon, 2005; Fujiwara and Kobayashi, 2005). These cytokines are critical to many aspects of the cytokine network operations in microbial immunity as well as microbial pathogenesis.

Macrophages also promote pathogenic processes associated with infection and autoimmune diseases. Recruitment of macrophages into tissues as a result of persistent microbial antigens (tuberculosis) or autoimmune antigens (rheumatoid arthritis) can lead to tissue changes and eventually organ failure and death. They are involved in promoting diseases such as rheumatoid arthritis, atherosclerosis, multiple sclerosis (Brown and Gordon, 2005; Kaperonis *et al.*, 2006; Ma *et al.*, 2004). The cytokines produced by these cells play an important role in pathogenesis and it is not surprising that anti-TNF therapy has become important in the treatment of rheumatoid arthritis (Feldmann and Maini, 2001).

1.3 T lymphocytes

T lymphocytes derived from the bone marrow, undergo further maturation in the thymus gland expressing unique α, β T cell receptors on the cell membrane (Figure 1.1). They can

exist in a naïve form and only mature following activation. The T cell receptor expressed on the naïve T lymphocytes is a complex of polypeptides expressing CD3 and either CD4 or CD8 receptor (Goldsby *et al.*, 2003c).

However, mature T cells usually express CD28, a receptor for the co-stimulatory B7 molecules present on B cells and other antigen-presenting cells (APC) and CD45, a signal-transduction molecule (Goldsby *et al.*, 2003c). These T cell receptors can only recognize specific antigen that is bound to the cell membrane proteins, MHC molecules that are further divided into two classes: MHC class I and II (Goldsby *et al.*, 2003d). T cells that express CD4 are usually restricted to recognizing antigen bound to MHC class II molecules whereas T cells expressing CD8 are restricted to recognition of antigen bound to MHC class I molecules (Goldsby *et al.*, 2003c).

1.3.1 Th cells and inflammation

Upon stimulation and under the influence of different environmental conditions , naïve T cells develop into at least three types of Th cells and T cells with regulatory properties (i.e. the Treg) (Figure 1.2) (Sakaguchi, 2008). There is interplay between these subpopulations to promote microbial killing and as well as in allergic and autoimmune diseases (Table 1.2). The skewing towards a Th cell type development seems to be controlled by the antigenic environment the cells encounter. These four T cell subtypes can be differentiated by the cytokines they produce and transcriptional factors which promote their differentiation from naïve T cells (Figure 1.2).

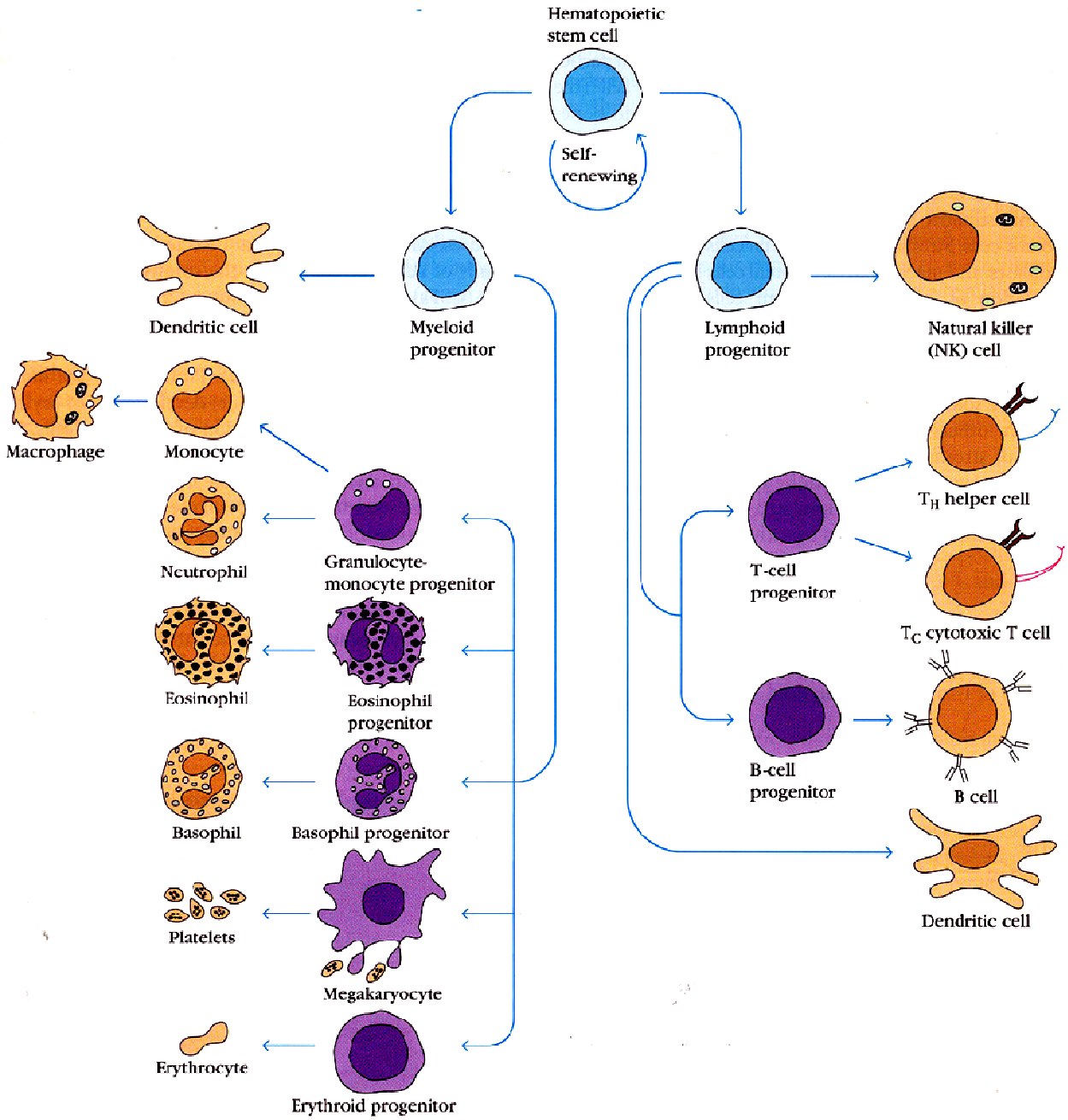


Figure 1.1 Hematopoiesis. The formation and development of the different functional leukocytes. Adapted from Goldsby *et al.*, (2003b).

Table 1.1 Microbial pattern recognition molecules.

Location	Family	Member	Selected microbial ligand
Serum	Collectins	Sp-A, Sp-D	Influenza A virus, herpes simplex virus, *Staphylococcus aureus*, *Escherichia coli*
		Mannose-binding lectin	HIV, influenza A virus, S.aureus, Neiserria meningitides, Chlamydia pneumonia, Candida albicans, Crytococcus neoformans, A. fumigates
	Pentraxins	C-reactive protein, serum amyloid P	S.aureus, E.coli, Streptococcus pyogenes, N. meningitides, A. fumigates, C. albicans, Plasmodium falciparum, influenza A virus, LPS
	Complement	C1q	Antibody-coated microbes, Listeria monocytogenes, Legionella pneumophila, E.coli, HIV
		C3	Microbial surfaces
	Lipid transferase	LBP	LPS
Membrane bound	Classic C-type lectins	Mannose receptor	Candida albicans, P. carinii, Mycobacterium tuberculosis, K. pneumonia, Lishmania donovani, HIV-1, zymosan
		DC-SIGN	HIV, Ebola virus, Leishmania spp.
	Non-classic C-type lectins	Dectin-1	Beta-Glucans, zymosan, Saccharomyces cerevisiae, C. albicans
	Leucine-rich proteins	CD14	E.coli, LPS, LTA, peptidoglycan
		Toll-like receptors (1-10)	LPS, LTA, zymosan, bacterial lipoproteins, peptidoglycan, viral proteins, fagellin, bacterial DNA
	Scavenger receptors	SR-A (I and II), LOX-1, MARCO	E.coli, S.aureus, M. tuberculosis, Enterococcus faecalis, N. meningitides, LPS, LTA, bacterial DNA
	Integrins	CR3, CR4	Complement-coated microbes, LPS, LPG, C. albicans, M. tuberculosis, C. neoformans
Intracellular	NODs	NOD-1, NOD-2	LPS, S. flexneri
	Interferon-induced proteins	PKR, OAS, ADAR1	Viral dsRNA
		Mx GTPase	Viral-protein complexes

Adapted from Brown and Gordon, (2005).

T helper cells can also be further subdivided into Th1, Th2 and Th17 (Figure 1.2). Th1 cells produce IFN-γ which is important for cell-mediated immune responses which are required for protection against intracellular microbial pathogens, and are regulated by the T-box transcription factor (T-bet) and Runt-related transcription factor 3 (Runx3) (Mowat and Gardside, 2005; Sakaguchi *et al.*, 2008). Th2, regulated by the trans-acting T cell specific transcription factor 3 (GATA-3), are defined by their production of IL-4 and role in humoral immunity and response against extracellular pathogens (Mowat and Gardside, 2005; Sakaguchi *et al.*, 2008). Th17s are regulated by the orphan receptor transcription factor (RORγt) and produce IL-17 (Sakaguchi *et al.*, 2008) (Table 1.2).

Tregs which are formally known as the suppressor T cells, can differentiate directly from the thymus without the aid of cytokines into nTregs or from naïve T cells under the influences of cytokines into iTreg (Figure 1.2) (Sakaguchi *et al.*, 2008). Tregs are known to control other T cells types and suppress either their activation in secondary lymphoid organs or their effector functions in peripheral tissues (Packard *et al.*, 2009). Regulatory T cells are often classified as $CD4^+$, $CD25^+$ and forkhead box P3 ($FoxP3^+$) cells with the special emphasise on the expression of the transcription factor $FoxP3^+$ which acts as an important regulator for Treg development and functions (Figure 1.2) (Sakaguchi *et al.*, 2008). Upon antigen exposure, Tregs become activated and exert antigen specific suppressor functions on the antigen (Table 1.2).

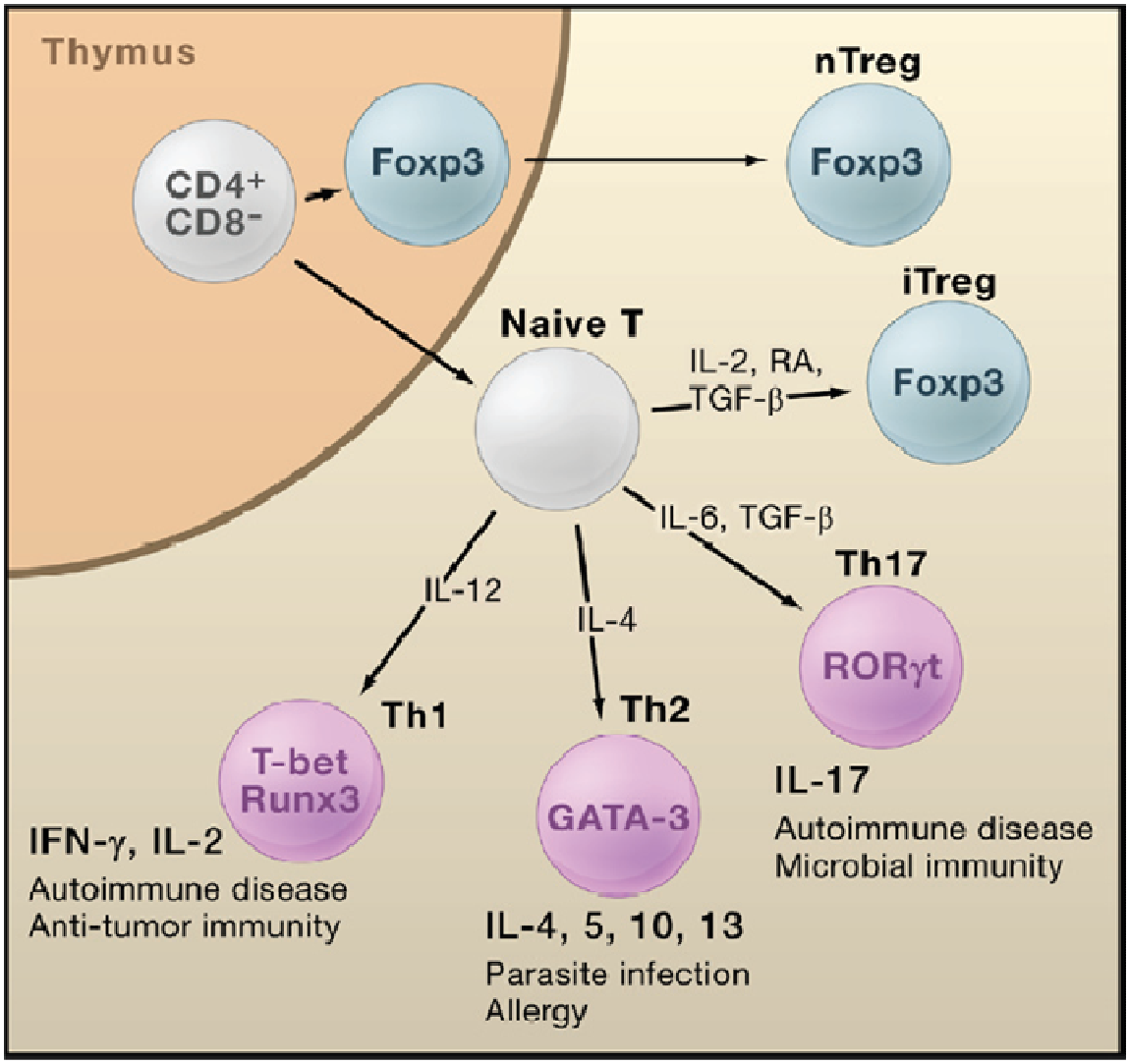


Figure 1.2 Differentiation and maturation of T helper and regulatory T cells. Cytokines and transcription factors that promote the differentiation of naïve T cells into Tregs and T helper cells are shown. The transcription factors T-bet and Runx3, GATA3, or RORγt are required for the differentiation of naïve T cells into Th1, Th2 and Th17 cells respectively, nTreg, nature Treg; iTreg, induced Treg; RA, retinoic acid (Adapted from Sakaguchi *et al.*, 2008).

Table 1.2 T cells and their functional role.

Cell type	Role and functional outcomes
Th1 cell	Inflammatory Production of cytokines which: • activates macrophages. • promotes the development of cytotoxic T cells. • helps B cells to make antibody. • induces class and subclass immunoglobulins switching. • induces monocyte and neutrophil infiltration. • inhibits Th2 responses.
Th2 cell	Anti-inflammatory or allergic inflammation • Production of cytokines which: • promotes basophil and eosinophil infiltration and activation. • promotes downstream switch of immunoglobulin class and subclass e.g. towards IgE and IgG_4. • inhibits macrophage functions.
Th17 cell	Inflammatory • Production of cytokines which promotes inflammation e.g. in rheumatoid arthritis. • Produces IL-17.
Tregs	Imunosuppression/ Anti-inflammatory • Compete with antigen-specific naïve T cells for DC. • Target dendritic cells including DC maturation, function and suppress DC maturation. • Inhibit cytokines include IL-10, IL-35 and transforming growth factor-β (TGF-β).

1.4 Neutrophils

The development of neutrophils from granulocyte-monocyte progenitor is regulated by a group of cytokines such as the granulocyte colony-stimulating factors (G-CSF), granulocyte macrophage colony-stimulating factors (GM-CSF) and IL-3 (Ferrante, 2005) (Figure 1.1). Neutrophils produced by hematopoiesis in the bone marrow are released into the peripheral blood and circulate for 7-10 h before migrating into the tissues, where they have a life span of only a few days. These cells are generally the first to arrive at sites of inflammation (Goldsby *et al.*, 2003c).

1.4.1 Neutrophil adhesion-selectins and integrins

Adhesion molecules on leukocytes facilitate their attachment to the endothelium and to tissue structures over which they migrate (Schmid-Schonbein, 2006) (Figure 1.3). The two general classes of adhesion molecules involved are the integrins and selectins that are either pre-expressed in both the plasma membrane and cytoplasmic granules, or may be newly synthesized upon stimulation of the cells (Schmid-Schonbein, 2006)

Initial attachment of leukocytes to endothelial membranes is facilitated by selectins P-, E- L (Schmid-Schonbein, 2006). These selectins are either stored in granules (P-selectins) or pre-expressed (E and L-selectin) and can be translocated rapidly to the cell surface in response to several inflammatory stimuli (Ulbrich *et al.*, 2003). The adhesive function of all selectins requires specialized counter-receptors and the interactions between P-selectin

glycoprotein ligand 1 (PSGL-1) and P-selectin is responsible for a major part of leukocyte rolling during inflammation (Ulbrich *et al.*, 2003). Neutrophil capture and rolling can be mediated by P-selectin binding PSGL-1 and activated by IL-8 (Figure 1.3). L-selectin located on neutrophil microvilli, interacts with PSGL-1 on endothelium to promote leukocytes capture to the endothelium (Ulbrich *et al.*, 2003; Burg and Pillinger, 2001).

In contrast, firm adhesion and spreading of cells on the endothelium is facilitated by integrins, a family of heterodimers, and tyrosine kinase receptors (Schmid-Schonbein, 2006). These molecules exists as dimers that contain an α and β subunit (Ulbrich *et al.*, 2003). Integrins are activated rapidly from a low-affinity to a high-affinity state following ligand binding and cell activation. The integrins used by leukocytes share a common β_2-chain (CD18) and four different α-chains (CD11a, CD11b, CD11c, CD11d) with CD11b/CD18 being one of the important integrins in the inflammatory reaction expressed on neutrophils, monocytes and macrophages (Schmid-Schonbein, 2006). Firm adhesion of the neutrophil to endothelial cells is confirmed when the integrins binds to the intracellular cell adhesion molecules (ICAM) or the vascular cell adhesion molecules (VCAM) found on the endothelium cells (Ulbrich *et al.*, 2003).

1.4.2 Neutrophil extravasations and transmigration

After firm adhesion via integrins, neutrophils transmigrate either between or directly through endothelial cells to arrive at extravascular sites of infection (Figure 1.3) (Witko-Sarsat *et al.*, 2000). Platelet endothelial cell adhesion molecule-1 (PECAM-1), a cell adhesion molecule of the Ig superfamily plays a role in transmigration (Witko-Sarsat *et al.*, 2000). PECAM-1 is expressed both on the neutrophil surface and at the endothelial cell junction to mediate

neutrophil extravasativia PECAM-1/PECAM-1 homophilic interaction (Witko-Sarsat *et al.*, 2000).

Neutrophil emigration from the vascular space and migration through the tissues are consequences of a regulated process involving the sequential release and compartmentalization of a wide variety of inflammatory mediators (Denson *et al.*, 1995). Early influx of neutrophils into an area of injury is predominantly mediated by IFN-γ, complement 5a (C5a) and leukotriene B_4 (LTB_4). IL-8 and IL-6 appear in a second wave of mediator activity, followed by IL-1β, GM-CSF, and TNF-α. In contrast, the concentration of IL-1α, IL-2 and IL-4 remains unchanged (Denson *et al.*, 1995). When neutrophils are exposed to a uniform concentration of chemoattractants, the speed or frequency of migration increases (Witko-Sarsat *et al.*, 2000). When the concentration of chemoattractants differs across the cell by as little as 0.1-1.0 %, ligand-linked receptors distribute asymmetrically, leading to a polarised response that causes directional movement, resulting in the net accumulation of neutrophils at sites of increasing concentration of attractants (Denson *et al.*, 1995).

1.4.3 Phagocytosis

Once the neutrophils arrive at the site of infection as explained above, they then proceed to phagocytose microbial pathogens. This process may be described in the following steps. Once receptors on the cell surface recognize and bind to the pathogen; signals are generated that induce actin polymerization under the membrane at the site of contact. Then actin-rich membrane extensions extend over the particle, the membranes fuse behind the particle, pulling it in toward the centre of the cell. Finally the phagolysosome matures via a series of

membrane fusion and fission events to become a phagolysosome (Figure 1.4) (Aderem, 2003). The phagolysosome is an acidic, hydrolytic compartment in which the pathogen is killed and digested (Aderem, 2003).

Neutrophils, like macrophages internalize opsonised particles bound via the Fc or complement receptors. While the internalization of complement opsonised particle involves a 'sinking in process', the Fc receptor promote the formation of pseudopods that surround the particle, termed phagosome (Lee *et al.*, 2003).

1.4.4 Fc receptors

Neutrophil phagocytosis depends upon direct contact with bacteria or other targets that have been opsonised by antibody and/or complement involving two different receptor classes, Fc receptors and complement receptors (Witko-Sarsat *et al.*, 2000). The main function of the Fc receptors is to recognize the Fc domain of IgG antibody providing a crucial linkage between the phagocytic effector cells and the lymphocytes that secrete antibody, as the macrophage/monocyte, neutrophil, and NK cell Fc-γRs confer the element of specific recognition on the effector cells (Table 1.3). There are three types of the Fc-γ receptors, namely Fc-γRI (CD64), Fc-γRIIA (CD32) and Fc-γRIIIB (CD16) (Ulbrich *et al.*, 2003). Fc-γRIII has no transmembrane portion but crosslinking Fc-γRIII enhances Fc-γRII-mediated phagocytosis by increasing actin filament assembly and by promoting of oxidants which augment the avidity and efficiency of Fc-γRII (Burg and Pillinger, 2001).

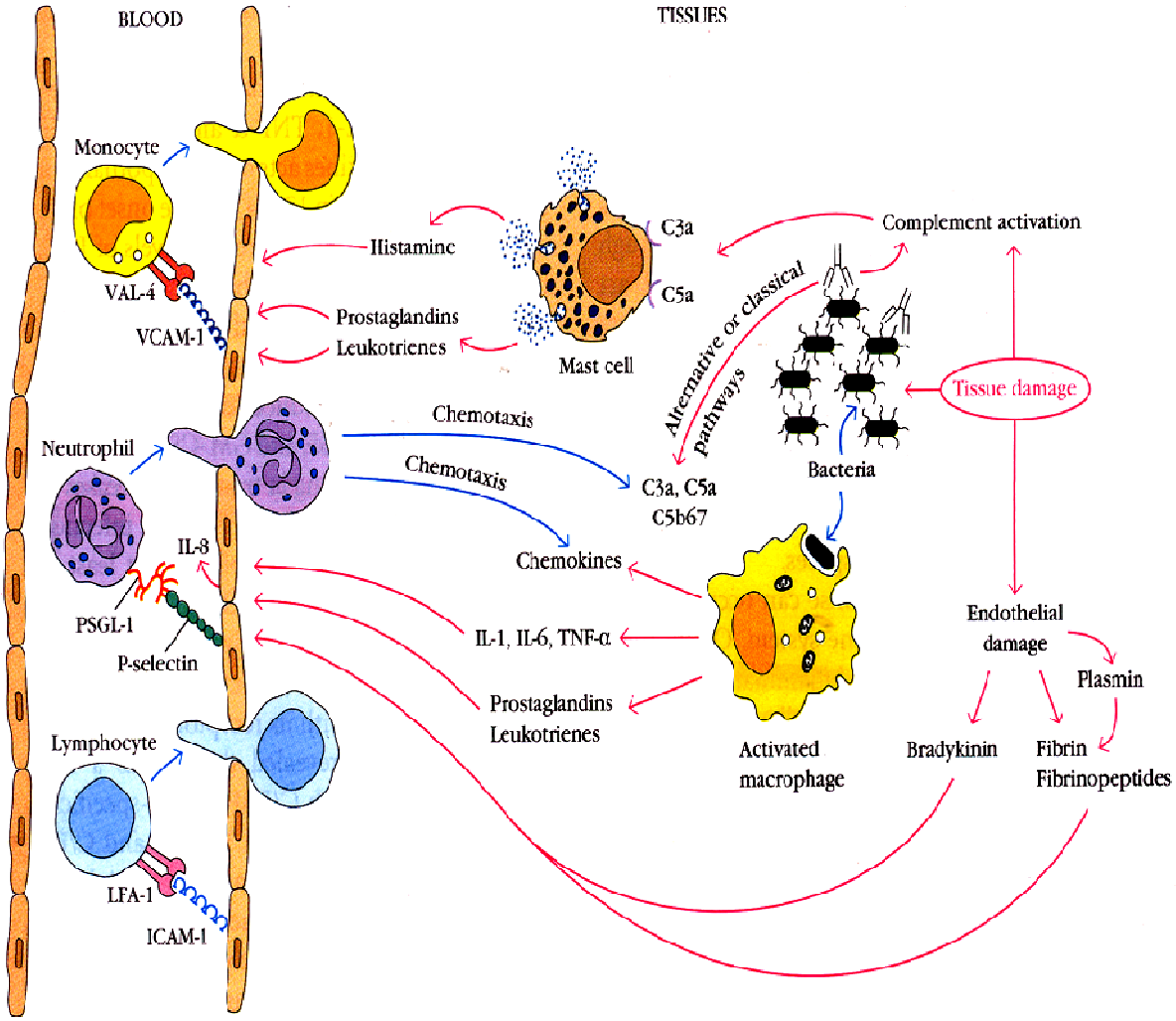


Figure 1.3 Overview of steps involved during an inflammatory condition. Tissue damage leads to the recruitment of leukocytes from the blood to the site of infection mediated by cytokines, chemokines, and chemoattractants. Adapted from Goldsby *et al.*, (2003d).

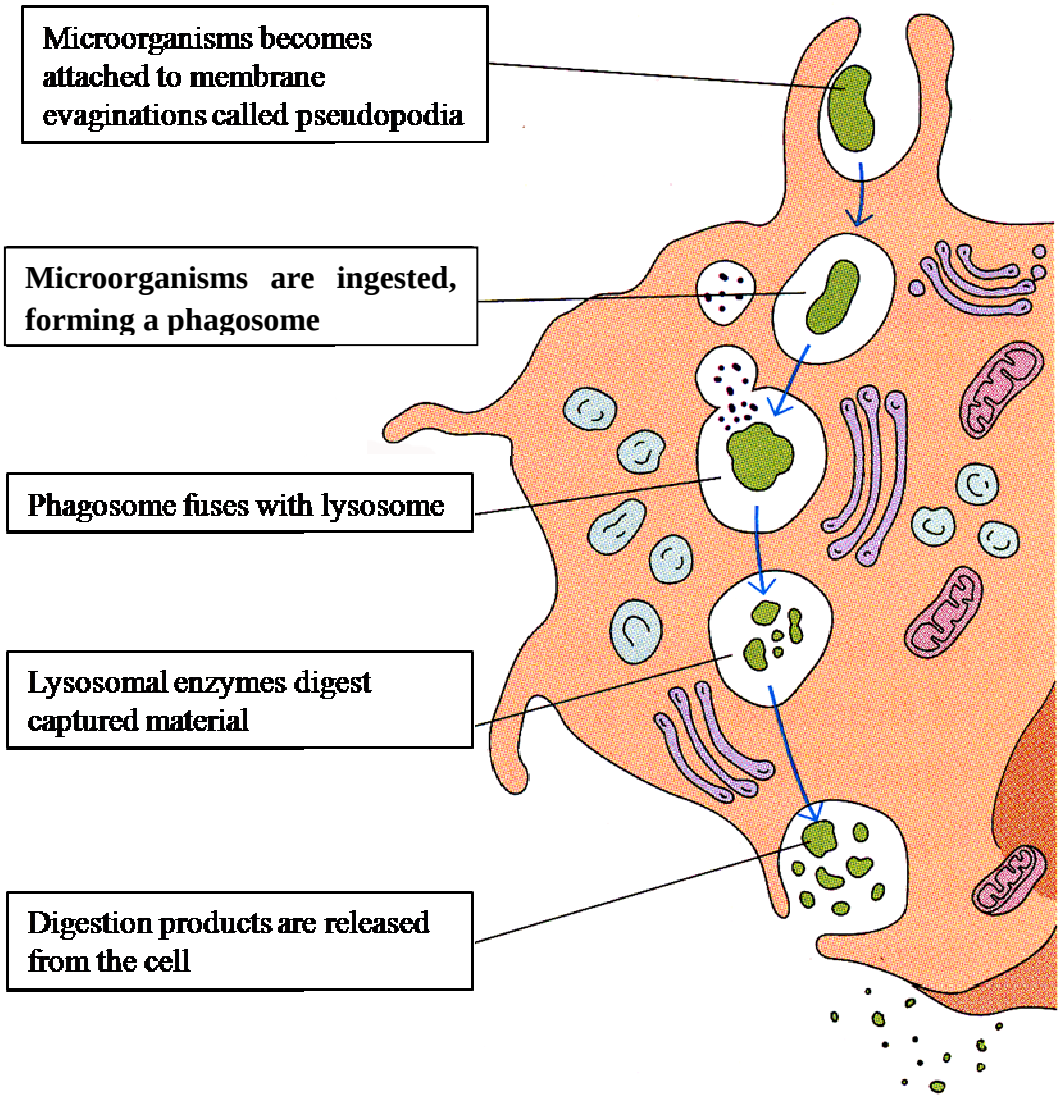


Figure 1.4 Steps involved in the phagocytosis of microorganisms. Adapted from Goldsby *et al.*, (2003d).

1.4.5 Receptors for complement (CR)

The activation of the complement cascade either through the classical, alternative or mannose-binding lectin (MBL) pathways causes proteolytic cleavage of these proteins, resulting in several ligands for receptors on leukocytes. These ligands may be divided into two categories: (a) soluble ligands, which cause chemotactic movement of leukocytes to sites of complement activation; and (b) surface-bound ligands, which function as opsonins. Activation of complement leads to the enzymatic cleavage of C3 and the deposition of C3b or C3bi on the microbial surface (Table 1.3) (Denson *et al.*, 1995). Complement-dependent neutrophil phagocytosis is mediated by complement receptor 3 (CR3) acting primarily on C3bi due to its high affinity for this component (Burg and Pillinger, 2001). CR1 and CR4 are also found to be important for recognition of bacteria by neutrophils. Phosphorylation of CR1 can trigger the "inside-out" signalling that activates CR3 binding capacity (Witko-Sarsat *et al.*, 2000) and CR4 directly binds to C3bi (Ferrante, 2005). However, binding of neutrophil CR3 to particles exclusively coated with C3bi is not sufficient to promote phagocytosis, and needs to be assisted by activation of other pathways e.g. using PMA or formyl-peptides (Brown, 1986). A complex service of signalling pathways activated during engulfment of opsonised targets lead to the fusion of protease-rich granules with the phagosome and the triggering of oxidative burst (Witko-Sarsat *et al.*, 2000; Ferrante, 2005).

1.4.6 Neutrophil degranulation

Neutrophil-derived microbicidal molecules are packed in granules that are released upon cell activation. Degranulation of vesicles into phagolysosomes or directly into extracellular space is a key process in the microbicidal activity of the cell. During phagocytosis, the

cytoplasmic granules approach the membrane of the developing phagocytic vacuole and plasma membrane where they fuse with either the membrane delivering granule contents to the phagocytic vacuole or with plasma membrane resulting in the extracellular secretion of granule proteins (Denson *et al.*, 1995) (Figure 1.4). There are two major granules, the azurophil (primary) and the specific type (secondary) (Segal, 2005). Azurophil granules, contains largely proteins and peptides directed toward microbial killing and digestion, whereas the specific granules contain unsaturated lactoferrin and stores for cell surface receptor and components which limit free radical reactions (Segal, 2005). Azurophil granules are characterized by the presence of myeloperoxidase and membrane-bound CD63. The specific granules contain lactoferrin and membrane-bound CD66b (Lee *et al.*, 2003).

A third type of granule has also been described, the tertiary granules which lack CD66 but contain gelatinase. A fourth group known as the secretory vesicles contain albumin and express alkaline phosphatase and CD35(Lee *et al.*, 2003). Degranulation involves the release of a wide range of anti-microbial enzymes into the phagosome. Such enzymes include myeloperoxidase, acid hydrolase and lysosomal proteases (Witko-Sarsat *et al.*, 2000)
The neutrophil uses a range of anti-microbial peptides and proteins to aid in the destruction of microorganisms. This includes the defensins, proteinase 3 (PR3), elastase, phospholipase A_2 (PLA_2) and metalloproteinase. Defensins are small cationic, antibiotic peptides that contain six cysteines in disulfide linkage and are major components of the azurophilic granules (Burg and Pillinger, 2001; Witko-Sarsat *et al.*, 2000). They act by inducing microbial membrane permeabilization and can regulate inflammatory process through binding of protease inhibitors (Burg and Pillinger, 2001).

Table 1.3 Functional neutrophil receptors involved in phagocytosis.

Receptor type	**Ligand**
FcγRI (CD64)	Fc domain of IgG
FcγRIIA (CD32)	Fc domain of IgG
FcγRIIIB (CD16)	Fc domain of IgG
FcαR (CD89)	Fc domain of IgA
CR1(CD35)	C3b
CR3 (CD11b/CD18)	C3bi
CR4 (CD11c/CD18)	C3bi

Adapted from Ferrante (2005).

Proteinase 3 can be found in both azurophilic and secretory granules and is often found on the membrane surface of secretory granules (Burg and Pillinger, 2001; Segal, 2005). The membrane bound PR3 is bioactive and insensitive to proteinase inhibitor and has been shown to enhance cleavage and activation of TNF and IL-1β (Burg and Pillinger, 2001). Elastase is another serine protease and similar to PR3 is capable of cleaving insoluble elastin and matrix proteins such as fibronectin, laminin and collagen (Witko-Sarsat *et al.*, 2000). Phospholipase A_2 (PLA_2) is a neutrophil granule protein that has very potent bactericidal activity against *S.aureus* (Burg and Pillinger, 2001). Lastly, metalloproteinases are calcium-requiring enzymes that function at neutral pH and are released in inactive proenzyme forms. These enzymes are required for neutrophil migration (Burg and Pillinger, 2001).

1.4.7 Neutrophil nicotinamide adenine nucleotide phosphate (NADP) H oxidases and reactive oxygen species

The killing of the microorganism involves the stimulation of the oxidative respiratory burst system, leading to the reduction of oxygen to superoxide anion, involving the NADPH oxidase, as well as the generation of hydrogen peroxide (Djaldetti *et al.*, 2002; Segal, 2005). The phagocyte NADPH oxidase is an essential component of the human cellular immune response which is required for microbial killing and belongs to the family of the NADPH oxidase (NOX) proteins (Lee *et al.*, 2003). However, oxidants generated by this system can also contribute to non-specific tissue damage associated with a variety of inflammatory diseases (Quinn, 2004). Because of the potential for tissue damage, the assembly of the NADPH oxidase from its components is highly regulated and involves several control points (Quinn, 2004).

Activation of the oxidase requires the assembly of at least five proteins with membrane-associated flavocytochrome b, which appear to contain all of the required redox components but cannot on their own catalyze the reaction (Quinn, 2004). While a fraction of the intrinsic components of the oxidase are present at the plasma (vacuole) membrane at the time of phagocytosis, most are delivered through fusion from intracellular storage sites in second granules (Borregaard and Cowland, 1997).

The activation of respiratory burst, leads to the activation of the NADPH oxidase, an enzymatic complex composed of cytosolic ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) and membrane proteins ($p22^{phox}$ and $gp91^{phox}$), which constitute a heterodimeric flavohemoprotein known as cytochrome b_{558} (Witko-Sarsat *et al.*, 2000). Cytochrome b_{558} is distributed between the plasma membrane and the membrane of specific granules and upon incorporation into the wall of the phagocytic vacuole, electrons are being released into the cytoplasm during the oxidation of NADPH to $NADP^+$ which counteracts cytosolic acidification (Figure 1.5) (Segal, 2005; Behe and Segal, 2007). Stimulation of neutrophils, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$, as well as a low molecular weight guanosine 5'-triphosphate (GTP)-binding protein, are translocated to assemble with the cytochrome b_{558} ($p22^{phox}$ and $gp91^{phox}$) in the membrane and form a complete and active NADPH oxidase (Roos *et al.*, 2003). During the respiratory burst, the oxidase mediates the translocation of electrons across the membrane lipid bilayer so as to reduce the oxygen present extracellularly (Lee *et al.*, 2003; Behe and Segal, 2007). Moreover, cations are being translocated in response to the electrical potential change by H^+ and/or K^+ influxes which help in granule secretion by increasing the ionic strength of the phagosomal lumen (Lee *et al.*, 2003).

This enzymatic complex is thus able to generate superoxide anion (O_2^-), which can dismutate into H_2O_2 (Witko-Sarsat *et al.*, 2000; Ferrante *et al.*, 1992; Dahlgren and Karlsson, 1999). There are three intermediates in the reduction of O_2, namely O_2^-, H_2O_2 and the hydroxyl radical ($OH^{\cdot}$) (Figure 1.5) (Witko-Sarsat *et al.*, 2000; Behe and Segal, 2007). The involvement of O_2^-, H_2O_2 and $OH^{\cdot}$ in tissue damage has been studied extensively especially with the role of oxygen reactive species in tissue damage in rheumatoid arthritis (Felson *et al.*, 1990; Mirshafiey and Mohsenzadegan, 2008; Moots, 2003; Rynes, 1993).

1.4.8 The H_2O_2-Myeloperoxidase-Halide system

The generation of superoxide anion via the activation of NADPH oxidase is the starting material for the production of a vast assortment of reactive oxygen intermediates, including halogenated oxidants generated through the myeloperoxidase (MPO) pathway (Witko-Sarsat *et al.*, 2000). MPO is a heme protein present in azurophil granules of neutrophils which is released upon cell activation into the phagolysosome or into the extracellular space (Dahlgren and Karlsson, 1999). MPO amplifies the toxic potential of H_2O_2 by producing reactive intermediates such as hypochlorous acid (HOCl) which can oxidise various molecules including amino acids, nucleotides, and hemoproteins (Witko-Sarsat *et al.*, 2000; Denson *et al.*, 1995; Burg and Pillinger, 2001). The MPO system is known to be responsible for the formation of tyrosyl radical and chloramination productions, the generation of tyrosine peroxide, reactive aldehydes, and the oxidation of serum proteins and lipoproteins (Witko-Sarsat *et al.*, 2000). The system has been shown to be important in microbial killing. MPO-deficient neutrophils show a slow rate of bacteria killing within the first hours but normalise later (Klebanoff and Rosen, 1978).

However recent studies from Segal's group have challenged the importance of MPO in microbial killing suggesting other mechanisms are involved including elastase and that the MPO system is unlikely to play a significant role in this killing (Reeves *et al.*, 2002; Segal, 2005). Studies using proteases knock-out mouse models such as elastase and cathepsin G or both enzymes showed that elastase is especially important in the killing of Gram-negative bacteria whereas cathepsin G is less important (Belaaouaj *et al.*, 1998; MacIvor *et al.*, 1999; Reeves *et al.*, 2002; Tkalcevic *et al.*, 2000). However, both enzymes are required to mediate the killing of microorganisms and the lack of both enzymes resulted in a defect in the killing of bacteria whilst displaying normal respiratory burst (MacIvor *et al.*, 1999; Reeves *et al.*, 2002). This further supported the idea that both NADPH oxidase acitivy as well as protease enzymes are required for effective microbial killing (Segal, 2005).

1.4.9 Neutrophil Priming

Some inflammatory mediators prime neutrophils for enhanced anti-microbial function (Ferrante, 2005). A broad array of inflammatory mediators, including chemotactic factors, endotoxin, cytokines, and certain lipids, can prime the neutrophil, and the primed state exists with respect to each of the major aspects of neutrophil function (Ferrante, 2005; Denson *et al.*, 1995). Primed neutrophils generate enhanced levels of reactive oxidants and have higher levels of degranulation and greater phagocytic activity (Ferrante *et al.*, 1993; Ferrante *et al.*, 1994). For example, the expression of cell-surface molecules, such as CR3 and cytochrome b, is very low in neutrophils found in blood, but this expression is rapidly up-regulated during priming e.g. by N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), GM-CSF (Edwards, 1994) and TNF (Onnheim *et al.*, 2008).

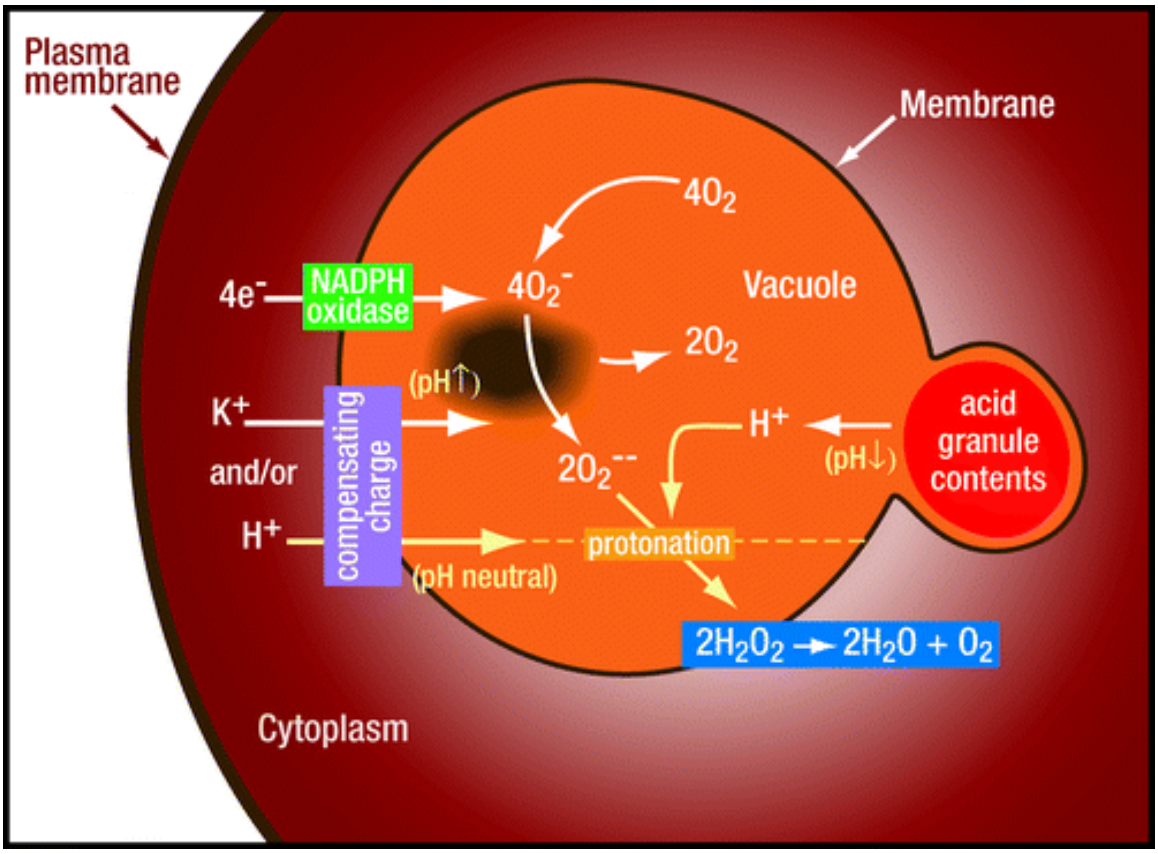


Figure 1.5 Generation of reactive oxygen species by neutrophils. Neutrophils undergoing phagocytosis generate reactive oxygen species so as to aid in the removal of the bacteria (Adapted from Segal, 2005).

Moreover, the priming effect of GM-CSF further confirmed by its effect of enhanced neutrophil respiratory burst partially involves the phosphorylation of $p47^{phox}$ (Dang *et al.*, 1999). This effect is only observed with GM-CSF but not with fMLP or PMA. The priming effect of TNF has also been studied and it primes neutrophil mediated cartilage damage depending on the cell-cartilage contact (Kowanko and Ferrante, 1996). Moreover, neutrophils primed with TNF and later challenged with PGE_2 were found to show reduced superoxide production measured via chemiluminescence (Li *et al.*, 1996). However, when challenged with fMLP, an enhanced response occurred (Zhang *et al.*, 1992; Ferrante *et al.*, 1992) and this similar result was also observed in TNF primed neutrophil response towards fMLP so to enhance oxidative burst (Elbim *et al.*, 1994). Other groups have also found that the priming of neutrophils by TNF occurs through its p55 receptor inducing the phosphorylation of $p47^{phox}$, a key element of the NADPH oxidase (Dewas *et al.*, 2003).

1.5 Chemoattractant receptors

Chemokines are small heparin binding proteins that direct the migration of circulating leukocytes to sites of inflammation, and function via activating specific G protein-coupled receptors (Lukacs and Harrison, 2007). There are more than 50 human chemokines described. These can be divided into three major families based on their structure and functional characteristics.

First and foremost is the CC chemokines which attract mononuclear cells found at sites of chronic inflammation (Charo and Taubman, 2004; Lukacs and Harrison, 2007). The most characterised one is MCP-1 (CCL2), an agonist for monocytes, T cells and basophils that plays a role in the recruitment of these cells (Uguccioni *et al.*, 1995; Mackay, 1996; Weber *et*

al., 1996). Other members of the family include proteins regulated on activation, normal T cell expressed and secreted (RANTES or CCL-5), macrophage inflammatory protein-1α (MIP-1α) (CCL3), and MIP-1β (CCL4) which are involved in the recruitment of monocytes, activation of T cells and mediate neutrophil migration (Charo and Taubman, 2004; Didier *et al.*, 1999; Ramos *et al.*, 2005). The second family of chemokines is the CXC family including IL-8 (CXCL8), which is an attractant for neutrophils, usually stored in granules of resting neutrophils and increases significantly upon stimulation (Pellme *et al.*, 2006). IL-8 is involved in acute pulmonary inflammation and known to activate monocytes (Charo and Taubman, 2004; Baggiolini *et al.*, 1997). The third family is represented by the CX3C family with one member, fractalkine (FK)(CX3CL1) (Charo and Taubman, 2004). Fractalkine is a type 1 transmembrane protein that acts as a cell-adhesion receptor to arrest cells under physiological flow conditions (Haskell *et al.*, 1999).

Other cellular mediators include chemoattractants that are activated and released at the site of infection such as fMLP, C5a and LTB_4. They also act as key factors in neutrophil migration and activation (Burg and Pillinger, 2001). Each chemoattractant has its own specific seven-transmembrane domain, heterotrimeric G protein coupled receptors (Burg and Pillinger, 2001; Karlsson and Dahlgren, 2002), e.g the activation mediated by fMLP via formyl peptide receptor (FPR) (Bylund *et al.*, 2003), C5a via C5a receptor (Karlsson and Dahlgren, 2002) and LTB_4 via LTB_4 receptor (Schultz *et al.*, 1991). The engagement of the receptors result in G protein activation followed by the dissociation of the α from the βγ G protein subunits which are important for phospholipase C, phosphatidylinositol-3 kinase (PI-3 kinase) and adenyl cyclase activation (Burg and Pillinger, 2001; Bylund *et al.*, 2003) . Phospholipase C can then hydrolyse membrane phospholipids resulting in the formation of inositol trisphosphate (IP3) and diacylglycerol (DAG) and the release of arachidonic acid (AA) (Burg

and Pillinger, 2001). IP3 also induces Ca^{2+} release from intracellular stores (Bylund *et al.*, 2003), which in conjunction with DAG lead to PKC activation. (Burg and Pillinger, 2001).

1.6 Cytokines and cytokine receptors

Cytokines are inflammatory mediators produced during inflammation and are produced following the stimulation of cells by a wide range of agents including LPS, cytokines, phagocytic particles, microorganisms, chemotactic factors, LTB_4, PAF, prostaglandin E2 (PGE2) and complement C5a (Ethuin and Chollet-Martin, 2005). They are produced mainly by lymphocytes and macrophages. While neutrophils have been shown to produce low amounts of cytokines, they can still modulate T and B cells as well as APC function. Some of the well studied cytokines includes TNF-α, IL-1, IL-6, IL-12, IFN-α, IL-4, IL-8, TGF-β, G-CSF, M-CSF, MCP-1 and MIP-2 (Ferrante, 2005). These cytokines act via well characterised cell surface receptors to induce a response to inflammation. The production of various cytokines by neutrophils depends on the stimulus the cell encounters such as bacteria, fungi, viruses, fMLP, LTB_4 PAF, C5a and cytokines themselves can also stimulate neutrophils (Ferrante, 2005). While the release of IL-8 is known to be important in neutrophil migration as well as recruiting T lymphocytes and basophils to the site of infection, MIP-1 is also known for its potent chemotactic properties for monocytes and T lymphocytes (Ferrante, 2005).

Neutrophils produce the pryogenic cytokines TNF and IL-1 and this may be one way in which the cells play a role in pathogenesis. TNF is produced by a number of cell types and is a potent mediator of host response to injury, inflammation, immunity and repair. Cellular responses such as adherence, superoxide production, phagocytosis, degranulation and efflux

of chloride ions are usually initiated following interaction of either or both TNFRI (55 kDa) and TNFRII (75 kDa) with the cytokine (Gasparini *et al.*, 2003; Ethuin and Chollet-Martin, 2005). TNF receptors are made up of 10 different transmembrane glycoproteins with cytokine-rich repeats in the extracellular region (Gruss, 1996). It is understood that the expression of these receptors decreases via shedding when neutrophils are exposed to inducing agents such as TNF, fMLP, PMA, A23187 endotoxin and GM-CSF (Dri *et al.*, 2000). Moreover, TNF is also found to enhance neutrophil adhesion molecule VCAM-1 which is in turn regulated by mitogen activated protein kinases (MAPKs) and NF-κB signalling molecules (Lee *et al.*, 2008). Under resting conditions, the amount of TNF expressed is minimal, however following cellular activation, the level of mRNA increases as well as the expression of TNF.

IL-1 is known to be produced in various cell types. The IL-1 family consists of 11 members of which IL-1α, IL-1β and IL-1 receptor antagonists (IL-1Ra) are the most investigated molecules. IL-1, unlike other cytokines exerts its effect both at the receptor level and intracellularly upon binding to IL-1 receptors (Dinarello, 2009). Other important actions of IL-1 are the induction of the production of prostaglandin E_2, activation of collagenase, and phospholipase A_2 as well as enhancing adherence of neutrophils, monocytes and lymphocytes (Ferrante *et al.*, 2005). It is also known to prime neutrophils for an increase in antimicrobial activity and the production of IL-1Rα can regulate the effect of IL-1 on neutrophils (Ferrante, 2005).

1.7 Inflammation and fatty acids

The immunomodulatory properties of short, medium and long carbon chain fatty acids have been widely reported. They modulate neutrophils, monocytes and lymphocytes responses by either inhibiting or stimulating the production of cytokines, chemokines, growth factors, reactive oxygen species and antibodies at non-toxic concentrations (Martins de Lima *et al.*, 2007; Calder, 1993; Calder *et al.*, 2002). Studies on the toxicity of these fatty acids on Jurkat (T lymphocytes) and Raji (B lymphocytes) cell lines have found that carbon chain length and number of double bonds plays an important role in cytotoxicity (Lima *et al.*, 2002). An increase in the number of carbons and double bonds resulted in a increased toxicity (Table 1.4) (Lima *et al.*, 2002). This finding was in contrast to those with J774 cells (macrophage cell line) where a difference in double bond did not result in a concentration dependent cytotoxicity (Table 1.4) (Martins de Lima *et al.*, 2006). This illustrates that the effects of these fatty acids may differ substaintially depending on cell and tissue type.

Martins de Lima *et al.*, (2006) have also found that high concentrations of fatty acids can mediate macrophage cell death, mainly due to necrosis. The mechanism of this was thought to be through the changes in cell mitochondrial transmembrane potential and intracellular neutral lipid accumulation (Martins de Lima *et al.*, 2006). One of the major action of these fatty acids is by activating directly or indirectly intracellular signalling molecules including protein kinase C, mitogen activated protein kinase, phospholipase A_2 and phosphatidyinositol 3 (Ferrante *et al.*, 2005). Fatty acids, in particular long chain fatty acids, inhibit T lymphocytes both *in vitro* and *in vivo* (Calder, 1993). The ability of long chain fatty acids to down-regulate Th1 cell cytokine production in chronic inflammation has also been reported (Calder *et al.*, 2002).

The importance of fatty acid chain length was also found in the ability of fatty acids to alter neutrophil function (Ferrante *et al.*, 2005). Several studies have reported that fatty acids, depending on their chain length, can mediate neutrophil cell death through reactive oxygen species (Miller *et al.*, 2005; Tedelind *et al.*, 2007; Wanten *et al.*, 2000b). This similar effect was also observed on human neutrophil migration (Wanten *et al.*, 2000b). Short chain fatty acids such as acetate, propionate, butyrate were found to inhibit LPS-induced TNF-α production but not IL-8 in neutrophils and inhibit the activation of the NF-κB pathway (Tedelind *et al.*, 2007). Furthermore, butyrate was also found to increase ICAM-1 and E-selectin expression but not on VCAM-1 and HLA-DR at a concentration of 2.5 to 5 mM (Miller *et al.*, 2005). Short chain fatty acids (6 carbons to 10 carbons) were found to have no effect on oxygen radical production by neutrophils compared to the stimulatory effect of medium-long chain fatty acids (12 carbons to 20 carbons) at a concentration of 1 mM (Wanten *et al.*, 2002; Ferrante *et al.*, 2005). Studies on other longer chain fatty acids such as palmitic acid, stearic acid, oleic acid (OA), linoleic acid, arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have been found to stimulate nitric oxide production at low concentrations of 1-10 μM and inhibit at higher concentrations (50-200 μM) (de Lima *et al.*, 2006).

Omega 3 polyunsaturated fatty acids are used as anti-inflammatory supplements, especially in patients with rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and in cardiovascular diseases (Calder *et al.*, 2002; Simopoulos, 2002). Some of the benefits from such supplements are due to DHA and EPA decreasing the levels of highly inflammatory eiconoids derived from AA and may be useful to lower the usage of anti-inflammatory drugs (Simopoulos, 2002).

1.8 Anti-malarials as anti-inflammatory agents

Developing appropriate anti-inflammatory agents remains a major challenge. Presently available agents have targeted mainly enzymes, G proteins coupled receptors, nuclear hormone receptors, cytokines, cytokine receptors and cell adhesion molecules and co-stimulatory molecules (Simmons, 2006). A summary of the specific anti-inflammatory targets is presented in Table 1.5.

Although a wide range of anti-inflammatory agents have been developed, the quest for effective drugs to treat chronic inflammatory conditions continues. Due to the broad usage of the present therapies, they are highly toxic and associated with significant side effects (Norman and Hickey, 2005).

Current treatments for inflammatory diseases have largely focused on trying to interrupt the synthesis of mediators that drive the host's response during injury. These include non-steroidal anti-inflammatory agents, steroids, glucocorticoids and anti-histamines (Gaestel *et al.*, 2009; Gilroy *et al.*, 2004).

More recently, treatments for chronic inflammatory diseases have been based on the inhibition of mediators that promote acute inflammation (Gilroy *et al.*, 2004; Ulbrich *et al.*, 2003) as well as the development of small molecular inhibitors of protein kinases in both acute and chronic inflammation (Gaestel *et al.*, 2009).

Table 1.4 Maximum concentration of fatty acid tolerated by human cell lines, Jurkat (T-lymphocyte), Raji (B-lymphocyte) and J774 (macrophages).

Fatty acids	Jurkat	Raji	J774
Propionic C3:0	>1 mM	>1 mM	ND
Butyric C4:0	800 µM	700 µM	ND
Caproic C6:0	>1 mM	>1 mM	ND
Caprylic C8:0	>1 mM	>1 mM	ND
Capric C10:0	400 µM	400 µM	ND
Lauric C12:0	200 µM	200 µM	ND
Myristic C14:0	150 µM	150 µM	ND
Palmitic C16:0	50 µM	100 µM	70 µM
Palmitoleic C16:1	100 µM	100 µM	ND
Stearic C18:0	50 µM	50 µM	70 µM
Oleic C18:1	250 µM	250 µM	100 µM
Elaidic C18:1	300 µM	400 µM	ND
Vacenic C18:2	200 µM	150 µM	ND
Linoleic C18:2	100 µM	100 µM	150 µM
γLinolenic C18:3	50 µM	50 µM	ND
Arachidonic C20:4	50 µM	25 µM	150 µM
Eicosapentaenoic C20:5	50 µM	25 µM	150 µM
Docosahexaenoic C22:6	50 µM	25 µM	150 µM

Adapted from Lima *et al.,* (2002) and Martins de Lima *et al.,* (2006). Abbreviation- ND- experiment not done.

However, the limitation of these therapies are evident since they also inhibit essential physiological processes, resulting in side effects or even leaving the patient susceptible to opportunistic infection (Gilroy *et al.*, 2004).

The usage of anti-malarials as immunosuppressive antimicrobial agents dates back to centuries with case studies reporting the effects of these anti-malarials in the 1940s (Meyer-Rohn, 1972; Randrianarivelojosia *et al.*, 2009). These have been widely used for therapeutic purposes in inflammatory diseases and also serve as a tool for examination of mechanisms of immunological functions (Thong and Ferrante, 1978, 1980). Of the various anti-malarial drugs developed, those with a 4-aminoquinoline structure such as amodiaquine, primaquine, quinine, mefloquine, CQ and HCQ have been found to have anti-inflammatory properties. However only CQ and HCQ have been successful in the treatment of rheumatic conditions (Jones and Jayson, 1984). Summaries of the effects of 4-aminoquinoline base compounds on leukocytes responses can be found in Table 1.6. While the different compounds exert their effects differently, it is evident that quinine, melfoquine, CQ and HCQ showed the most promising effects as an anti-inflammatory agent and only CQ and HCQ were found to be more effective at a lower concentration that is applicable to clinical application.

Table 1.5 Summary of anti-inflammatory targets.

Target class	Specific targets
Enzymes	COX-1 and 2 IMPDH Capase 3
G protein coupled receptors	CysLT1 H1
Nuclear hormone receptors	Corticosteroids
Cytokines and cytokines receptors	TNF-α and TNFRII IL-1β and IL-1RA IL-2 and IL-2R IFN-α_2 IFN-β_1 IFN-γ
Cell interaction molecules (adhesion and co-stimulatory molecules)	LFA and CD11a CD2 and LFA-3 VLA-4 and CD49d $CTLA_4$ and Ig

Adapted from Simmons (2006) with modifications. Abbreviations-IMPDH-inosine monophosphate dehydrogenase, LFA-leukocyte functional antigen, VLA-very late antigen and CTLA- cytotoxic T lymphocyte-associated antigen.

Table 1.6 Summary of effects of quinoline based compounds on neutrophil functions.

Neutrophil functions	**CQ**	**HCQ**	**Amodiaquine**	**Quinine**	**Melfoquine**	**References**
Chemotaxis	√	ND	X	√/X	√/X	(Ferrante *et al.*, 1986; Labro and Babin-Chevaye, 1988; Kharazmi *et al.*, 1983)
Adherence	√	√	ND	√	√	(Ferrante *et al.*, 1986)
Respiratory burst	√	√	√	√	√	(Ferrante *et al.*, 1986; Hurst *et al.*, 1987; Neal *et al.*, 1987; Kharazmi *et al.*, 1983; Labro and Babin-Chevaye, 1988)
Degranulation	X	ND	ND	ND	√/X	(Neal *et al.*, 1987; Bates and Ferrante, 1988; Ferrante *et al.*, 1986)
MPO activity	√	ND	ND	√	ND	(Labro and Babin-Chevaye, 1988; Ferrante *et al.*, 1986)
Phagocytosis	√	ND	X	ND	√	(Labro and Babin-Chevaye, 1988; Jones and Jayson, 1984)

Abbreviation: ND-not done, √-inhibition observed, X-no inhibition

1.8.1 Chloroquine

CQ is a 4-aminoquinoline anti-malarial that contains a 7-chloroquinoline-substituted ring system with a flexible pentadiamino side chain (Figure 1.6A). These compounds can be readily synthesised, are inexpensive to produce, and are generally well tolerated with acceptable toxicity profiles for treatment of acute infections (O'Neill *et al.,* 1998). The medication is available in tablets of 125 mg or 250 mg as chloroquine phosphate and is known to have potent blood schizonticide activity, effective against the erythrocytic forms of all strains of *Plasmodia,* and has also been useful in the treatment of rheumatoid arthritis, systemic lupus erythematosus and various dermatological diseases (Adams *et al.*, 1983; Isaacson *et al.*, 1982). Moreover the anti-HIV1 effect of CQ has also been reported where it suppresses HIV-1 replication in T cell lines (Tsai *et al.*, 1990).

CQ is a weak base which tends to accumulate down the pH gradient so that its presence in the parasite is 10,000 times greater than in the red blood cell (O'Neill *et al.*, 1998). The weak base effects of CQ on parasite vesicular pH and/or its ability to concentrate in the parasite, facilitates the killing of *Plasmodium* and limiting undesirable side effects (Schlesinger *et al.*, 1988). The action of CQ is limited to the stages of the parasite that are actively involved in haemoglobin degradation (O'Neill *et al.,* 1998). Moreover, it has been found that although CQ highly concentrates within acidic compartments, its site of action is extralysosomal (Weber *et al.,* 2002).

1.8.1.1 Effects of CQ on neutrophils

The effects of CQ on neutrophil functions have been extensivly documented. It has been reported by Raghoebar *et al.,* (1986) that neutrophils accumulate CQ to a greater extent than mononuclear cells due to the large numbers of acidic organelles present in neutrophils, reaching a steady state after 60 min at 37 °C (Raghoebar *et al.*, 1986). *In vitro* treatment of neutrophils with high concentrations (194 µM) of CQ did not result in cell death (French *et al.,* 1987; Labro and Babin-Chevaye, 1988) but exerted a concentration dependant inhibitory effect on neutrophil chemotaxis (Kharazmi *et al.*, 1983; Ferrante *et al.*, 1986) (Table 1.6). The ability of CQ to inhibit neutrophil functions was further confirmed by showing that it inhibited phagocytosis of zymosan particles (Jones and Jayson, 1984; Labro and Babin-Chevaye, 1988) as well as superoxide production induced by fMLP and PMA (Hurst *et al.*, 1987).

One study examining the inhibitory effect of CQ on superoxide production induced by opsonised zymosan, found that it does not act on Mo-1 expression (an epitope on CR3) suggesting that it does not inhibit surface receptors expression (Hurst *et al.,* 1988). With further findings suggesting that it inhibits PMA induced superoxide production in neutrophils (Hurst *et al.,* 1988). Therefore, it has been proposed that chloroquine inhibits activation of the PKC signalling pathway by either competing for the membrane phospholipid regulatory site of PKC, thereby reducing availability of this essential cofactor or it impairs the recruitment of components of the NADPH oxidase from specific granules to the cell membrane (Hurst *et al.*, 1988).

1.8.1.2 Effect of CQ on monocytes/macrophages

CQ markedly inhibits LPS-induced synthesis and release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in human macrophages (Karres *et al.*, 1998). This down-regulatory effect on cytokine production occurs at the transcriptional level and may be caused by disturbance of the signalling through the weak base property of CQ (Karres *et al.*, 1998). Another finding suggested that the effect of CQ on TNF production in RAW 264.7 murine macrophages occurred at the pro-TNF level, inhibiting the maturation of pro-TNF into the mature 17 kDA TNF, namely at the post-translational step (Jeong and Jue, 1997). In the same study, it was found that treating macrophages with CQ (150 μM) at -2, 0 and 2 h before or after LPS challenge resulted in an inhibition on TNF (Jeong and Jue, 1997). The mechanism of the inhibitory effect of CQ on IL-1β and IL-6 production in macrophages stimulated with LPS is different from that of TNF in that it reduces the levels of IL-1β and IL-6 mRNA by decreasing their stability (i.e. accelerating its decay) (Jang *et al.*, 2006).

Apart from the inhibitory effect of CQ seen on cytokine production, Ziegler and Unanue, (1982) suggested that CQ also altered macrophage antigen-handling events as observed by its inhibition of *Listeria* uptake by macrophages treated with CQ 30 min prior to *Listeria* encounter. A lesser effect was seen if CQ was added post bacterial interaction. CQ was also found to inhibit calcium release from macrophage intracellular stores by blocking inositol trisphosphate (IP3) from binding to its receptor (Misra *et al.*, 1997). In purified mouse macrophages treatment with CQ inhibited the release of arachidonic acid and generation of the eicosanoid PGE2 production induced following stimulation with PMA (Bondeson and Sundler, 1998).

1.8.1.3 Effect of CQ on lymphocyte responses

The effect of CQ on lymphocyte proliferation has been previously investigated. The drugs inhibit both antigen-(tetanus toxoid-TT or paraphenylenediamine-PPD) and mitogen-(phytohaemagglutinin-PHA) induced lymphocyte proliferation in a dose dependent manner with EC_{50} values of 6.9, 11.6 and 20.8 µM respectively (Hugosson *et al.*, 2002) . The greater inhibitory activity of CQ on TT is likely to be due to an effect on APCs and the requirement to process antigen through the lysosomal pathway, a target for CQ due to the lysosomotropic effects of the drug (Hugosson *et al.*, 2002). Moreover, its weak base properties are likely to contribute to the inhibition of T cell proliferation and IL-2 production (Landewe *et al.*, 1995).

Morphological studies of the effects of CQ on lymphocytes suggested that it induces a dose dependent increase of autophagosomes, affecting the lysosomal function which contributes to the immunosuppressive properties of CQ (Jones and Jayson, 1984). As a lysosomotropic agent, it also affects lysosomal acidification, resulting in a decrease of the invariant chain dissociation from the MHC II molecule (Blum and Cresswell, 1988). The inhibition of endosomal antigen processing of exogenous proteins by CQ is thought to be due to the raising of intralysosomal pH and inhibiting the response of $CD4^+$ T cells (Kalish and Koujak, 2004).

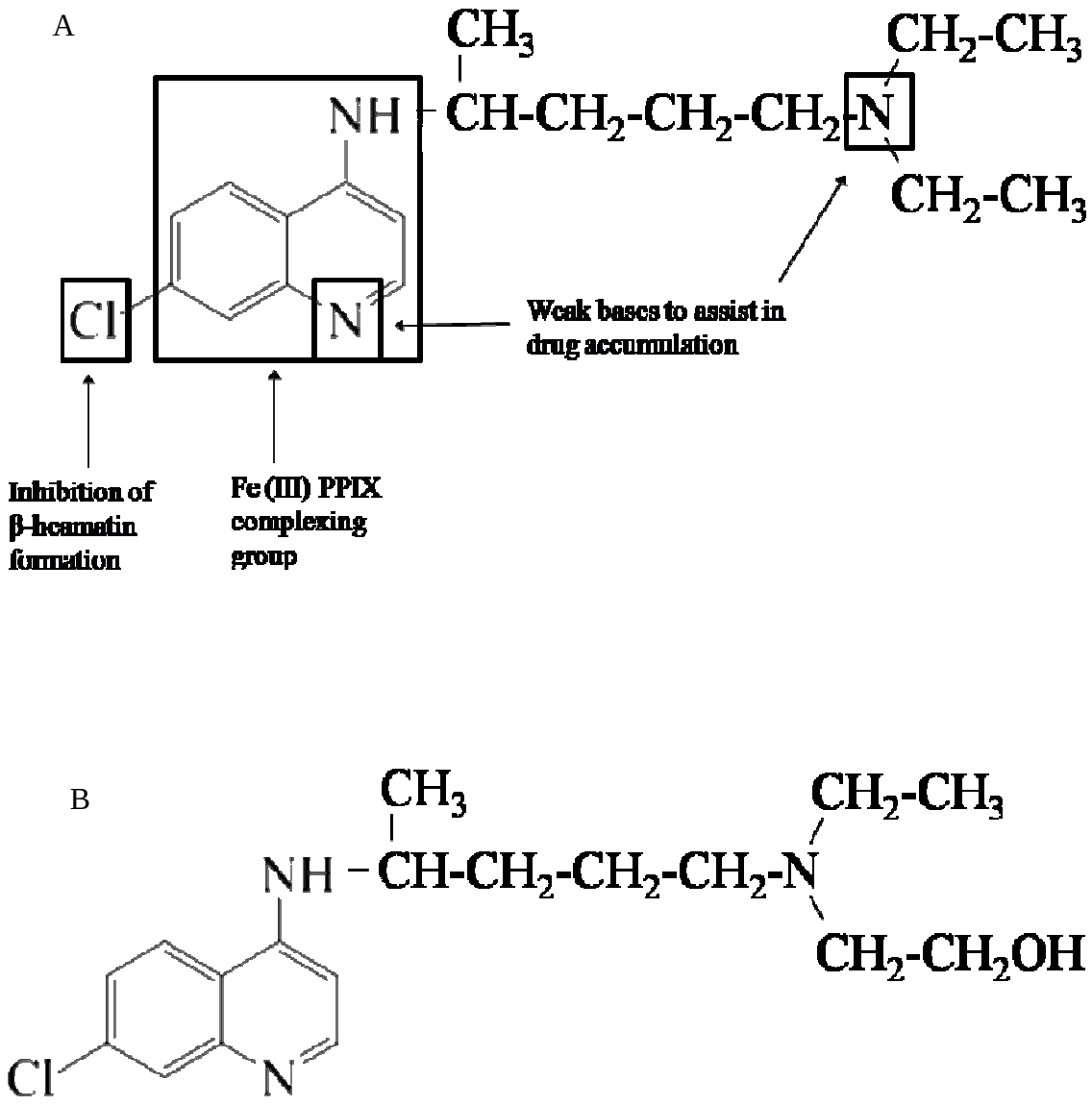


Figure 1.6 Chemical structure of CQ (A) and HCQ (B). Adapted from Egan *et al*., (2000).

1.8.2 Hydroxychloroquine

HCQ is an amphiphilic drug prepared via beta hydroxylation of chloroquine (i.e. introduction of a hydroxyl group into one of the N-ethyl groups of chloroquine) (Figure 1.6B) (O'Neill *et al.*, 1998). The hydroxyl function group is thought to reduce the toxicity by introducing a readily available functionality that could undergo gluccoronidation and thus detoxification and excretion (O'Neill *et al.*, 1998). HCQ has been proven to be three times less toxic than chloroquine in animal models of gross toxicity and achieves higher blood levels after a single oral dose (O'Neill *et al.*, 1998). This drug is used in the treatment of malaria, rheumatoid arthritis, and systemic lupus erythematosus, and is on the market under the name of Plaquenil (Sanofi Pharmaceuticals, New York) (Marquardt and Albertson, 2001). The drug is available as tablets of 200 mg of HCQ phosphate each containing 155 mg of HCQ.

1.8.2.1 Effect of HCQ on neutrophils

Although not as extensively studied as chloroquine, HCQ has been shown to affect leukocyte function. Hurst *et al.*, (1987) found that there was a dose dependent inhibition of fMLP-stimulated neutrophil superoxide production by HCQ. It caused no effect on fMLP-receptor binding and was proposed that HCQ inhibits fMLP stimulated hydrolysis of phosphioinositides thus inhibiting the production of NADPH which is important for superoxide production (Table 1.6). The drug inhibited neutrophil superoxide production induced by opsonised zymosan particles as well as that induced by PMA and fluoride, with maximum of 40 to 50 % inhibition at 100 μM (Hurst *et al.*, 1988). HCQ has no effect on the expression of CR3 on neutrophil surfaces, similar to CQ.

1.8.2.2 Effect of HCQ on monocytes/macrophages and T lymphocytes

HCQ is found to cause inhibition of IL-1α and IL-6 in monocytes stimulated by LPS at a post-transcriptional level by either inhibiting the translation of the two cytokines or by altering the enzymes involved in the process (Sperber *et al.*, 1993). In contrast, it has no effect on TNF production by monocytes and no effect on IL-2, IL-4 and IFN-γ production in T cells (Sperber *et al.*, 1993). HCQ was also found to inhibit the release of arachidonate induced by zymosan in murine macrophages suggesting an effect on phospholipase C, similar to the effects of CQ (Bondeson and Sundler, 1998).

1.8.3 Clinical use of anti-malarials in inflammatory diseases

CQ and HCQ are widely used in the treatment of rheumatoid arthritis and systemic lupus erythematosus (Chen *et al.*, 2005). The mechanisms of action of these two drugs has been linked to their ability to alkalinise macrophage lysosomes (Poole and Ohkuma, 1981), stabilise lysosomal membranes, inhibit receptor recycling, inhibit phospholipase A_2 (Bondeson and Sundler, 1998) and inhibit leukotriene formation from human and guinea pig lung tissue (Kench *et al.*, 1985). While the above studies investigated the mechanisms by which these drugs exert their inhibitory effects, some results have been contradictory and require further studies to resolve the findings.

1.8.3.1 Drug metabolism and toxicity of CQ and HCQ

CQ is highly lipophilic with a diacidic base and accumulates within the acidic compartments of the cell leading to an increase in lysosomal pH, hence, it exerts toxic effects at high doses (O'Neill *et al.*, 1998). The drug diffuses through the plasma membrane and preferentially concentrates in acidic cytoplasmic vesicles which influence cell endocytosis, exocytosis, phagocytosis, antigen presentation and iron metabolism (Chen *et al.*, 2005). Also, oral administration of therapeutic doses of CQ may cause psychosis, delirium, personality changes, and depression. There is ganglio-side storage of CQ in the nervous tissue (Chen *et al.*, 2005). Clinical symptoms of chloroquine toxicity range from reversible cardiac muscle damage to irreversible retinopathy (Jaeger *et al.*, 1987). The toxicity associated with CQ occurs not only because of its accumulation but due to its long-half life and high plasma concentrations (O'Neill *et al.*, 1998). It has been estimated that retinal toxicity occurs in approximately 10 to 20 % of patients who receive CQ therapy and up to 3 % of patients who receive HCQ therapy (Finbloom *et al.*, 1985). Cases of CQ overdoses usually result in serious rapid symptoms seen within 30 min and death within 1 to 3 h of ingestion due to cardiac arrest (Marquardt and Albertson, 2001). Oral administration of CQ usually results in a rapid absorption from the gastrointestinal tract to the blood with a high bioavailability of between 80 and 90 % (Chen *et al.*, 2005). The metabolic degradation of CQ investigated by others has confirmed that CQ undergoes degradation and oxidization and only a small fraction of the drug is secreted as carboxylic acid (McChesney *et al.*, 1966). The metabolic degradation of CQ *in vivo* is outlined in Figure 1. 7.

HCQ has been reported as a drug with a low margin of safety and a mortality rate in overdose studies from adults ranging from 10-30 % with symptoms observed between 30 min to 4.5 h after ingestion (Marquardt and Albertson, 2001). Some clinical symptoms reported include drowsiness, dizziness and visual disturbances to seizures, apnea, arrhythmias, and hypotension (Marquardt and Albertson, 2001). The drug mainly accumulates in tissues, especially the liver and has a half-life of approximately 8 weeks (Temprano *et al.*, 2005). The biotransformation of HCQ differs from CQ only in that it produces two first-stage metabolites instead of one (McChesney, 1983).

Extracellularly, CQ/HCQ are present in a protonated form and are incapable of crossing the plasma membrane (Savarino *et al.*, 2003). However, their non-protonated portion can enter the intracellular compartment and become protonated, therefore enabling accumulation within acidic organelles such as the endosome, golgi vesicles, and the lysosomes (Savarino *et al.*, 2003).

Studies in human volunteers revealed differences in excretion of CQ and HCQ, while three times as much CQ (18 %) appeared in urine compared with faeces (8 %), three times more HCQ was found in faeces (24 %) than urine (6 %) (O'Neill *et al.*, 1998). This reflects the differences in metabolism between CQ and HCQ (O'Neill *et al.*, 1998). Results from previous research on the effects of these two drugs have concluded that 400 mg of HCQ is safer than 250 mg of CQ (Rynes, 1997), but despite the influences of both CQ and HCQ, these two drugs have the potential to cause serious toxicity issues.

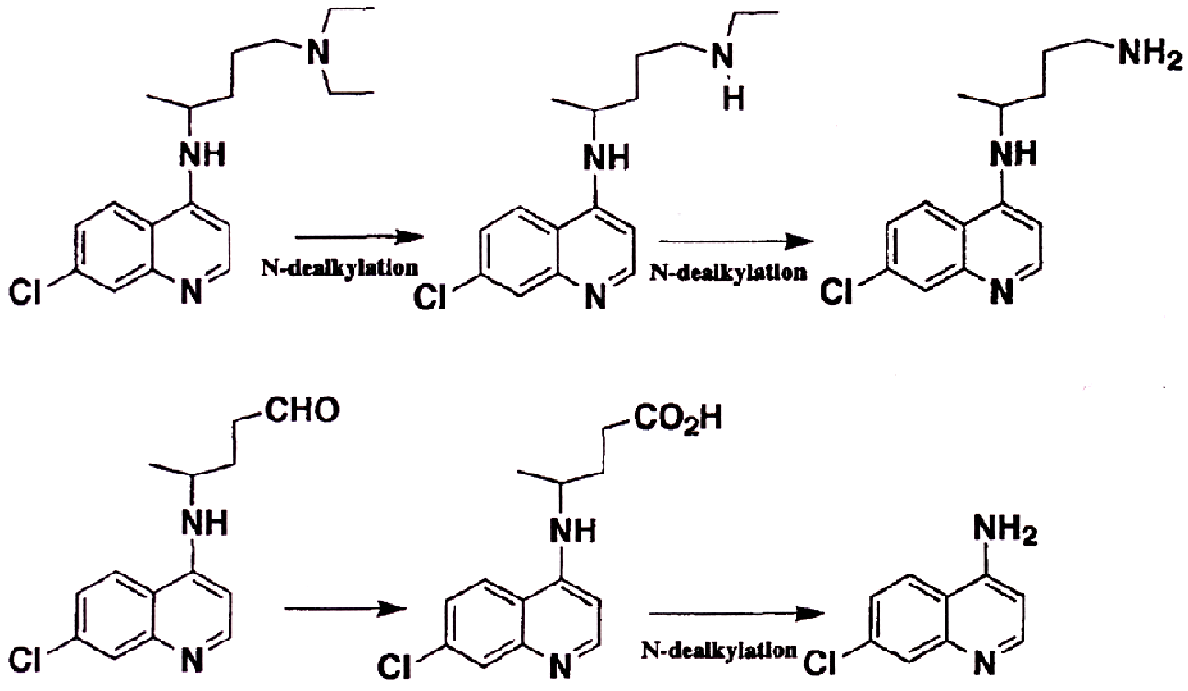


Figure 1.7 Metabolism of chloroquine *in vivo*. Adapted from O'Neill *et al.*, (1998).

1.9 Aims and hypothesis

Our group has previously been interested in the anti-inflammatory properties of quinoline compounds commonly used as anti-malarial agents. This includes quinine, chloroquine, mefloquine and primaquine. One of the activities of these compounds was found to inhibit neutrophil function, a component of acute inflammation and also chronic inflammation (Benna *et al.*, 1991). While patients with certain inflammatory disorders such as rheumatoid arthritis have been shown to benefit from the CQ derivative, HCQ, it has remained of limited use in the treatment of these diseases. Scherbel *et al.*, (1958) reported that the use of CQ and HCQ resulted in neuromuscular reactions; blurring of vision, throbbing and unilateral headaches; gastrointestinal reactions; nausea, anorexia, vomiting, bloating, abdominal cramps, heartburn and loss in weight and dermatologic reactions; dryness of the skin, itching, urticaria, morbilliform eruptions, increased pigmentation and graying or bleaching of the hair. Moreover, neuromyotoxicity (Stein *et al.*, 2000), cardiac toxicity (Veinot *et al.*, 1998), retinopathy (Browning, 2002) and fetal abnormalities (Temprano *et al.*, 2005) have also been reported in patients administering either CQ or HCQ in a long term treatment regime.

1.9.1 Hypothesis

By adding a fatty acid to the side chain of the quinoline group, structural elements are generated which lead to the development of anti-inflammatory agents with selectivity for immune cells activation pathway targets. Furthermore activity of the newly synthesised compounds would be related to specific structural elements of the fatty acid side chain.

1.9.2 Aims

1.9.2.1 General

To attempt to identify a new class of anti-inflammatory agents based on a quinoline structural element.

1.9.2.2 Specific

To synthesise a series of quinoline based compounds bearing fatty acids of different carbon length on the side chain (NT compound).

To examine the effects of the NT compounds on neutrophil functions of adherence, chemotaxis, respiratory burst and cytokine production.

To study the effects of the compounds on lymphocyte and macrophage responses.

To compare their activity with that of CQ and HCQ.

To examine whether any effects are related to responses induced by specific cell agonists.

To try to understand the bases for any effects caused by the NT compounds.

To determine whether key NT compounds have *in vivo* efficacy in inflammatory mouse models.

2.0 CHAPTER 2 MATERIAL AND METHODS

2.1 Reagents, biochemicals and antibodies

Ficoll 400, percoll and lymphoprep were obtained from Pharmacia Biotech (Uppsala, Sweden). Lucigenin and dimethyl sulphoxide (DMSO) were from Merck KGaA (Darmstadt, Germany). Modified-Wright Giemsa were obtained from Hema-Tek Stain Pak (Bayer, USA), and mounting medium from Surgipath (Victoria, Australia). Roswell Park Memorial Institute (RPMI-1640), hanks balanced salt solution (HBSS) and foetal bovine serum (FBS) and L-glutamine were from JRH Biosciences (Lenexa, KS, USA). Lauric acid (LA), chloroquine (CQ), hydroxychloroquine (HCQ), arachidonic acid (AA), leukotriene B_4 (LTB_4), A23187, interleukin 8 (IL-8), complement 5a (C5a), HEPES, pyridoxal phosphate (PyrP), palmitoyl co-enzyme A (PCoA), L-serine, bis-nitrophenyl phosphate (NBPP), magnesium (Mg^{2+}), ATP, β-mercaptoethanol, trypan blue, folin and ciocalteau's phenol reagent, fMLP, PMA, sodium diatrizoate, formaldehyde and rose bengal were from Sigma, Aldrich Pty. Ltd, (Sydney, Australia). Angiografin was obtained from Schering AG (Berlin, Germany). GM-CSF and TNF-α were purchased from Prospec-Tany Technogene Ltd., (Rehovot, Israel). Ammonia solution was from AJAX chemical (NSW, Australia). Penicillin/streptomycin was from Commonwealth Serum Laboratories (CSL) (Victoria, Australia). Isoton II and bovine serum albumin (BSA) were purchased from Beckman Coulter (Fullerton, CA). Phytohaemagglutinin (PHA) was purchased from Murex Diagnostics (Dartford, U.K). Tetanus Toxoid was from Calbiochem (Darmstadt, Germany). Anti-CD3 and anti-CD28 antibodies were purchased from eBioscience (San Diego, CA). Anti-mouse IgG_1, (HRP conjugated) and PE-labelled mouse anti-human CD11b antibody were from Becton Dickinson (San Jose, CA). Anti-mouse IgG_1 (FITC), FITC-labelled mouse anti-human anti-CD120a and anti-CD120b were purchased from Monosan (Uden, Netherland). Fatty acid chlorides were purchased from Nu Chek Prep (Elysan, Minnesota).

2.2 Chemical experimental sections

Experimental sections described in 2.2, 2.3 and 2.4 were carried out by the organic chemist, Dr. Neil Trout. Melting points were determined on a Buchi melting point apparatus in sealed glass capillaries and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini-300 MHz spectrometer (Palo Alto, CA). The probe temperature of the instrument was 295 ± 2 K. All ^{1}H and ^{13}C NMR spectra were recorded in deuterated chloroform ($CDCl_3$) as solvent at 300 and 75 MHz, respectively, with chloroform ($CHCl_3$) (7.26 ppm) for ^{1}H and $CDCl_3$ (77.0 ppm) for ^{13}C as the internal reference. Analytical thin-layer chromatography (TLC) was performed on silica gel plates and visualized with ultraviolet (UV) absorbance. Solvents and reagents were obtained from commercial sources and were used without purification, unless noted. Reactions were carried out under an inert atmosphere of nitrogen. Elemental analysis and compound purity were determined via NMR performed by Monash University, Melbourne, Australia.

2.3 Synthesis of base structure 4-amino-7-chloroquinoline (NT0)

Methods were followed as described previously (Vippagunta *et al.*, 1999). A mixture of 4, 7-dichloroquinoline (10 g, 0.05 mmol) and phenol (80 g, 0.85 mol) were heated and stirred together up to 145 °C under N_2 when anhydrous ammonia gas was bubbled into the solution for 10 min. The ammonia was stopped and the resulting mixture heated further to 180 °C (internal temperature), then the ammonia was re-administered for 2 h. After this time, the mixture was cooled slowly to room temperature to which ether and water were added. The ether mixture was washed with 15 % sodim hydroxide (NaOH) solution (2 x 50 ml) followed by water. The ether extracts were dried and evaporated to dryness to yield the crude amine.

The residue was re-dissolved in diethyl ether (250 ml) and washed with 2 M hydrochloride (HCl) solution (2 x 100 ml). The aqueous layer was then basified with 15 % NaOH solution and then re-extracted with ether, dried and removed *in vacuo* to afford the pure-amine as an off-white solid (5.20 g, 58 %), mp 147-148 °C, (148.5-149.5 ºC). The compound was characterised by ^{1}H and ^{13}C NMR and all spectra agreed with those previously published (Vippagunta *et al.*, 1999). It was used without further purification.

2.4 Synthesis of 4-amino-7-chloroquinoline-fatty acid conjugates (NT1-13)

To the above solution of amine in dry distilled tetrahydrofuran (THF) (4 ml) containing a spatula amount of oven dried K_2CO_3 or 1 equivalent of DBU under N_2 at 0 °C was added the appropriate fatty acid chloride drop wise (2 equivalents). In order to generate a variety of compounds of interest, different commercially available fatty acid chlorides were added to the above solution. These are detailed in Table 2.1. After 5 min, the mixture was allowed to warm up to room temperature and refluxed for 48 h under N_2. The reaction was then quenched with the slow addition of water and the mixture extracted with CH_2Cl_2. The organic extracts were washed with $NaHCO_3$ solution, dried, filtered and removed by rotary evaporation to yield the crude product. The residue was chromatographed on silica gel eluting with 10-40 % ethyl acetate in hexane. Appropriate fractions were taken and visualised on thin layer chromatography (TLC) plates under UV light for identification. The fractions were evaporated to dryness and the identity of the compound was further confirmed by ^{1}H and ^{13}C NMR.

2.5 Preparation of NT compounds

The dry compound was dissolved in chloroform (1 ml) and then transferred into a pre-weighed glass tube. This was then dried under nitrogen and the final weight was determined. A stock solution was then prepared in chloroform including an ethanol working stock of 20 mM. All stocks were then gased briefly with nitrogen, the lid was sealed with parafilm and stored in 4 °C. The compound was further diluted either with RPMI or HBSS prior to experiments to ensure so that the final concentration of ethanol in the solution did not exceed 0.1 %.

2.6 Preparation of lauric acid

Chloroform stocks and an ethanol working stock of lauric acid were prepared. All stocks were gased with nitrogen and the lid sealed with parafilm and stored in 4 °C. A further dilution from the ethanol stock was performed either with RPMI or HBSS prior to adding to cells, to ensure the final concentration of ethanol in the solution did not exceed 0.1 %.

2.7 Preparation of CQ and HCQ

Working stocks of CQ and HCQ were prepared as previously described (Weber *et al.,* 2002). Briefly, CQ and HCQ stocks were prepared in HBSS, the lid sealed with parafilm, and stored in -70 °C until future usage.

Table 2.1 List of substrates required for the synthesis of Quinoline-Fatty Acid conjugates.

Compounds	Amount of 4-amino-7-chloroquinoline	Substrates	Amount of substrate
NT1	120 mg, 0.67 mmol	Decanoyl chloride	200 µl
NT2	100 mg, 0.56 mmol	Stearoyl chloride	200 µl
NT3	120 mg, 0.67 mmol	Undecenoyl chloride	250 µl
NT4	100 mg, 0.56 mmol	Hexanoyl chloride (caproyl chloride)	200 µl
NT5	100 mg, 0.56 mmol	Butyroyl chloride (tetranoyl chloride)	200 µl
NT6	120 mg, 0.67 mmol	Octadecenoyl chloride (85%) (oleoyl chloride)	250 µl
NT7	115 mg, 0.65 mmol	Propanoyl chloride	200 µl
NT	110 mg, 0.62 mmol	Dodecanoyl chloride (lauroyl chloride)	200 µl
NT9	120 mg, 0.67 mmol	Nonadecanoyl chloride	200 µl
NT10	150 mg, 0.84 mmol	Pivalolyl chloride	250 µl
NT11	120 mg, 0.67 mmol	Pentanoyl chloride (valeroyl chloride)	200 µl
NT12	120 mg, 0.67 mmol	Nonaoyl chloride	200 µl
NT13	100 mg, 0.56 mmol	11-eicosenoyl chloride	250 µl

Complete chemical structure can be found in Chapter 3, Figure 3.1-14.

2.8 Animals

Male BALB/c mice aged between 6-8 weeks with an average weight of 20-25 g were purchased from Adelaide University Animal House, Adelaide and Animal Resource Center, Perth. The animals were housed in the Women's and Children's Hospital's animal house. The study was approved by the CYWCH Animal Ethics Committee and the University of Adelaide Animal Ethics Committee.

2.9 Isolation of human peripheral blood mononuclear cells and neutrophils.

Human mononuclear leukocytes and neutrophils were purified from blood of healthy donors by the rapid single step method as previously described (Ferrante *et al.*, 1982). Briefly, 6 ml of peripheral blood was layered onto 4 ml of Hypaque-ficoll medium containing 8 % Ficoll 400 and adjusted to a density of 1.114 by the addition of sodium diatrozoate and angiograffin. The preparation was then centrifuged at 600 g for 35 min to allow leukocytes to separate. PBMCs were harvested from the top band and neutrophils from the lower band. The cells were washed three times in RPMI-1640 for PBMCs and in HBSS for neutrophils. The cells were of > 98 % purity and > 99 % viability as judged by their ability to exclude trypan blue.

2.10 Preparation of TNF-rich medium (TNF-RM)

TNF-RM was prepared as previously described (Bates *et al.*, 1991). Briefly, PBMCs (20 mls of 4 x 10^7) in RPMI-1640 supplemented with L-glutamine (200 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) were mixed in a culture flask (Greiner Bio-One,

Germany) with (20 mls of 2 x 10^8) *Staphylococcus aureus* (*S. aureus* NCTC 6571, Oxford strain) in RPMI-1640 supplemented with 5 % of human heat inactivated AB serum. The cells were then incubated at 37 °C with 5 % CO_2 for 24 h and the resultant TNF-RM was obtained by centrifugation at 2600 g for 10 min, filtering through 0.20 µm pore filter (Sartorius Stedium Biotech, Goettinger, Germany) and aliquoted for storage at -70 °C until further usage. The amount of TNF in this preparation was 39 ng/ml.

2.11 Neutrophil function assays

A limitated number of key neutrophil functional responses were used to assess the immunomdulatory properties of the compounds, these promote neutrophil accumulation at infection sites (adherence and chemotaxis) and micribal killing/tissue damage (generation of oxygen derive reactive species and cytokine production). These assays have been widely used in our Laboratory and have both been standardized and published previously. They represent internationally widely accepted assays for determining neutrophil functional responses.

2.11.1 Adherence assay

The adherence assay was carried out as described previously (Bates *et al.*, 1993). Briefly, a 96 well flat bottom microtiter plate (Greiner Bio-One, Germany) was coated with 250 µl of 10 % autologous plasma for 30 min at 37 °C/ 5 % CO_2 followed by washing three times with HBSS. To 100 µl neutrophils (5 x 10^5 cells), was added the stimulus (20 µl) and HBSS to give a final volume of 200 µl and cells. Then the cells were allowed to adhere for either 30 min at 37 °C/ 5 % CO_2 and unbound cells were removed by washing the wells twice with

HBSS (200 µl). The adhered cells were then stained with 0.25 % Rose Bengal (w/v), a dye that rapidly stains predominantly the cell nucleus, for 5 min at room temperature (Feenstra and Tseng, 1992). Excess dye was then removed by washing three times with HBSS. The dye was then released by adding 100 µl 50% ethanol in PBS. Cell adhesion was determined by the amount of dye present in each well. This is done by reading the plate at an absorbance of 570 nm using a Dynatech MR5000 plate reader. Parallel wells were run in the absence of neutrophils and results are expressed after subtraction of absorbance values from wells without neutrophils.

2.11.2 Chemotaxis

Neutrophil chemotaxis was measured using the under-agarose method as described previously (Ferrante *et al.*, 1980). Briefly, equal volumes of agarose (1 %, w/v) and double strength RPMI-1640 supplemented with 10 % heat-inactivated FCS were mixed and allowed to set in 60 mm culture dish (Geriner Bio-One, Germany) and sets of 3 wells were made. Then 5 µl aliquots of fMLP (0.4 µM), neutrophils (2×10^5), and 0.1 % DMSO (v/v) were added to the outer, central and inner wells, respectively. The dish was then incubated at 37 °C for 90 min in a humidified atmosphere of 5 % CO_2 in air. Neutrophil migration was measured from the edge of the central well to the cells leading front migrating towards either the fMLP (chemotaxis) or DMSO (random migration) containing well under an inverted Leitz microscope fitted with a grid eyepiece graticule.

2.11.3 Chemiluminescence assay

Neutrophil superoxide production was assayed by measuring lucigenin (9, 9'-bis N-methyl-acridinium nitrate)-dependent chemiluminescence assay (Hii *et al.*, 1999) via a luminometer (Autolumat Plus LB 953, Berthold Australia, Bundoora, Australia). This assay had been shown privously to measure superoxide production (Dahlgren *et al.*, 1985; Gyllenhammar, 1987; Alves *et al.*, 2003). To 100 µl (1 x 10^6) neutrophils was added the stimulus (100 µl), lucigenin (500 µl of 250 µM) and HBSS to a total volume of 1 ml. The cells were placed in a luminometer and the resulting chemiluminescence measured. Results are expressed as peak chemiluminescence produced, unless specified otherwise.

2.12 Analysis of cytokine and chemokine gene expression by quantitative real-time PCR

2.12.1 Preparation of samples for RNA isolation

Neutrophils (1 ml of 1 x 10^7) were pretreated with various concentrations of the compound (1 ml) for 1 h and then stimulated with 2 ml TNF (100 U) in the presence of autologous plasma (200 µl) for 30 minutes at 37 °C/ 5 % CO_2. After stimulation, samples were pelleted and stored at -70 °C until RNA isolation.

2.12.2 Isolation of RNA

Total RNA was extracted from 1 x 10^7 neutrophils using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Australia). The neutrophil samples were lysed and homogenized in 600 µl RLT buffer supplement with β-mercaptoethanol (10 µl/ml) to which

ethanol (600 µl of 70 %) was then added. The lysate (700 µl each time) was then loaded onto the RNease Mini spin column, spun for 15 sec at ≥ 8000 g. The RNA was washed once in RW1 buffer (700 µl-15 sec), twice with RPE (500 µl-15 sec followed by 2 min) and transferred into a new collection tube to which RNase free water (30 µl) was added. The purified RNA was then eluted by centrifugation for 1 min at > 8000 g and stored at –70 °C until required for cDNA synthesis. Purity of the RNA samples was assessed by determining the optical density at 260 nm and 280 nm expressed as a ratio via UV/VIS SP8001 Spectrometer (Metertech, Taiwan).

2.12.3 Synthesis of cDNA (Reverse Transcription)

Single-stranded cDNA was synthesised from total RNA isolated earlier using iScript cDNA Synthesis Kit according to the manufacturer's instructions (BioRad, NSW, Australia). A master mixed was prepared containing iScript Reaction Mix (4 µl) which contains a unique blend of oligo(dT) and random hexamer primers, iScript Reverse Transcriptase which is RNase H+ (1 µl), RNA (1 µg) and RNA free distilled water up to a final volume of 20 µl. cDNA was synthesized under the following conditions: 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min.

Firstly, oligo(dT) primer containing additional sequences at the 3'end anneals to the mRNA. Reverse transcriptase then extends from the annealed oligo(dT) primer along the mRNA template until it reaches the 5' end where the enzyme's terminal transferase activity attaches several additional nucleotides onto the newly synthesized strand of cDNA, ie. copying of the mRNA sequence into the cDNA sequence. This resulted in a single-strand cDNA that can

then be converted into double-strand cDNA for construction of cDNA libraries

2.12.4 Quantitative real-time PCR

Real time PCR was performed in the iCycler iQ™ 96 well PCR plate using iQ™ Sybr® Green Supermix (BioRad, NSW, Australia) according to the manufacturer's directions. Primers for cytokines were initially designed using Invitrogen Oligo Perfect Designer website and then synthesized by Invitrogen (Carlsbad, CA, USA) (Table 2.2). The synthesised cDNA was then amplified in duplicate in a PCR 96 well plate (Bio-Rad, CA, USA). Each well contained iQ SYBR Green supermix (10 μl), cDNA (1 μl) and each specific primer pair or the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (500 nM). The amplification conditions used were as follow: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C s for 30 s and hold at 4 °C. A standard curve was also performed in each run for the cDNA of the diluted control. All standard curves and data were analysed using Bio-Rad iQ5 Standard Edition V2.0.148.060623 (Bio-Rad, CA, USA). Fold change values were calculated in which ΔCt = the Ct for cytokine gene minus the Ct of the housekeeping gene, GAPDH: ΔΔCt = the ΔCt of stimulated cells at different concentration minus the ΔCt of unstimulated cells.

Table 2.2 Primer sequences

Genes	Forward primer (5’ to 3’)	Reverse primer (5’ to 3’)
GAPDH	GAG TCA ACG GAT TTG GTC GT	GAC AAG CTT CCC GTT CTC AG
IL-1β	TCA TTG CTC AAG TGT CTG AAG C	TCC TGG AAG GAG CAC TTC AT
IL-8	TCT GTG TGA AGG TGC AGT TTT G	AAT TTC TGT GTT GGC GCA GT

2.13 Regulation of 3-ketosphinganine synthase

2.13.1 Microsomal isolation

Microsomal isolation was performed as described previously (Williams *et al.*, 1984). Briefly, mice were first euthanized and liver (1-2 g) was removed and stored in ice-cold buffered sucrose (1 g/ml) containing sucrose (0.25 M), Tris-HCl buffer (10 mM-pH 7.5) and EDTA (1 mM). Mouse liver was then washed 2-3 times with buffered sucrose, minced, and homogenized. The homogenized solution was then centrifuged at 7300 g for 10 min at 4 °C. Supernatant was collected and spun further at 100,000 g for 60 min at 4 °C. The resultant supernatant was removed and the pellet containing crude microsomes was re-suspended in buffered sucrose (1 g/ml). Microsomes were then stored at -70 °C and protein concentration was determined by the Lowry’s protein determination method.

2.13.2 Lowry's protein determination

The amount of protein from microsomal isolation was measured via Lowry's protein determination. Protein standards were prepared fresh for each assay, by serially diluting 1 mg/ml BSA with H_2O, resulting in 6 standards (0 µg, 3.125 µg, 6.25 µg, 12.5 µg, 25 µg, and 50 µg). Unknown samples were diluted 10x before being assayed. All standards and known samples were assayed in 50 µl aliquots. To start the reaction, 150 µl of solution 1 [Lowry's solution (2 % Na_2CO_3, 1 % SDS, 0.4 % NaOH, 0.16 % Na/K tartrate): $CuSO_2$, 100:1] was added to all tubes and left to stand at room temperature for 15min. Then 15 µl of solution 2 (H_2O:Folin and Ciocalteau's Phenol Reagent, 1:1) was added. After 10 min at room temperature, 180 µl from each tube was transferred to a 96 well plate and the optical density (OD) measured at 540 nm in a plate reader. The OD of the protein standards was used to create a standard curve from which the protein concentration of the unknown samples was determined.

2.13.3 Serine palmitoyl transferase (SPT) assay

The enzyme substrate assay was performed as described previously (Holleran *et al.*, 1990; Williams *et al.*, 1984). Briefly, a reaction mix containing pyridoxal phosphate (PyrP) (50 µM), palmitoyl-coenzyme A (PCoA) (150 µM), [G-^{3}H] L-serine (1.0 mM-sp act 45,000-50,000 dpm/nmol) and microsomal protein (50-200 µg) was added to the assay buffer (HEPES- 100 mM-pH 8.3 at 20 °C, DTT-5.0 mM and EDTA-2.5 mM- pH 7.4) containing either Mg^{2+} (1mM), ATP (1mM), NBPP (1mM), NT8 (100 µM) or diluent up to a final volume of 100 µl. The assay mix was then incubated for 10 min at 37 °C in a water bath

with gentle shaking to initiate the reaction. Ice cold NH_4OH (0.5 M-200 µl) was added to terminate the reaction, followed by the addition of chloroform-methanol solution (1:2) (15 ml), sphingosine base (25 µg) and NH_4OH (0.5 M-2 ml). The organic phase was then obtained by centrifugation for 10 min at 600 g and washing twice with deionized water (2 ml), made basic with NH_4OH. The enzymatic activity was then quantitated from the organic phase (500 µl) using a Beckman LS-1800 scintillation counter and results were expressed as pmoles of 3-keto-sphinganine (3KDS) formed per min per mg of protein. Total SPT activity was obtained by multiplying the specific activity by the total protein.

2.14 Lymphoproliferation

Whole mononuclear cells or T lymphocytes (50 µl of 5 x 10^5) were stimulated with various stimuli at 37 ºC / 5 % CO_2 for either 2 or 5 days in a 96-well U-bottom plate (Linbro; Flow Laboratories, Virginia, USA). Six hours prior to harvesting 1 µCi [*methyl*-^{3}H] thymidine (25 Ci/mmol; Amersham Life Sciences, Buckinghamshire, England) diluted in RPMI-1640 supplemented with 5 % human AB serum was added to each well. Supernatants (200 µl) were removed and stored in -70 °C for cytokine determination. Cells were then harvested onto glass fibre filters (Wallac, Turku, Finland) using a FilterMate Harvester (PerkinElmer, Victoria, Australia) and allowed to air dry before 3.5 ml of scintillation cocktail was added. The amount of radioactivity present was then measured using Wallac MicroBeta JET counter (PerkinElmer, Victoria, Australia) expressed as disintegration per minute (DPM).

2.15 Cytokine determination

IFN-γ, IL-1, IL2, IL-6, IL-10, lymphotoxin and TNF-α levels were determined using the cytometric bead array (BD Cytometric Bead Array Flex Sets System; BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Briefly, 50 µl of capture bead suspension and 50 µl were added to an equal amount of sample or standard dilution of 1:1, 1:4, 1:64, 1:256 and 1:1024 in a 96 well U-bottom plate. It was then incubated for 1 h at room temperature in the dark and in some cases the sample was diluted 1:20 with diluent if necessary. The phycoerythrin (PE) detection reagent (50 µl) was added to the wells and incubated in the dark at room temperature for a further 2 h. Subsequently, samples were washed and centrifuged at 200 g at room temperature for 5 min. The supernatant was discarded and 150 µl of wash buffer was added. Samples were analysed on a BD FACSArray bioanalyser (BD Biosciences, CA, USA) (Figure 2.1). Quality control beads were used prior to reading the samples to ensure the instrument was standardised. Examples of the standard curves are shown in Figure 2.2, 2.3.

2.16 Flow cytometry

2.16.1 CR3 up regulation and expression

The presence and up-regulation of the complement receptor (CR3) in neutrophils treated with NT8 was determined by flow cytometry. In the FACS tubes (Evergreen Scientific, Los Angeles, USA), neutrophils (100 µl of 1 x 10^6) were incubated with TNF-RM for 1 h in 37 °C / 5 % CO_2.

Figure 2.1 BD FACSArray BioAnalyser

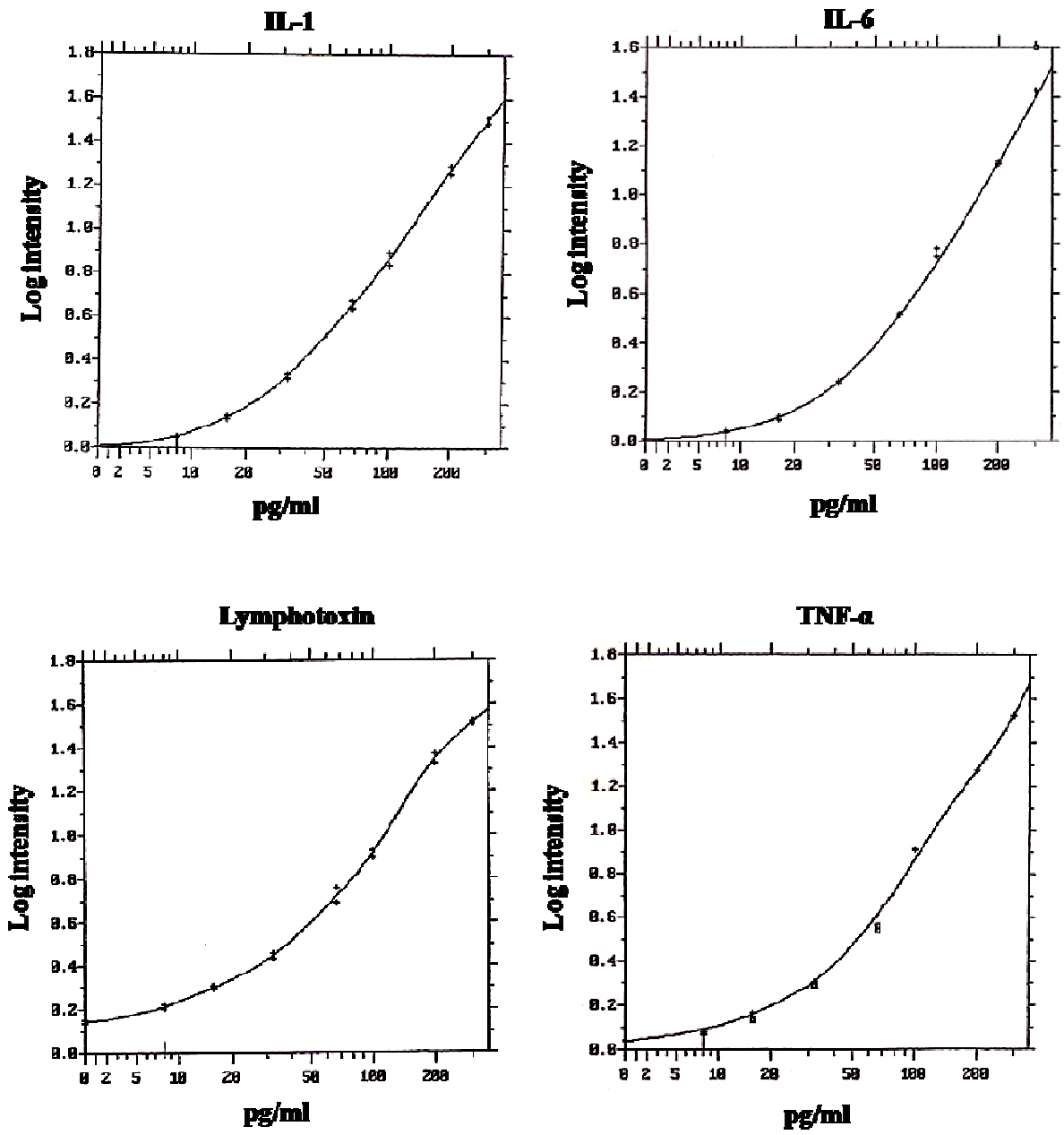


Figure 2.2 Examples of standard curves for human IL-1, IL-6, lymphotoxin and TNF-α production. Standard curves were generated for IL-1, IL-6, lymphotoxin and TNF-α using the BD CBA Flex Set System.

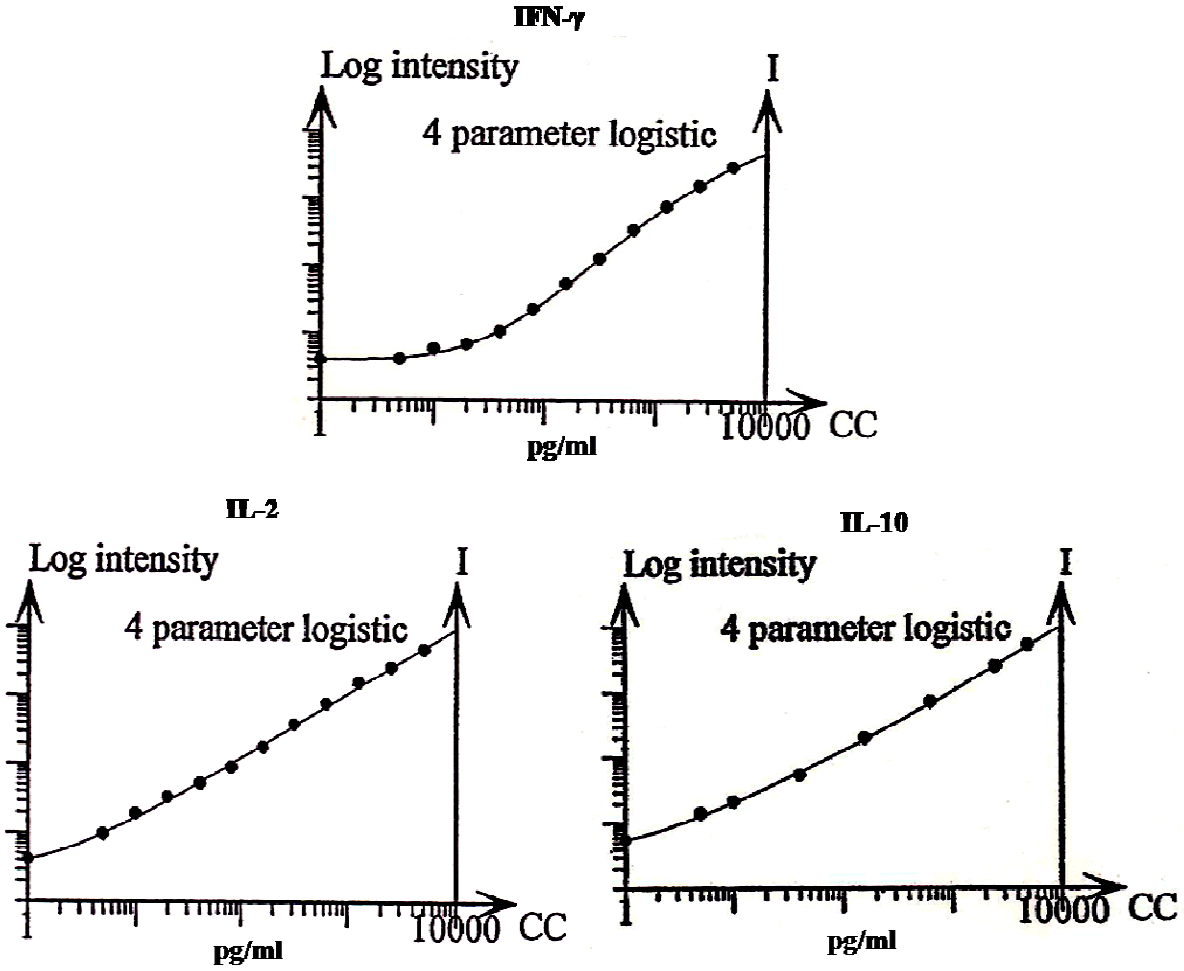


Figure 2.3 Examples of standard curves for human IFN-γ, IL-2 and IL-10 production. Standard curves were generated for IFN-γ, IL-2 and IL-10 using the BD CBA Flex Set System.

The pellet was re-suspended in 200 µl of Isoton II supplement with 0.1 % (w/v) BSA, and 2 µl of IgG_1 isotype control or 2 µl of PE-labelled anti-CD11b antibody was added. Tubes were then incubated on ice for 30 min in the dark and excess antibodies were then removed by washing twice with Isoton II. Cells were then fixed with formaldehyde 0.1 % and stored in the dark at 4 °C until analysed by a flow cytometer (FACScan, Becton Dickinson, CA, USA).

2.16.2 CD120a/CD120b expression

The effect of NT8 on surface receptor TNFRI and TNFRII was measured by flow cytometry. Neutrophils (100 µl of 1 x 10^6) were treated with NT8 for 1 h and then incubated with anti-CD120a, anti-CD120b or isotype control anti-mouse IgG_1 for 30 min in dark. Cells were then washed, pelleted and incubate further with 2 µl of anti-mouse fluorescein isothiocyanate (FITC) for 30 min in dark at 4 °C. Excess antibodies were removed by washing twice with Isoton II and fixed with formaldehyde 0.1 % and then stored in the dark 4 °C until analysis via the flow cytometer.

2.17 Lipopolysaccharide-induced peritoneal inflammation

Male BALB/c mice were housed under a 12 h light-dark cycle in cages with unlimited access to food and water in a temperature controlled room for at least 5 days prior to usage. LPS-induced peritoneal inflammation was carried out as previously described (Ferrante *et al.*, 2006). Briefly, mice were injected intraperitoneally (ip) with different doses of NT8 for various times before or after LPS challenge. PBS was injected as control and after 24 h,

the animals were euthanized and peritoneal cavity washout was performed. The cavity was first injected with 2 ml of HBSS and after mixing, 1 ml of the exudates was collected. The number of leukocytes was then determined by a combination of performing a cell count in a haemocytometer and differential count on Giemsa stained smears of the cell exudates.

Slides of cellular infiltrates were prepared by centrifugation of the cells collected from the washout (vehicle- 200 µl or LPS challenged-100 µl) and fixed with a drop of methanol followed by Giemsa staining.

2.18 Statistics

All data were presented as the mean ± standard error of the mean (SEM) unless stated otherwise. Statistical significance was assessed using analysis of variance (ANOVA). Values were considered statistically significant when $p<0.05$. All statistical analyses were performed using Graphpad Instat software or Graphpad Prism software. For any issues regarding statistics which were beyond the experience of the Department statataticians from the University of Adelade were available for consultation.

3.0 CHAPTER 3 SYNTHESIS OF NT COMPOUNDS

3.1 Introduction

For the past decades, there has been a major interest in using anti-malarials such as chloroquine and hydroxychloroquine as anti-inflammatory agents. Clinically, the use of these drugs in rheumatoid arthritis and lupus erythematosus patients can be traced back to the mid 1950s (Page, 1951; Scherbel *et al.*, 1957; Morand *et al.*, 1992). Even with its popularity and demand, there is still a need for the development of new drugs, mainly, due to the emergence of CQ resistance strains of malarial parasites and side effects reported from patients taking these drugs (Blair *et al.*, 2006; Dittrich *et al.*, 2005; Djimde *et al.*, 2001; Stein *et al.*, 2000; Veinot *et al.*, 1998; Scherbel *et al.*, 1958).

A major effort has been made in the synthesis of new compounds with greater anti-malarial or anti-inflammatory properties. The main approach is by either altering the side chain that is linked to the 4-amino-7-chloroquinoline ring structure or by altering the ring structure itself so to reduce its cytotoxicity (Ridley *et al.*, 1996; Madrid *et al.*, 2004; Solomon *et al.*, 2007). HCQ, a CQ derivative different from CQ only by an additional hydroxyl group, has been found to be less toxic due to the fact that a lower dosage is required during treatment, indicating that it is a more effective compound compared to CQ (Mackenzie, 1983).

Both CQ and HCQ tend to accumulate quickly in the acidic compartments reaching a steady state after 60 min at 37 °C due to the fact that they are weak bases (Raghoebar *et al.*, 1986). The accumulation of the two compounds leads to an increase in pH within intracellular vacuoles, altering the behavior of lysosomes and interfering with the vesicle fusion process so that cells do not undergo normal pinocytosis, exoplasmosis and phagolysosomal fusion (Gonzalez-Noriega *et al.*, 1980; Tietze *et al.*, 1980). Moreover, the quinoline ring structure

allows CQ to pass through the lipid bilayer into the cell, and once inside the cell protonation occurs allowing the diprotonated form to pass back through the lipid layer (Naisbitt *et al.*, 1997).

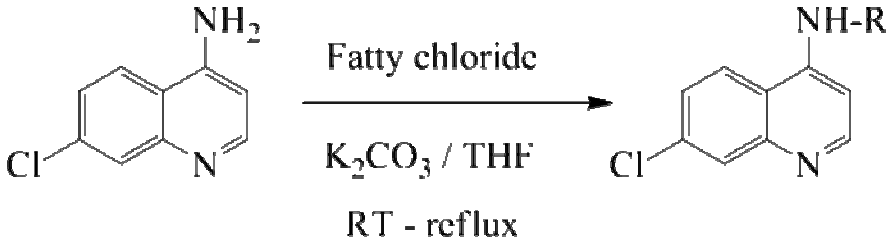
It has also been well studied that CQ and HCQ can form a π- π stacking interaction with the porphryin system of FE(III)FPIX complex so that the complex is no longer toxic to the parasite in the presence of protein (Egan, 2005; Fitch, 1983; Stocks *et al.*, 2002). In addition to that, they have also been found to exhibit an inhibitory effect on some key neutrophil functions such as a adherence, respiratory burst, phagocytosis and cytokine production (Ferrante *et al.*, 1986; Hurst *et al.*, 1987, 1988; Jones and Jayson, 1984; Karres *et al.*, 1998) as well as the antigen processing and presentation processes in macrophages and dendritic cells as discussed in detail in Chapter 1 (Fox, 1993).

In an attempt to improve the therapeutic potential of quinoline based drugs we aimed to synthesize a group of novel quinoline-fatty acid based compounds (Scheme 3.1). The employment of a fatty acid side chain, is expected to makes the compound more soluble and facilitate their uptake by cells. After a series of chemical syntheses and analyses, as mentioned in Chapter 2.2-4, 13 compounds were successfully generated. All these compounds contained fatty acid side chains of different lengths/ saturated and unsaturated (Figure 3.1-3.14). It is expected that these compounds will lead to some unique biological properties as a result of the type of fatty acid side chains.

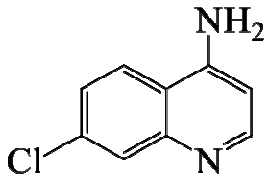
3.2 Results

All the NT compounds were synthesized from the starting 4-amino-7-chloroquinoline. Different fatty chains (length and saturation) were attached to the amine through a coupling reaction with the appropriate fatty acid chloride resulting in 13 quinoline fatty acid conjugates, (Figures 3.1-14). All the compounds were fully characterized by ^{1}H, ^{13}C NMR, GCMS and high resolution MS with purities greater than 98 %. In general, the coupling reaction worked well but yields were not optimized as the desired conjugate was easily purified by column chromatography thus eluting ahead of the starting amine. The conjugates synthesised were all stable at room temperature and there was no evidence of oxidation which was seen with greater unsaturation of the fatty chain (not presented). Overall the NMR data showed that all compounds synthesized were of greater than 95 % purity (Figure 3.1.1-3.1.14).

Scheme 3.1 Synthetic scheme of the NT compounds



Compound	R
NT0	H
NT1	$-CO(CH_2)_8CH_3$
NT2	$-CO(CH_2)_{16}CH_3$
NT3	$-CO(CH_2)_8CH=CH_2$
NT4	$-CO(CH_2)_4CH_3$
NT5	$-CO(CH_2)_2CH_3$
NT6	$-CO(CH_2)_7CH=CH(CH_2)_7CH_3$
NT7	$-COCH_2CH_3$
NT8	$-CO(CH_2)_{10}CH_3$
NT9	$-CO(CH_2)_{17}CH_3$
NT10	$-CO(CH_3)_3$
NT11	$-CO(CH_2)_3CH_3$
NT12	$-CO(CH_2)_7CH_3$
NT13	$-CO(CH_2)_9CH=CH(CH_2)_7CH_3$



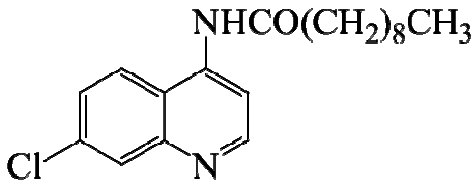
NT0

Figure 3.1 Chemical structure of the synthesised NT0 compound. Synthesis of NT0 was followed as previously published (Vippagunta *et al.*, 1999). An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-amino-7-chloroquinoline. Yield: 58 %; melting point: 147-148 °C; molecular weight: 179.6262.

NMR Data

The compound was characterised by ^{1}H and ^{13}C NMR and all spectra agreed with those previously published (Vippagunta *et al.*, 1999). It was used without further purification.

HRMS calc. for $C_9H_8ClN_2O$: 179.0375, obs. 179.0373.



NT1

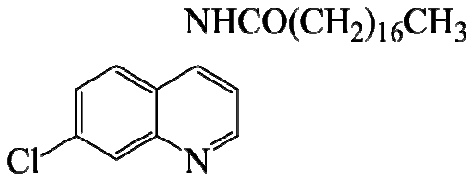
Figure 3.2 Chemical structure of the synthesized NT1 compound. The crude product was obtained by adding decanoyl chloride to the solution of 4-amino-7-chloroquinoline as previously described above in section 2.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-decanamido-7-chloroquinoline. Yield: 31 %; melting point: 97-98 °C; molecular weight: 333.8755.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.76 (1H, d), 8.45 (1H, NH-bs), 8.2 (1H, d), 8.05 (1H, d), 7.85 (1H, d), 7.45 (1H, dd), 2.55 (t), 1.80 (m), 1.30 (bs), 0.85 (bt).

^{13}C NMR ($CDCl_3$-77 ppm): 172.45 (CO), 151.71, 148.73, 141.16, 135.55, 128.71, 127.21, 121.31, 118.64, 111.61 (9 aromatics, 1 carbonyl = 10 C), 37.82, 33.83, 31.78, 29.38, 29.32, 29.19, 25.41, 22.60, 14.04 (9 aliphatic carbons).

R_f =0.43 (40 % EtOAc/hexane), HRMS calc. for $C_{19}H_{26}ClN_2O$: 333.1734, obs. 333.1736.



NT2

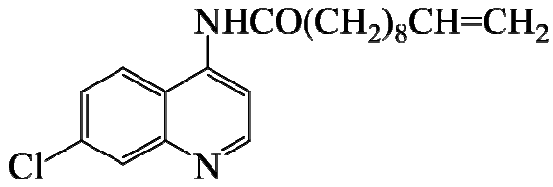
Figure 3.3 Chemical structure of the synthesized NT2 compound. The crude product was obtained by adding stearoyl chloride to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-steramido-7-chloroquinoline. Yield: 14 %; melting point: 94-95 °C; molecular weight: 446.0882.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.70 (1H, bs, NH), 8.40 (1H, s), 8.20 (1H, d), 7.95 (1H, s), 7.75 (1H, d), 7.40 (1H, dd), 2.55 (t), 1.75 (m), 1.25 (bs), 0.89 (bt).

^{13}C NMR ($CDCl_3$-77 ppm): 172.25 (CO), 151.69, 148.60, 141.08, 135.60, 128.70, 127.27, 121.00, 118.44, 111.39 (9 aromatics, 1 carbonyl = 10 C), 37.90, 31.89, 29.66, 29.62, 29.58, 29.44, 29.32, 29.21, 25.40, 22.65, 14.08 (1 aliphatic resonances for 17 carbons).

R_f =0.47 (40 % EtOAc/hexane), HRMS calc. for $C_{27}H_{41}ClN_2O$, 445.2986, obs. 445.2986.



NT3 (n-1)

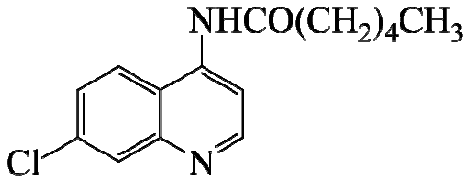
Figure 3.4 Chemical structure of the synthesized NT3 compound. The crude product was obtained by adding undecenoyl chloride to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-undecenamido-7-chloroquinoline. Yield: 28 %; melting point: 87-88 °C; molecular weight: 345.8862.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.78 (1H, d), 8.26 (1H, bs), 8.17 (1H, d), 8.05 (1H, d), 7.76 (1H, d), 7.41 (1H, dd), 5.79 (1H, m), 5.01-4.91 (2H, m), 2.55 (2H, t), 2.00 (2H, m), 1.78 (2H, m), 1.36 (10H, m).

^{13}C NMR ($CDCl_3$-77 ppm): 172.20 (CO), 152.11, 149.14, 140.62, 139.03, 135.33, 129.13, 127.16, 120.91, 118.61, 114.15, 111.66 (9 aromatics, 1 carbonyl, 2 alkene = 12 C), 37.82, 33.69, 29.22, 29.22, 29.15, 28.97, 28.79, 25.37 (8 aliphatic carbons).

R_f =0.38 (30% EtOAc/hexane), HRMS calc. for $C_{20}H_{26}ClN_2O$, 345.1734, obs. 345.1737.



NT4

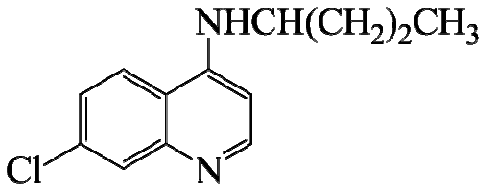
Figure 3.5 Chemical structure of the synthesized NT4 compound. The crude product was obtained by adding hexanoyl chloride (caproyl chloride) to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR.. The chemical name assigned for this compound was 4-hexanamido-7-chloroquinoline. Yield: 39 %; melting point: 119-120 °C; molecular weight: 277.7692.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.72 (1H, bd), 8.55 (1H, bs), 8.12 (1H, d), 8.00 (1H, d), 7.80 (1H, d), 7.38 (1H, dd), 2.50 (2H, t), 1.75 (2H, m), 1.31 (2H, m), 0.89 (3H, bt).

^{13}C NMR ($CDCl_3$-77 ppm): 172.48 (CO), 151.87, 148.99, 140.91, 135.42, 128.84, 127.14, 121.29, 118.80, 111.91 (9 aromatics, 1 carbonyl = 10C), 37.70, 31.30, 25.08, 22.32, 13.83 (5 aliphatic carbons).

R_f =0.36 (40 % EtOAc/hexane), HRMS calc. for $C_{15}H_{18}ClN_2O$, 277.1108, obs. 277.1106.



NT5

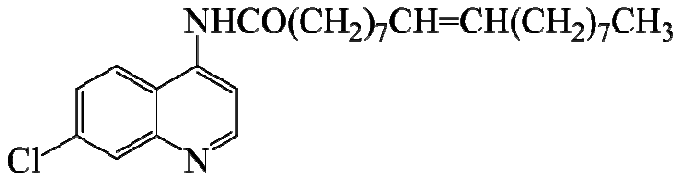
Figure 3.6 Chemical structure of the synthesized NT5 compound. The crude product was obtained by adding butyroyl chloride (tetranoyl chloride) to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR.. The chemical name assigned for this compound was 4-butanamido-7-chloroquinoline. Yield 46 %; melting point: 126-127 °C; molecular weight: 249.7161.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.70 (2H, bd), 8.10 (1H, d), 7.95 (1H, d), 7.82 (1H, d), 7.35 (1H, dd), 2.52 (2H, t), 1.78 (2H, sext), 1.00 (3H, t).

^{13}C NMR ($CDCl_3$-77 ppm): 172.44 (CO), 151.58, 148.69, 141.22, 135.53, 128.50, 127.14, 121.55, 118.83, 111.92 (9 aromatics, 1 carbonyl = 10 C), 39.50, 18.85, 13.63.

R_f =0.21 (40 % EtOAc/hexane), HRMS calc. for $C_{13}H_{14}ClN_2O$, 249.0795, obs. 249.0797.



NT6 (n-9)

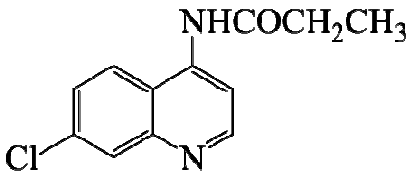
Figure 3.7 Chemical structure of the synthesized NT6 compound. The crude product was obtained by adding octadecenoyl chloride (85 %) (oleoyl chloride) to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-octadec-9-en-amido-7-chloroquinoline. Yield: 23 %, oil; molecular weight: 444.0723.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.80 (1H, d), 8.25 (1H, d), 8.20 (1H, bs), 8.08 (1H, s), 7.80 (1H, d), 5.35 (2H, m), 7.48, 2.58 (2H, t), 2.05 (m), 1.81 (m), 1.30 (bs), 0.90 (3H, bt).

^{13}C NMR ($CDCl_3$-77 ppm): 172.12 (CO), 151.93, 148.85, 140.91, 135.54, 130.06, 129.60, 128.97, 127.29, 120.91, 118.47, 111.41 (9 aromatics, 1 carbonyl, 2 alkene = 12 C), 37.93, 31.87, 29.73, 29.66, 29.48, 29.28, 29.23, 29.17, 29.07, 27.20, 27.12, 25.38, 22.64, 14.07.

R_f =0.76 (40 % EtOAc/hexane), HRMS calc. for $C_{27}H_{40}ClN_2O$, 443.2829, obs. 443.2826.



NT7

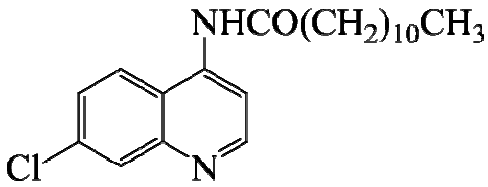
Figure 3.8 Chemical structure of the synthesized NT7 compound. The crude product was obtained by adding propanoyl chloride to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-propanamido-7-chloroquinoline. Yield: 53 %; melting point: 140-141 °C; molecular weight: 235.0638.

NRM Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.78 (1H, d), 8.20 (1H, bs), 8.05 (1H, d), 7.78 (1H, d), 7.45 (1H, dd), 2.59 (2H, q), 1.32 (3H, t).

^{13}C NMR ($CDCl_3$-77 ppm): 172.67 (CO), 152.17, 149.11, 140.64, 135.39, 129.19, 127.24, 120.76, 118.50, 111.45 (9 aromatics, 1 carbonyl = 10 C), 30.94, 9.39.

R_f =0.23 (40 % EtOAc/hexane), HRMS calc. for $C_{12}H_{12}ClN_2O$, 235.0638, obs. 235.0631.



NT8

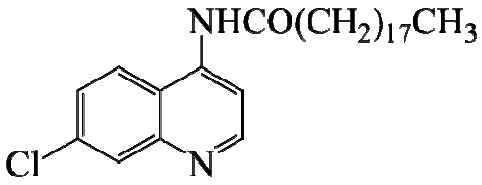
Figure 3.9 Chemical structure of the synthesized NT8 compound. The crude product was obtained by adding dodecanoyl chloride (lauroyl chloride) to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-dodecanamido-7-chloroquinoline. Yield: 25 %; melting point: 78-79 °C; molecular weight: 361.9287.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.65 (2H, bs), 8.15 (1H, d), 7.90 (1H, s), 7.75 (1H, d), 7.30 (1H, dd), 2.55 (t), 1.78 (m), 2.58 (2H, t), 1.30 (bs), 0.90 (t).

^{13}C NMR ($CDCl_3$-77 ppm): 172.64 (CO), 151.03, 147.91, 141.61, 135.70, 127.81, 127.11, 121.47, 118.38, 111.48 (9 aromatics, 1 carbonyl = 10 C), 37.69, 31.86, 29.63, 29.58, 29.47, 29.39, 29.36, 29.29, 29.25, 25.42, 24.21, 22.64, 14.06.

R_f =0.44 (40 % EtOAc/hexane), HRMS calc. for $C_{21}H_{30}ClN_2O$, 361.2047, obs. 361.2052.



NT9

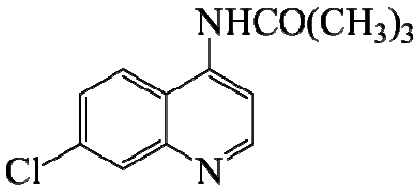
Figure 3.10 Chemical structure of the synthesized NT9 compound. The crude product was obtained by adding nonadecanoyl chloride to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-nonadecanamido-7-chloroquinoline. Yield: 16 %; melting point: 120-121 °C; molecular weight: 460.1148.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.78 (1H, bs), 8.22 (2H, bs), 8.06 (1H, s), 7.80 (1H, d), 7.45 (1H, dd), 2.60 (t), 1.80 (dt), 1.30 (bs), 0.90 (bt).

^{13}C NMR ($CDCl_3$-77 ppm): 172.18 (CO), 152.00, 149.00, 140.82, 135.48, 129.07, 127.27, 120.91, 118.49, 111.50 (9 aromatics, 1 carbonyl = 10 C), 37.93, 31.89, 29.66, 29.62, 29.57, 29.44, 29.32, 29.21, 25.41, 22.65, 14.08 (18 aliphatic carbons).

R_f =0.47 (40 % EtOAc/hexane), HRMS calc. for $C_{28}H_{44}ClN_2O$, 459.3142, obs. 459.3144.



NT10

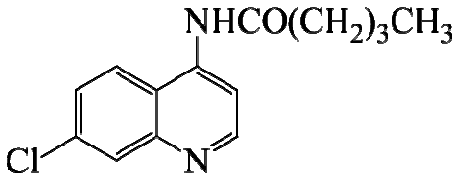
Figure 3.11 Chemical structure of the synthesized NT10 compound. The crude product was obtained by adding pivalolyl chloride to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-pivalamido-7-chloroquinoline. Yield: 27 %; melting point: 150-151 °C; molecular weight: 263.7426.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.80 (1H, bs), 8.25 (2H, m), 8.07 (1H, s), 7.65 (1H, bd), 7.50 (1H, d), 1.44 (9H, s).

^{13}C NMR ($CDCl_3$-77 ppm): 177.00, 151.89, 148.75, 140.84, 135.47, 129.14, 127.41, 120.36, 118.76, 111.31 (9 aromatics, 1 carbonyl = 10 C), 40.52, 27.56 (5 aliphatic carbons, 4 in the tert-butyl group at 27.56).

R_f =0.30 (40 % EtOAc/hexane), HRMS calc. for $C_{14}H_{16}ClN_2O$, 263.0951, obs. 263.09453.



NT11

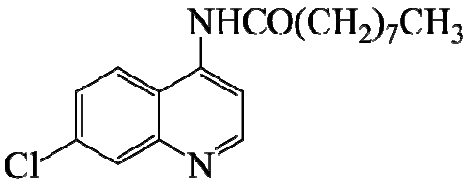
Figure 3.12 Chemical structure of the synthesized NT11 compound. The crude product was obtained by adding pentanoyl chloride (valeroyl chloride) to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-pentaamido-7-chloroquinoline. Yield: 28 %; melting point: 139-140 °C; molecular weight: 263.7426.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.78 (1H, bs), 8.40 (1H, bs), 8.19 (1H, d), 8.03 (1H, bs), 7.82 (1H, dd), 2.56 (2H, t), 1.76 (2H, pt), 1.41 (2H, st), 0.95 (3H, s).

^{13}C NMR ($CDCl_3$-77 ppm): 172.38 (CO), 151.66, 148.68, 141.11, 135.56, 128.65, 127.22, 121.26, 118.65, 111.68 (9 aromatics, 1 carbonyl = 10 C), 37.54, 27.43, 22.30, 13.74 (4 aliphatic carbons).

R_f =0.34 (40 % EtOAc/hexane), HRMS calc. for $C_{14}H_{16}ClN_2O$, 263.0951, obs. 263.09474.



NT12

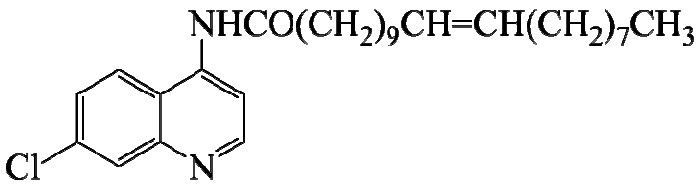
Figure 3.13 Chemical structure of the synthesized NT12 compound. The crude product was obtained by adding nonanoyl chloride (pelargonoyl chloride) to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. Nonanoyl chloride was prepared from the treatment of nonanoic acid with $SOCl_2$ at 60 °C for 15 h. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-nonanamido-7-chloroquinoline. Yield: 28 %; melting point: 100-101 °C; molecular weight: 319.8490.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.74 (1H, bs), 8.58 (1H, bs), 8.18 (1H, d), 8.00 (1H, bs), 7.85 (1H, d), 7.39 (1H, dd), 2.55 2H, t), 1.75 (2H, quin), 1.30 (1H, bs), 0.88 (3H, s).

^{13}C NMR ($CDCl_3$-77 ppm): 172.50 (CO), 151.59, 148.66, 141.24, 135.61, 128.59, 127.22, 121.40, 118.72, 111.75 (9 aromatics, 1 carbonyl = 10 C), 37.81, 31.73, 29.26, 29.19, 29.07, 25.41, 22.56, 14.02 (8 aliphatic carbons).

R_f =0.54 (40 % EtOAc/hexane), HRMS calc. for $C_{18}H_{24}ClN_2O$, 319.1577, obs. 319.15719.



NT13 (n-11)

Figure 3.14 Chemical structure of the synthesized NT13 compound. The crude product was obtained by adding 11-eicosenoyl chloride to the solution of 4-amino-7-chloroquinoline as previously described above in section 1.3. An initial confirmation of the yielded product was by TLC and later by ^{1}H and ^{13}C NMR. The chemical name assigned for this compound was 4-eicos-11-en-amido-7-chloroquinoline. Yield: 30 %; melting point: 28-29 °C; molecular weight: 472.1255.

NMR Data

^{1}H NMR ($CHCl_3$-7.26 ppm): δ 8.75 (1H, bd), 8.38 (1H, bs), 8.19 (1H, d), 8.05 (1H, bs), 7.80 (1H, d), 7.40 (1H, d), 5.35 (2H, bm), 2.55 (2H, t), 2.00 (3H bm), 1.76 (2H, quin), 1.31 (25H, bs), 0.95 (3H, s).

^{13}C NMR ($CDCl_3$-77 ppm): 172.27 (CO), 151.93, 148.98, 140.87, 135.47, 129.93, 129.73, 128.97, 127.20, 121.07, 118.64, 111.67 (9 aromatics, 1 carbonyl, 2alkene = 12 C), 37.85, 31.85, 29.72, 29.47, 29.40, 29.32, 29.27, 29.22, 27.17, 25.41, 22.63, 14.06 (12 aliphatic resonances for 17 carbons).

R_f =0.49 (40 % EtOAc/hexane), HRMS calc. for $C_{29}H_{44}ClN_2O$, 471.3142, obs. 471.31312.

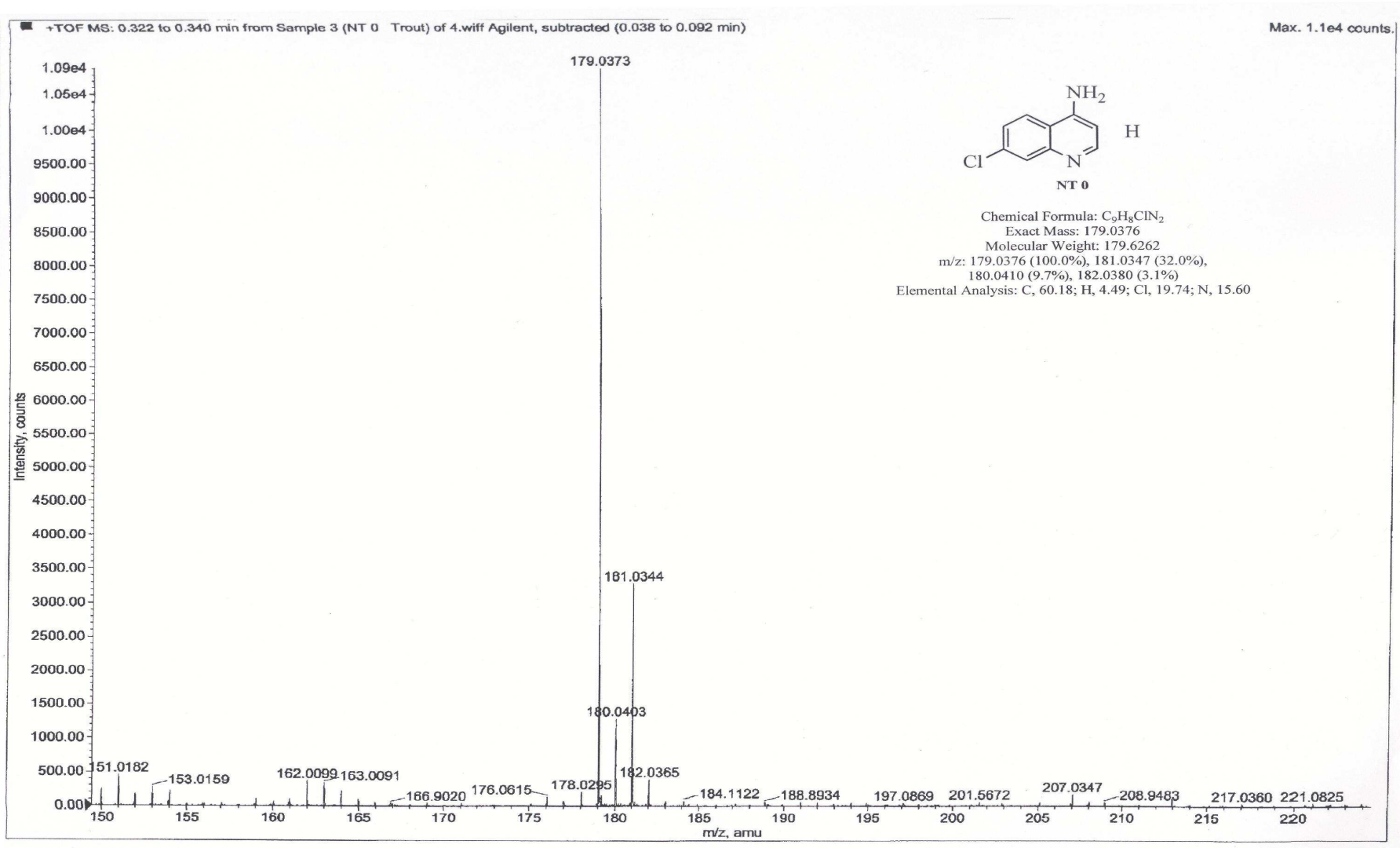


Figure 3.1.1 NMR data for NT0.

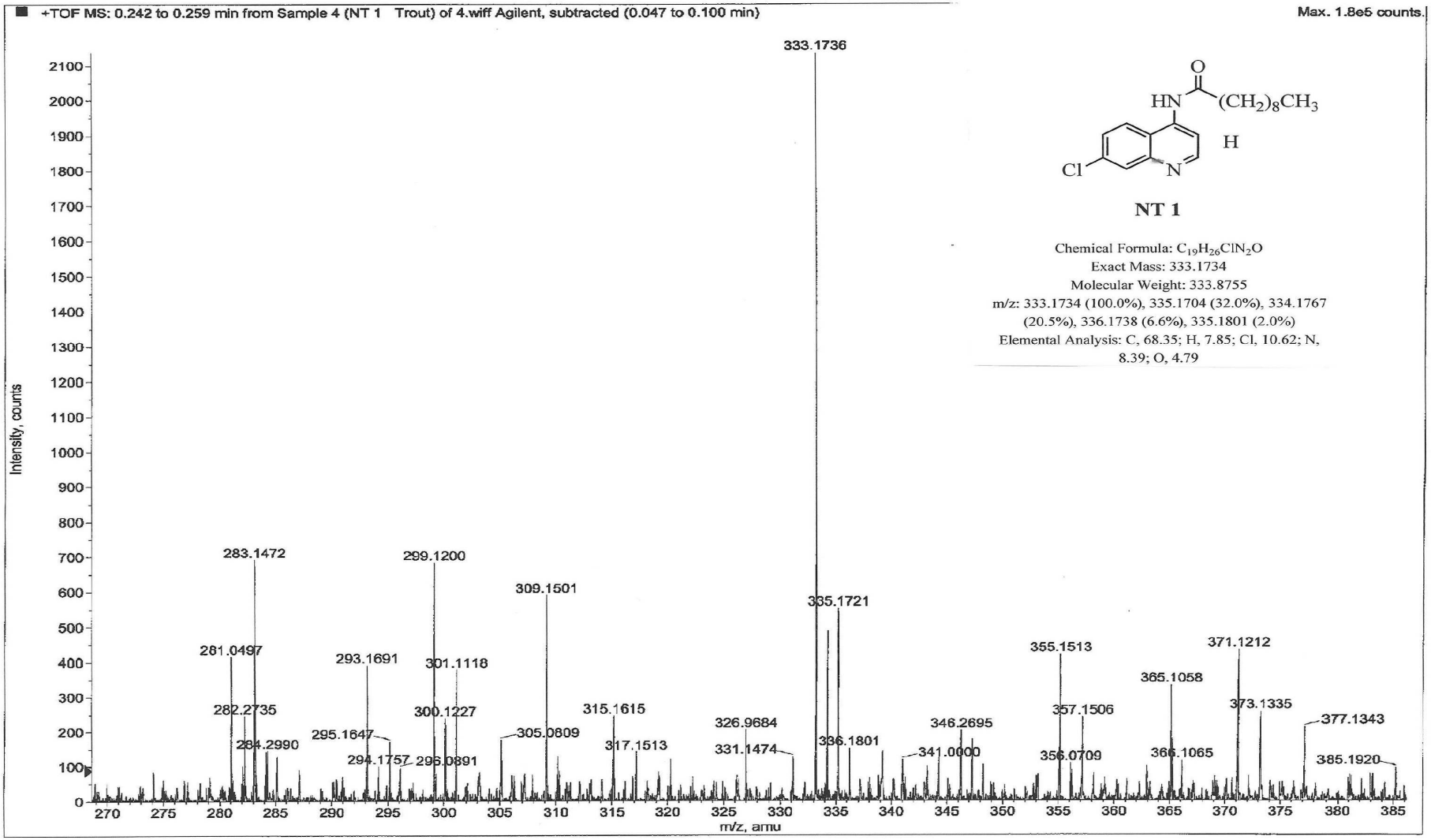


Figure 3.2.1 NMR data for NT1.

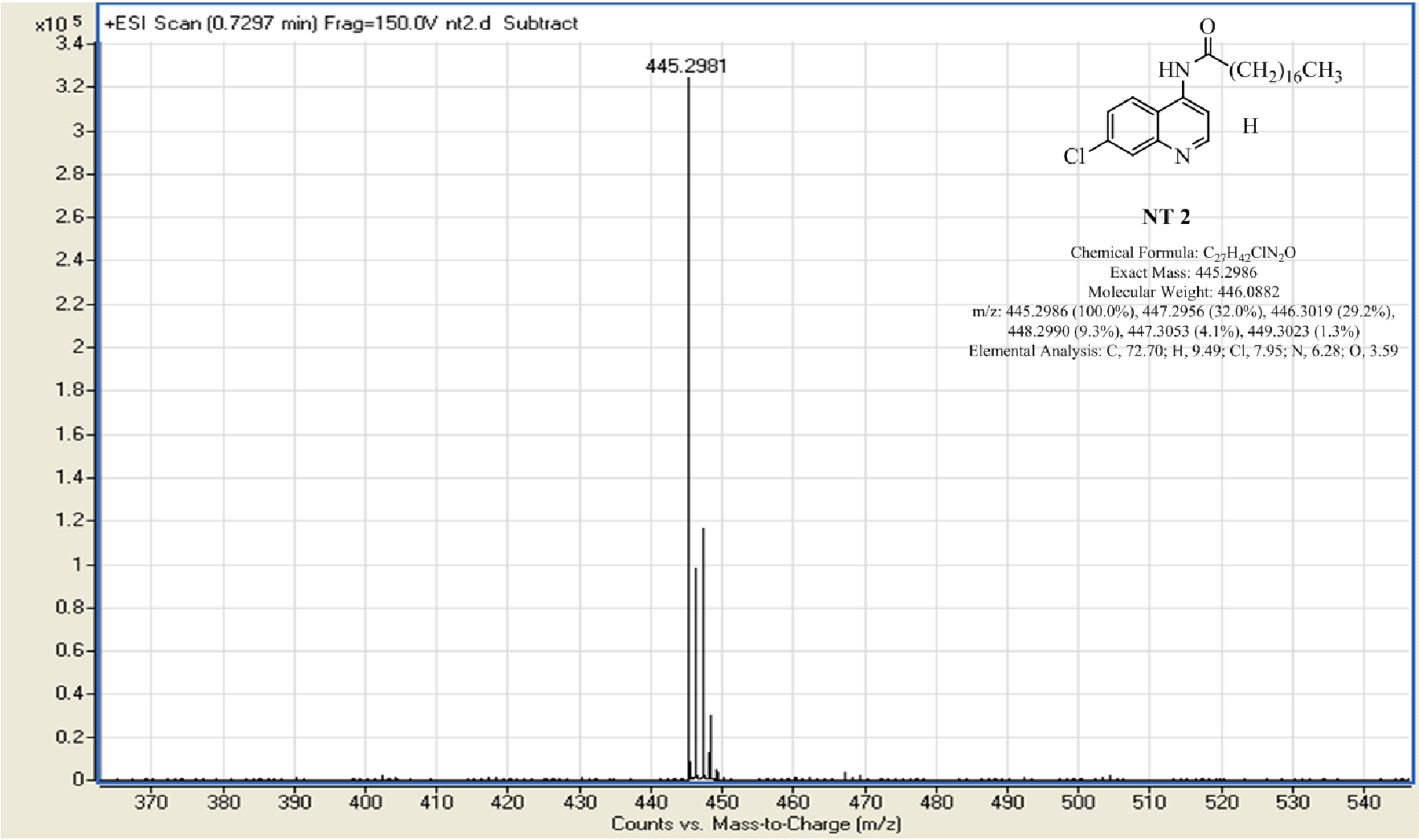


Figure 3.3.1 NMR data for NT2.

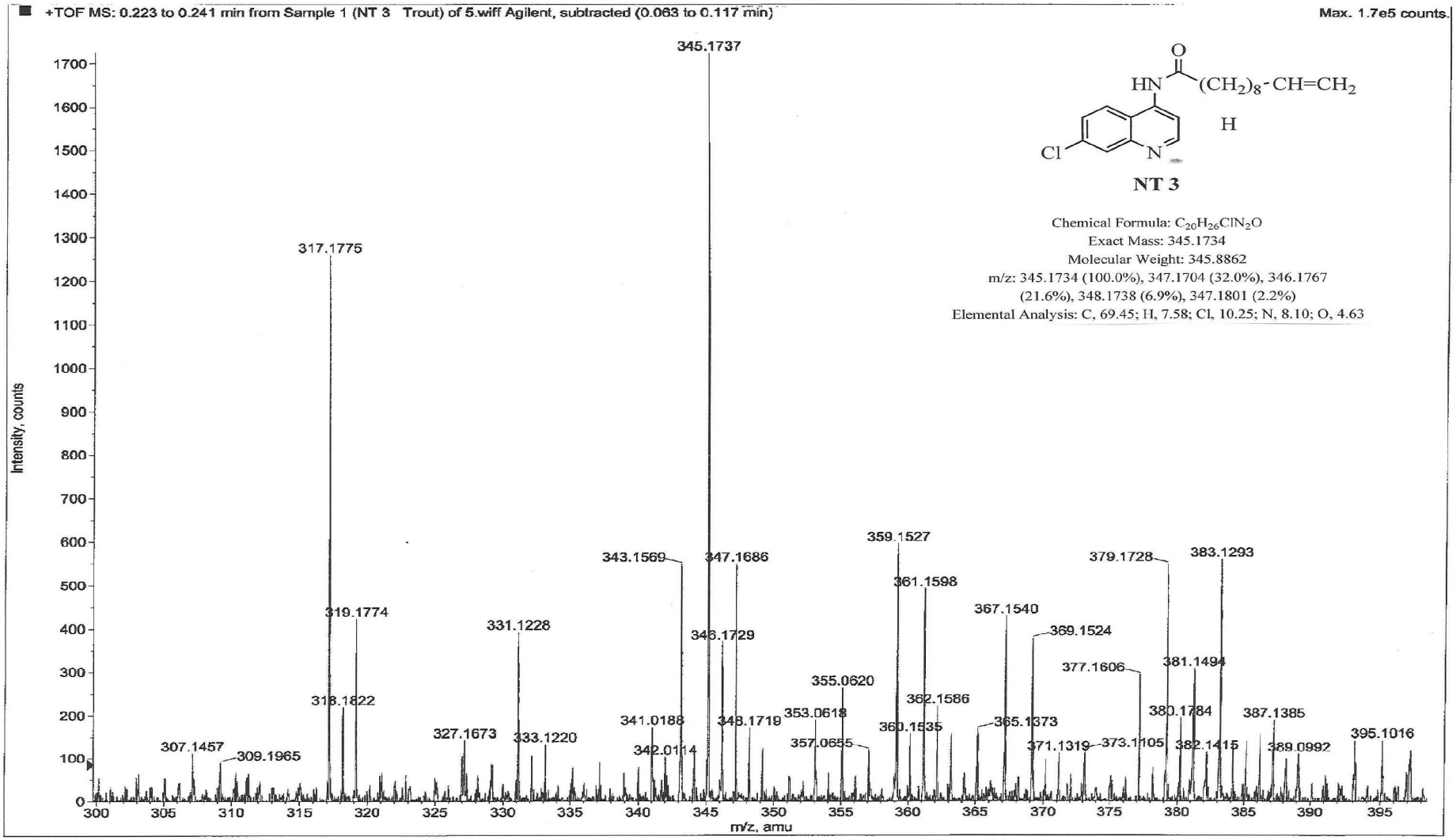


Figure 3.4.1 NMR data for NT3.

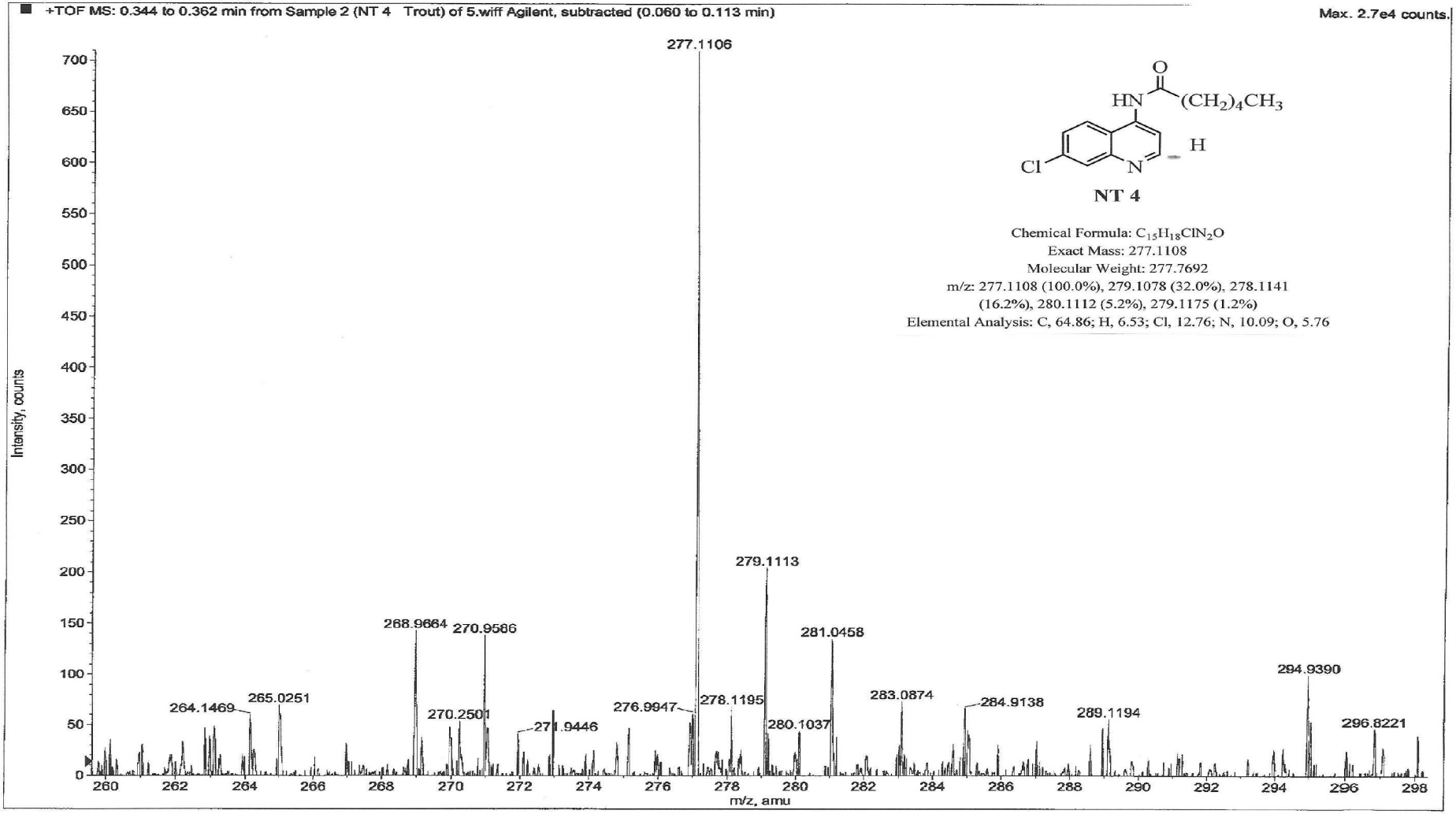


Figure 3.5.1 NMR data for NT4.

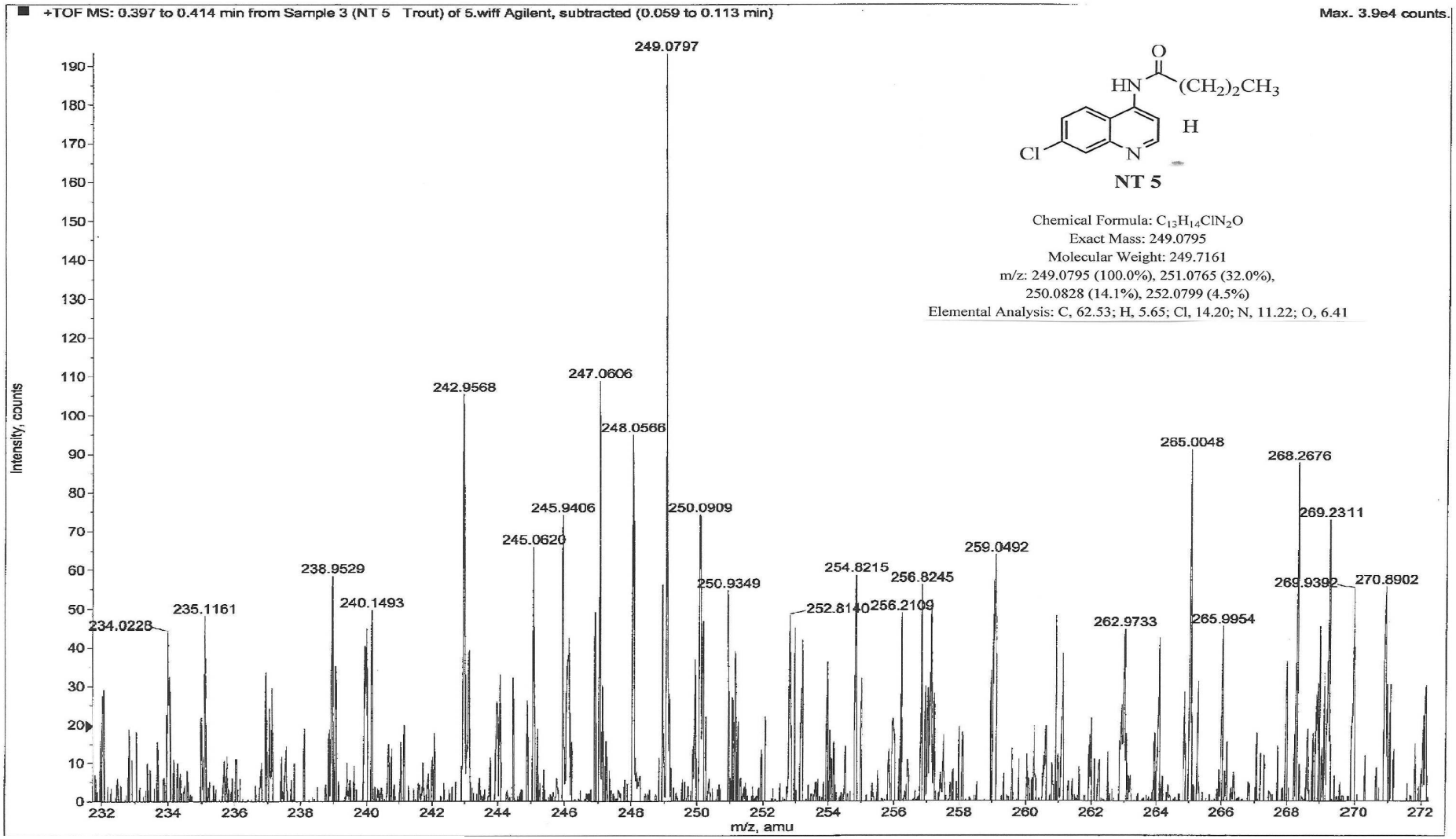


Figure 3.6.1 NMR data for NT5.

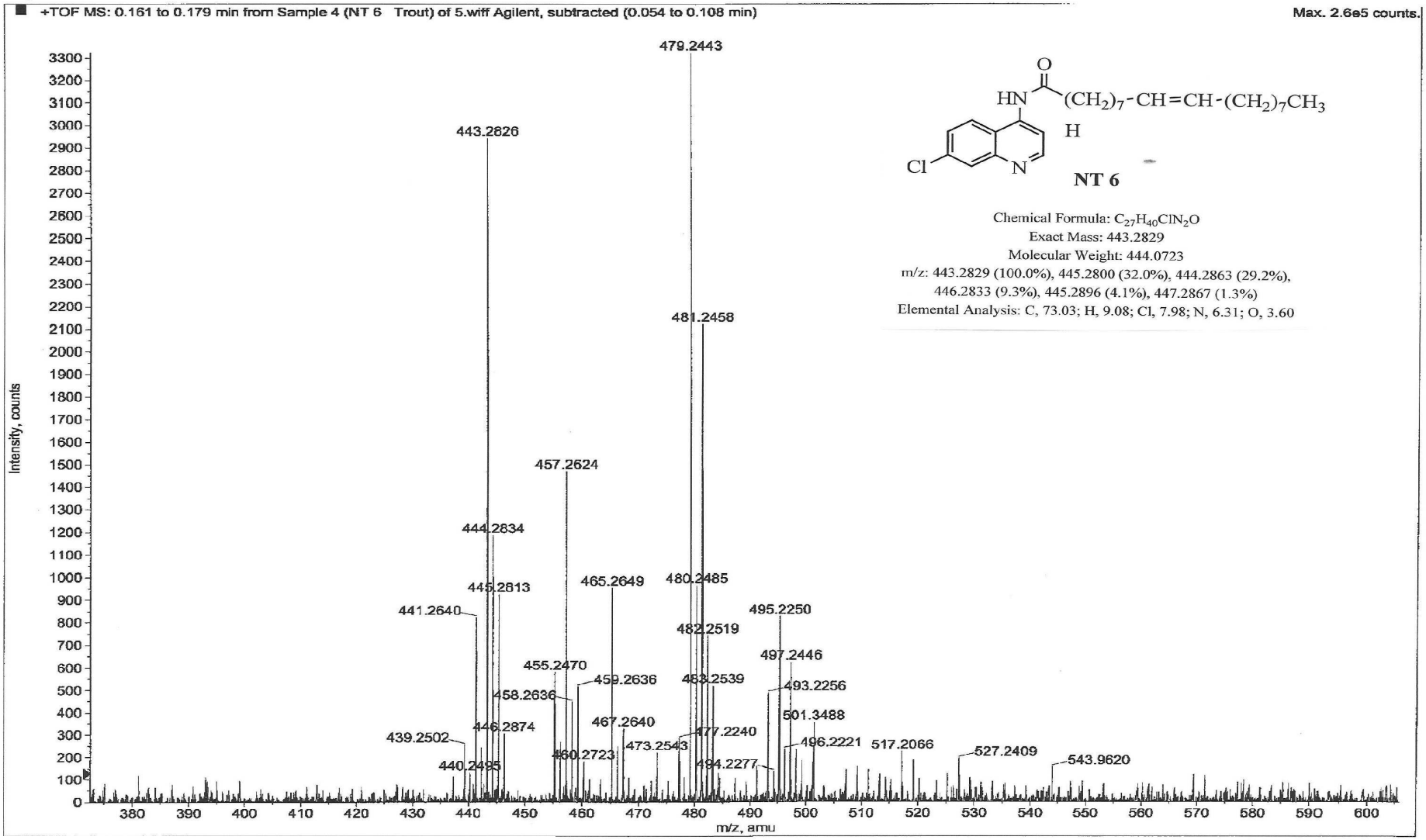


Figure 3.7.1 NMR data for NT6.

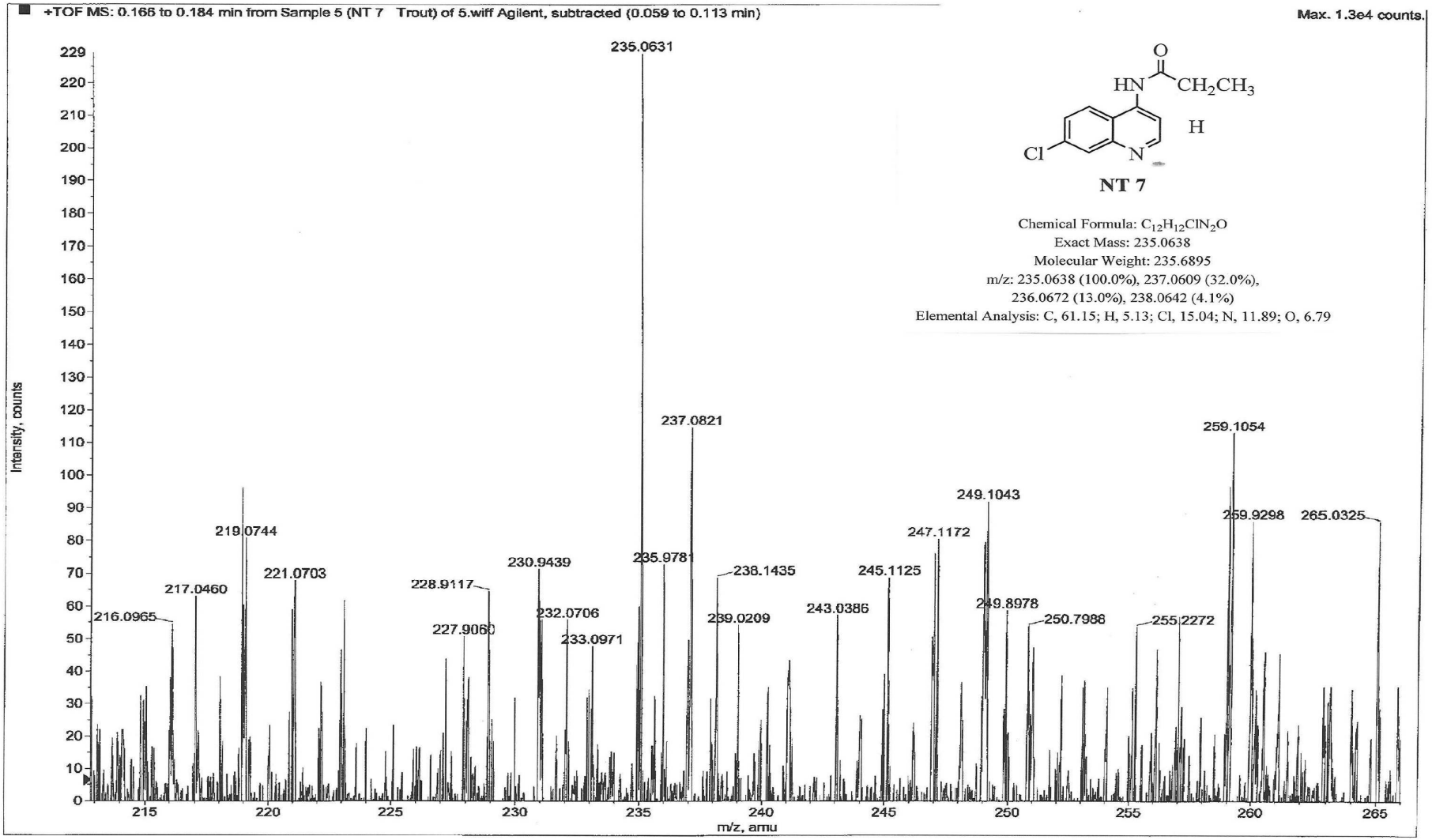


Figure 3.8.1 NMR data for NT7.

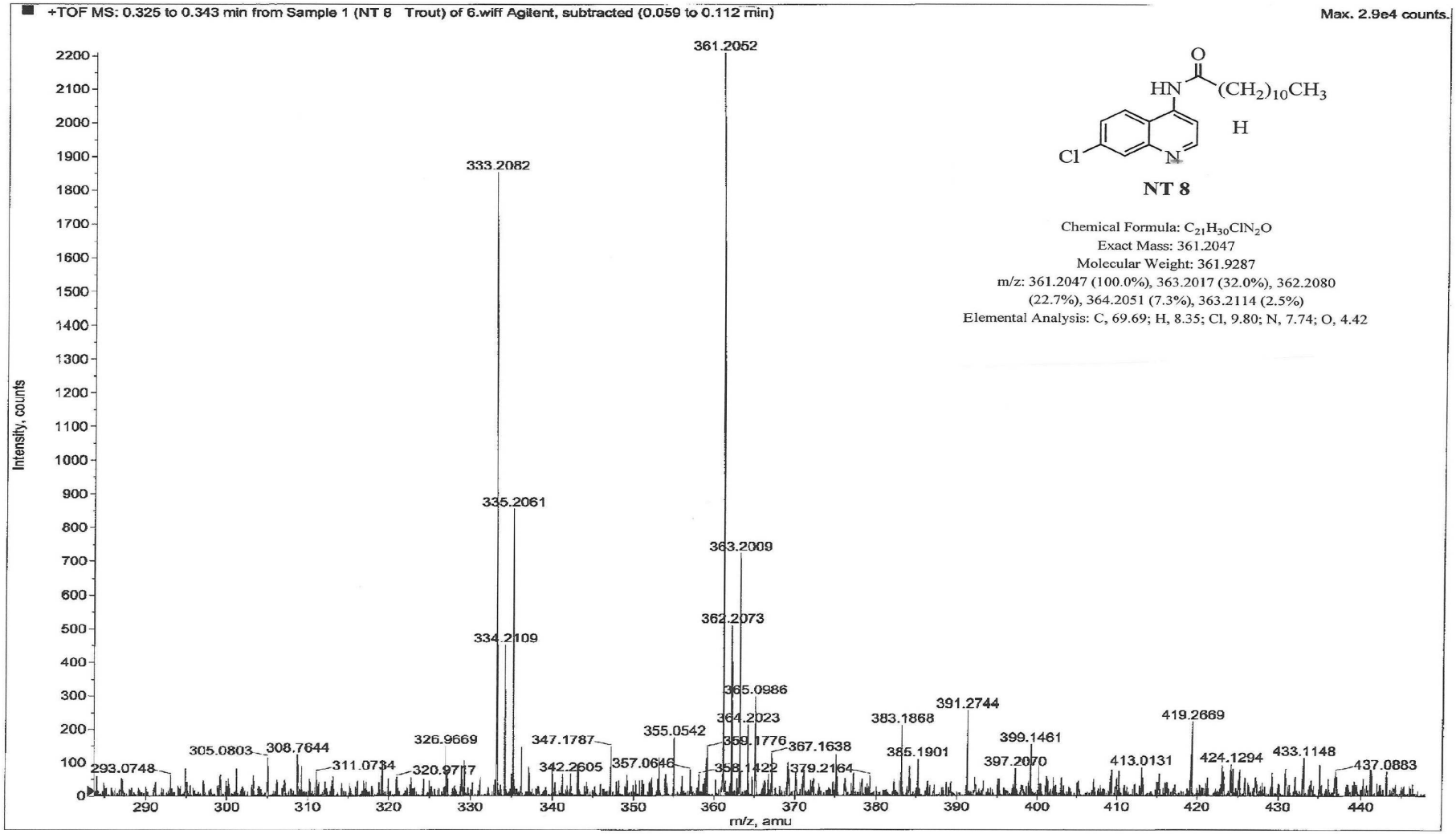


Figure 3.9.1 NMR data for NT8.

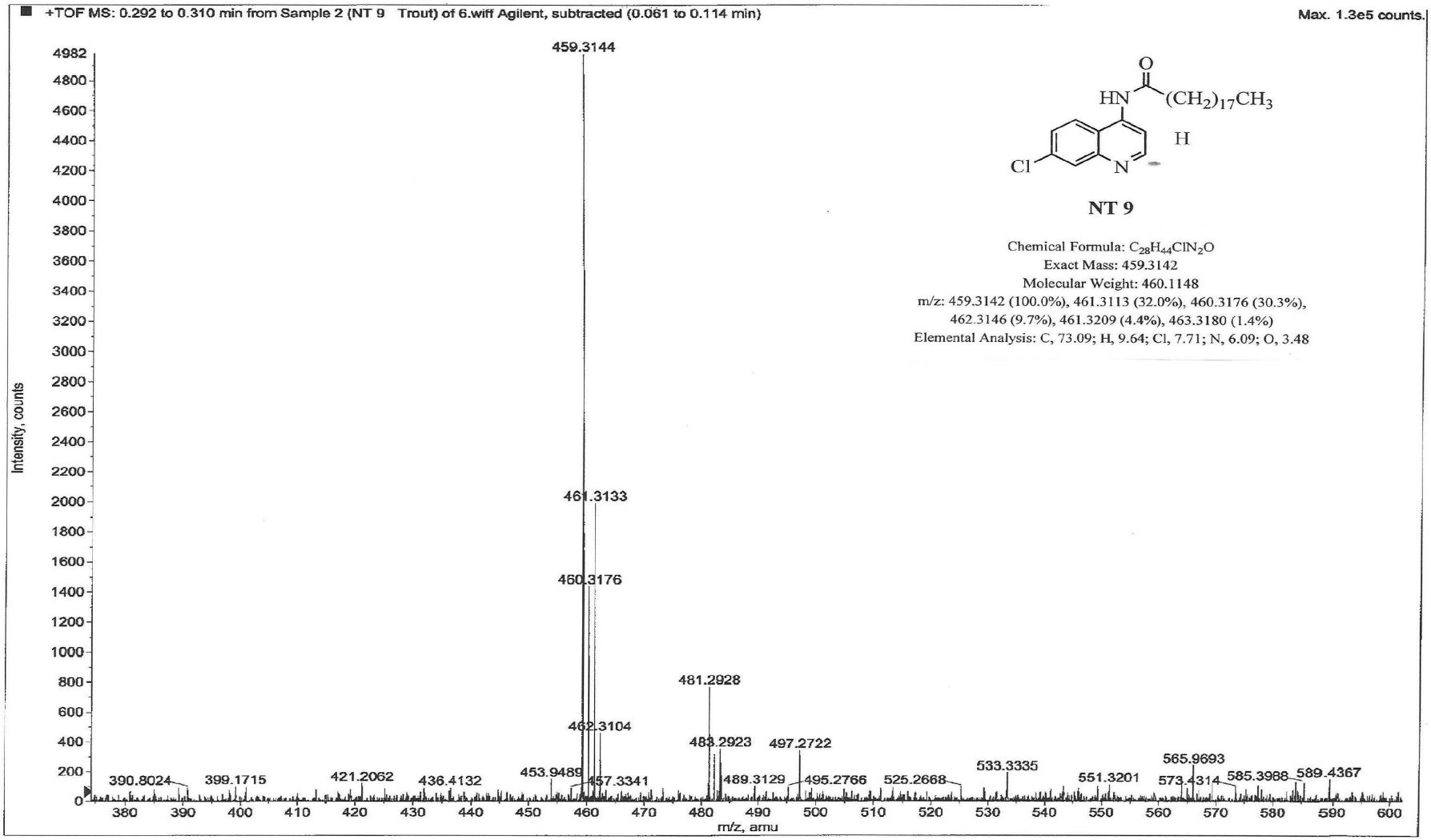
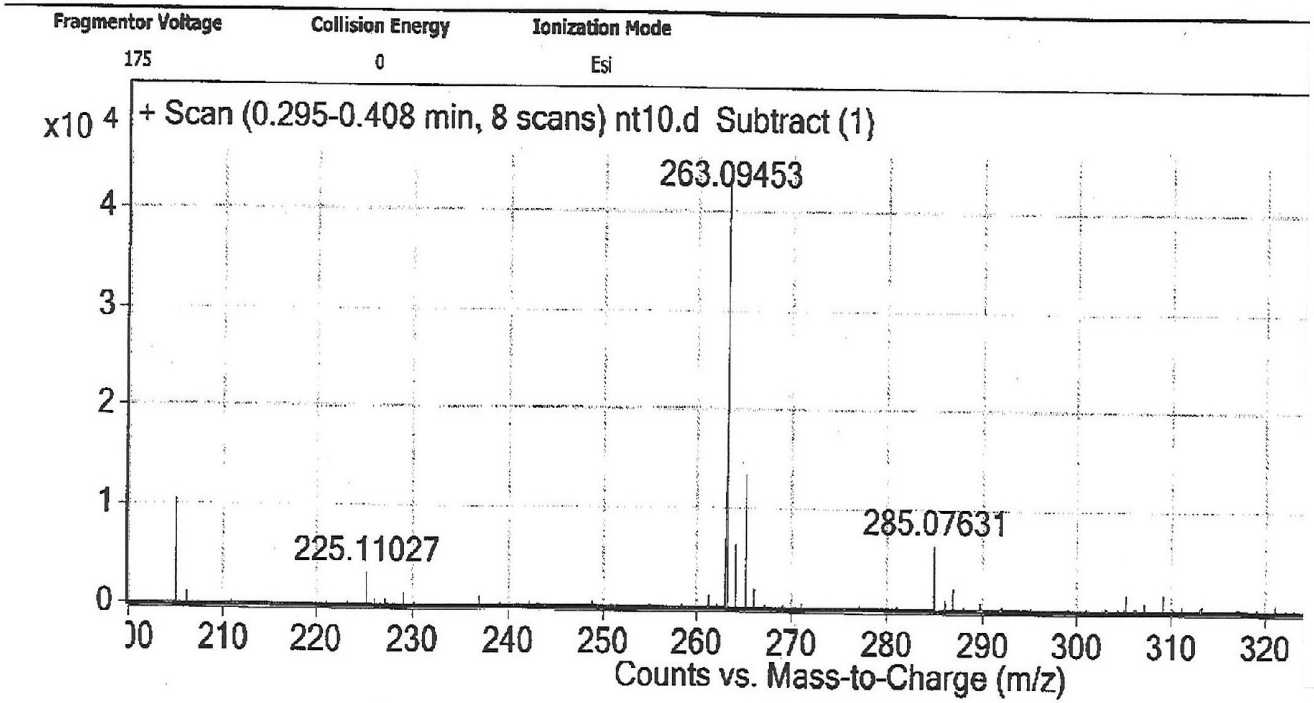
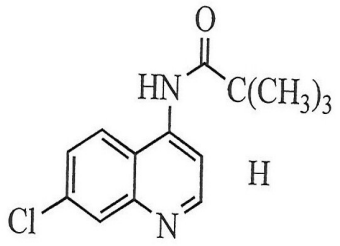


Figure 3.10.1 NMR data for NT9.

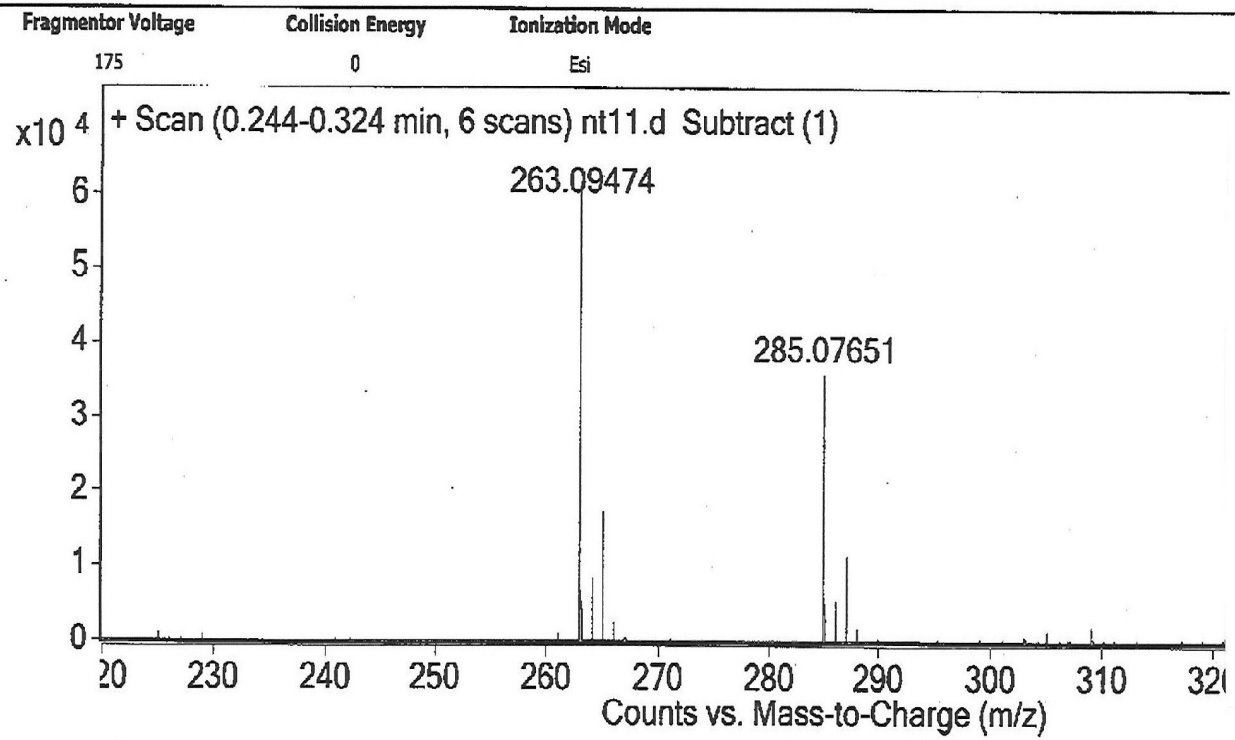


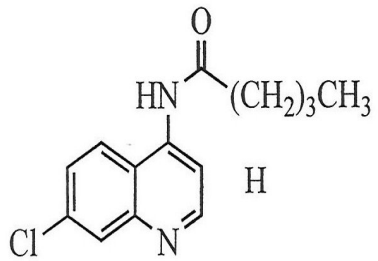


NT 10

Chemical Formula: $C_{14}H_{16}ClN_2O$
Exact Mass: 263.0951
Molecular Weight: 263.7426
m/z: 263.0951 (100.0%), 265.0922 (32.0%), 264.0985 (15.1%), 266.0955 (4.8%), 265.1018 (1.1%)
Elemental Analysis: C, 63.76; H, 6.11; Cl, 13.44; N, 10.62; O, 6.07

Figure 3.11.1 NMR data for NT10.





NT 11

Chemical Formula: $C_{14}H_{16}ClN_2O$

Exact Mass: 263.0951

Molecular Weight: 263.7426

m/z: 263.0951 (100.0%), 265.0922 (32.0%), 264.0985 (15.1%), 266.0955 (4.8%), 265.1018 (1.1%)

Elemental Analysis: C, 63.76; H, 6.11; Cl, 13.44; N, 10.62; O, 6.07

Figure 3.12.1 NMR data for NT11.

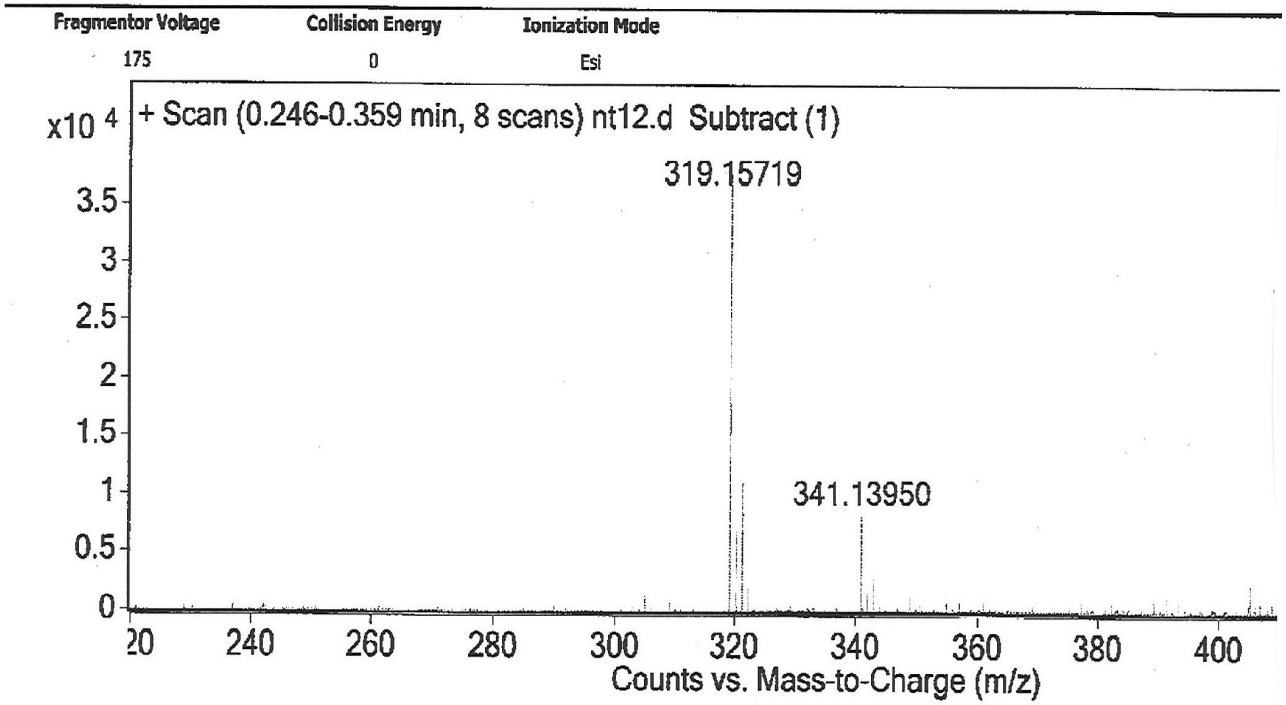
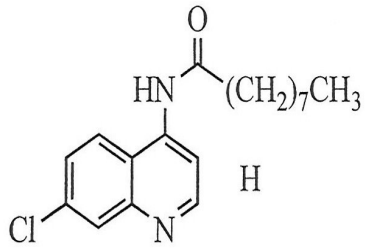


Figure 3.13.1 NMR data for NT12.



NT 12

Chemical Formula: $C_{18}H_{24}ClN_2O$
Exact Mass: 319.1577
Molecular Weight: 319.8490
m/z: 319.1577 (100.0%), 321.1548 (32.0%), 320.1611 (19.5%), 322.1581 (6.2%), 321.1644 (1.8%)
Elemental Analysis: C, 67.59; H, 7.56; Cl, 11.08; N, 8.76; O, 5.00

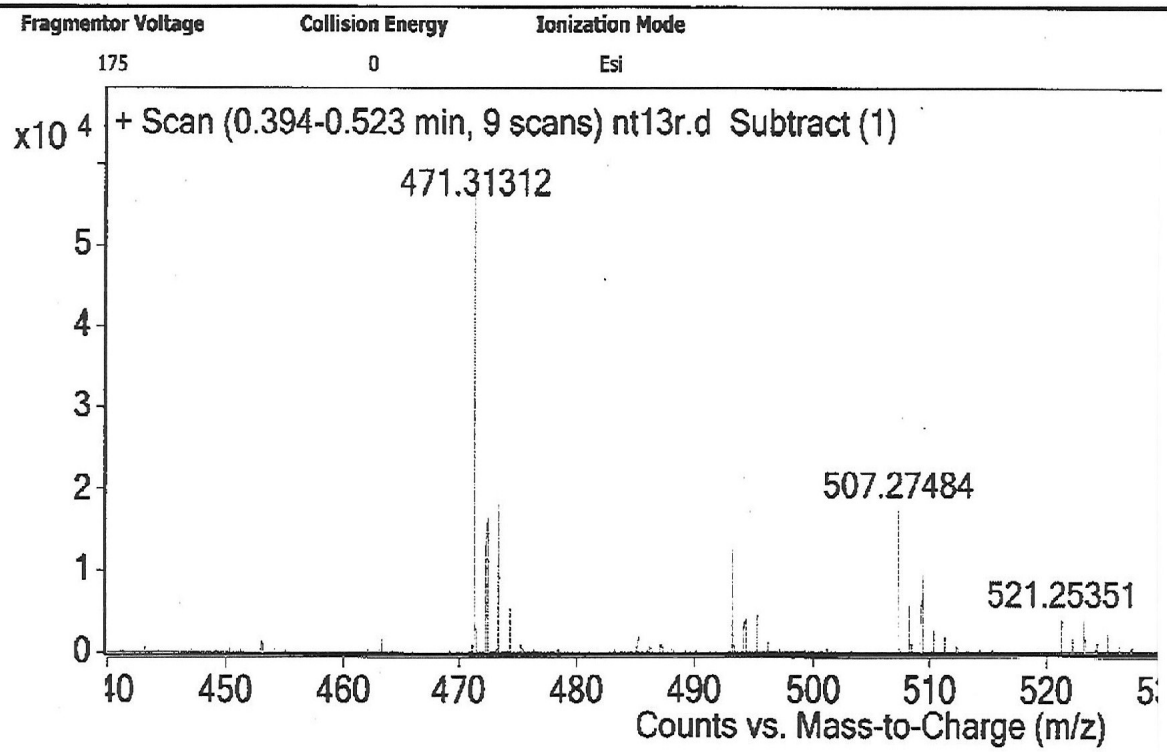
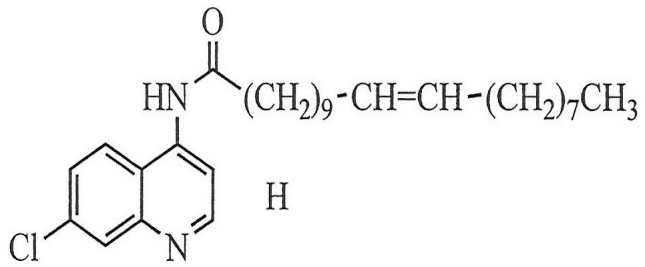


Figure 3.14.1 NMR data for NT13.



NT 13

Chemical Formula: $C_{29}H_{44}ClN_2O$

Exact Mass: 471.3142

Molecular Weight: 472.1255

m/z: 471.3142 (100.0%), 473.3113 (32.0%), 472.3176 (31.4%), 474.3146 (10.0%), 473.3209 (4.7%), 475.3180 (1.5%)

Elemental Analysis: C, 73.77; H, 9.39; Cl, 7.51; N, 5.93; O, 3.39

3.3 Summary

An aromatic base quinoline structure (NT0) with a 7-chloro group has been synthesized according to Vippagunta *et al.*, (1999). From that base structure, thirteen different NT compounds have been synthesised each bearing a different fatty acid side chain attached to the NH_2 group at C_4 of the aromatic ring. All fatty acid chains possess different lengths and degrees of saturation. Thirteen compounds with different carbon chain length of 3 to 20 carbons were prepared in a yield ranging from 14-58 %. They can be further subdivided into the saturated group: NT1, 2, 4, 5, 7, 8, 9, 10, 11 and 12 and the unsaturated group: NT 3, 6 and 13. Of all the 13 compound synthesised, only one compound displayed an oil appearance (NT6), which is different to the other compound that exists as powder form. Finally, it is also observed that compounds with a longer side chain tend to be less soluble compared to the shorter ones.

4.0 CHAPTER 4 THE EFFECTS OF NT COMPOUNDS ON NEUTROPHIL ADHERENCE

4.1 Introduction

Neutrophil migration and accumulation at inflammatory foci is upregulated by their ability to adhere to the endothelium as discussed in Chapter 1.4.1-2. The process involves receptor ligand interaction. An initial loose-rolling adhesion of the neutrophils to the receptors on the endothelial cells involves the L-selectins on the leukocytes and the E- and P-selectins on the endothelial cell (Ferrante, 2005). This then results in the activation of the ICAM on the endothelial cell as well as the integrins (CD11a, CD11b and CD11c/CD18) on neutrophils thereby promoting firm adhesion (Witko-Sarsat *et al.*, 2000; Ferrante, 2005). Neutrophil adherence is also known to involve other receptors such as the Fc receptors, and the adenosine receptors depending on the stimulus they encounter (Witko-Sarsat *et al.*, 2000; Lantz *et al.*, 1994; Cronstein *et al.*, 1992). Interestingly, Cronstein *et al.*, (1992) found that engagement of adenosine A_1 receptors promoted neutrophil adherence to the endothelium whereas the engagement of A_2 receptors resulted in the inhibition of neutrophil adherence to fibrinogen coated surfaces. As you have an interest in integrins eg CR3, CD11b/CD18, you should mention and reference these.

During inflammation inflammatory mediators are generated which bind to neutrophils and regulate their ability to adhere to the endothelium. These include cytokines such as TNF and GM-CSF and eicosanoids such as LTB4 (refs)

Previous studies have demonstrated that a series of quinoline based compounds including quinine, quinacrine, CQ, pryimethamine and melfoquine inhibited neutrophil adherence (Ferrante *et al.*, 1986). The aim of the present studies was to examine the effects of adding a fatty acid to the side chain of the quinoline structure on neutrophil adherence with particular

emphasis on the role of the structure of the fatty acid used.

4.2 The effect of NT compounds on adherence induced by TNF rich medium (TNF-RM)

To enable us to gain an understanding of the activity conferred by altering the fatty acid side chain on the quinoline group, we examined all 13 compounds for their ability to alter the adherence of stimulated neutrophil activity. A previously established strong inducer of neutrophil adherence was used, medium derived from *S.aureus* stimulated PBMC cultures which has high TNF activity (TNF-RM) (Bates *et al.*, 1991).

Neutrophils (5 x 10^5/ml) were pre-treated with NT compounds, CQ or HCQ for 1 h and then stimulated with TNF-RM. After 30 min, the ability of neutrophils to adhere to plasma coated plastic surfaces was determined. The compounds were examined at three concentrations, 5, 20 and 50 µM. The results showed that the compounds varied in their ability to inhibit neutrophil adherence (Figure 4.1-16, Table 4.1). NT1, NT3 and NT8 were the most active. Interestingly neither CQ nor HCQ were active at these concentrations. At the 50 µM concentration significant inhibition was seen with NT2, NT5, NT7, NT10 NT11 and NT12 (Table 4.2).

Under the concentrations tested, the cells showed a viability of > 99 % as judged by their ability to exclude trypan blue dye and the finding that these have no inhibitory effects at 50 µM for all compounds on basal neutrophil adherence (Table 4.3). The most effective was NT8, a compound with a quinoline base attached to a 12 carbon (lauric acid) side chain, inhibiting at 20 µM, followed by NT1, a compound with a 10 carbon fatty chain inhibiting at

20-50 µM, and NT3, containing an 11 carbon unsaturated fatty chain which inhibited at 20-50 µM. Interestingly, all these three compounds consist of a medium length fatty acid side chain of 10-12 carbons which has been shown previously with structurally similar 4-aminoquinolines tends to be an effective anti-malarial agent (Krogstad *et al.*, 1988; Ridley *et al.*, 1996).

Of all the compounds tested, only NT1, NT3 and NT8 exert a concentration dependent inhibitory effect on adherence reaching a 62, 59 and 85 % inhibition respectively at 50 µM (Figure 4.1-4.16).

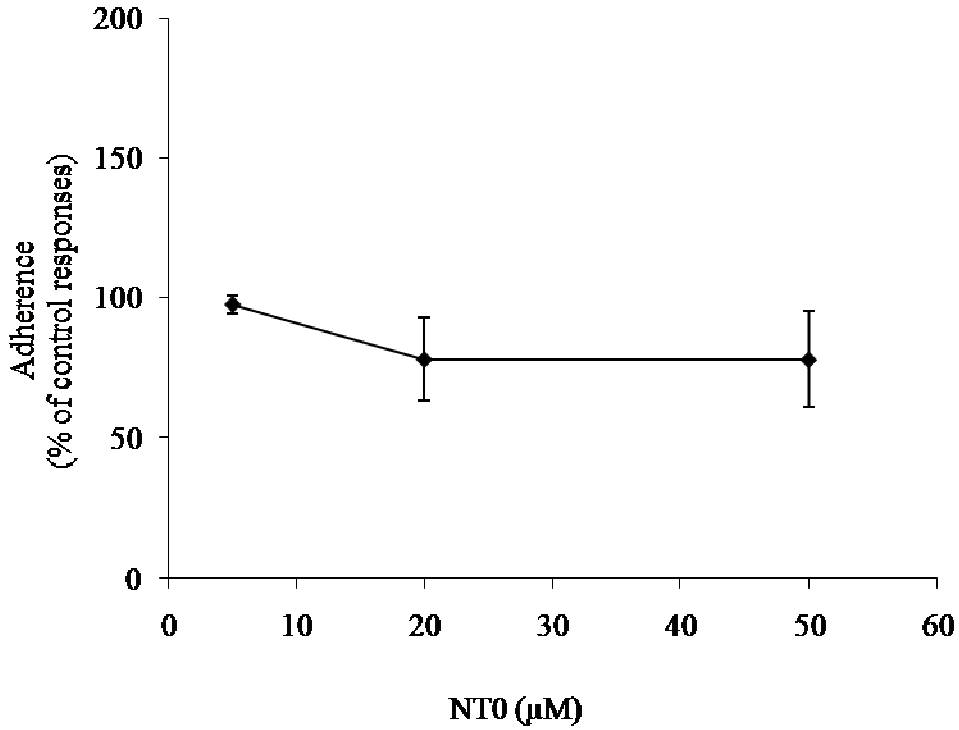


Figure 4.1 Effect of NT0 ($C_9H_8ClN_2$) on neutrohil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT0 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.09 ± 0.00 and 0.29 ± 0.03 respectively.

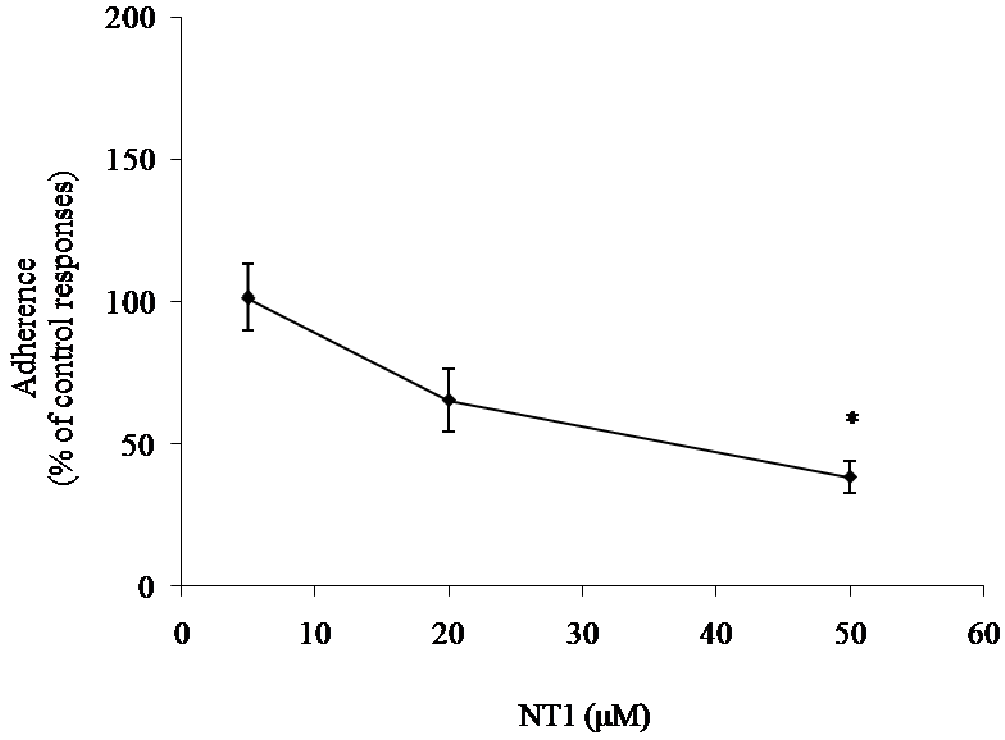


Figure 4.2 Effect of NT1 ($C_{19}H_{26}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT1 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.08 ± 0.00 and 0.41 ± 0.08 respectively. Significance of difference between control and NT1 treated cells: *$p<0.01$ (Tukey-Kramer multiple comparisons test).

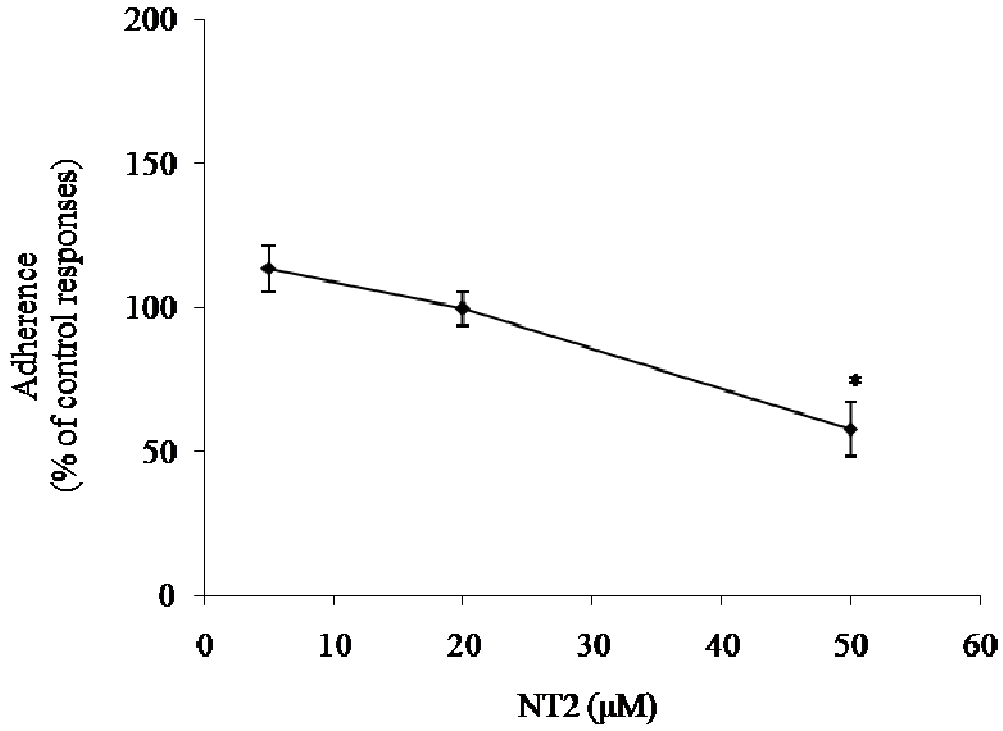


Figure 4.3 Effect of NT2 ($C_{27}H_{42}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT2 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.08 ± 0.01 and 0.44 ± 0.10 respectively. Significance of difference between control and NT2 treated cells: *$p<0.01$(Tukey-Kramer multiple comparisons test).

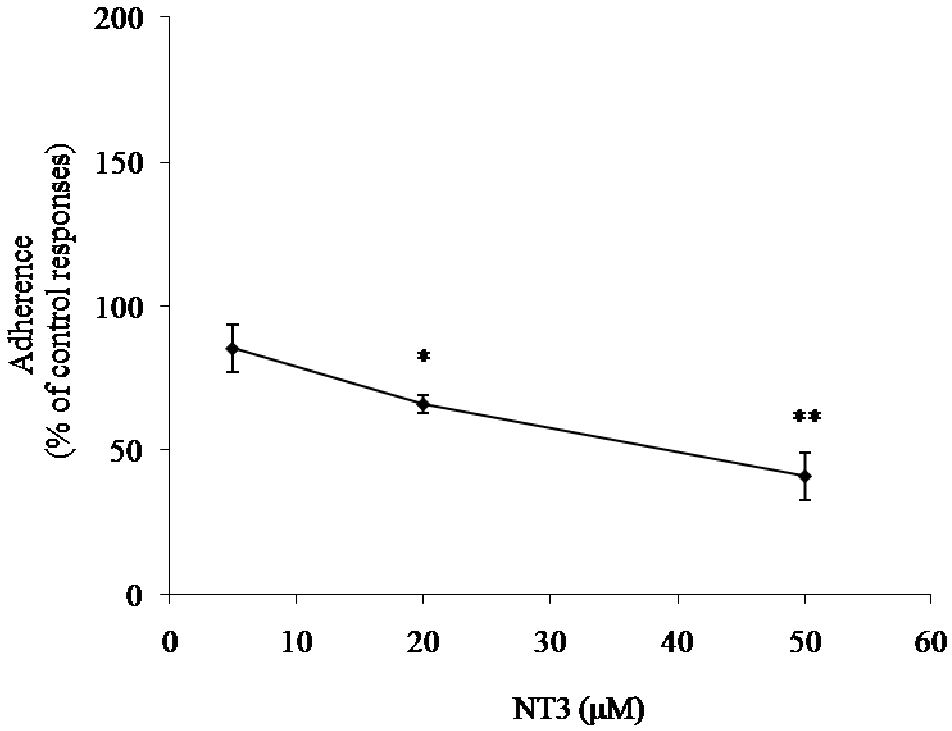


Figure 4.4 Effect of NT3 ($C_{20}H_{26}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT3 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.09 ± 0.01 and 0.33 ± 0.11 respectively. Significance of difference between control and NT3 (20 µM) treated cells: *$p<0.01$; between control and NT3 (50 µM) treated cells: **$p<0.001$ (Tukey-Kramer multiple comparisons test).

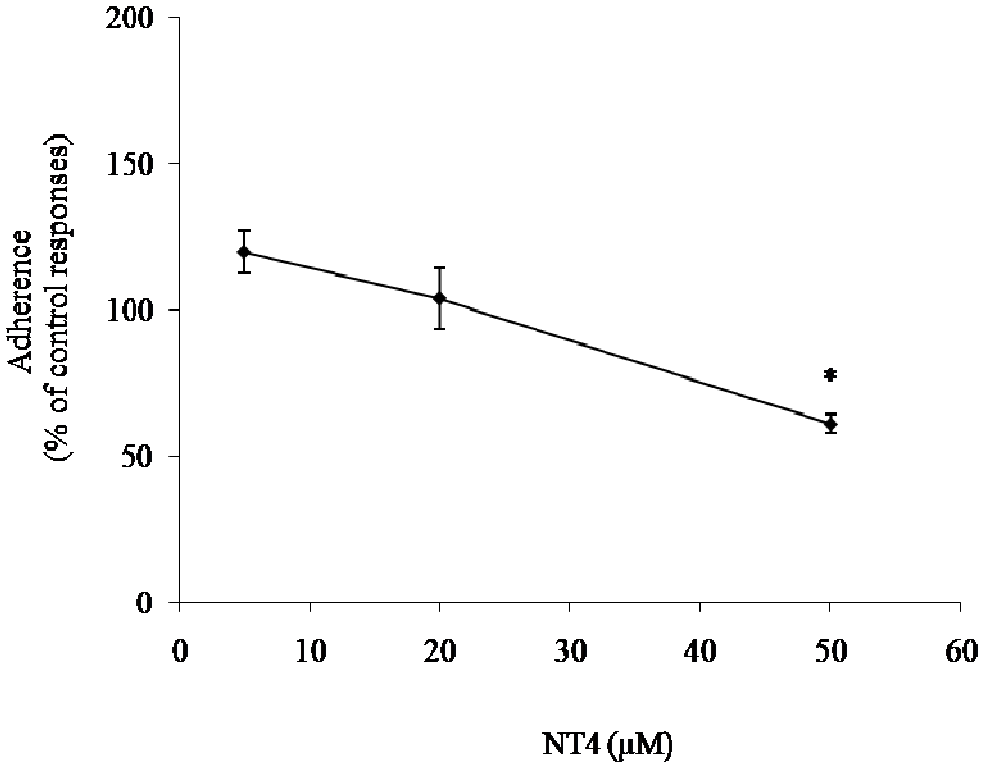


Figure 4.5 Effect of NT4 ($C_{15}H_{18}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT4 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.08 ± 0.01 and 0.40 ± 0.11 respectively. Significance of difference between control and NT4 (50 µM) treated cells: *$p<0.01$ (Tukey-Kramer multiple comparisons test).

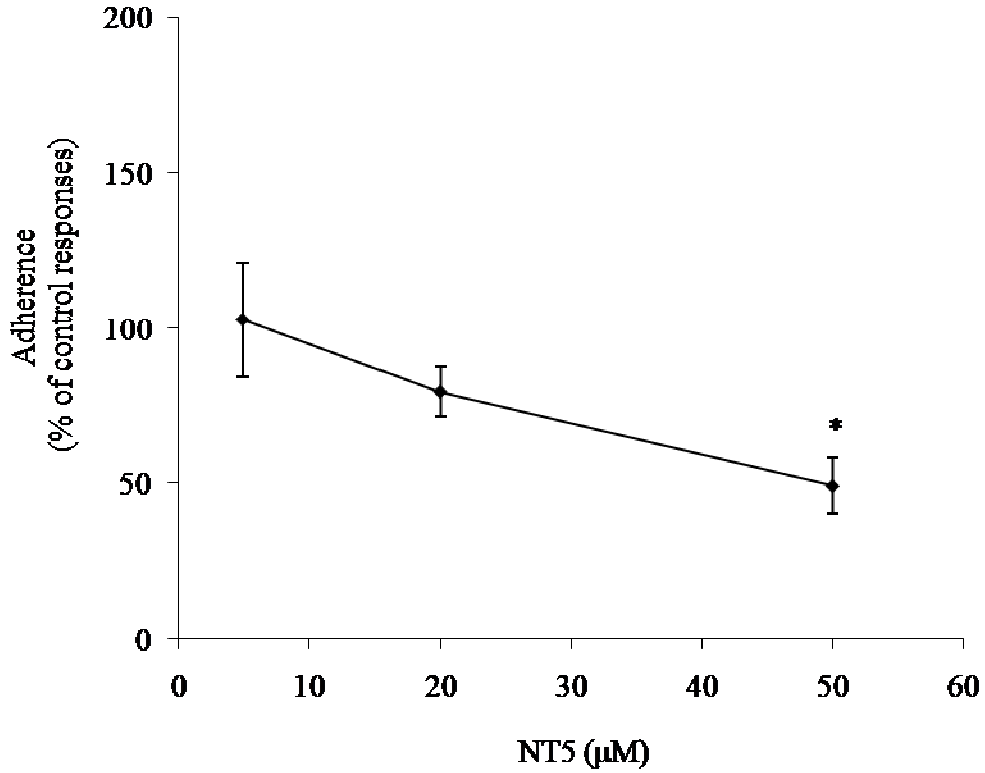


Figure 4.6 Effect of NT5 ($C_{13}H_{14}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT5 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.09 ± 0.00 and 0.42 ± 0.10 respectively. Significance of difference between control and NT5 (50 µM) treated cells: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

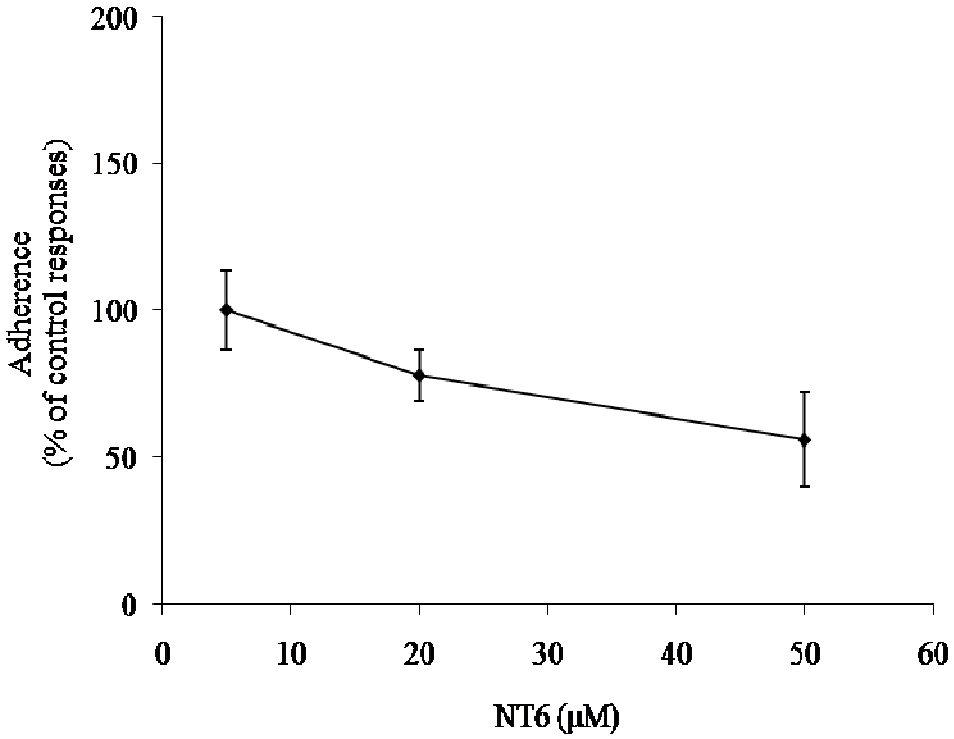


Figure 4.7 Effect of NT6 ($C_{27}H_{40}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT6 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.07 ± 0.00 and 0.30 ± 0.10 respectively.

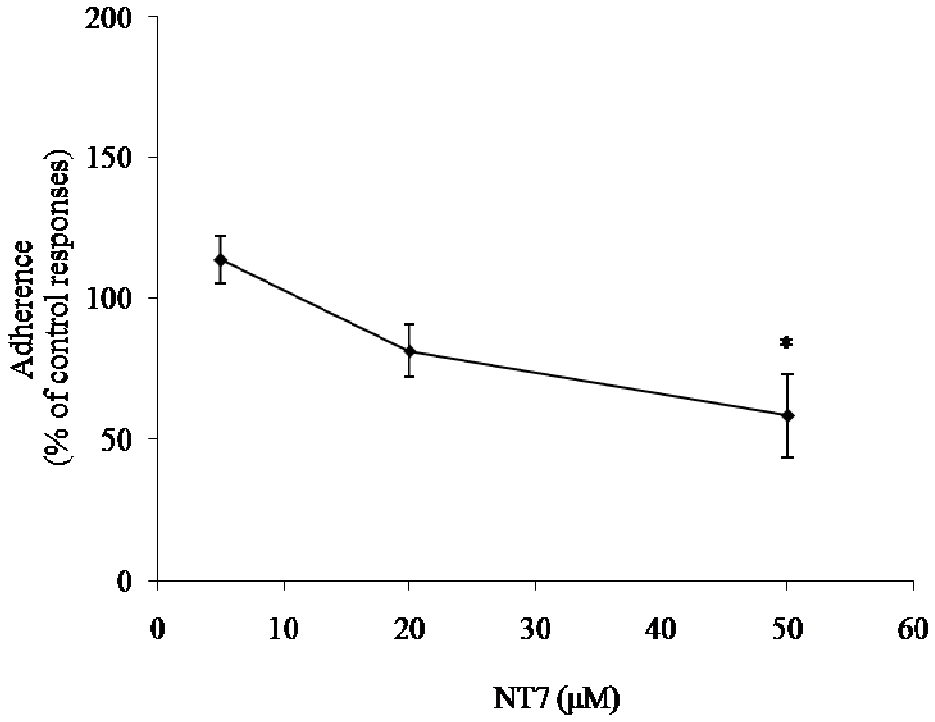


Figure 4.8 Effect of NT7 ($C_{12}H_{12}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT7 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.09 ± 0.01 and 0.34 ± 0.10 respectively. Significance of difference between control and NT7 (50 µM) treated cells: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

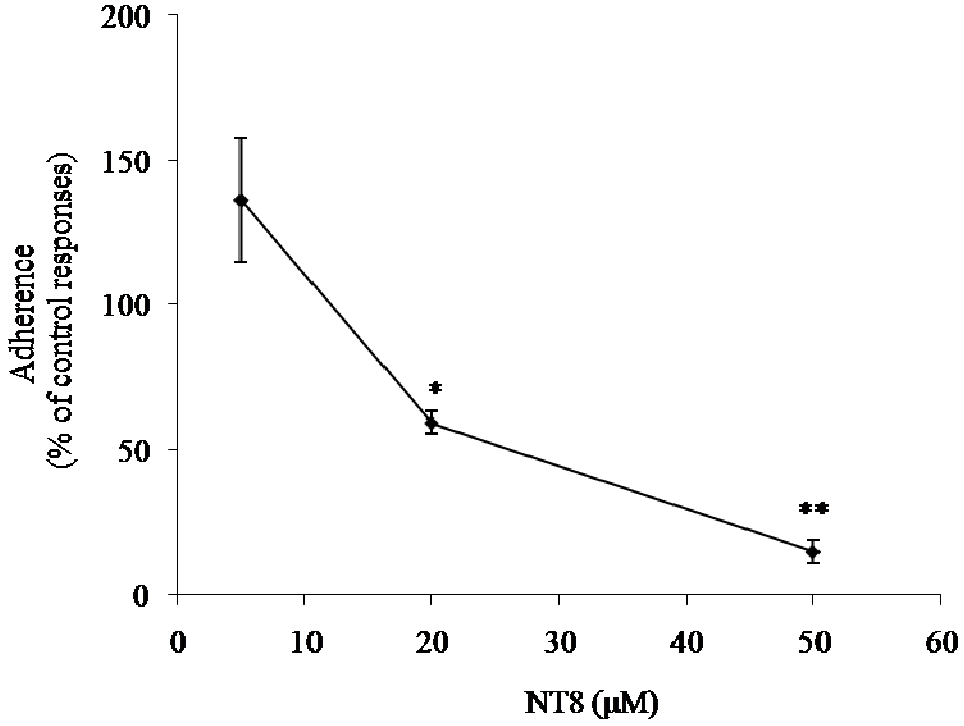


Figure 4.9 Effect of NT8 ($C_{21}H_{30}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT8 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The data are presented as ± SEM of 4 experiments. The basal and stimulated OD 570 nm values ± SEM were 0.07 ± 0.01 and 0.41 ± 0.10 respectively. Significance of difference between control and NT8 (20 µM) treated cells: *$p<0.01$; between control and NT8 (50 µM) treated cells: **$p<0.001$ (Tukey-Kramer multiple comparisons test).

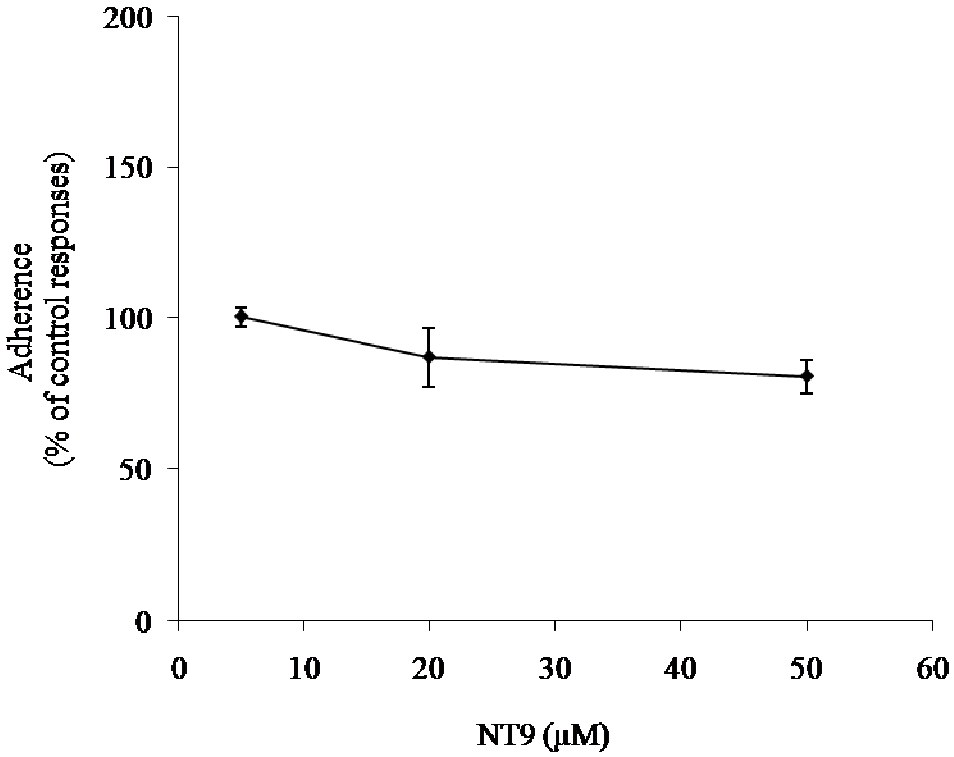


Figure 4.10 Effect of NT9 ($C_{28}H_{44}ClN_2O$) on neutropihl adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT9 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.09 ± 0.06 and 0.24 ± 0.09 respectively.

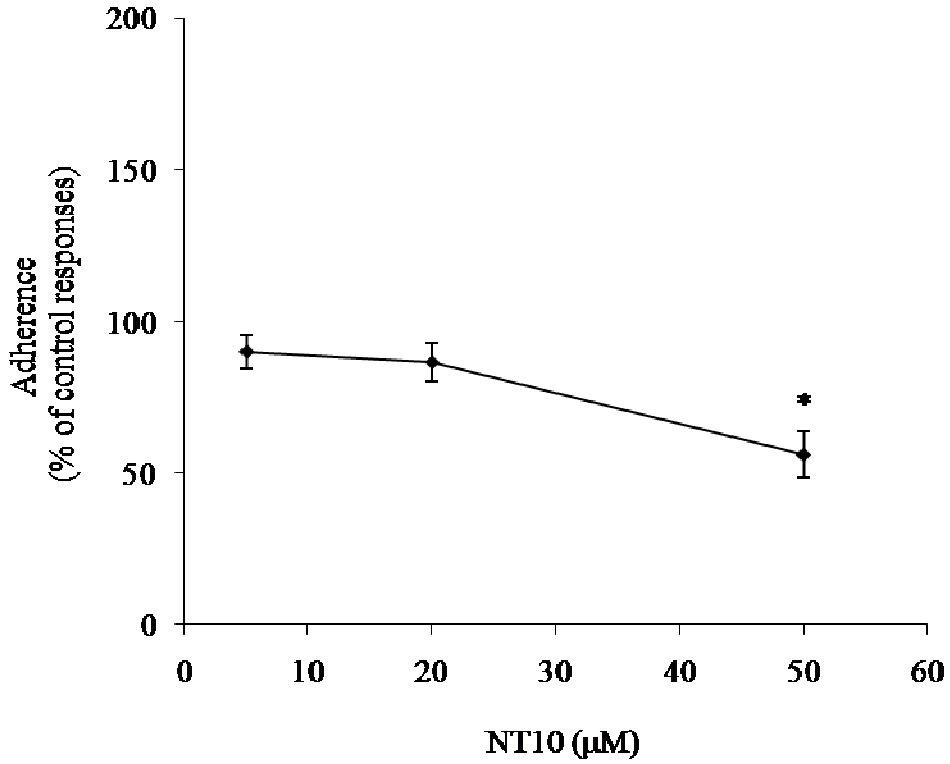


Figure 4.11 Effect of NT10 ($C_{14}H_{16}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of N10 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.09 ± 0.01 and 0.39 ± 0.15 respectively. Significance of difference between control and NT10 (50 µM) treated cells: *$p<0.001$ (Tukey-Kramer multiple comparisons test).

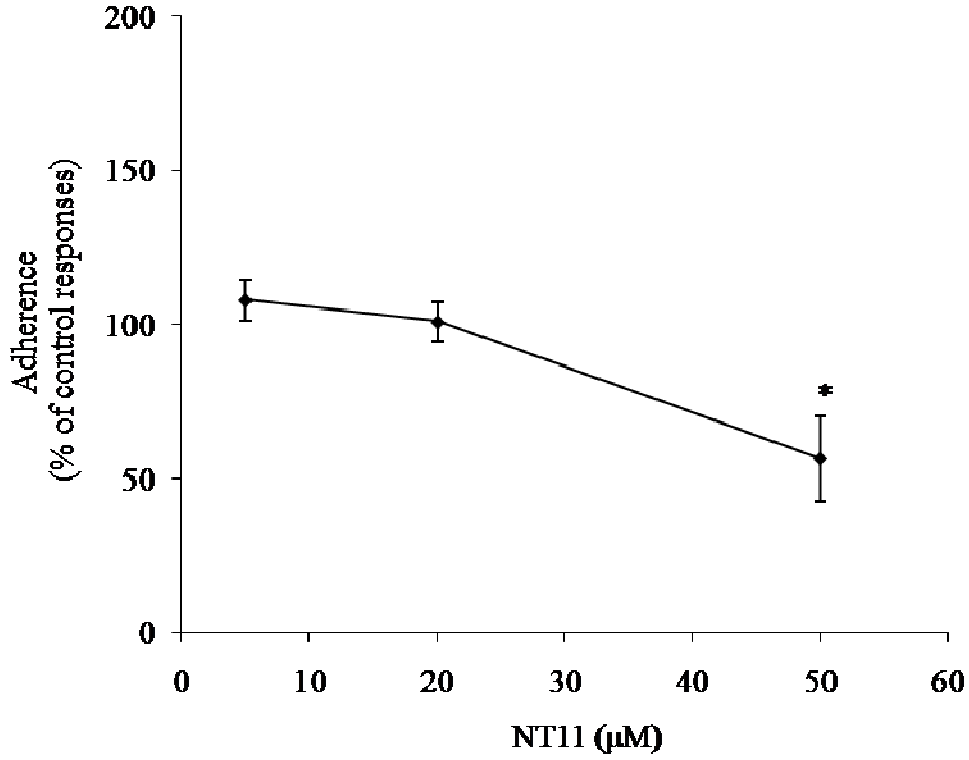


Figure 4.12 Effect of NT11 ($C_{14}H_{16}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT11 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.09 ± 0.08 and 0.46 ± 0.13 respectively. Significance of difference between control and NT11 (50 µM) treated cells: * $p<0.05$ (Tukey-Kramer multiple comparisons test).

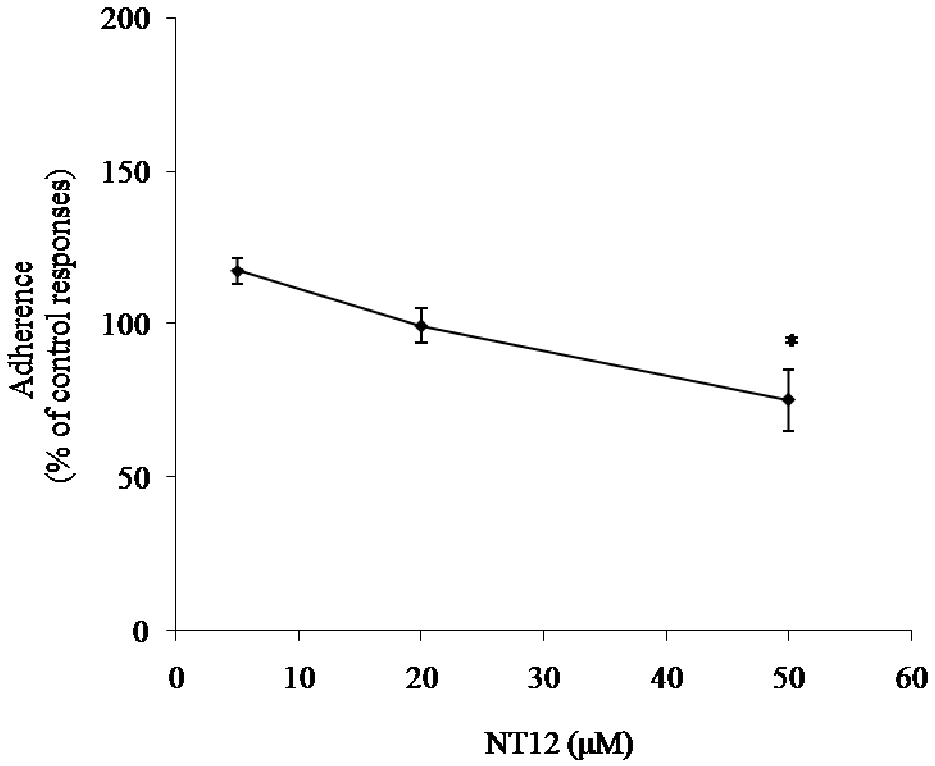


Figure 4.13 Effect of NT12 ($C_{18}H_{24}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT12 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.09 ± 0.01 and 0.28 ± 0.09 respectively. Significance of difference between control and N12 (50 µM) treated cells: *$p<0.01$ (Tukey-Kramer multiple comparisons test).

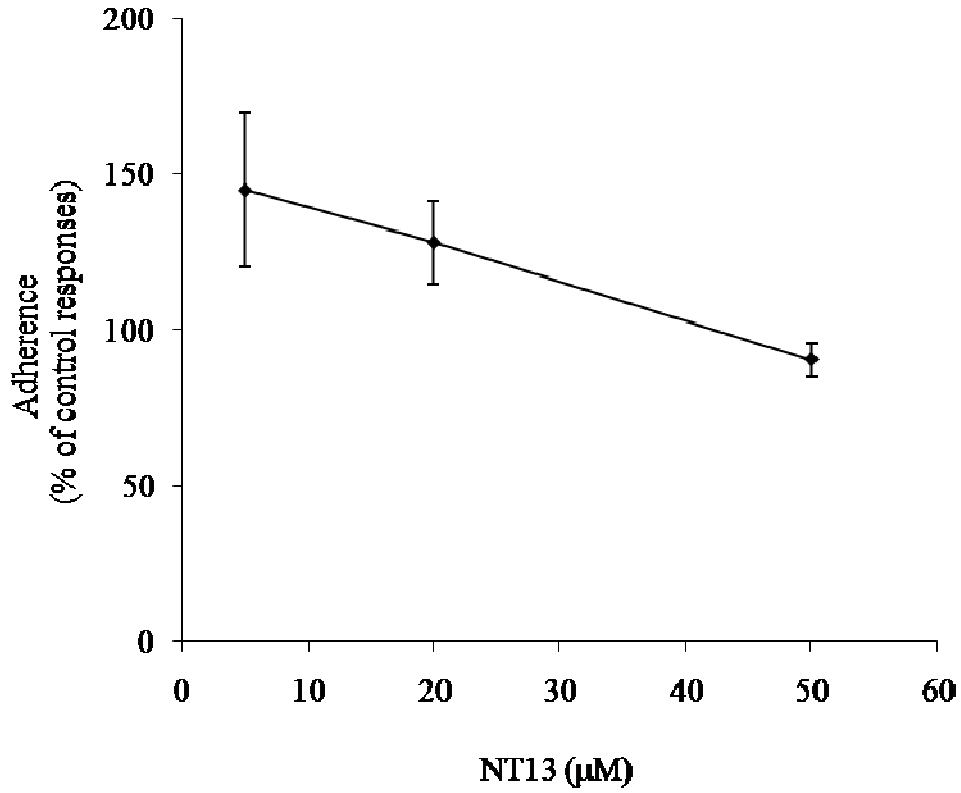


Figure 4.14 Effect of NT13 ($C_{29}H_{44}ClN_2O$) on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT13 and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.08 ± 0.01 and 0.40 ± 0.13 respectively.

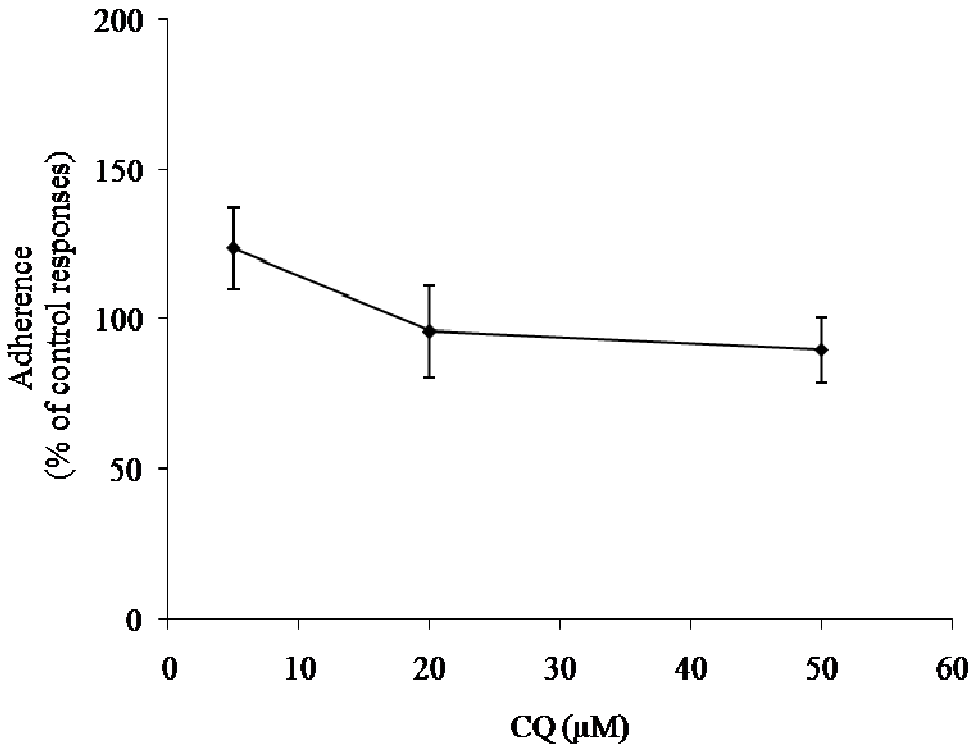


Figure 4.15 Effect of CQ ($C_{18}H_{26}ClN_3 \cdot 2H_3PO_4$)on neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of CQ and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.08 ± 0.00 and 0.49 ± 0.16 respectively.

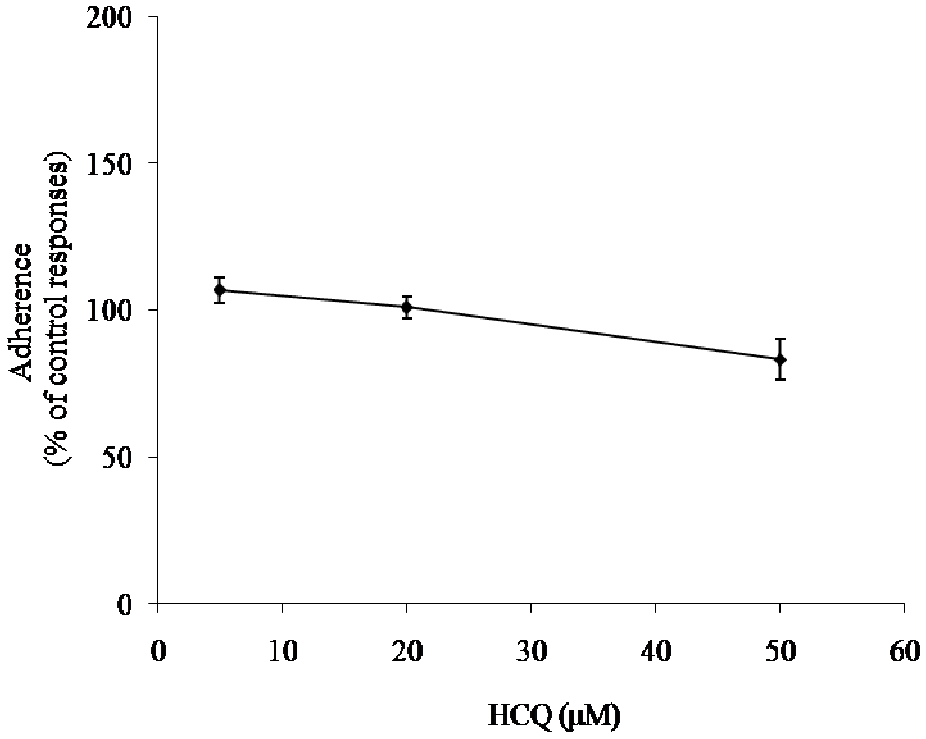


Figure 4.16 Effect of HCQ ($C_{18}H_{26}ClN_3O \cdot H_2SO_4$) on adherence assay. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of HCQ and then stimulated by adding 20 µl of TNF-RM (39 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are presented as ± SEM of 4 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.08 ± 0.01 and 0.46 ± 0.10 respectively.

Table 4.1 Summary effects of 4-amino-7-chloroquinoline based compounds on neutrophil adherence assay as shown by IC_{50} comparisons.

Compounds	**IC_{50} (μM)**
0	>50
1	38
2	>50
3	39
4	>50
5	>50
6	>50
7	>50
8	25
9	>50
10	>50
11	>50
12	>50
13	>50
CQ	>50
HCQ	>50

The IC_{50} values calculated for each compound were based on the minimal concentration required to reach a 50 percent inhibition from Figure 4.1-16. The values generated were based on adherence stimulated with TNF-RM from 4 experiments, each conducted with neutrophils from a different donor.

Table 4.2 Summary of the inhibitory effects of 4-amino-7-chloroquinoline based compounds on neutrophil adherence at 50 µM concentration.

Compounds	% inhibition
0	22
1	62
2	42
3	59
4	39
5	51
6	44
7	42
8	85
9	19
10	44
11	44
12	25
13	10
CQ	10
HCQ	17

The percentage inhibition calculated for each compound (50 µM) was based on the values generated from adherence assay stimulated with TNF-RM from 4 experiments, each conducted with neutrophils from a different donor.

Table 4.3 Summary of the effects of 4-amino-7-chloroquinoline based compounds on neutrophil basal adherence at 50 µM concentration.

Compounds	**% of control basal values ± SEM**
0	128 ± 6.15
1	106 ± 1.56
2	106 ± 7.49
3	130 ± 18.53
4	129 ± 12.96
5	120 ± 13.92
6	107 ± 2.46
7	111 ± 3.92
8	104 ± 3.24
9	123 ± 17.73
10	106 ± 4.78
11	100 ± 3.89
12	107 ± 8.99
13	98 ± 8.45
CQ	106 ± 4.58
HCQ	112 ± 7.84

The percentage of control calculated for each compound (50 µM) was based on the basal adhesion values of compound treated cells divided by basal adhesion values of vehicle. Data obtained from 4 experiments, each conducted with neutrophils from a different donor.

4.3 Comparison effects of NT8 to NT0 and lauric acid.

Since NT8 is a compound comprised of a 4-amino-7-chloroquinoline base (NT0) attached to a lauric acid side chain (12 carbon fatty acid), these two base compounds were also examined for effects on the neutrophil adherence response. Short, medium and long chain fatty acids of different degree of unsaturation have been found to have a range of effects on neutrophil functions (Naccache *et al.*, 1984; Wanten *et al.*, 2002). Naccache *et al.*, (1984) found that lauric acid stimulated rabbit neutrophil aggregation induced by fMLP. Moreover Wanten *et al.*, (2002) found that medium chain saturated fatty acids including lauric acid, induced oxygen radical production in neutrophils and that a mixture of long chain/medium chain triglyceride or pure medium chain triglycerides enhancesd neutrophil β_2 integin expression, adhesion and degranulation.

The neutrophils were treated with either NT8, lauric acid or NT8 and after (?) were stimulated with the TNF-RM. The adhesion response was measured. Ther results showed that at concentrations up to 50 µM, NT8 but neither the base compound NT0 nor lauric acid inhibited the neutrophil adherence response. Thus thee inhibitory effects of NT8 are a unique property of this compound (Figure 4.17).

4.4 Effect of NT8 on TNF induced neutrophil adherence

In another set of studies, recombinant TNF was used to stimulate neutrophil adherence. Similarly, the inhibitory effect of NT8 was also observed when TNF was used as a stimulus instead of TNF-RM (Figure 4.18).

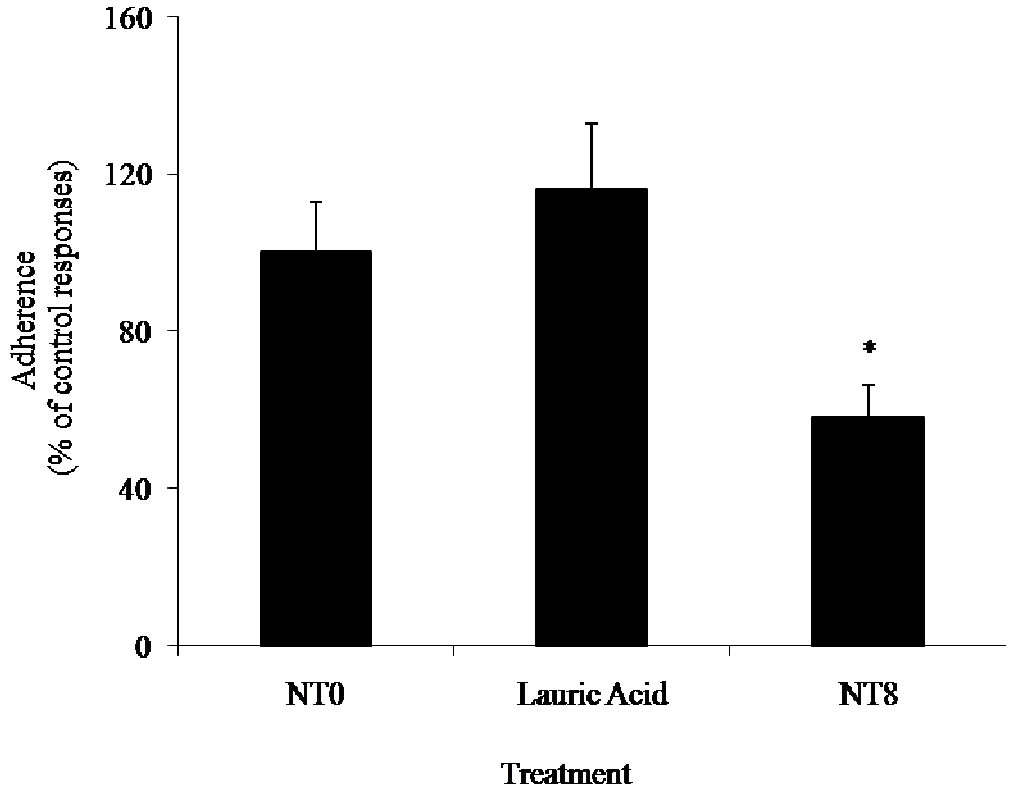


Figure 4.17 Comparison of effects of NT8, NT0 and lauric acid on TNF-RM stimulated neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated as above with 20 μM of NT8, NT0 or lauric acid and then tested for adherence in response to TNF-RM. The data are expressed as % of the control responses and presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and TNF-RM + NT8: *$p<0.05$; between control and TNF-RM + NT8: *$p<0.05$ (Dunnett: compare all vs control).

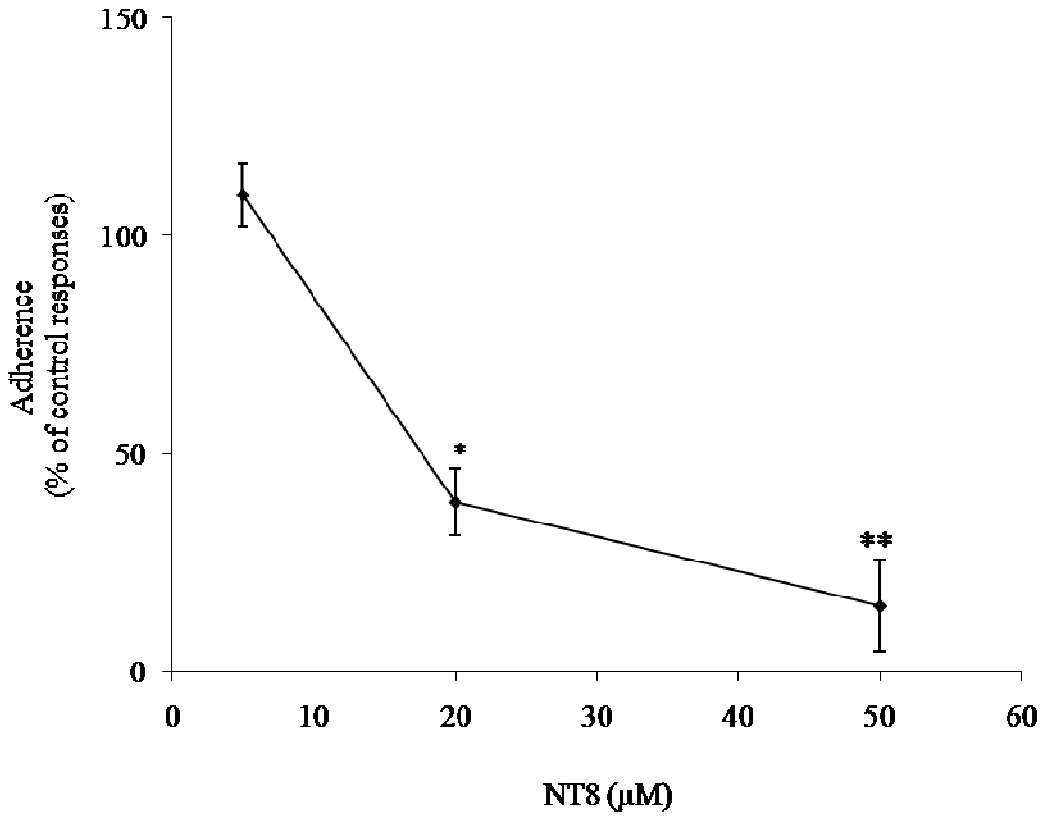


Figure 4.18 Effect of NT8 on TNF-mediated neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with varying concentrations of NT8 and then stimulated by adding 20 µl of recombinant TNF (100 U/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. Results represent the mean data ± SEM of 3 experiments, each conducted with neutrophils from a different donor. The basal and stimulated OD 570 nm values ± SEM were 0.08 ± 0.01 and 0.27 ± 0.05 respectively. Significance of difference between control and NT8 (20 µM) treated cells; between control and NT8 (50 µM) treated cells: *$p<0.01$, **$p<0.001$ respectively (Tukey-Kramer multiple comparisons test).

4.5 Summary

We have now demonstrated that these new quinoline compounds have biological effects. Exposure of neutrophils to these compounds resulted in a decrease in the ability of neutrophils to respond to TNF-induced adherence. The type of fatty acid added to the quinoline side chain made a significant difference to this activity, which ranged from no effect to a highly significant effect over a concentration range of 5-50 μM (Table 4.1). The most effective of the 13 compounds were NT1, NT3 and NT8. Since NT8 had the greatest inhibitory effect, further studies were conducted with the compound.

The data demonstrated that the activity of NT8 was due to the new structure synthesised as neither of the individual components, NT0 or lauric acid showed inhibitory activity. The inhibitory effects were seen when TNF-RM was used as the stimulator as well as recombinant TNF. However, the effects of NT8 on neutrophils stimulated with recombinant TNF were more pronounced. This suggested that NT8 may have preference for TNF-induced responses. Under the different concentrations used in the experiments, the cells maintained normal viability tested by their ability to exclude trypan blue.

5.0 CHAPTER 5 INHIBITORY EFFECTS OF NT8 ON TNF-INDUCED NEUTROPHIL RESPIRATORY BURST, MIGRATION INHIBITION AND CYTOKINE PRODUCTION

5.1 Introduction

Having established that NT8 was the most effective compound in inhibiting neutrophil adhesion (Chapter 4), we sought to gain a better understanding of the effects of NT8 on other neutrophil functions relevant to the inflammatory response, the oxygen-dependent respiratory burst, migration inhibition and cytokine production. Previous studies using quinoline derived inhibitors have found that primaquine and quinacrine inhibited the fMLP induced superoxide production at a concentration rage of 50-250 μM (Neal *et al.*, 1987). Chloroquine, under the same condition, was only affective at 1 mM (Neal *et al.*, 1987). Moreover Hurst *et al.,* (1988) reported that both CQ and HCQ showed an inhibitory effect on zymosan-, PMA- and fluoride-induced superoxide generation in neutrophil at 100 μM. The inhibitory effect of CQ was extended to chemotaxis. Kharazmi *et al*., (1983) found that CQ inhibited chemotaxis at concentrations attained in clinical situations.

Neutrophils are also known to produce cytokines that are important in activating other cell types. A wide range of cytokines such as IL-1, TNF, IFN-γ, IL-8, etc, have been shown to be produced by neutrophils. In this manner, neutrophils may also control the adaptive immune response. Because of the importance of TNF in the inflammatory mediator network, it was the aim of this studt to examine the effects of NT8 on TNF-induced respiratory burst, cell migration inhibition and cytokine production.

5.2 Effect of NT8 on the oxygen-dependent respiratory burst

It is widely accepted that the respiratory burst can be measured by the lucigenin-dependent chemiluminescence assay which measures reactive oxygen species (ROS), in particular superoxide (Alves *et al.*, 2003). This assay method was used to examine the effects of NT8 on the respiratory burst in neutrophils as described in Chapter 2.11.3.

The cells were pre-treated with either 20 or 50 μM of NT8 for 1 h and then stimulated with TNF-RM. The resultant chemiluminescence produced was measured. The data presented in Figure 5.1 and 5.2 show that TNF-RM induced a marked respiratory burst (6 fold increase in chemiluminescence). NT8 had no direct effect on the response but inhibited the ability of TNF-RM to induce a response, by approximately 50 % at 50 μM concentration. No effect was seen at 20 μM. The kinetics of the chemiluminescence response is presented in Figure 5.2. The data demonstrates that NT8 caused both a delay in response and a reduction in peak rate of chemiluminescence production at 50 μM.

5.3 The effects of NT0 and lauric acid on the neutrophil respiratory burst

To see if the activity seen at 50 μM of NT8 was due to the unique structure of the generated molecule, the effects of the individual components, NT0 and lauric acid on neutrophil respiratory burst was examined. The data showed that while neutrophils treated with NT8 were not significantly depressed in their response to TNF-RM, neither NT0 nor lauric acid had any effect when examined at 50 μM concentration (Figure 5.3).

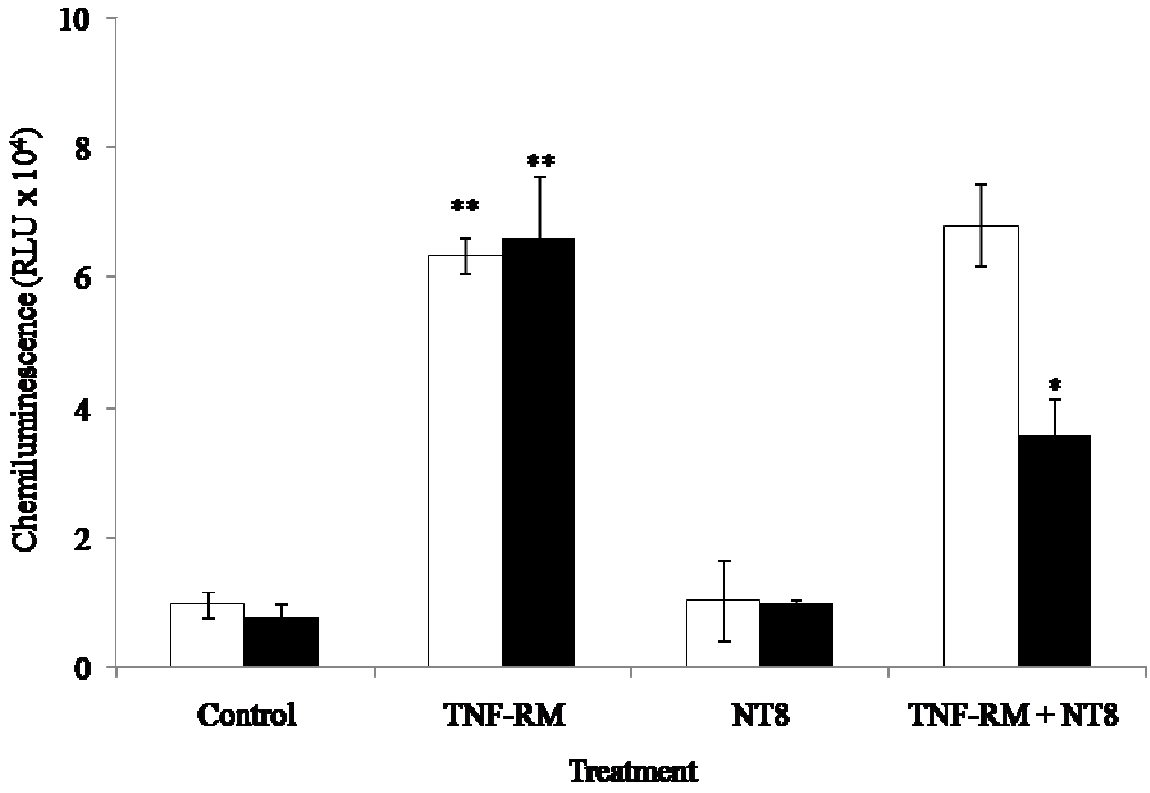


Figure 5.1 Effect of NT8 on the oxygen-dependent respiratory burst induced by TNF-RM. Neutrophils (5 x 10^6/ml) were pre-treated with either 20 μM (□) or 50 μM (■) NT8 for 1 h and then stimulated by adding 100 μl of TNF-RM (39 ng/ml). The resultant chemiluminescence was then measured. The data represent initial peak rates of chemiluminescence and are presented as the mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and TNF-RM treated cells: **$p<0.01$ and between stimulated and stimulated cells pretreated with NT8: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

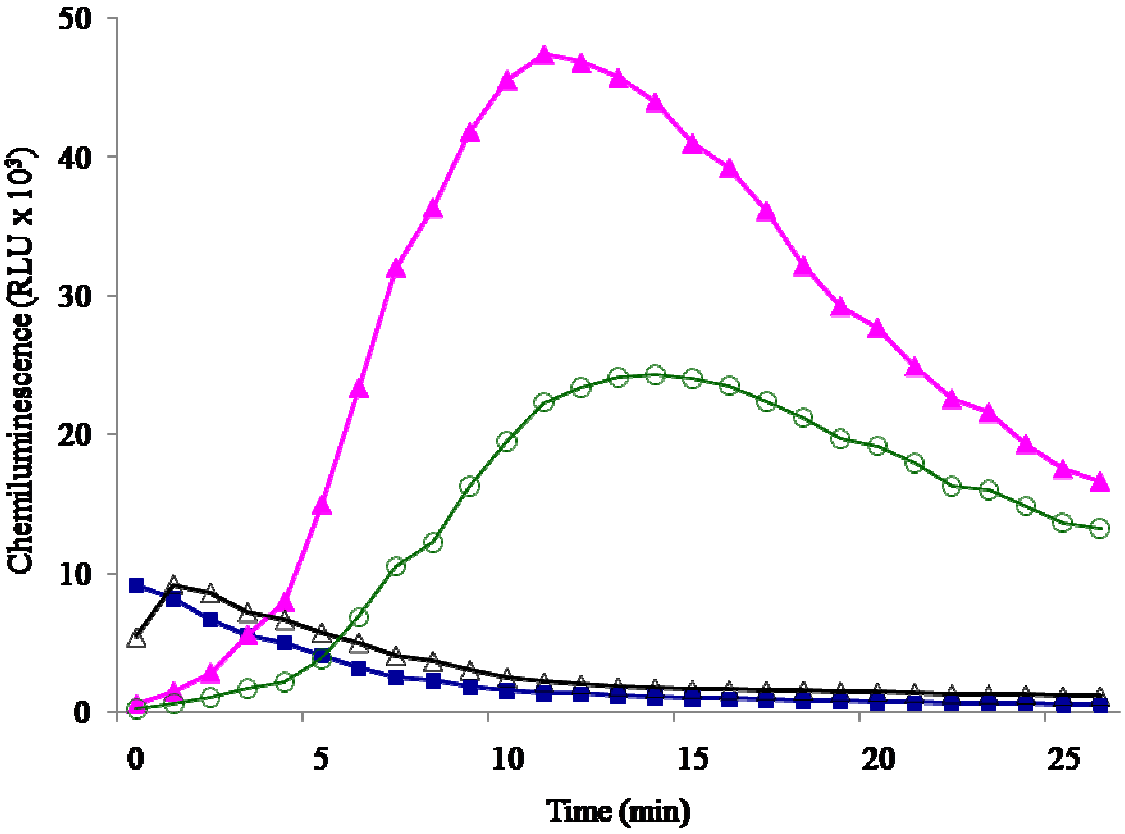


Figure 5.2 Effect of NT8 on the kinetics of TNF-RM induced change in chemiluminescence. The results are a representative experiment of data presented in Figure 5.1. Neutrophils + diluents (■), neutrophils + TNF-RM (▲), neutrophils + NT8 (Δ) and neutrophils + TNF-RM + NT8 (○).

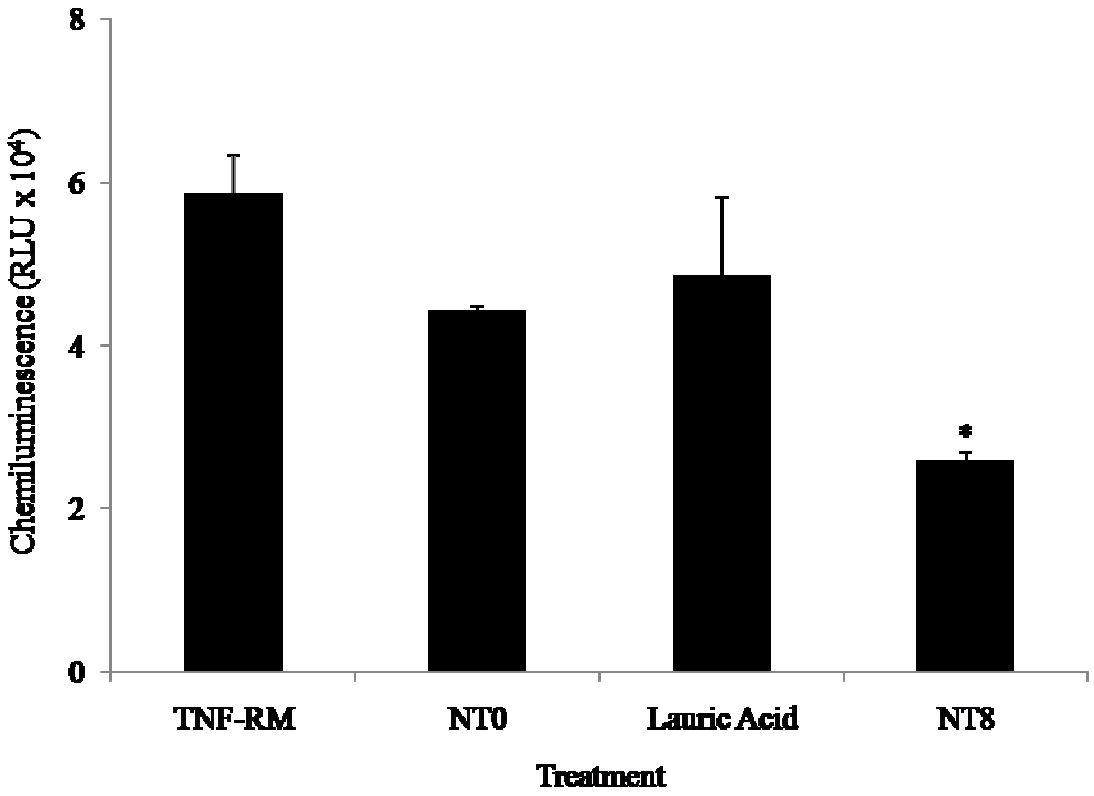


Figure 5.3 Effect of NT0 and lauric acid on the neutropil respiratory burst. Neutrophils (5 x 10^6/ml) were pre-treated with 50 μM of NT8, NT0 or lauric acid for 1 h and then stimulated by adding 100 μl of TNF-RM (39 ng/ml) and the resultant chemiluminescence measured. The data represent initial peak rates of chemiluminescence and are presented as the mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between TNF-RM and TNF-RM + NT8 treated group: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

5.4 The effect of NT8 on the respiratory burst induced by recombinant TNF

While the TNF-RM represents a medium culture that mimics cytokine combination most likely to be found in bacteria-induced inflammation, the modulatory effects of NT8 can be better defined using TNF. In these experiments the neutrophils were pre-treated with 20 or 50 μM of NT8 and then stimulated with recombinant TNF. The results showed that TNF caused direct stimulation of the neutrophil respiratory burst of approximately 4 fold above the base activity (Figure 5.4, 5.5). At 50 μM but not 20 μM, NT8 caused significant suppression of this response (Figure 5.4, 5.5). Examination of kinetics of chemiluminescence produced showed that NT8 caused a delay in response and a decreased peak initial rate (Figure 5.5).

5.5 The effects of CQ and HCQ on the neutrophil respiratory burst response

Since the basis for the use of NT8 compound is its relationship to CQ and HCQ as anti-inflammatory agents, we examined the effects of these agents on the neutrophil respiratory burst (chemiluminescence). The results showed that neither CQ nor HCQ at 50 μM significantly inhibited the TNF-induced neutrophil respiratory burst (Figure 5.6). The kinetics of the responses are shown in Figure 5.7. The kinetics of rates of chemiluminescence produced were also not affected by either CQ or HCQ.

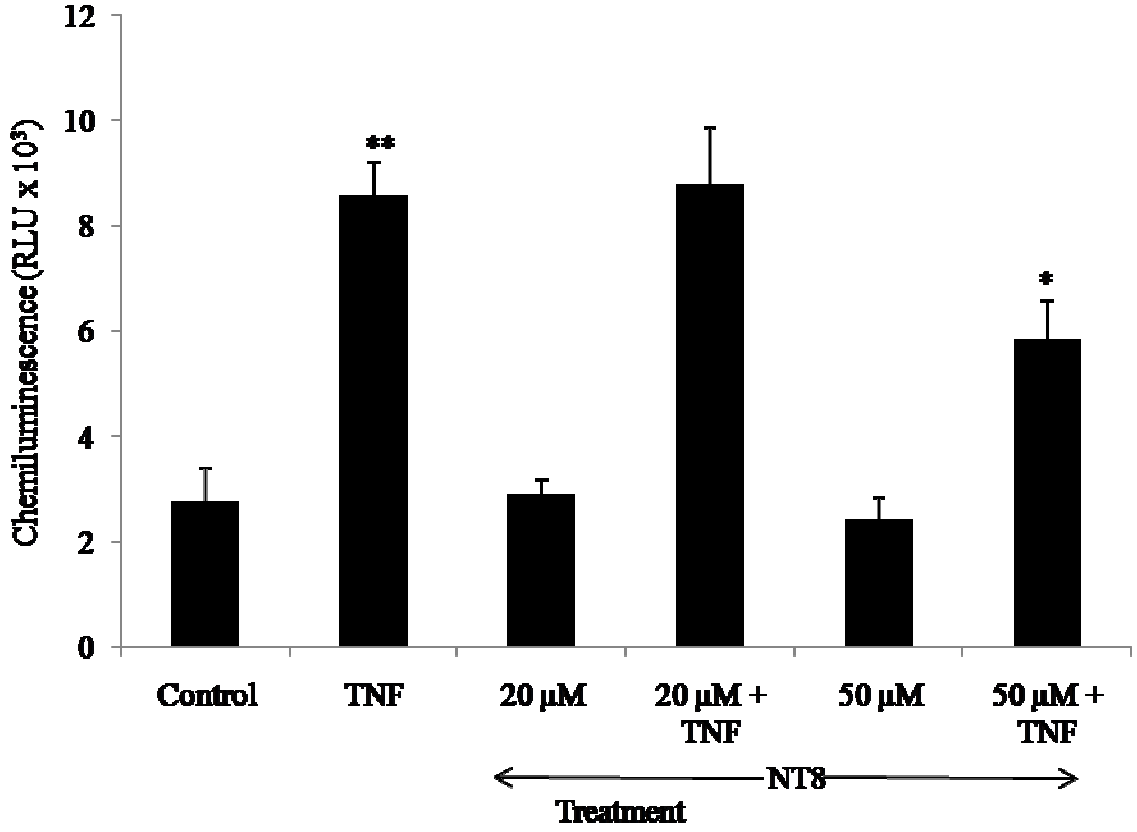


Figure 5.4 Effect of NT8 on TNF-induced chemiluminescence. Neutrophils (5 x 10^6/ml) were pre-treated with either 20 or 50 µM NT8 for 1 h and then stimulated by adding 100 µl of recombinant TNF (100 U/ml). The resultant chemiluminescence was measured. The data represent initial peak rates of chemiluminescence and are presented as the mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and TNF treated cells: **$p<0.001$ and between stimulated and stimulated cells pretreated with NT8 (50 µM): *$p<0.05$ (Tukey-Kramer multiple comparisons test).

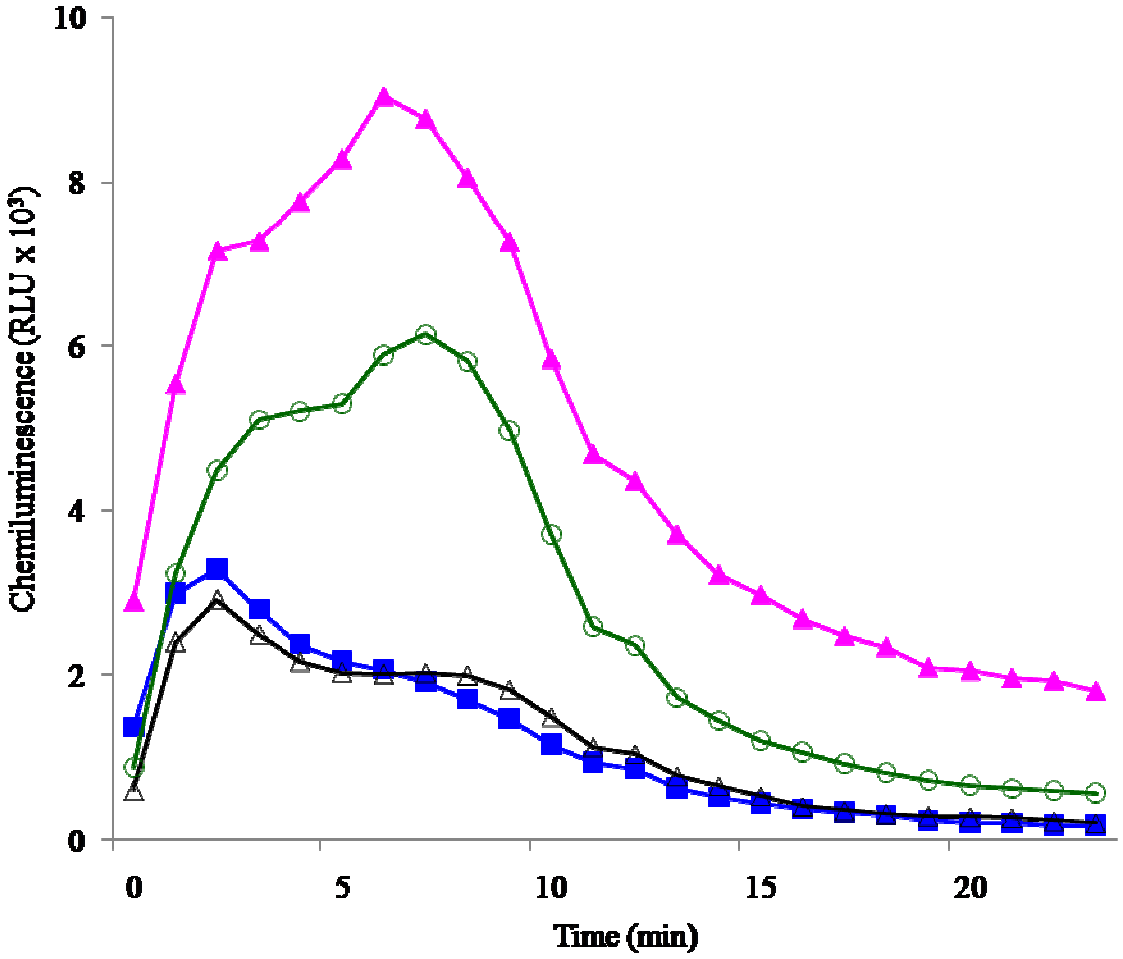


Figure 5.5 Effect of NT8 on the kinetics of TNF-induced change in chemiluminescence. The results are a representative experiment of data presented in Figure 5.4. Neutrophils + diluents (■), neutrophils + TNF (▲), neutrophils + NT8 (Δ) and neutrophils + TNF + NT8 (○).

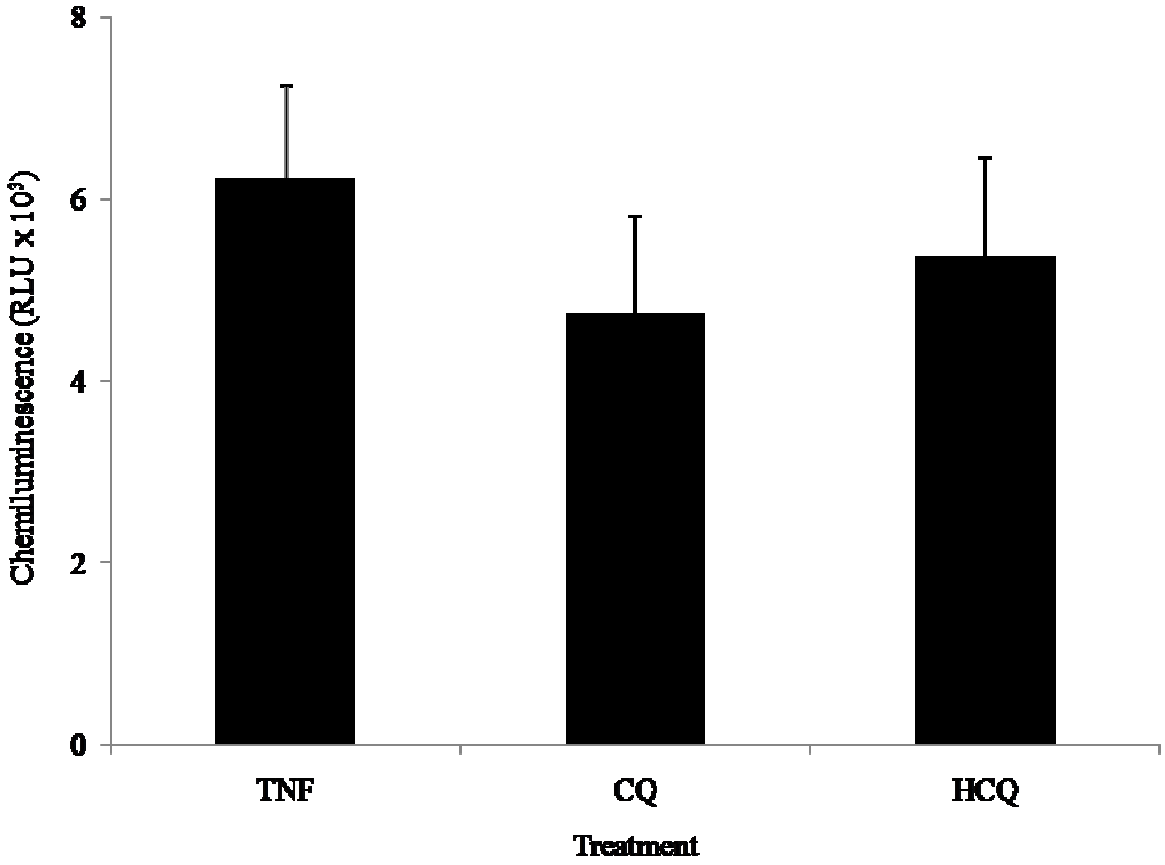


Figure 5.6 Effect of CQ and HCQ on TNF-induced neurophil respiratory burst. Neutrophils (5 x 10^6/ml) were pre-treated with 50 µM of either CQ or HCQ for 1 h and then treated with 100 µl of TNF (100 U/ml). The resultant chemiluminescence production was measured in a luminometer. The data represent initial peak rates of chemiluminescence and are presented as the mean ± SEM of 4 experiments, each conducted with neutrophils from a different donor.

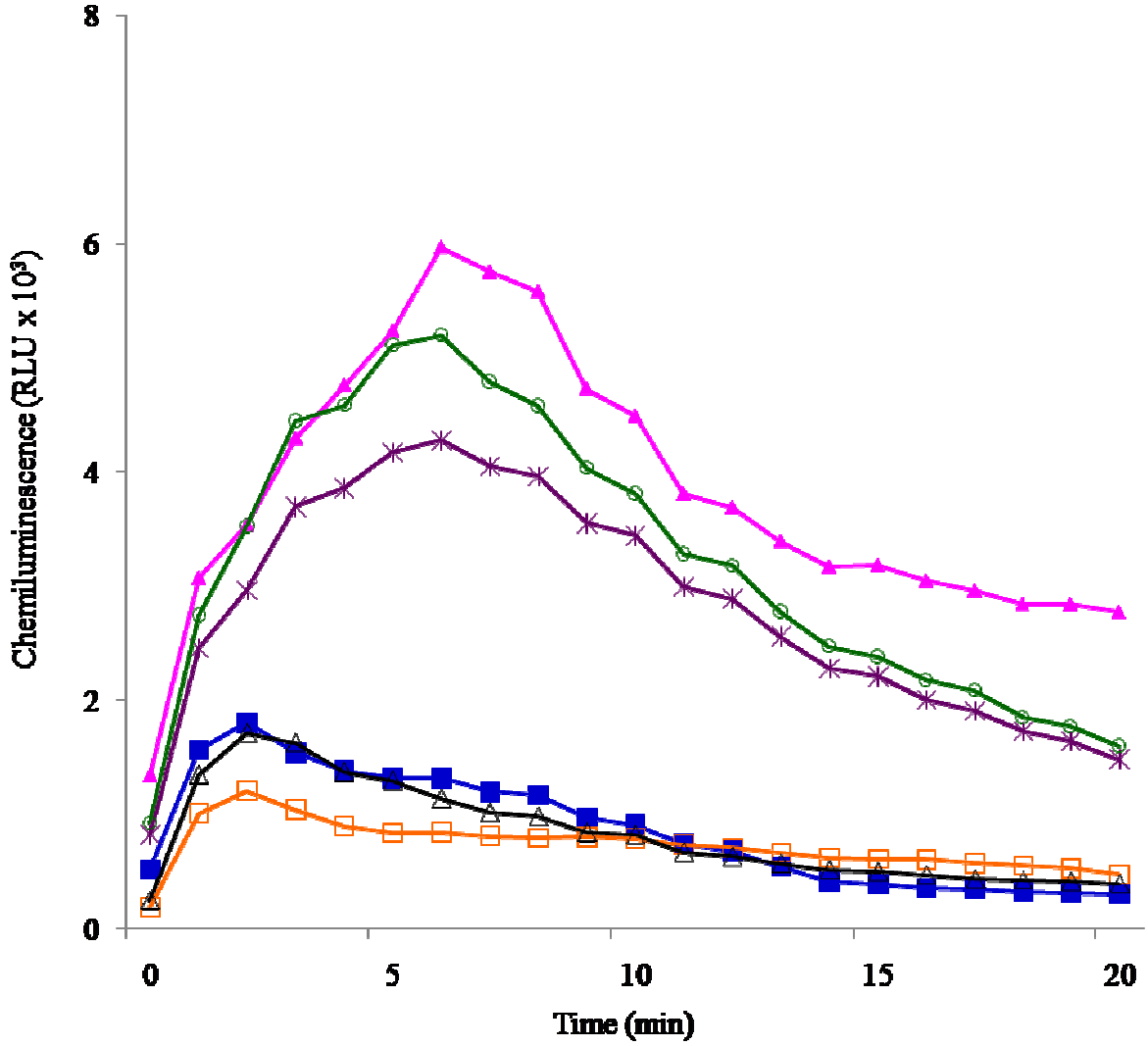


Figure 5.7 Effect of CQ and HCQ on the kinetics of TNF-induced change in chemiluminescence. The results are a representative experiment of data presented in Figure 5.6. Neutrophils + diluents (■), neutrophils + TNF (▲), neutrophil + CQ (□), neutrophil + TNF + CQ (×), neutrophils + HCQ (Δ) and neutrophils + TNF + HCQ (○).

5.6 Effect of NT8 on neutrophil random movement

In our experiment, random migration was assessed using the agarose method described in Chapter 2.11.2. Neutrophils were allowed to migrate across the agarose supplemented with heat inactived fetal calf serum. Our results showed that treating neutrophils with either 20 or 50 µM NT8 had no significant effect on random migration (Figure 5.8). The base compounds NT0 and lauric acid as well as CQ showed no inhibitorys effect at 50 µM.

5.7 Effect of NT8 on the neutrophil migration inhibiton induced by TNF

Another major function of TNF is its ability to inhibit neutrophil migration (Ferrante *et al.*, 1988) which presumably represents a mean by which cells are retained at the inflammatory foci. The effect of NT8 on TNF-induced migration inhibition was examined. Neutrophils were treated with TNF (300 U) for 30 min and then examined for migration. TNF inhibited random migration (Figure 5.9). NT8 had no effect on the ability of TNF to induce migration inhibition at both 20 and 50 µM (Figure 5.9, 5.10). TNF also inhibits the fMLP-induced chemotactic migration of neutrophils. The effect of NT8 on this response was examined. The neutrophils were pre-treated with NT8 and then with TNF for 30 min prior to examination of chemtactic migration in response to fMLP. The results showed that NT8 had no significant effect on the ability of TNF to inhibit neutrophil migration induced by fMLP (Figure 5.9, 5.10).

These results suggested that the cell migration inhibition properties of TNF were not affected by NT8. Again demonstrating some selective effects of NT8 on the TNF-induced adherence response of neutrophils.

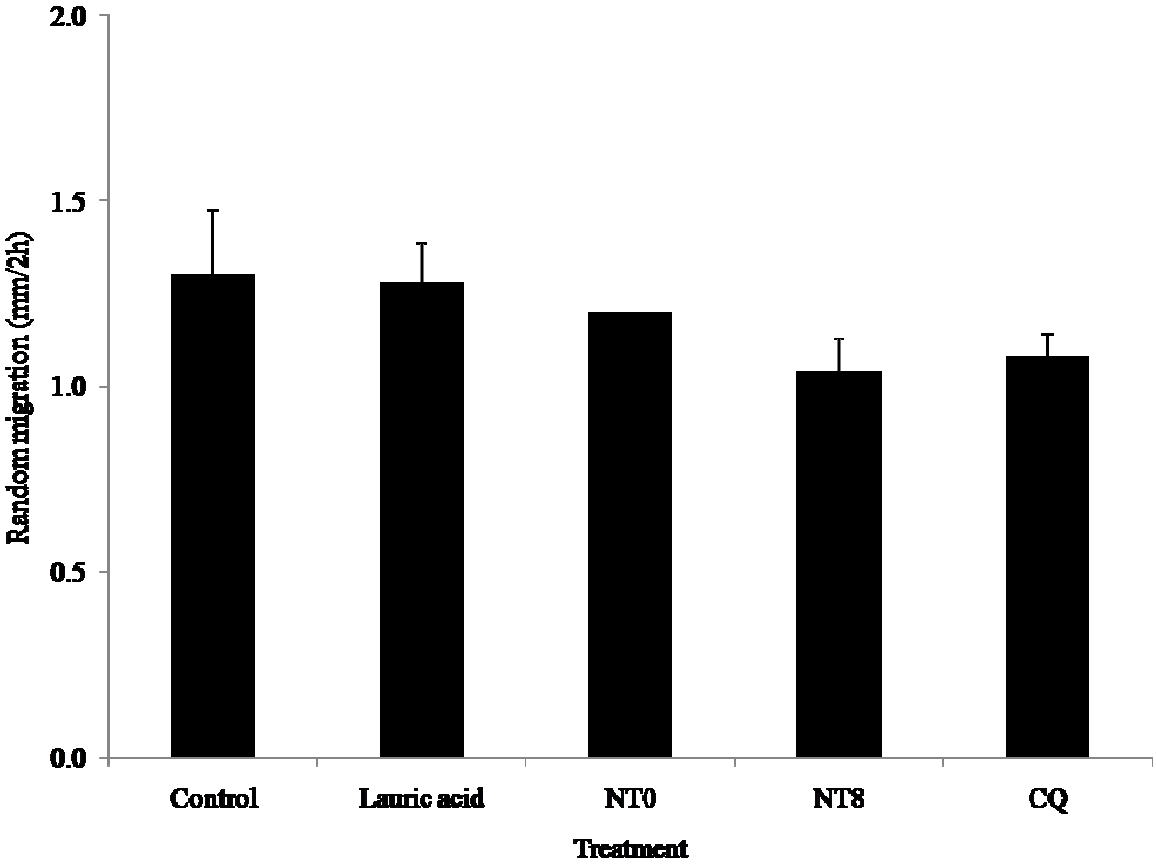


Figure 5.8 Comparison of effect of NT0, lauric acid and NT8 on neutrophil random migration. Neutrophils (5 x 10^6/ml) were pre-treated with either lauric acid, NT0, NT8 or CQ at 50 μM for 1 h and then tested for random migration. Data are expressed as the mean ± SEM from 3 experiments, each conducted with neutrophils from a different donor.

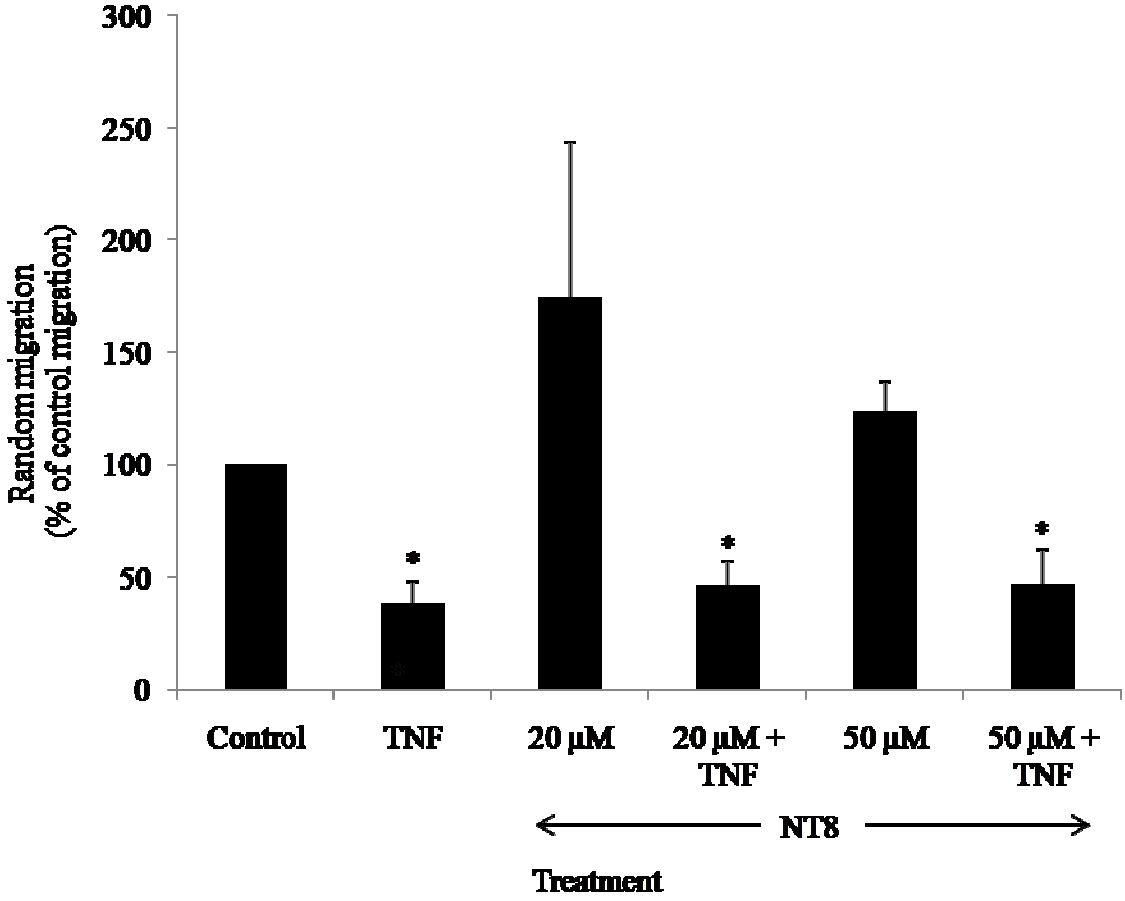


Figure 5.9 Effect of NT8 on TNF-induced inhibition of neutrophil random migration. Neutrophils (5 x 10^6/ml) were pre-treated with NT8 at 20 or 50 µM for 1 h and then stimulated by adding 160 µl of TNF (300 U/ml) for 30 min. The random migration was then measured. Data are expressed as % of control migration ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and TNF treated groups with and without the NT8 treatment: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

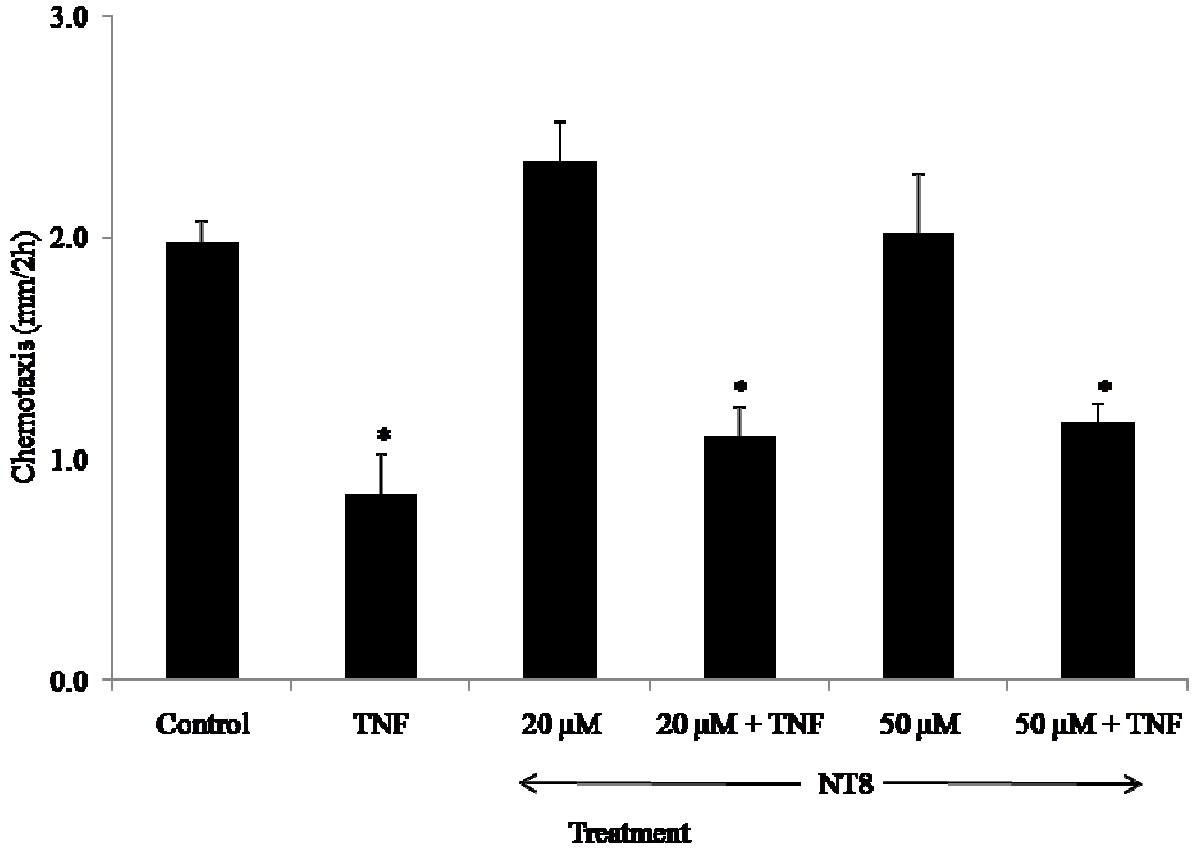


Figure 5.10 Effect of NT8 on TNF-induced inhibition of neutrophil chemotaxis. Neutrophils (5 x 10^6/ml) were pre-treated with NT8 at 20 or 50 µM for 1 h and then stimulated by adding 160 µl TNF (300 U/ml) for 30 min. Migration in response to fMLP was then measured. Data are expressed as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and TNF treated groups with and without the NT8 treatment: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

5.8 Effect of NT8 on TNF-induced IL-1β and IL-8 production by neutrophils

Cytokines play an important role as mediators of various inflammatory conditions. There is little known however about the effects of chloroquine on neutrophil cytokine production. Previous reports have examined the effects of CQ on LPS induced production of cytokines in PBMCs. The agents inhibited TNF-α, IL-1β and IL-6 production (Karres *et al.*, 1998; Picot *et al.*, 1993; Weber and Levitz, 2000). Since our previous results showed that NT8 inhibits TNF induced adherence and chemiluminescence, the effects of NT8 on neutrophil function was extended to cytokine production.

Neutrophils were pre-treated with NT8 and then stimulated with TNF. After 2 h, the amount of IL-1β and IL-8 mRNA was measured using the methods described in Chapter 2.12. The results showed that NT8 inhibited the production of both IL-1β and IL-8 at 20 and 50 μM of the compound (Figure5.11, 5.12). The individual compounds NT0 and lauric acid did not suppress IL-1β and IL-8 mRNA production (Figure 5.13, 5.14).

5.9 Effect of CQ on TNF-induced IL-1β and IL-8 production in neutrophils

The effects of CQ on neutrophil cytokine production have not been previously investigated; therefore we also examined the effects. Results showed that CQ did not cause a significant decrease in IL-1β and IL-8 mRNA production (Figure 5.15, 5.16).

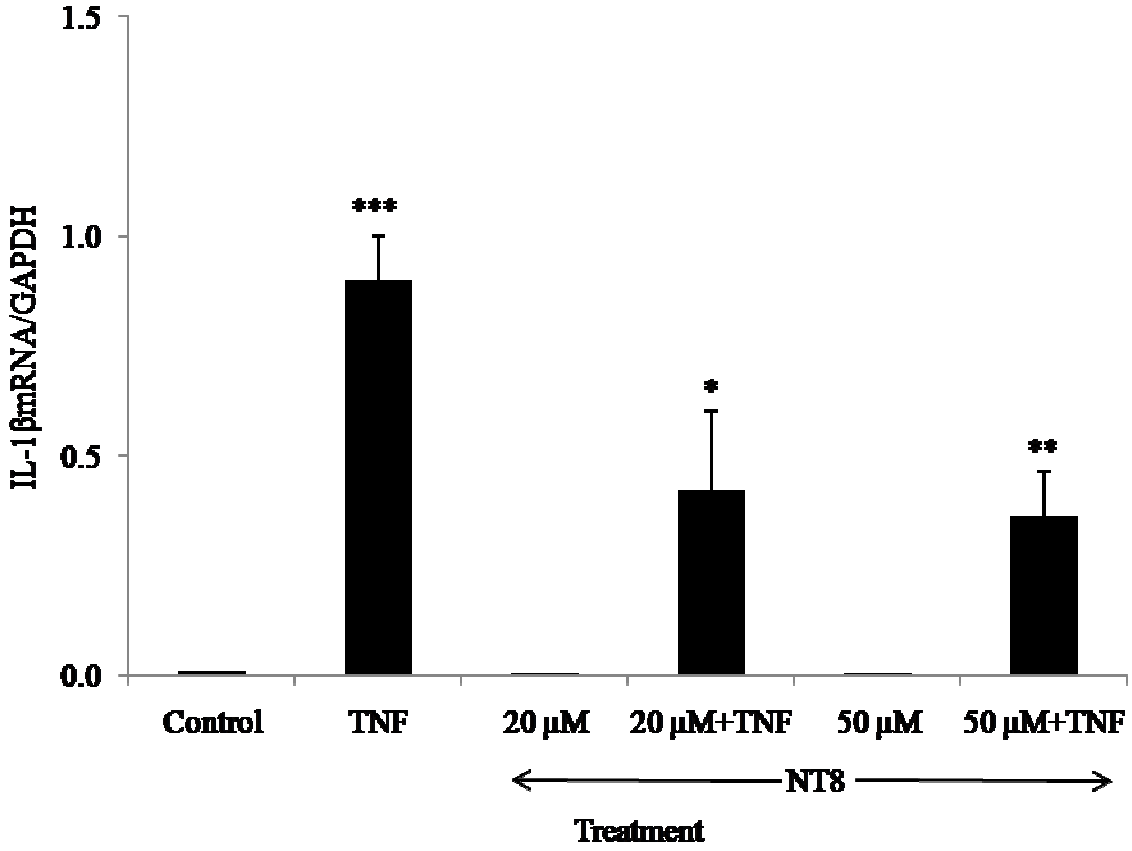


Figure 5.11 Effect of NT8 on TNF-induced IL-1β mRNA in neutrophils. Neutrophils (5 x 10^6/ml) were pre-treated with either 20 or 50 µM of NT8 and then stimulated with TNF for 1 h. Levels of IL-1β were determined by quantitative real-time PCR. The housekeeping gene GAPDH was used to normalize the samples. Data are expressed as amount of mRNA produced ± SEM from 3 experiments. Significance of difference between control and TNF treated cells and between stimulated and stimulated cells pretreated with NT8: *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Tukey-Kramer multiple comparisons test).

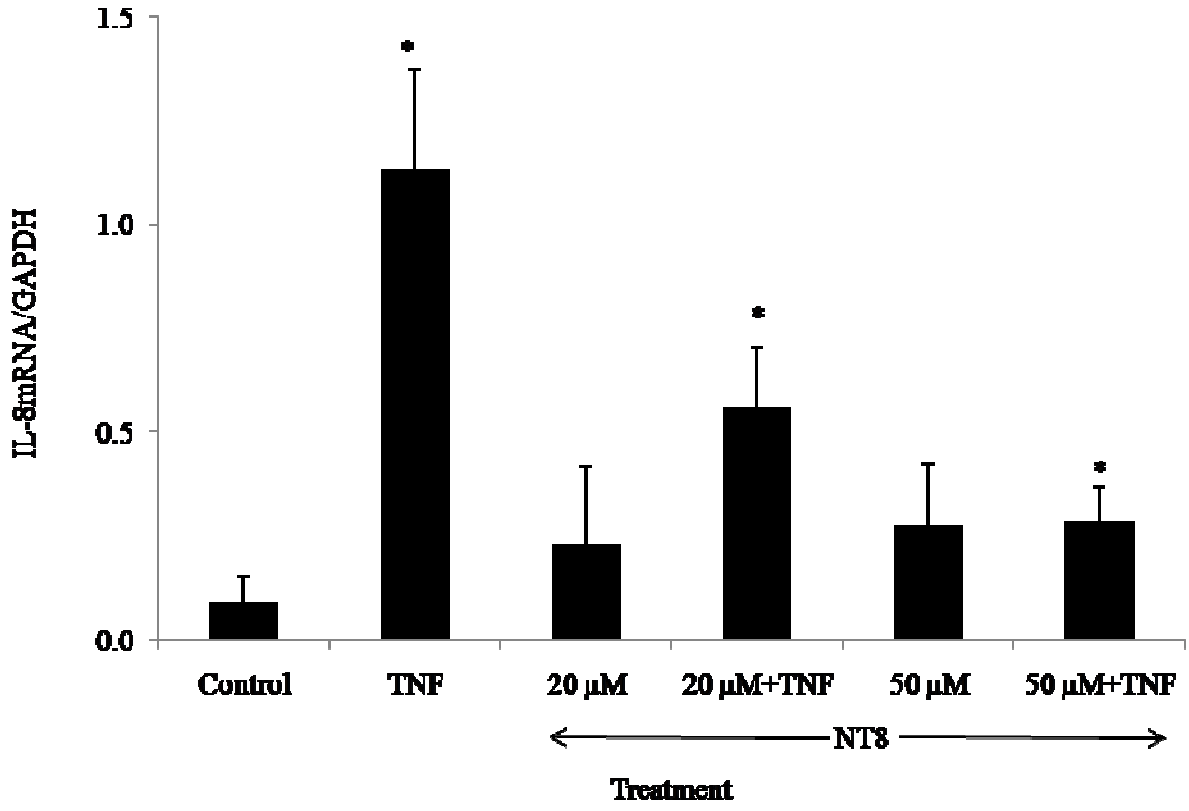


Figure 5.12 Effect of NT8 on TNF-induced IL-8 mRNA in neutrophils. Neutrophils (5 x 10^6/ml) were pre-treated with 20 or 50 µM of NT8 and then stimulated with TNF for 1 h. Levels of IL-8 were determined by quantitative real-time PCR. The housekeeping gene GAPDH was used to normalize the samples. Data are expressed as amount of mRNA produced ± SEM from 3 experiments. Significance of difference between control and TNF treated cells and between stimulated and stimulated cells pretreated with NT8: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

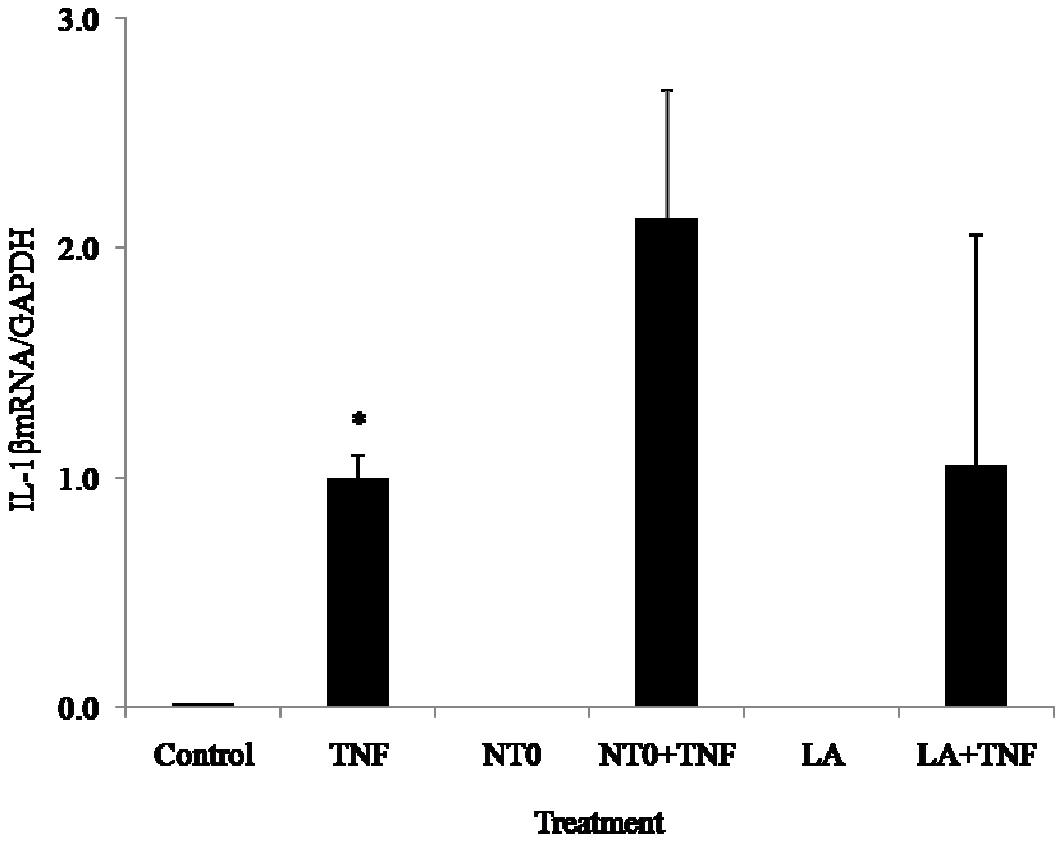


Figure 5.13 Effect of NT0 and LA on TNF-induced IL-1β mRNA in neutrophils. Neutrophils (5 x 10^6/ml) were pre-treated with either lauric acid or NT0 (50 μM) and then stimulated with TNF for 1 h. Levels of IL-1β were determined by quantitative real-time PCR. The housekeeping gene GAPDH was used to normalize the samples. Data are expressed as amount of mRNA produced ± SEM from 3 experiments. Significance of difference between control and TNF: *$p<0.05$.

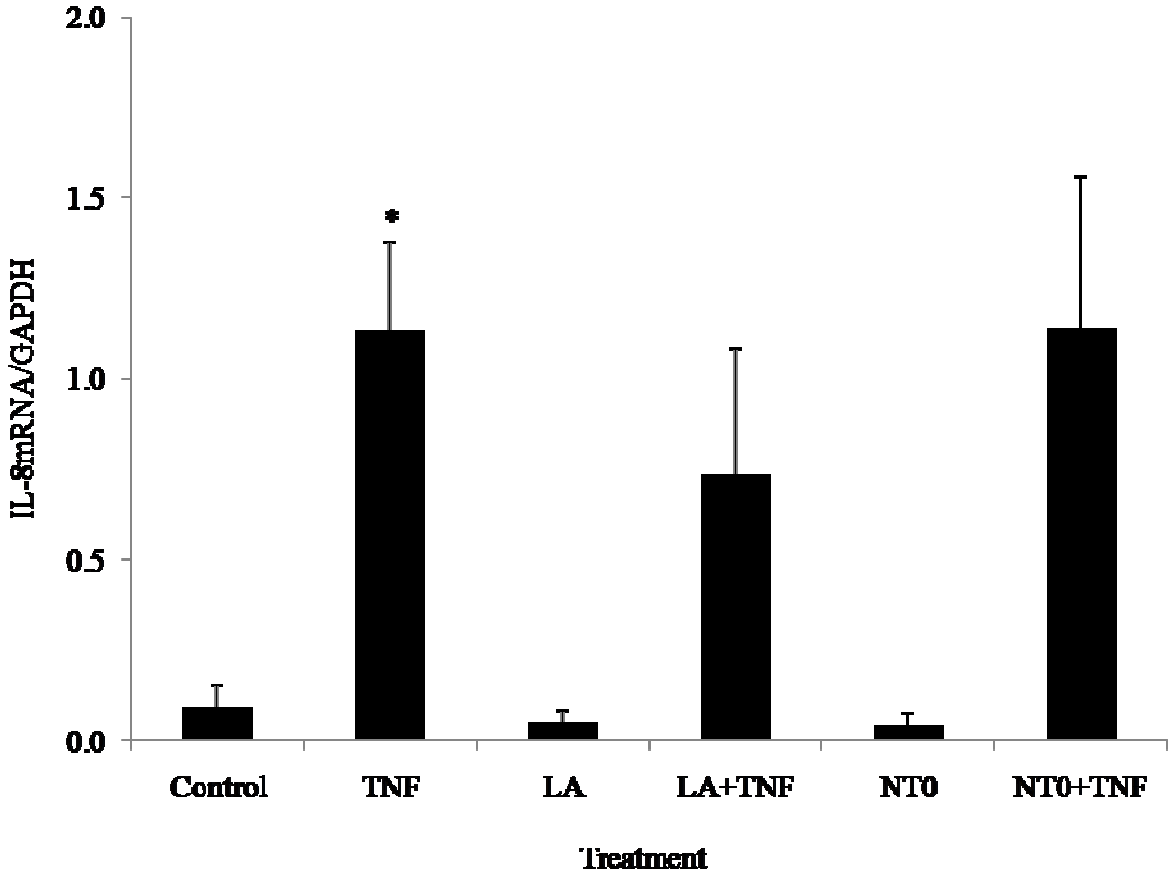


Figure 5.14 Effect of LA or NT0 on TNF-induced IL-8 mRNA in neutrophils. Neutrophils (5 x 10^6/ml) were pre-treated with either lauric acid or NT0 (50 μM) and then stimulated with TNF for 1 h. Levels of IL-8 were determined by quantitative real-time PCR. The housekeeping gene GAPDH was used to normalize the samples. Data are expressed as amount of mRNA produced ± SEM from 3 experiments. Significance of difference between control and TNF: *$p<0.05$.

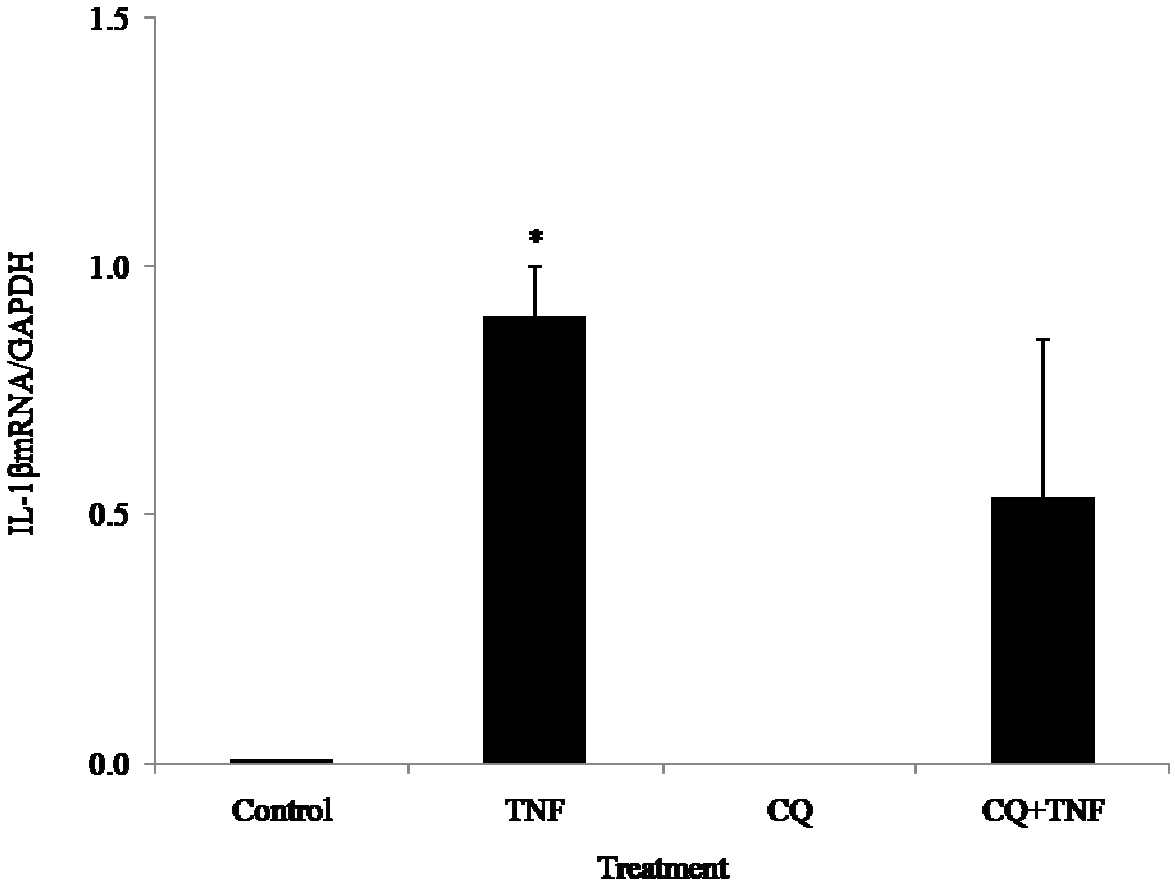


Figure 5.15 Effect of CQ on TNF-induced IL-1β mRNA in neutrophils. Neutrophils (5 x 10^6/ml) in RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 % AB serum were pre-treated with CQ (50 μM) and then stimulated with TNF for 1 h. Levels of IL-1β were determined by quantitative real-time PCR. The housekeeping gene GAPDH was used to normalize the samples. Data are expressed as amount of mRNA produced ± SEM from 3 experiments. Significance of difference between control and TNF: *$p<0.05$.

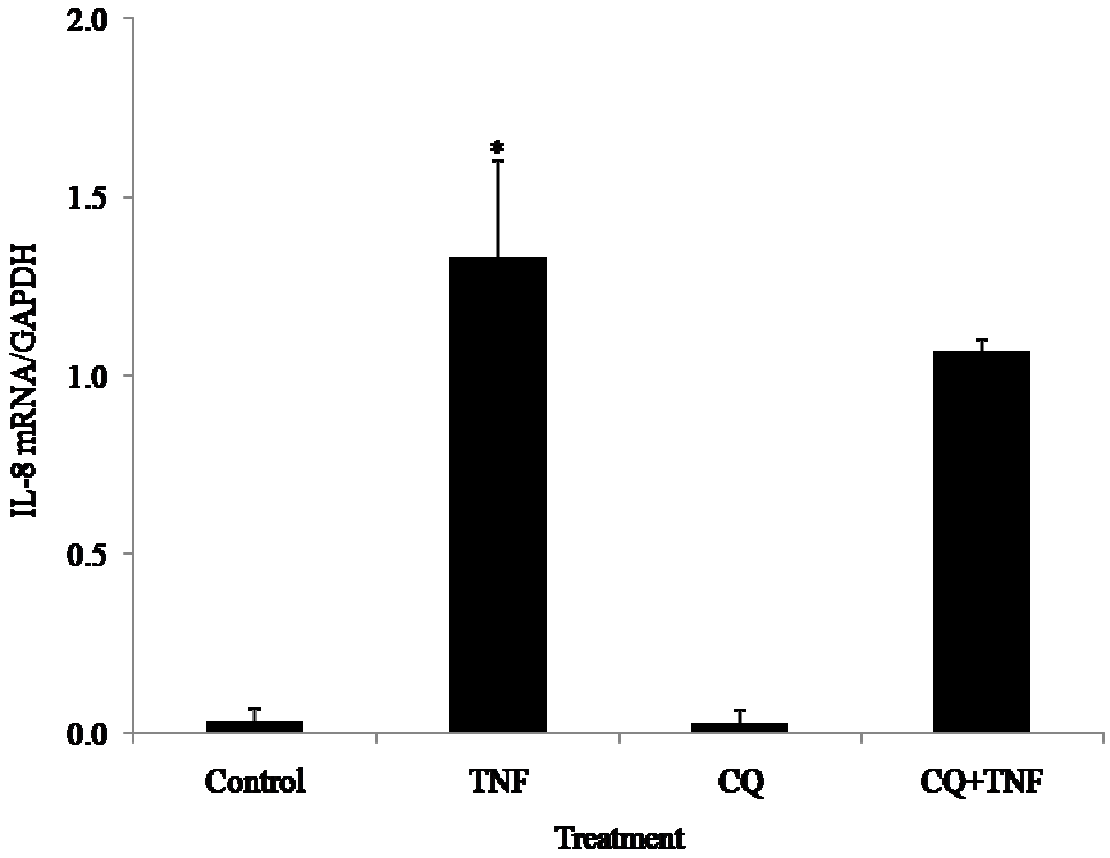


Figure 5.16 Effect of CQ on TNF-induced IL-8 mRNA in neutrophils. Neutrophils (5 x 10^6/ml) in RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 % AB serum were pre-treated with CQ (50 μM) and then stimulated with TNF for 1 h. Levels of IL-8 were determined by quantitative real-time PCR. The housekeeping gene GAPDH was used to normalize the samples. Data are expressed as amount of mRNA produced ± SEM from 3 experiments. Significance of difference between control and TNF: *$p<0.05$.

5.10 Summary

NT8 significantly inhibited the TNF or TNF-RM induced respiratory burst in neutrophils. However, this was evident only at 50 µM NT8. At the same concentration neither NT0 nor lauric acid had any effect. It is also interestingly that CQ and HCQ had no effect on this neutrophil function at 50 µM.

Results also showed that NT8 had no effect on the TNF-induced inhibition of neutrophil random migration or chemotactic migration.

In addition to this, the data demonstrated that the TNF response of neutrophils in relation to production of IL-1β and IL-8 mRNA is highly sensitive to treatment with NT8 but not CQ.

6.0 CHAPTER 6 SELECTIVITY OF THE INHIBITORY EFFECTS OF NT8 FOR THE TNF-INDUCED RESPONSES

6.1 Introduction

To gain a better understanding of how NT8 affects neutrophil functions, we took several approaches to elucidate aspects of the mechanisms involved. Results from chapters 4 and 5 focused mainly on responses to TNF. By using other agonists, it becomes possible to not only characterize better the properties of NT8 but gain some insights as to how NT8 induces its effects since cell activation will occur through different receptors and usually involve different sets of intracellular signaling cascades. Our next approach is to find out whether or not the inhibitory effects were selective for TNF. A range of agonists are available to undertake this study and includes fMLP, LTB_4, IL-8, C5a, GM-CSF and LPS-induced responses. These agonists have been established previously to stimulate neutrophil functions by different as well as ovelapping pathways (see below) via cell surface receptors. In contrast, other agonicts such as PMA which acts on intracellular PKC level, A23187 (calcium ionophore) have been shown to mobilize cellular calcium and arachidonic acid which causes neutrophil activation by diverse mechanisms (Ferrante *et al.*, 2005). The neutrophil functions to be examined here restricted to adhesion, chemotaxis and respiratory burst. It was the aim of this investigation to determine as to whether or not the NT8 effects were selective for response to TNF or affect responses induced by these other agonists.

6.2 Alteration of the adherence response induced by other surface receptor acting agonists

A diverse range of agonists which act on receptors on neutrophil surfaces representing endogenous (LTB_4, C5a, IL-8 and GM-CSF) and exogenous (fMLP and LPS) were used to try to extablish the selective action of NT8 for the inhibition of the TNF-induced neutrophil adherence response. All experiments were performed as described in Chapter 2.11.1 for the different agonists.

6.2.1 fMLP

The synthetic bacterial peptide fMLP acts via a high and low affinity receptor on neutrophils and leads to the stimulation of several responses including cell adhesion (Kettritz *et al.*, 2004; Bylund *et al.*, 2003). Treatment of cells with fMLP increased their adhesion to serum coated plastic surface. Experiments were conducted in a similar manner to those in which TNF was used as the cell agonist (Chapter 4). Neutrophils were pre-treated for 1 h with 0, 20 or 50 µM of NT8. Other cells were treated with 50 µM of either NT0 or lauric acid. Following pre-treatment, the cells were challenged with 0.1 µM fMLP for 30 min and then stained to quantitate the amount of neutrophil adherence.

The results presented in Figure 6.1 demonstrate that, unlike the effects on the TNF-induced adherence, the response to fMLP was not inhibited at 20 µM NT8. However at 50 µM, there was approximately an 80 % reduction in this response. The fMLP response was not affected by either NT0 or lauric acid (Figure 6.1).

6.2.2 LTB_4

LTB_4 is formed from the metabolism of arachidonic acid via the 5-lipogenease pathway (Ferrante *et al.*, 2005). Biosynthesis of leukotrienes involves the release of AA from membrane phospholipids by Ca^{2+}-dependent cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) and its conversion into 5-hydroperoxyeicosatetraenoic acid and subsequently leukotriene A_4, catalyzed by 5-lipoxygenase (Zarini *et al.*, 2006). LTA_4 is then enzymatically converted to LTB_4 by LTA_4-hydrolase (Zarini *et al.*, 2006). This product is a major inflammatory mediator which stimulates cells by acting on its surface receptor. LTB_4 has previously been found to cause rapid neutrophil adhesion (Dahlen *et al.*, 1981).

The effect of NT8 on the LTB_4-induced neutrophil adherence response was examined. Neutrophils were pre-treated with 0, 20 or 50 µM of NT8, NT0, or lauric acid and then examined for adherence in response to LTB_4. The results showed that the LTB_4-induced adherence was significantly inhibited at 20 and 50 µM concentration of NT8. While NT0 had no effect on the adherence response to LTB_4, lauric acid caused significant inhibition of the response at 50 µM (Figure 6.2).

6.2.3 GM-CSF

GM-CSF is a growth factor that mediates proliferation of granulocytes, macrophages, erythrocytes, megakaryocytes and eosinophil progenitors (Jubinsky *et al.*, 1994). It has been reported to stimulate a number of neutrophil functions including adherence and phagocyte activity and inhibit neutrophil migration (Jubinsky *et al.*, 1994; Zhou *et al.*, 2003).

The investigations on the effect of NT8 were extended further to examine its effects on the GM-CSF-induced neutrophil adherence response. The neutrophils were pre-treated for 1 h with 0, 20, or 50 µM of NT8. Another set of cells was treated with either NT0 or lauric acid, and the response to GM-CSF measured. The results showed that the GM-CSF induced neutrophil adhesion was inhibited at 50 but not 20 µM NT8 (Figure 6.3). Under these conditions there was no effect with either NT0 or lauric acid.

6.2.4 IL-8

IL-8 is a prototypic member of the CXC subfamily of chemokines that stimulates neutrophil responses (Knall *et al.*, 1996). It exerts its effect by binding to the IL-8 receptor which is a seven-transmembrane spanning receptor coupled to specific G proteins (Knall *et al.*, 1996). It has also been reported that IL-8 causes rapid neutrophil adhesion (Detmers *et al.*, 1991).

The effect of NT8 on IL-8 induced neutrophil adherence was investigated. Results showed that neutrophils pre-treated with 50 µM of NT8 caused 55 % decrease in adherence in response to the IL-8 (Figure 6.4). At these concentrations, NT0 and lauric acid showed no inhibitory effect (Figure 6.4).

6.2.5 C5a

C5a is generated during the activation of the complement cascade. This product is a chemotactic factor for both neutrophils and macrophages, but also stimulates the respiratory burst, degranulation and cell adhesion (Guo and Ward, 2005; Smith *et al.*, 1979).

Using C5a as a neutrophil stimulator of adhesion, we examined the effects of NT8 on this response. The neutrophils were pre-treated with 0, 20, and 50 µM of NT8 and then tested for their response to C5a. Results showed that NT8 inhibited neutrophil adherence at 50 but not 20 µM (Figure 6.5). At the same time, neither NT0 nor lauric acid showed any inhibitory effect.

6.2.6 Lipopolysaccharide

LPS from gram negative bacteria is known to stimulate neutrophil functions including adherence (Aida and Pabst, 1991; Worthen *et al.*, 1992). Neutrophils were pre-treated for 1 h with 0, 20, or 50 µM of NT8, lauric acid or NT0 and measured for adherence in response to LPS. The results showed that only at 50 µM did NT8 inhibit significantly neutrophil adherence (Figure 6.6).

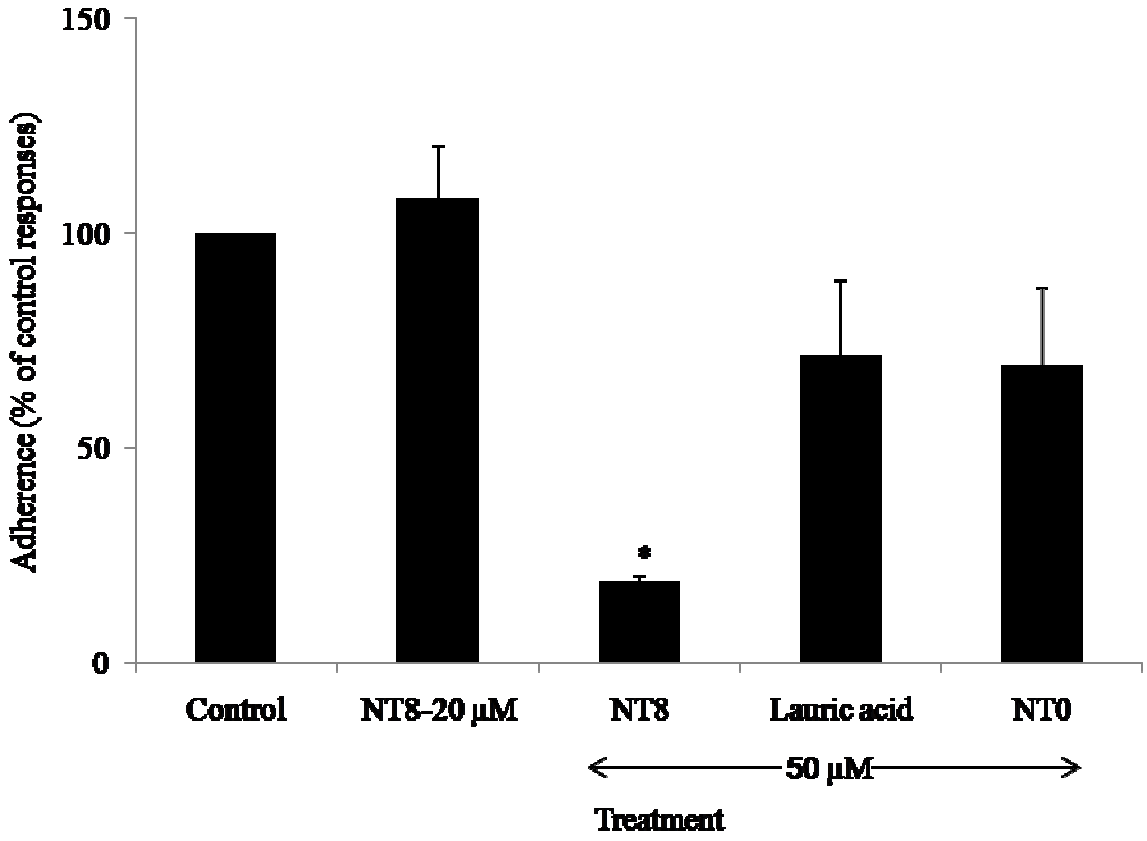


Figure 6.1 Effect of NT8 on fMLP-induced neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated for 1 h with either 20 µM or 50 µM NT8, 50 µM lauric acid or 50 µM NT0 and then stimulated by adding 20 µl of fMLP(1 µM). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are expressed as % of the control responses and presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. The base and stimulated OD 570 nm values ± SEM for the non-stimulated and stimulated controls were 0.07 ± 0.00 and 0.32 ± 0.04 respectively. Significance of difference between control and 50 µM: *$p<0.01$ (Dunnett: compare all vs control).

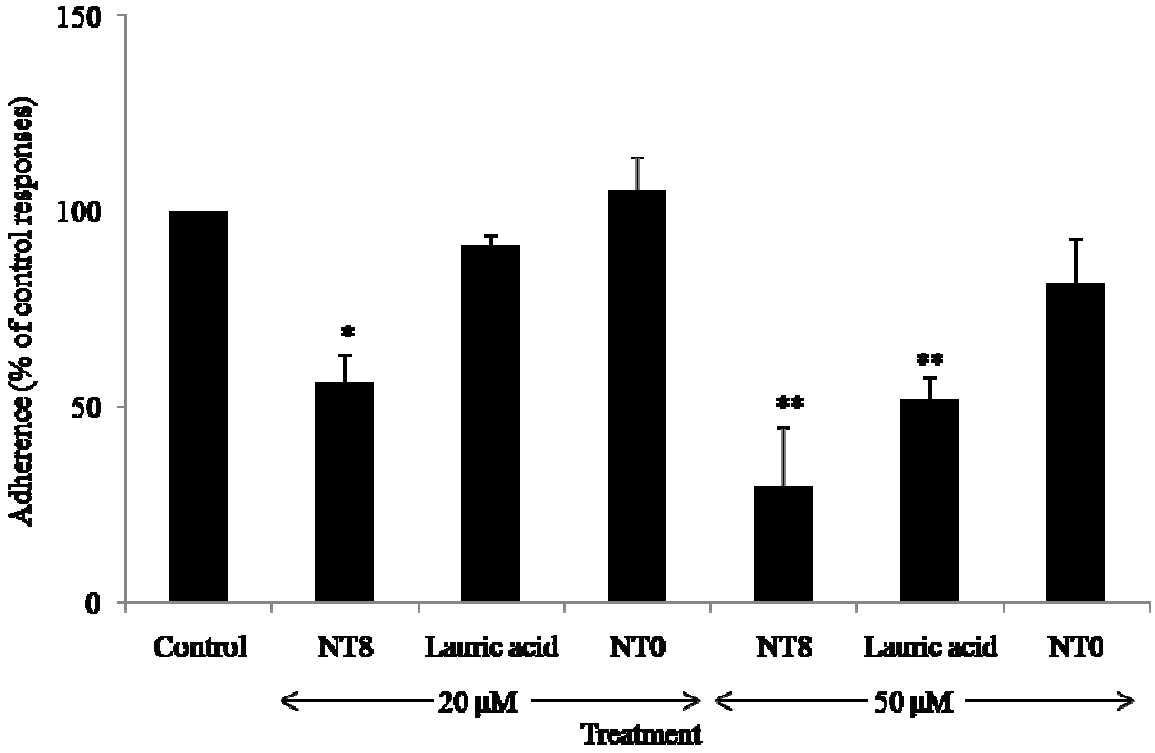


Figure 6.2 Effect of NT8 on LTB_4-induced neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated for 1 h with either 20 or 50 µM of NT8, lauric acid or NT0 and then stimulated by adding 20 µl of LTB_4 (0.1 µM). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are expressed as % of the control responses and presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. The base and stimulated OD 570 nm values ± SEM for the controls were 0.08 ± 0.01 and 0.24 ± 0.03 respectively. Significance of difference between control and 20 µM NT8, control and 50 µM NT8 and control and lauric acid (50 µM): *$p<0.05$, **$p<0.01$(Tukey-Kramer multiple comparisons test).

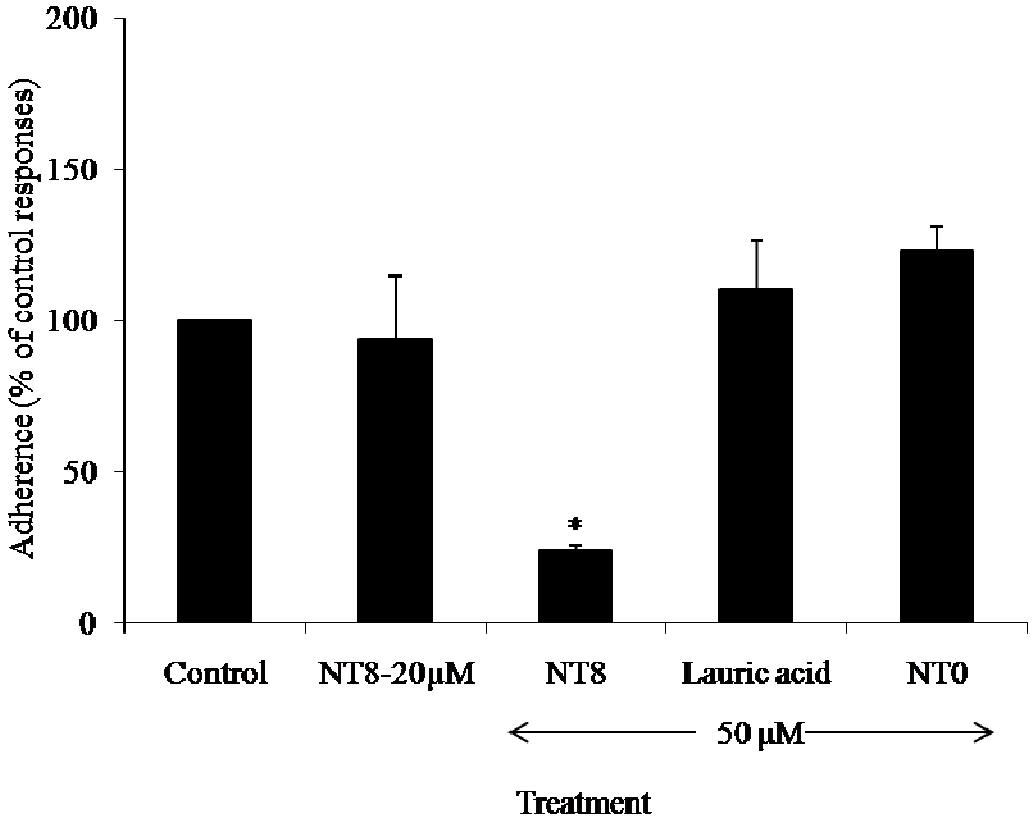


Figure 6.3 Effect of NT8 on GM-CSF- induced neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated for 1 h with either 20 µM or 50 µM NT8, 50 µM lauric acid or 50 µM NT0 and then stimulated by adding 20 µl of GM-CSF (20 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are expressed as % of the control responses and presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. The base and stimulated OD 570 nm values ± SEM for the controls were 0.08 ± 0.00 and 0.21 ± 0.04 respectively. Significance of difference between control and NT8 (50 µM): *$p<0.01$ (Dunnett: compare all vs control).

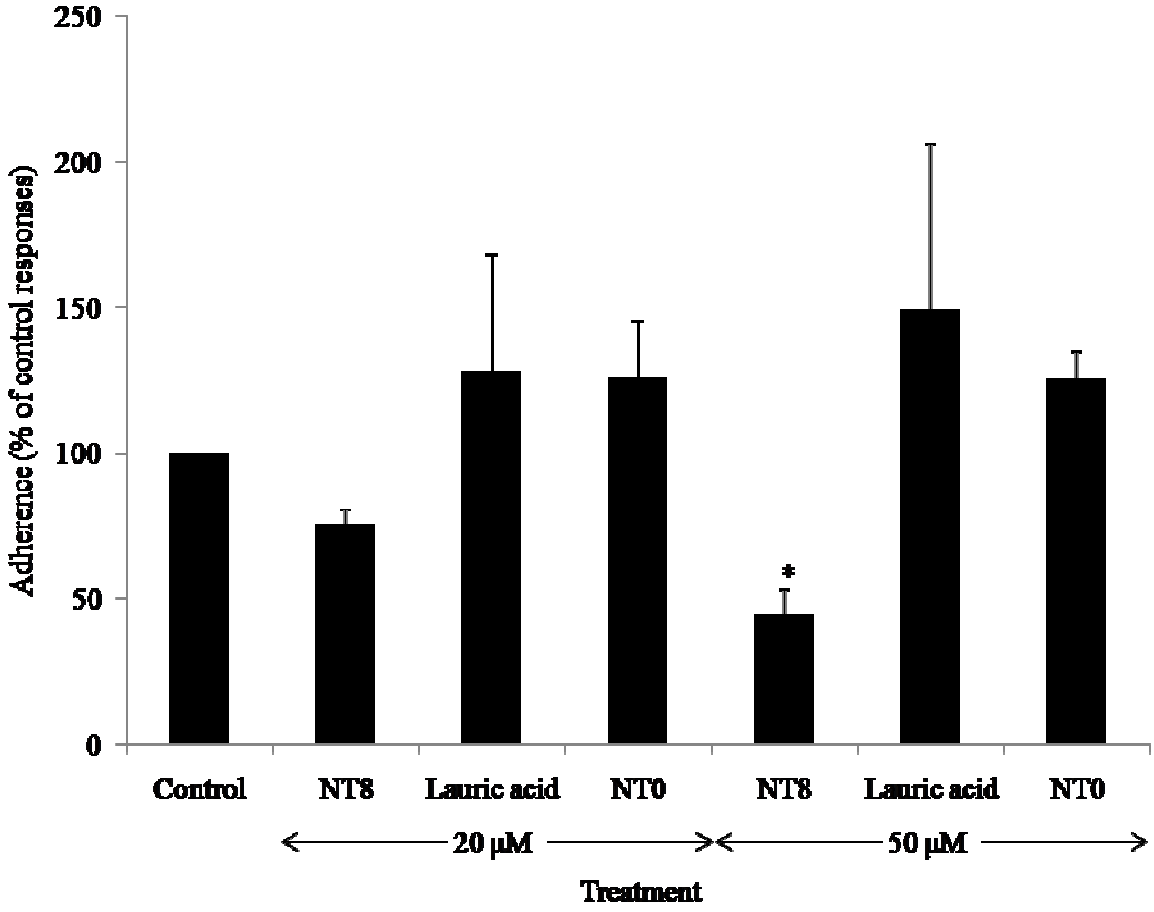


Figure 6.4 Effect of NT8 on IL-8-induced neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated for 1 h with either 20 or 50 µM of NT8, lauric acid or NT0 and then stimulated by adding 20 µl of IL-8 (0.1 µM). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are expressed as % of the control responses and presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. The base and stimulated OD 570 nm values ± SEM for the controls were 0.08 ± 0.00 and 0.18 ± 0.03 respectively. Significance of difference between control and 50 µM: *$p<0.01$ (Tukey-Kramer multiple comparisons test).

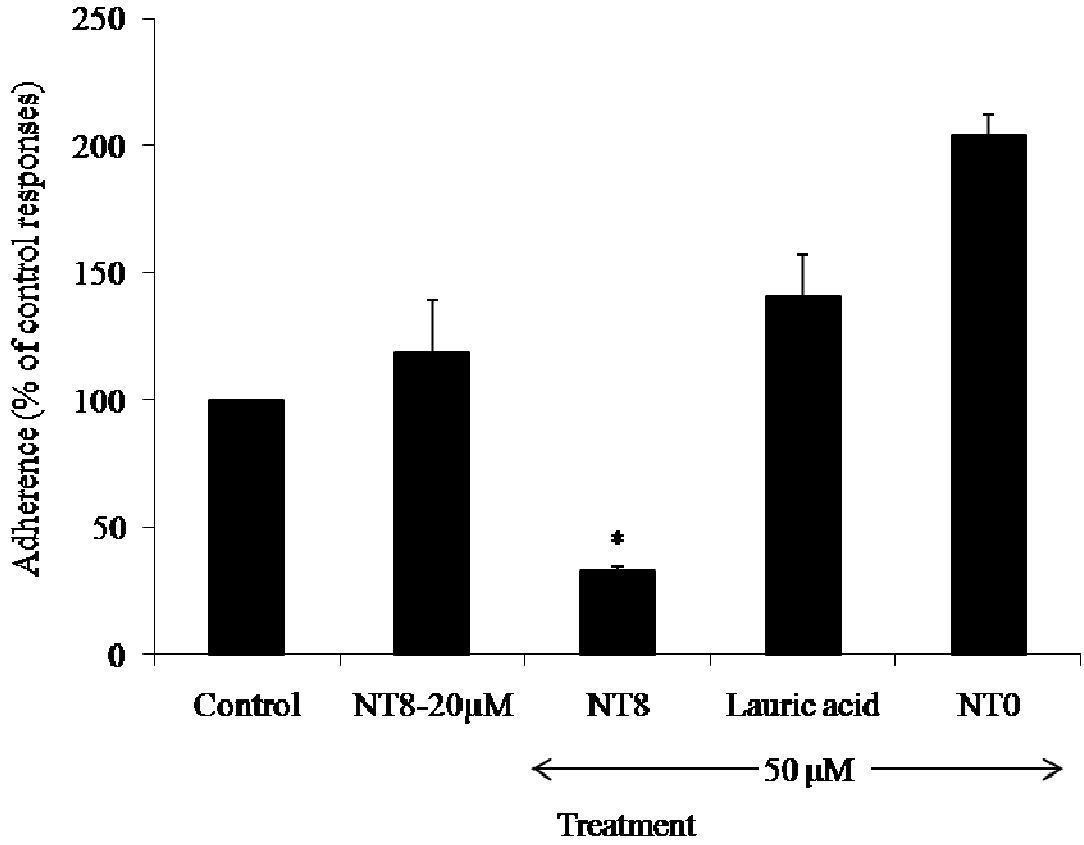


Figure 6.5 Effect of NT8 on C5a-induced neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated for 1 h with either 20 µM or 50 µM NT8, 50 µM lauric acid or 50 µM NT0 and then stimulated by adding 20 µl of C5a (0.1 µM). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are expressed as % of the control responses and presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. The base and stimulated OD 570 nm values ± SEM for the controls were 0.08 ± 0.00 and 0.33 ± 0.07 respectively. Significance of difference between control and 50 µM NT8: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

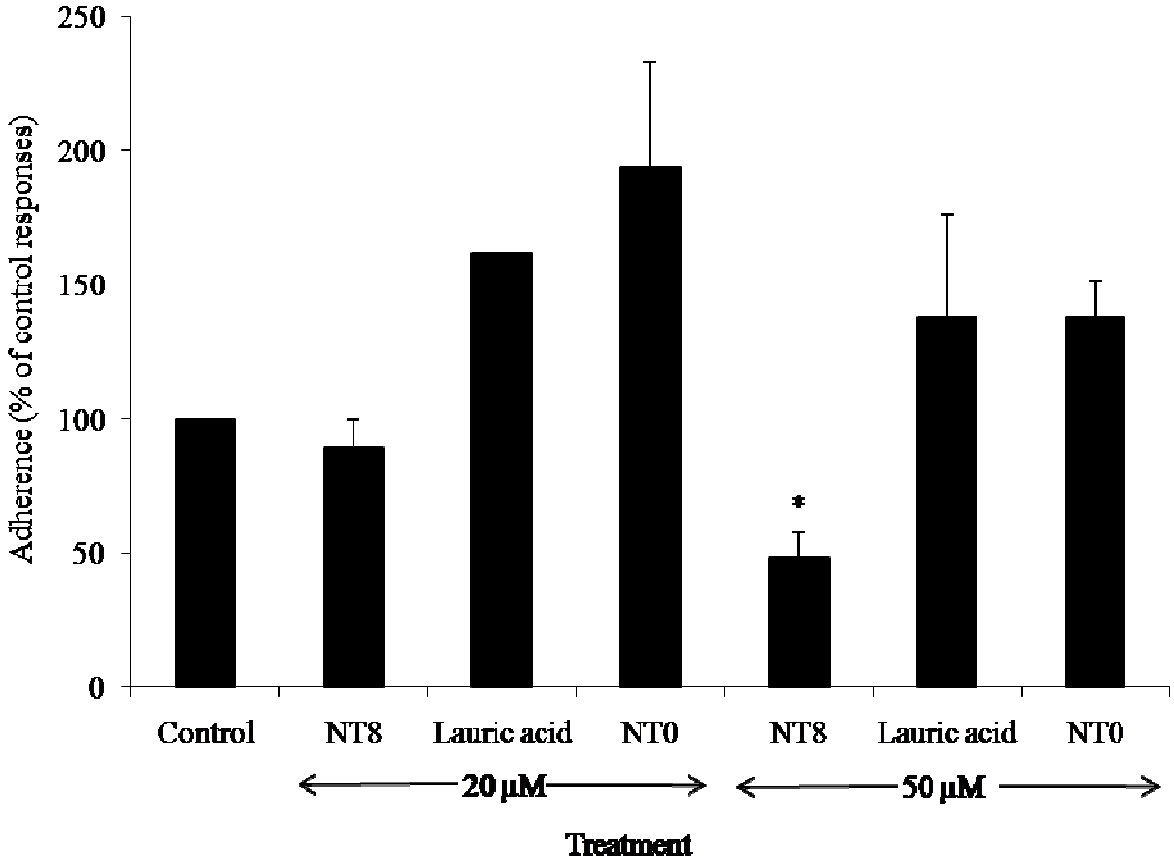


Figure 6.6 Effect of NT8 on LPS-induced neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated for 1 h with either 20 or 50 µM of NT8, lauric acid or NT0 and then stimulated by adding 20 µl of LPS (10 ng/ml). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are expressed as % of the control responses and presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. The base and stimulated OD 570 nm values ± SEM for the controls were 0.07 ± 0.00 and 0.24 ± 0.03 respectively. Significance of difference between control and 50 µM NT8: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

6.3 The effect of NT8 on neutrophil chemotaxis

The chemotactic response enables neutrophils to infiltrate and accumulate at sites of infection. The exogenous chemoattractant fMLP, which promotes the recruitment of neutrophils from the blood to the inflammatory foci was used to asses the sensitivity of this neutrophil response to NT8.

Using the migration under agarose method, the effects of NT8 on neutrophil chemotaxis to fMLP was examined. Neutrophils were treated with 0, 20, or 50 µM of NT8 for 1 h and then examined for migration towards fMLP. The effects of NT8 were also compared to treatment with 50 µM of either NT0 or lauric acid.

The results demonstrated that NT8 did not inhibit neutrophil chemotaxis at both concentrations and at the same time there was also no effect observed on CQ treated cells in the fMLP-induced chemotaxis (Figure 6.7). This suggests that apart from being receptor selective, NT8 inhibits the adherence but not chemotactic response to fMLP ie. is function selective also. There also was no effect on random migration.

Previous studies found that CQ and its derivatives have a different effects on random migration and chemotaxis (Ferrante *et al.*, 1986). Ferrante *et al*., (1986) demonstrated that CQ inhibited random migration by 15 % at 40 µM and chemotaxis (to fMLP) only by 12 %. Kharazmi *et al*., (1983) found that chemotaxis towards casein was markedly inhibited by CQ, reaching a complete inhibition at 100 µM. However Labro and Babin-Chevaye (1988) found that the inhibitory effect of CQ on either random migration or chemotaxis was dependent on the method used to measure these functions. The inhibitory effects of CQ on

random migration, fMLP or activated serum induced migration was only observed when the compound was directly incorporated into the agarose but not when the cells were only pre-treated with the compound, even at concentration of 200 µM (Labro and Babin-Chevaye, 1988).

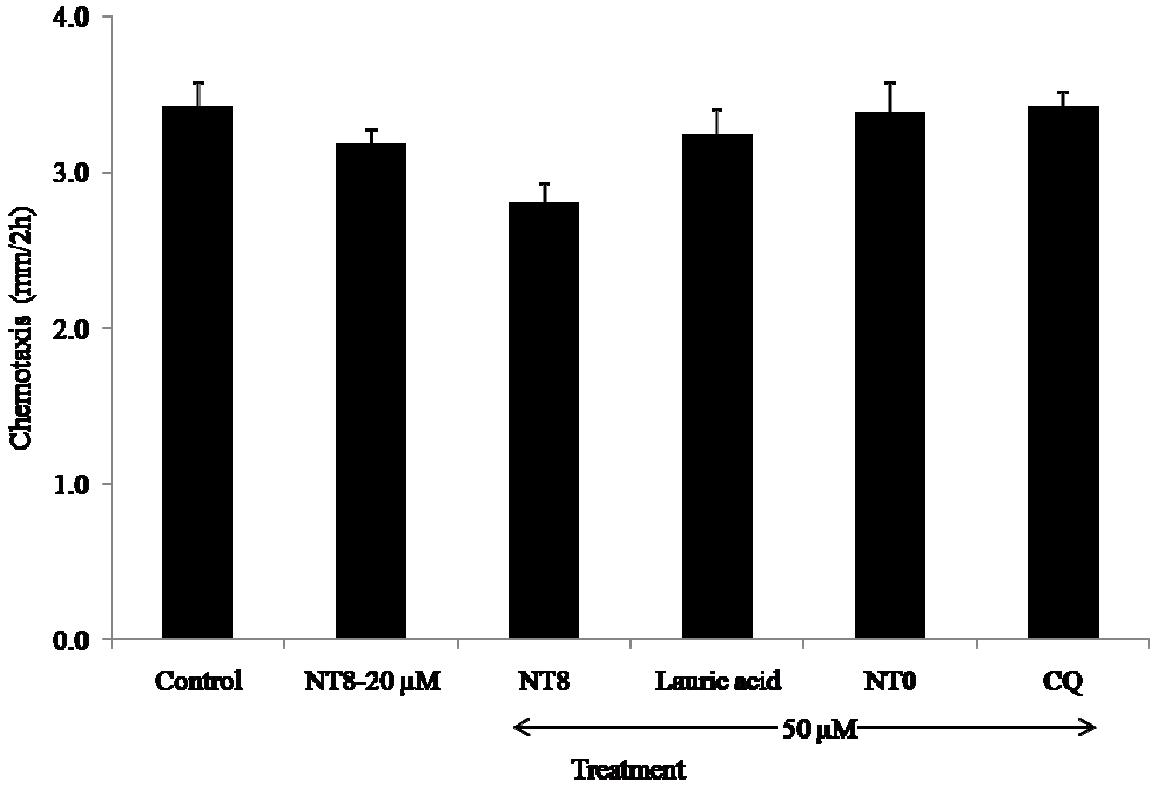


Figure 6.7 Effect of NT8 on fMLP-induced neutrophil chemotaxis. Neutrophils (5 x 10^6/ml) were pre-treated with either 20 µM, 50 µM NT8, 50 µM lauric acid or 50 µM NT0 and then tested for migration in response to fMLP. Data are presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor.

6.4 Oxygen-dependent respiratory burst

Apart from the importance of the release of oxygen derivative reactive species (ODRS) by neutrophils in defense against microbial pathogens, these ODRS play a role in tissue damage in inflammatory diseases such as rheumatoid arthritis (Hadjigogos, 2003; Mirshafiey and Mohsenzadegan, 2008). Since we have an interest in developing NT8 and its derivatives as anti-inflammatory agents, we investigated its effects on the ability of neutrophils to produce ODRS. The response was measured as lucigenin-dependent chemiluminescence which has been shown to be an assay for superoxide production by neutrophils (Gyllenhammar, 1987).

6.4.1 fMLP

The effect of NT8 on fMLP mediated lucigenin dependent respiratory burst was investigated. At higher concentrations than those which stimulate chemotaxis, fMLP induces a modest respiratory burst activity in neutrophils (Figure 6.8). Neutrophils were pre-treated with 0, 20 or 50 µM of NT8 and after 1 h were examined for their ability to undergo the respiratory burst activity induced by fMLP. The data showed that NT8 had no effect on the basal response but inhibited the fMLP-induced response at 50 µM (Figure 6.8) but not at 20 µM (data not shown). The kinetics of the generation of chemiluminescence in fMLP stimulated neutrophils is shown in Figure 6.9. These showed that the inhibition was also reflected in the total amount of chemiluminescence produced (area under curve). NT8 had no effect on the basal respiratory activity of the cell (Figure 6.8).

6.4.2 LTB_4

LTB_4 has been found to induce a number of functional changes in neutrophil. Apart from acting as a chemotatic agent and stimulating neutrophil adherence, it also stimulates the oxygen-dependent respiratory burst activity (Schultz *et al.*, 1991). When the effect of NT8 on LTB_4 mediated enhanced chemiluminescence response was examined, the data showed that NT8 at 50 μM did not inhibit the response (Figure 6.10, 6.11). The kinetics of the generation of chemiluminescence in LTB_4 stimulated neutrophils are shown in Figure 6.11. This is interesting in terms of the strong inhibitory effects of NT8 on the LTB_4-induced neutrophil adherence (6.2.2)

6.4.3 GM-CSF

GM-CSF is a neutrophil stimulator and plays an important role in the activity of neutrophil during inflammation (DiPersio *et al.*, 1988; Gomez-Cambronero *et al.*, 2003). This includes the ability to stimulate ODRS production. The effectsof NT8 on this respose induced by the cytokine was examined. Cells were treated with 50 μM NT8 for 1 h and then challenged with GM-CSF. The results showed that while NT8 at 50 μM inhibited the respiratory burst response induced by GM-CSF, this did not reach statistical significance (Figure 6.12, 6.13).

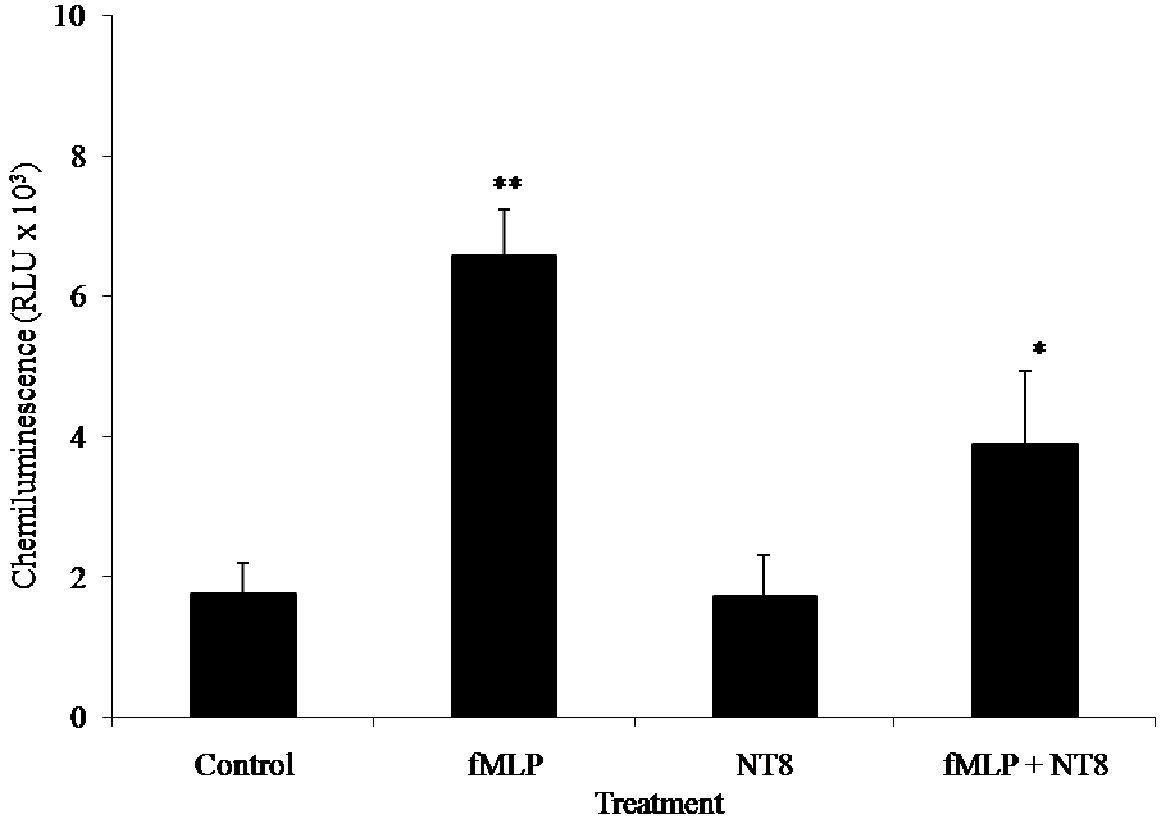


Figure 6.8 Effect of NT8 on fMLP-induced chemiluminescence. Neutrophils (5 x 10^6/ml) were pre-treated with NT8 (50 µM) for 1 h and then stimulated by adding 100 µl of fMLP (0.1 µM). The resultant chemiluminescence production was measured in a luminometer. The data represent initial peak rates of chemiluminescence and are presented as the mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and fMLP: **$p<0.001$ and between fMLP and fMLP + NT8: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

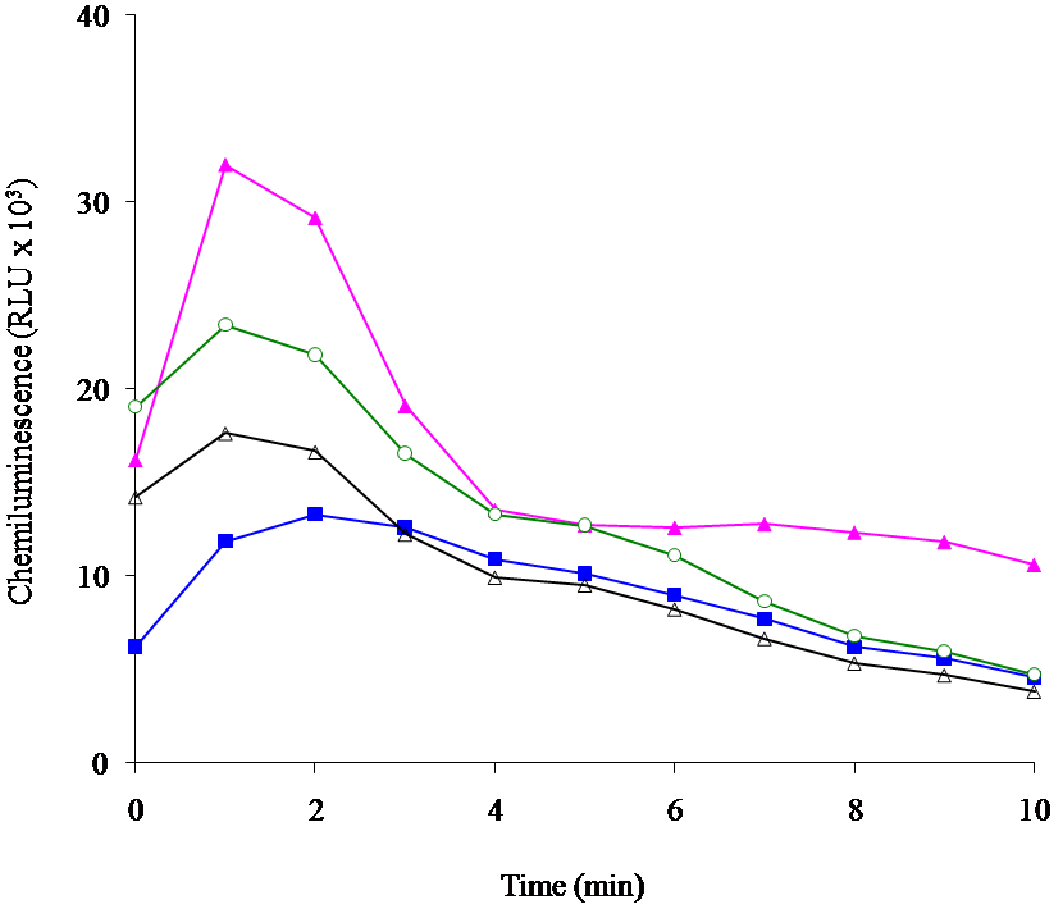


Figure 6.9 Effect of NT8 shown as kinetics of fMLP-induced change in chemiluminescence. Data is a representative experiment of results shown in Figure 6.8. The data is presented as relative light units. Neutrophils + diluents (■), neutrophils + fMLP (▲), neutrophils + NT8 (Δ) and neutrophils + fMLP + NT8 (○).

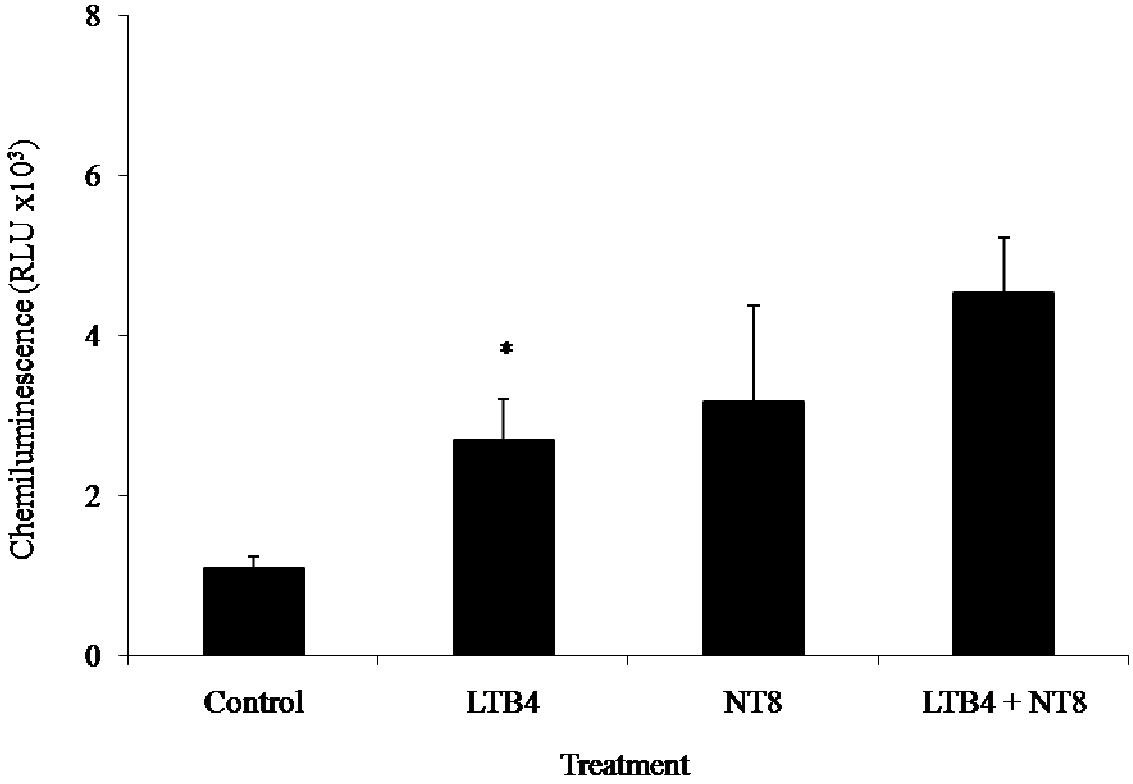


Figure 6.10 Effect of NT8 on LTB_4-induced chemiluminescence. Neutrophils (5 x 10^6/ml) were pre-treated with NT8 (50 μM) for 1 h and then stimulated by adding 100 μl of LTB_4 (0.1 μM). The resultant chemiluminescence production was measured in a luminometer. The data represent initial peak rates of chemiluminescence and are presented as the mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and LTB_4: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

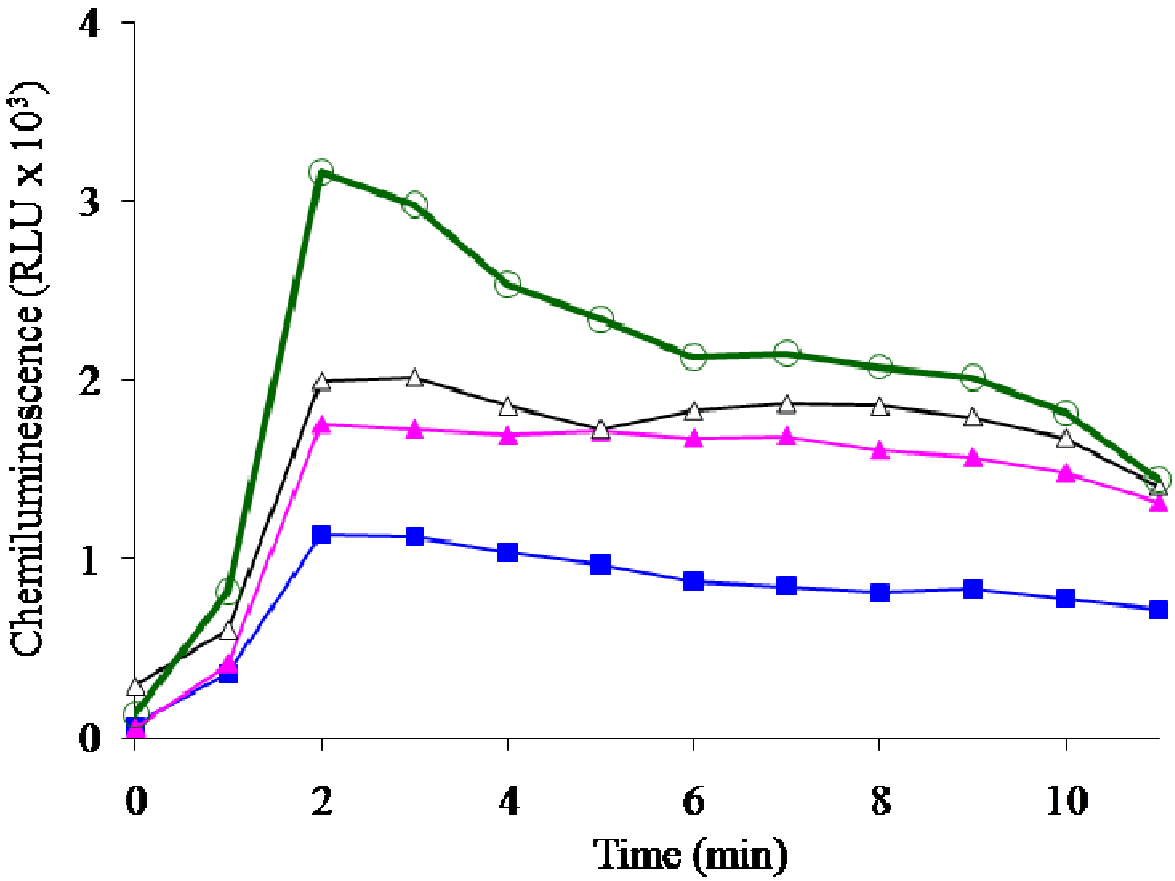


Figure 6.11 Effect of NT8 shown as kinetics of LTB_4-induced change in chemiluminescence. Data is a representative experiment of results shown in Figure 6.10. The data is presented as relative light units. Neutrophils + diluents (■), neutrophils + LTB_4 (▲), neutrophils + NT8 (Δ) and neutrophils + LTB_4 + NT8 (○).

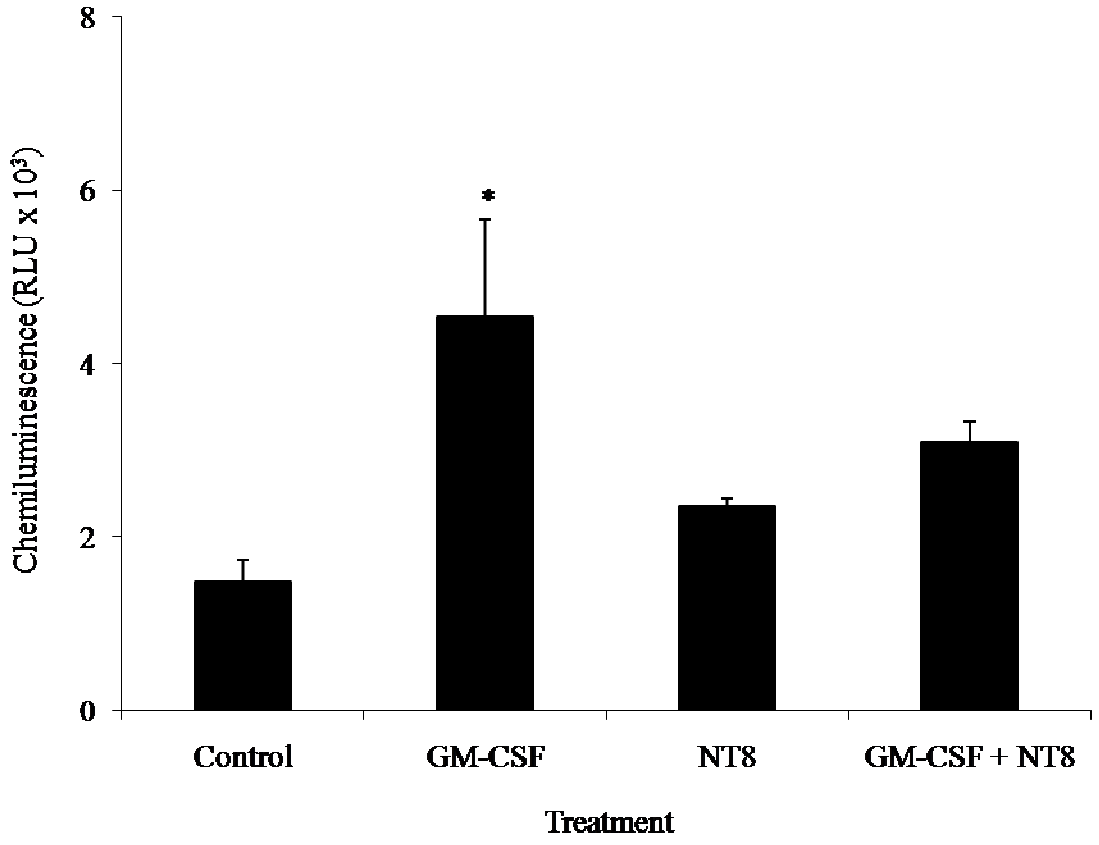


Figure 6.12 Effect of NT8 on GM-CSF-induced chemiluminescence. Neutrophils (5 x 10^6/ml) were pre-treated with NT8 (50 μM) for 1 h and then stimulated by adding 100 μl of GM-CSF (40 ng/ml). The resultant chemiluminescence production was measured in a luminometer. The data represent initial peak rates of chemiluminescence and are presented as the mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and GM-CSF: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

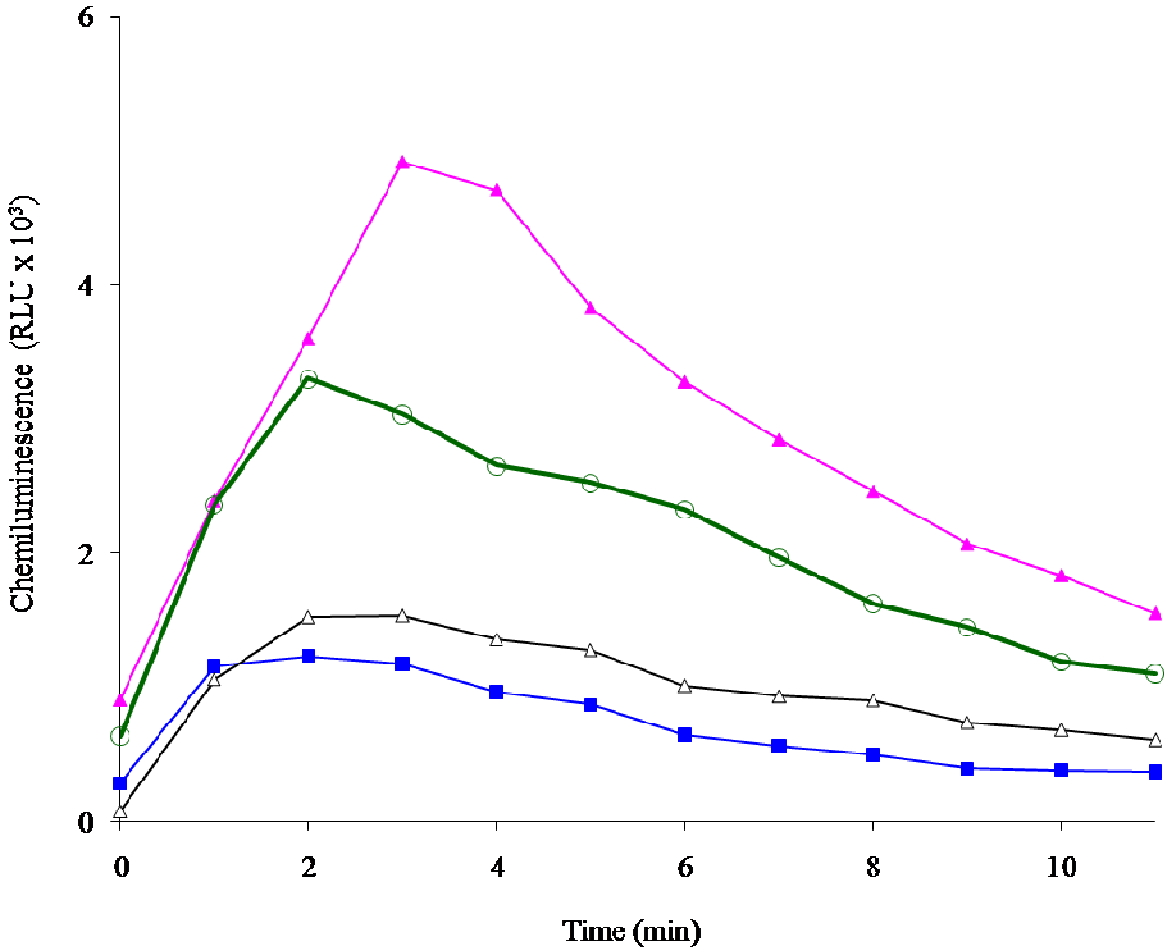


Figure 6.13 Effect of NT8 shown as kinetics of GM-CSF-induced change in chemiluminescence. Data is a representative experiment of results shown in Figure 6.12. The data is presented as relative light units. Neutrophils + diluents (■), neutrophils + GM-CSF (▲), neutrophils + NT8 (Δ) and neutrophils + GM-CSF + NT8 (○).

6.5 Effects on arachidonic acid induced neutrophil responses.

Arachidonic acid (AA) is a free fatty acid metabolized via the cycloxygenase and lipoxygenase pathways to produce biologically active products eicosanoids, namely prostaglandins, leukotrienes and lipoxins (Krischer *et al.*, 1998). However, it has also been demonstrated that AA is a strong neutrophil agonist that induces neutrophil migration inhibition, degranulation, adhesion and activation of the oxygen-dependent respiratory burst (Ferrante *et al.*, 2005).

Since AA represents an agonist with its own unique properties and is generated during cell activation in the inflammatory reaction, we examined whether NT8 altered the neutrophil stimulation by AA. Neutrophils were pretreated with 0, 20 or 50 µM of NT8 and then examined for AA induced adherence. The results showed this NT8 inhibited the AA-induced neutrophil adherence only at 50 µM (77 % inhibition). The treatment with 50 µM of either NT0 or lauric acid had no effect on the AA response (Figure 6.14).

The effect of NT8 on AA mediated neutrophil chemiluminescence was also examined. AA at 30 µM resulted in a significant increase in chemiluminescence but the pre-treatment of neutrophils with NT8 (50 µM) for 1 h did not inhibit the neutrophil respiratory burst response (Figure 6.15, 6.16). The results again demonstrated that NT8 has selectivity for inhibition of the neutrophil adhesion response.

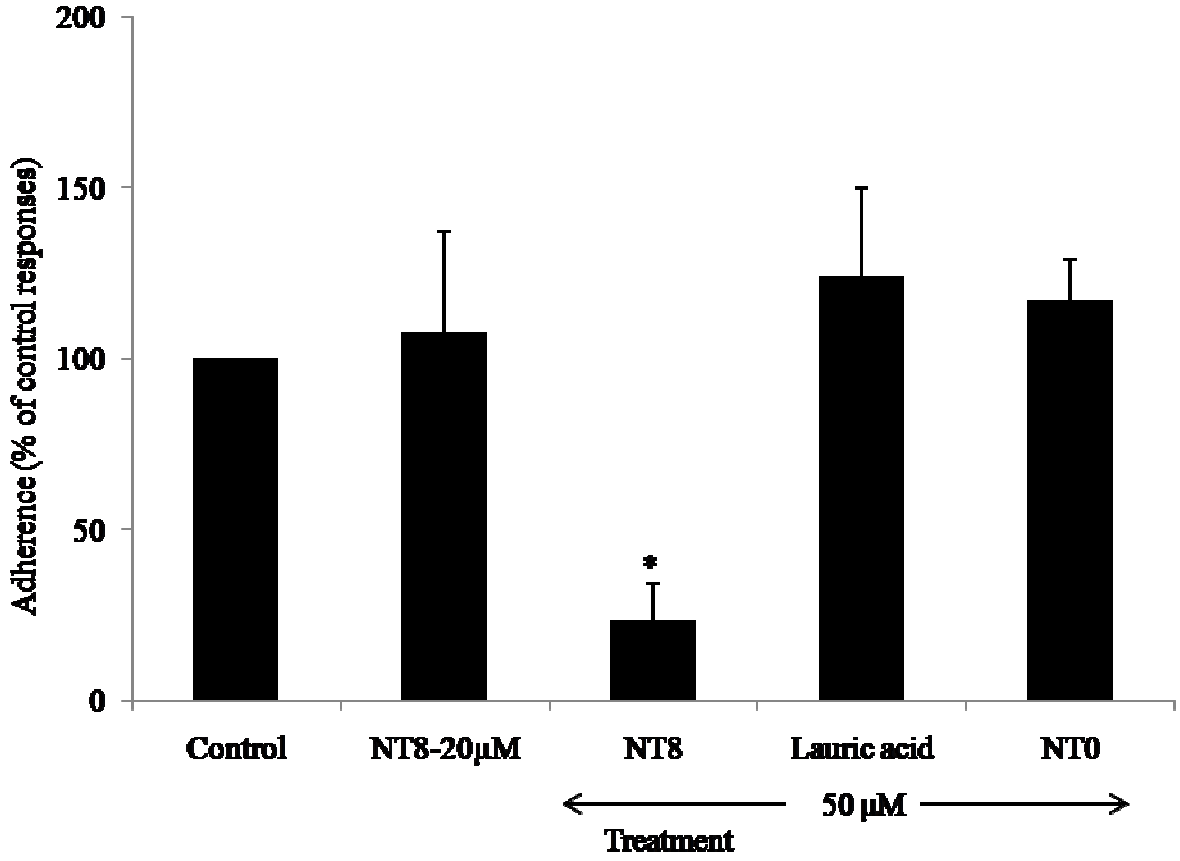


Figure 6.14 Effect of NT8 on AA-induced neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with either 20 µM or 50 µM NT8, 50 µM lauric acid or 50 µM NT0 and then stimulated by adding 20 µl of AA (30 µM). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are expressed as % of the control responses and presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. The base and stimulated OD 570 nm values ± SEM for the controls were 0.07 ± 0.01 and 0.31 ± 0.02 respectively. Significance of difference between control and 50 µM NT8: *$p<0.01$ (Tukey-Kramer multiple comparisons test).

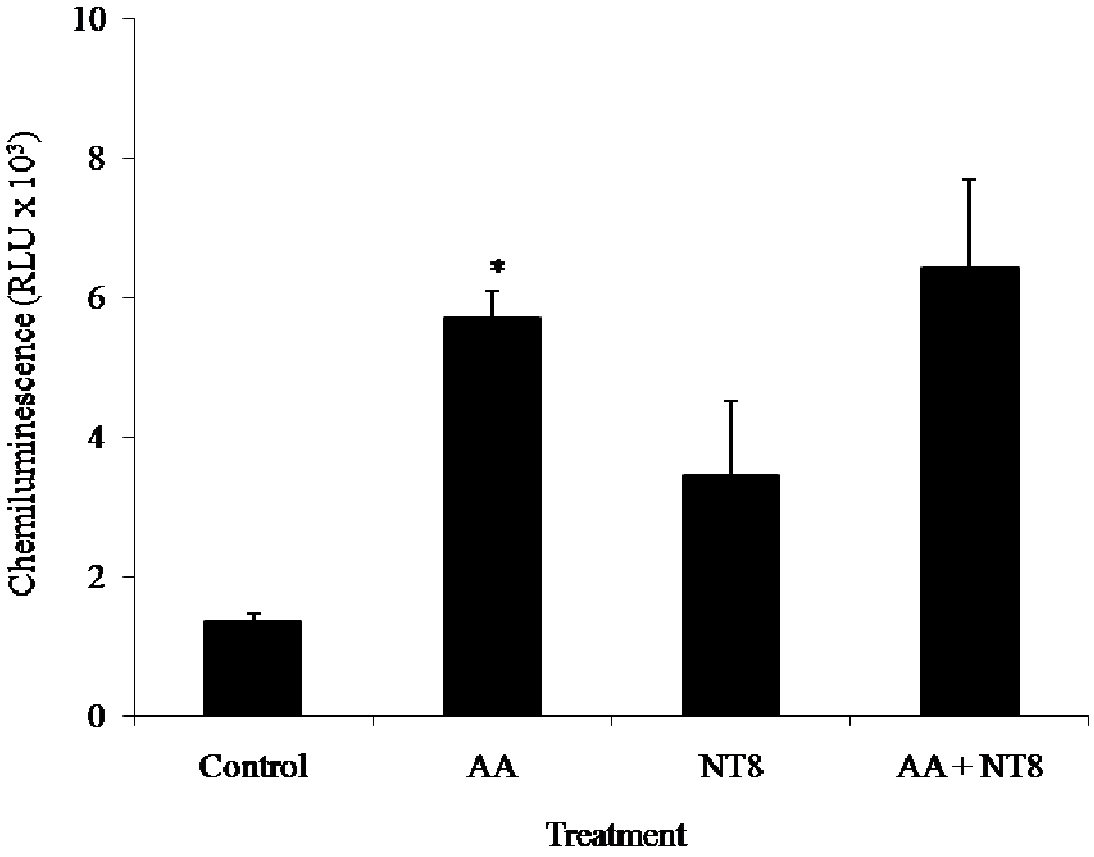


Figure 6.15 Effect of NT8 on AA-induced chemiluminescence. Neutrophils (5 x 10^6/ml) were pre-treated with NT8 (50 μM) for 1 h and then stimulated by adding 100 μl of AA (30 μM). The resultant chemiluminescence production was measured in a luminometer. The data represent initial peak rates of chemiluminescence and are presented as the mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and AA: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

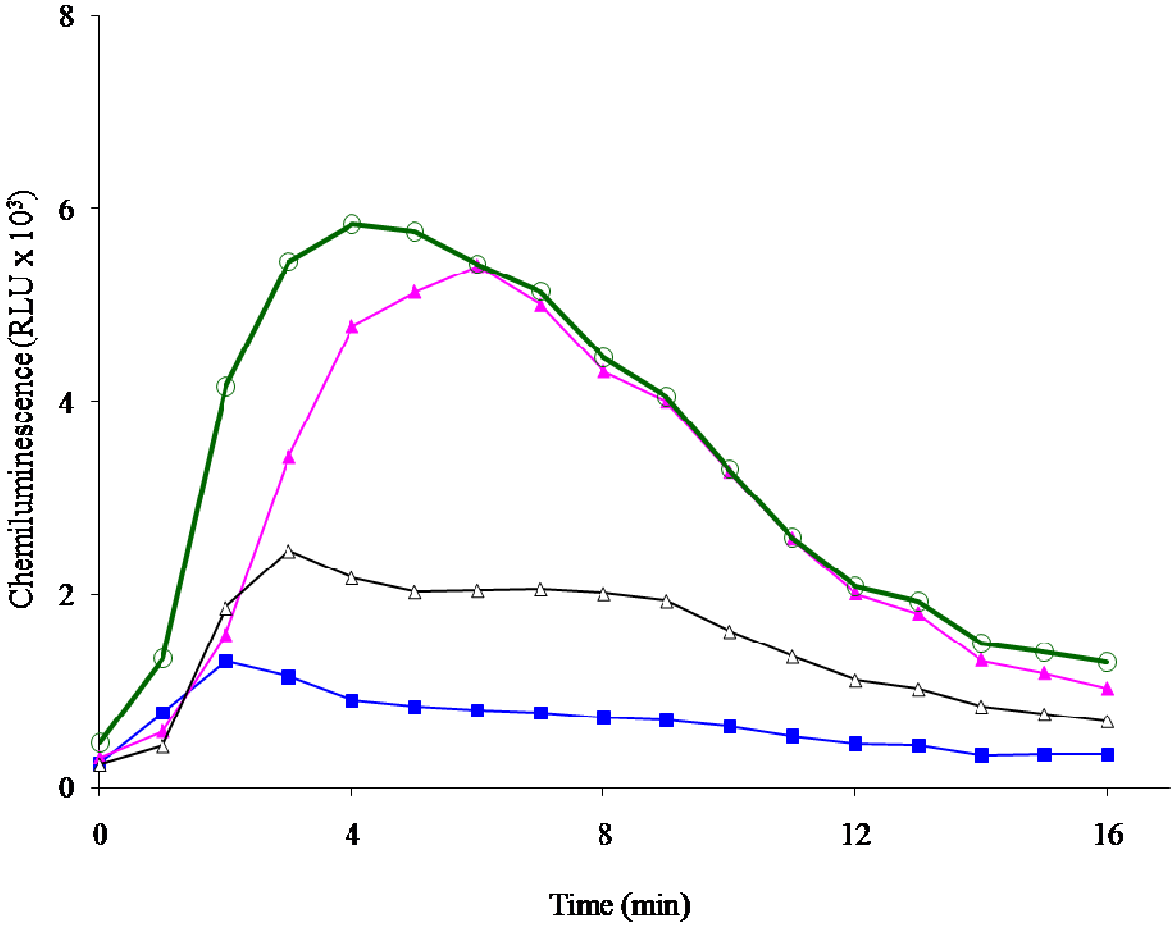


Figure 6.16 Effect of NT8 shown as kinetics of AA-induced change in chemiluminescence. Data is a representative experiment of results shown in Figure 6.15. The data is presented as relative light units. Neutrophils + diluents (■), neutrophils + AA (▲), neutrophils + NT8 (Δ) and neutrophils + AA + NT8 (○).

6.6 The effect of NT8 on agonists which act at the post receptor level, PMA and A23187

One way of gaining some understanding of the pathways targeted by NT8 is by using an agonist which acts at the post surface receptor level to stimulate the response. In these investigations we have chosen two agents, PMA which acts on PKC, and A23187 which mobilizes intracellular calcium. Both of these agents have been shown to stimulate both neutrophil adhesion and respiratory burst responses (Davies *et al.*, 1990; Guo and Ward, 2005; Lew *et al.*, 1986; Myers *et al.*, 1990).

6.6.1 Adherence

In these investigations, neutrophils were pretreated with either 20 or 50 µM of NT8, 50 µM NT0 or lauric acid. The results presented in Figure 6.17, show that the PMA-induced adherence was significantly depressed at 50 µM NT8. There was no corresponding inhibition either with NT0 or lauric acid.

Stimulating an increase in intracellular Ca^{2+} has been shown to enhance neutrophil adherence, and calcium ionophore has been used to induce this response (Davies *et al.*, 1990). Calcium ionophore (A23187) selectively aids in the transport of Ca^{2+} across the natural and artificial membranes so that calcium can accumulate leading to enhanced neutrophil adhesion (Lew *et al.*, 1986). In our study, neutrophils were pre-treated with either 20 or 50 µM of NT8 and then stimulated with A23187 to induce the cell adhesion response. The results showed that NT8 caused an 80 % inhibition of A23187-induced neutrophil adherence at 50 µM (Figure 6.18).

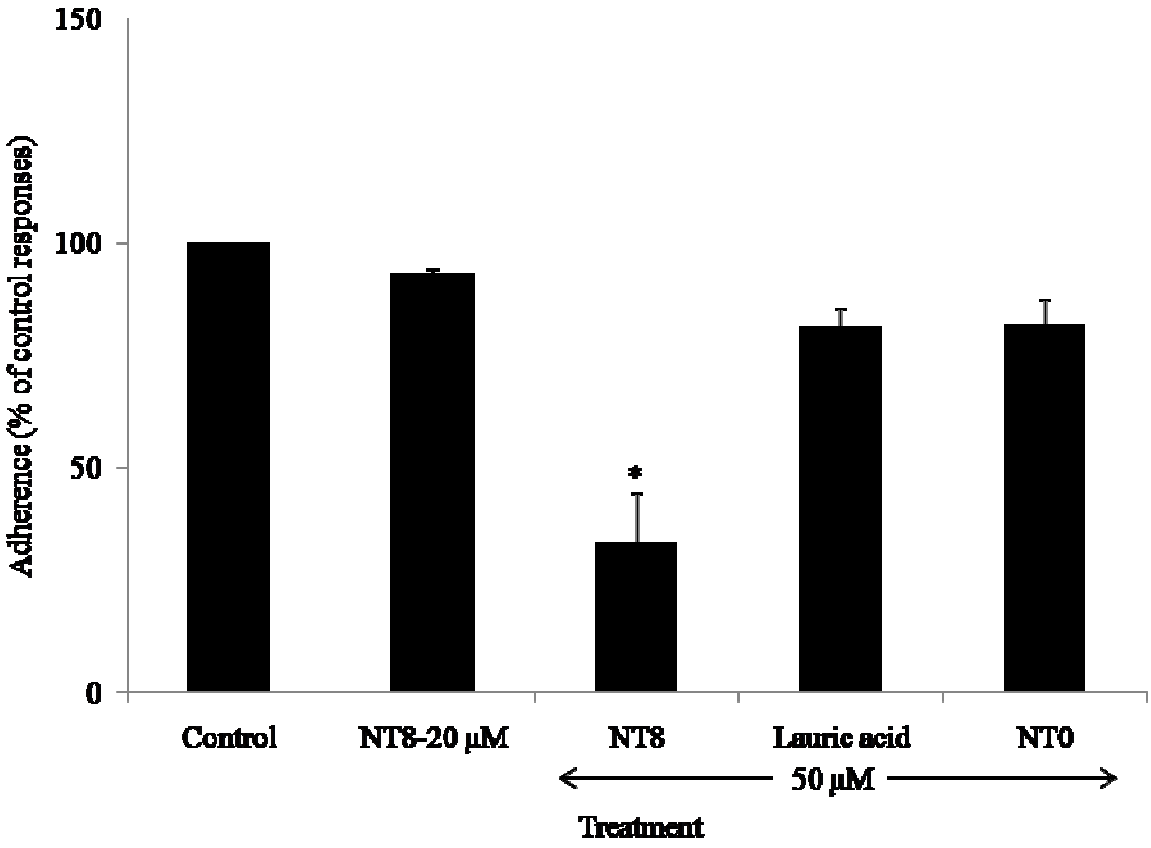


Figure 6.17 Effect of NT8 on PMA-induced neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with either 20 µM or 50 µM NT8, 50 µM lauric acid or 50 µM NT0 and then stimulated by adding 20 µl of PMA (0.1 µM). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are expressed as % of the control responses and presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. The base and stimulated OD 570 nm values ± SEM for the controls were 0.07 ± 0.01 and 1.02 ± 0.09 respectively. Significance of difference between control and 50 µM NT8: *$p<0.01$ (Dunnett: compare all vs control).

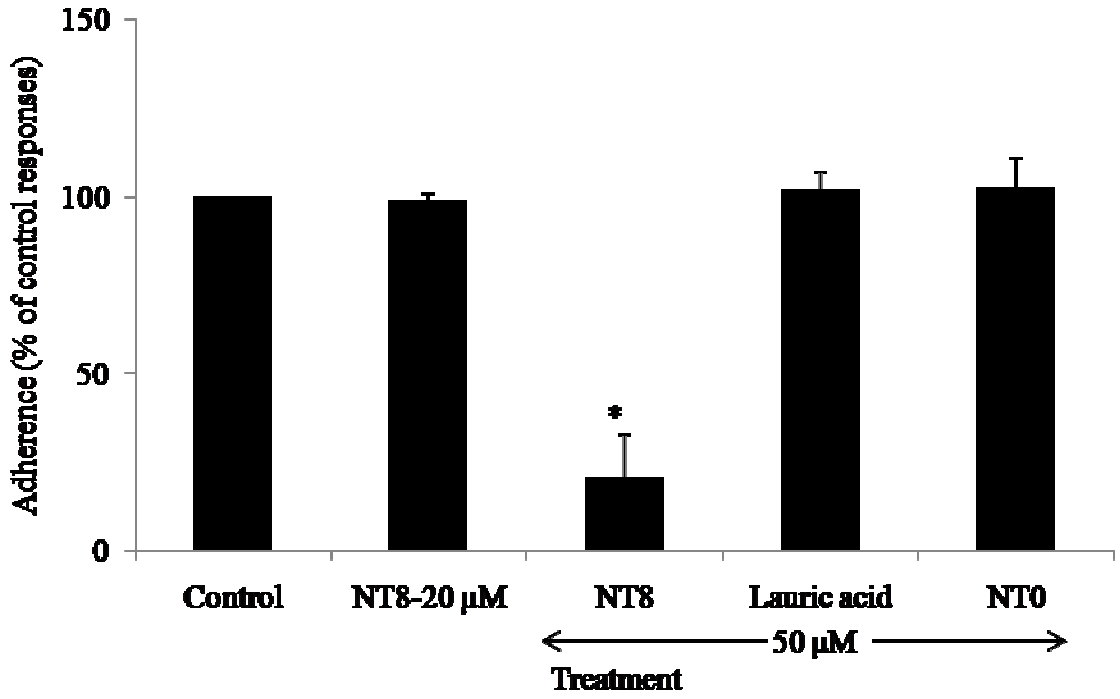


Figure 6.18 Effect of NT8 on A23187-induced neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with either 20 µM or 50 µM NT8, 50 µM lauric acid or 50 µM NT0 and the stimulated by adding 20 µl of A23187 (10 µM). Adherence was measured by staining the cells with Rose Bengal and reading the plates at OD 570 nm. The data are expressed as % of the control responses and presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. The base and stimulated OD 570 nm values ± SEM for the controls were 0.08 ± 0.00 and 1.08 ± 0.16 respectively. Significance of difference between control and 50 µM NT8: *$p<0.01$ (Tukey-Kramer multiple comparisons test).

6.6.2 Oxygen-dependent respiratory burst

PMA stimulates the neutrophil respiratory burst by acting on PKC (Allard *et al.*, 1999). The effect of NT8 on PMA mediated oxygen-dependent respiratory burst was examined by the lucigenin-dependent chemiluminescence assay. The neutrophils were pre-treated with 50 μM of NT8 and the response to PMA was examined. The data showed that there was no inhibition in the response induced by this agonist (Figure 6.19). This contrasts with the effects on the adherence response (Figure 6.17). The kinetics of the generation of chemiluminescence in PMA stimulated neutrophils are shown in Figure 6.20. These demonstrated that the total chemiluminescence produced was similarly not affected by NT8.

The A23187-induced neutrophil respiratory burst was also measured in cells which had been pre-treated with NT8 at 50 μM. NT8 did not cause any inhibition of the respiratory burst (Figure 6.21) and this was confimed in terms of assessing the kinetics of the generation of chemiluminescence by A23187 (Figure 6.22).

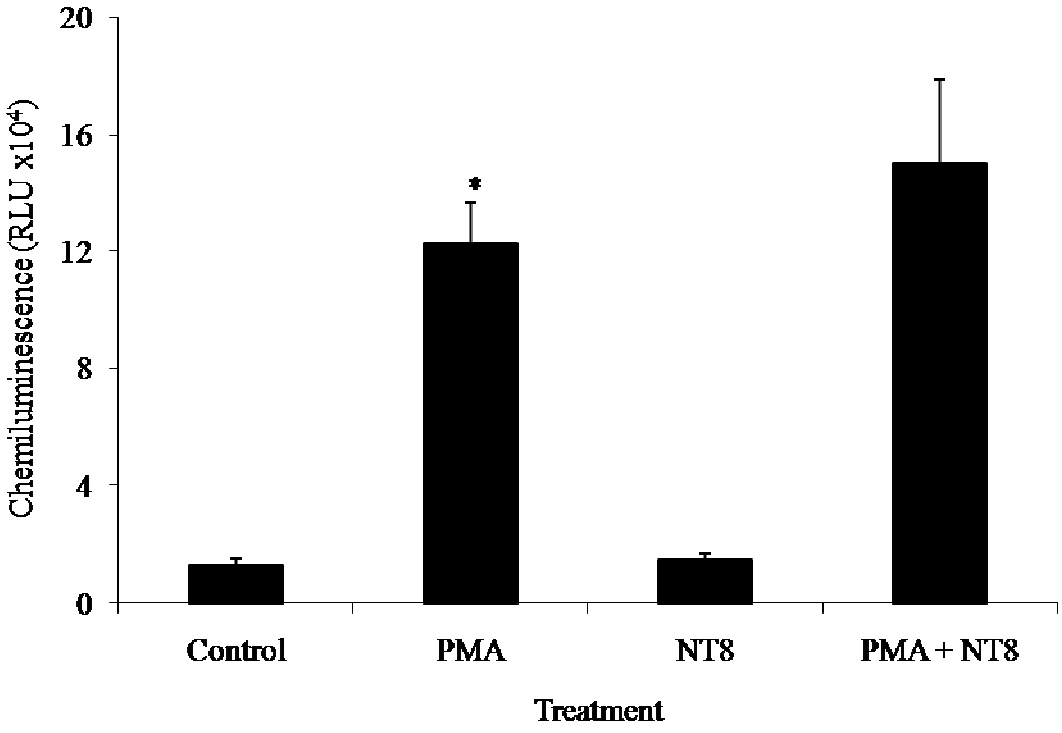


Figure 6.19 Effect of NT8 on PMA-induced chemiluminescence. Neutrophils (5 x 10^6/ml) were pre-treated with NT8 (50 μM) for 1 h and then stimulated by adding 100 μl of PMA (0.1 μM). The resultant chemiluminescence production was measured in a luminometer. The data represent initial peak rates of chemiluminescence and are presented as the mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and PMA: *$p<0.01$ (Tukey-Kramer multiple comparisons test).

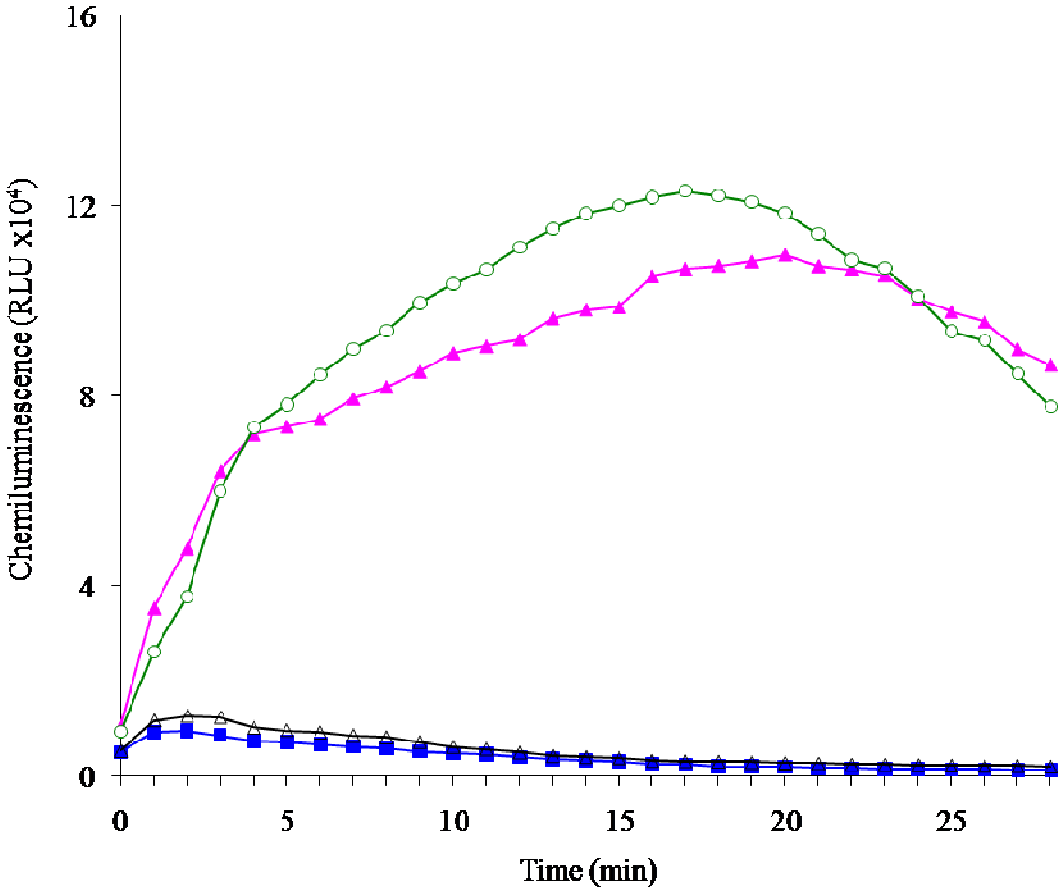


Figure 6.20 Effect of NT8 shown as kinetics of PMA-induced change in chemiluminescence. Data is a representative experiment of results shown in Figure 6.19. The data is presented as relative light units. Neutrophils + diluents (■), neutrophils + PMA (▲), neutrophils + NT8 (Δ) and neutrophils + PMA + NT8 (○).

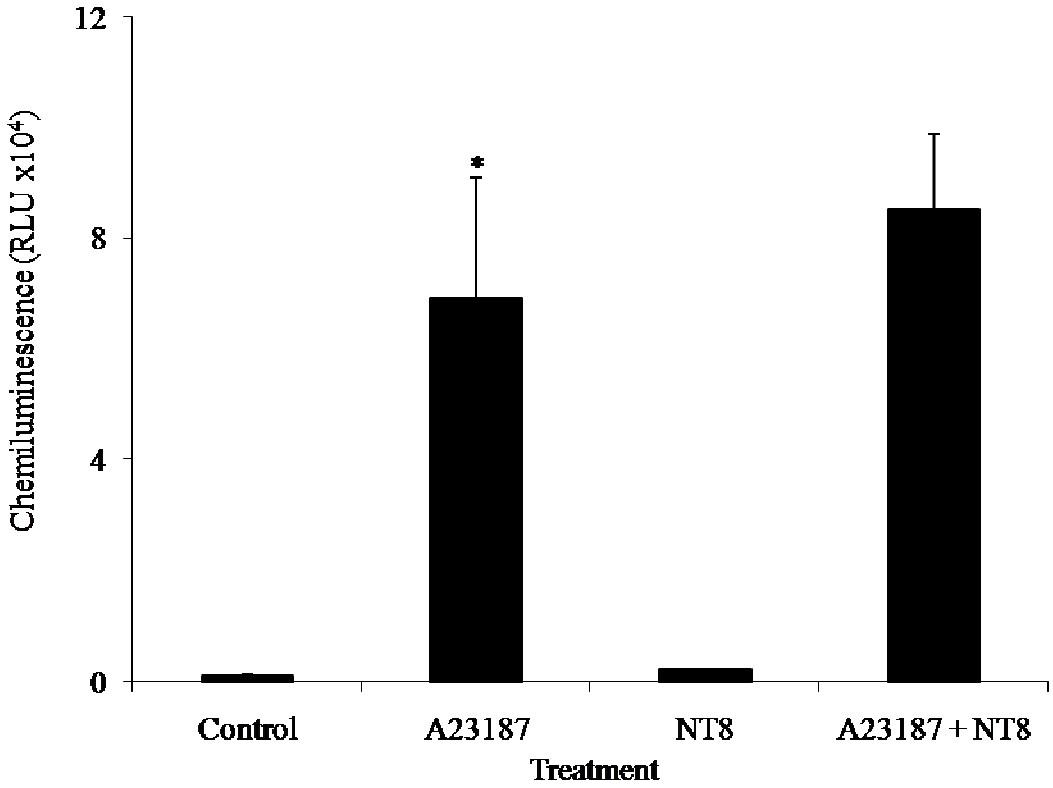


Figure 6.21 Effect of NT8 on A23187-induced chemiluminescence. Neutrophils (5 x 10^6/ml) were pre-treated with NT8 (50 μM) for 1 h and then stimulated by adding 100 μl of A23187 (10 μM). The resultant chemiluminescence production was measured in a luminometer. The data represent initial peak rates of chemiluminescence and are presented as the mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and A23187: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

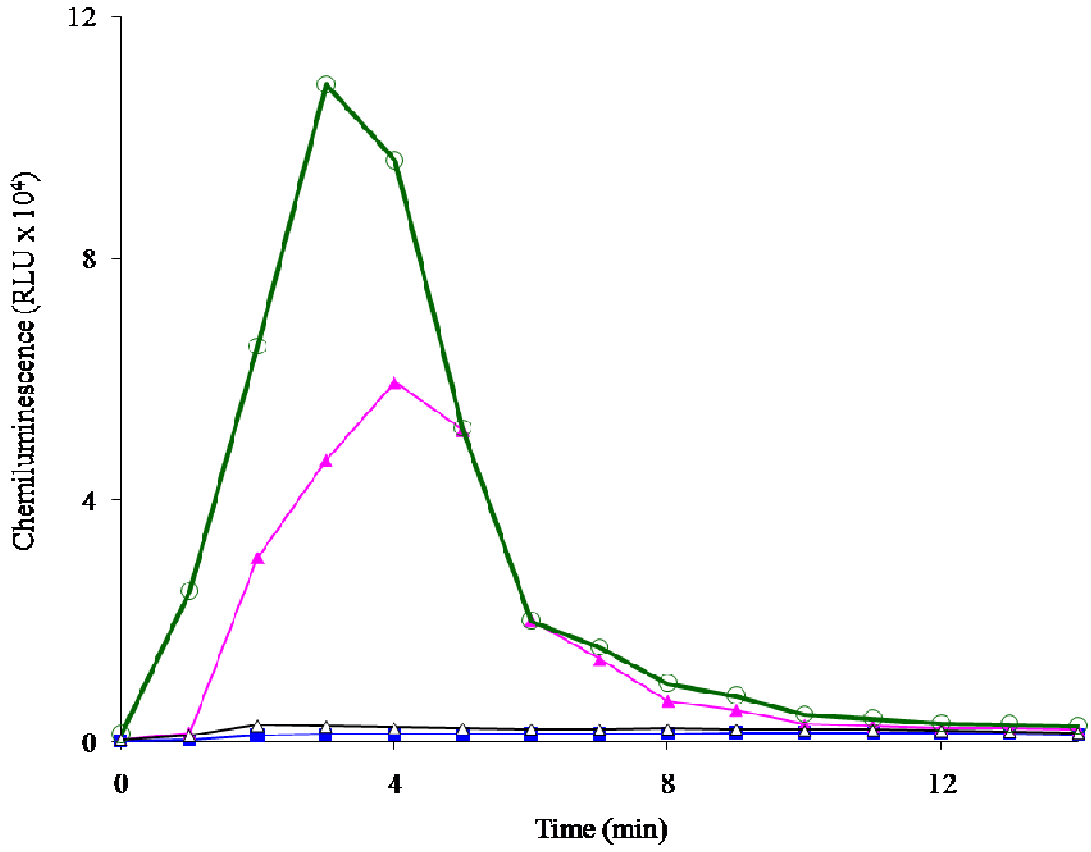


Figure 6.22 Effect of NT8 shown as kinetics of A23187-induced change in chemiluminescence. Data is a representative experiment of results shown in Figure 6.21. The data is present as relative light units. Neutrophils + diluents (■), neutrophils + A23187 (▲), neutrophils + NT8 (Δ) and neutrophils + A23187 + NT8 (○).

6.7 The effects of NT8 are irreversible

To gain further insights into the mechanisms of NT8 affects, we examined whether the inhibitory effects were reversible by washing the cells after the pretreatment. Neutrophils were pre-treated with either 20 or 50 µM of NT8 and then washed three times with medium before stimulating them with either TNF-RM or recombinant TNF. Cell adherence was measured. The results showed that the effect was not reversible by washing (Figure 6.23). Similar results were obtained when recombinant TNF was used as the stimulator (Figure 6.23).

6.8 Effects of NT8 on agonist induced up regulation of CR3 expression.

It is well established that neutrophil agonists increase the surface expression of the functional cell surface receptors such as, CR3. This receptor plays a role in neutrophil adhesion to plasma coated surfaces (Schleiffenbaum *et al.*, 1989; Powell *et al.*, 1997). It was therefore of interest to examine whether NT8 caused a reduction in the ability of TNF to induce an increase in expression of this receptor. To follow the expansion of CR3 we used a monoclonal antibody against the CD11b peptide of this receptor and used flow cytometry analysis as described in Chapter 2.16 (Moghaddami *et al.*, 2003). When neutrophils were pre-treated with 50 µM of NT8 for 1 h and then stimulated with TNF-RM, there was no reduction in CR3 expression (Figure 6.24). A histogram of the results obtained from a representative experiment is shown in Figure 6.25.

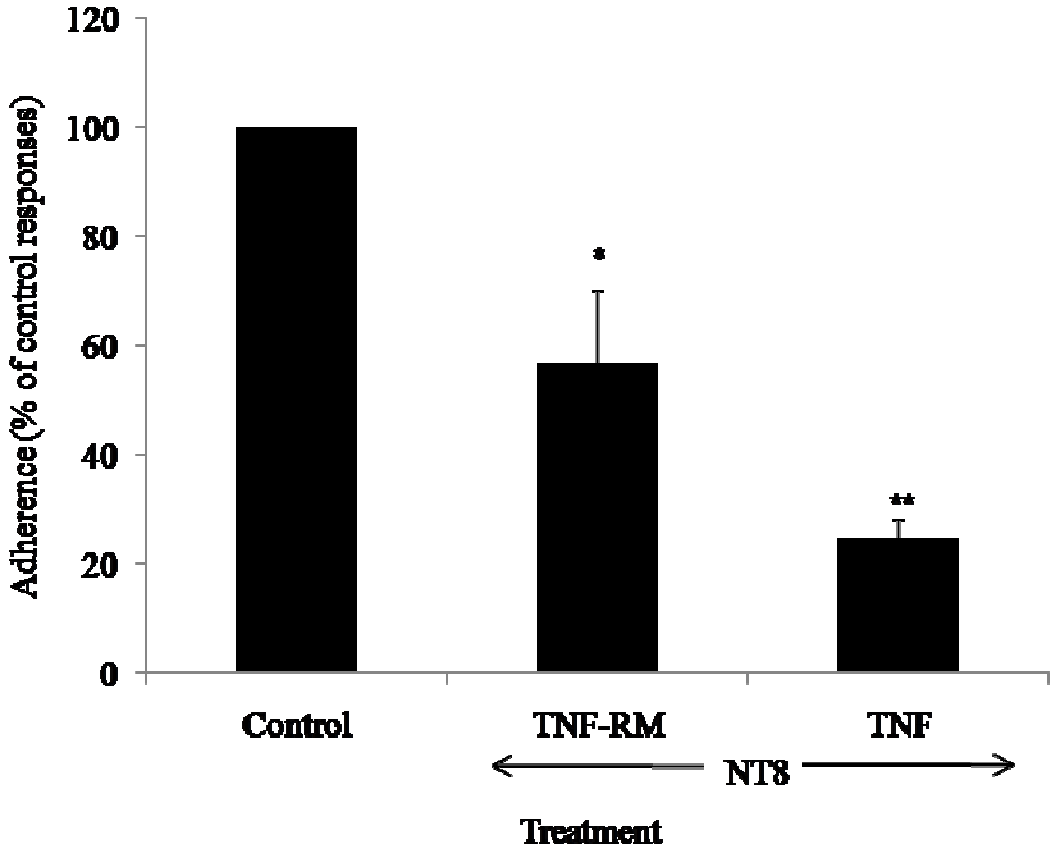


Figure 6.23 Irreversible effect of NT8 on TNF-RM/TNF mediated neutrophil adherence. Neutrophils (5 x 10^6/ml) were pre-treated with 50 μM of NT8 for 1 h and prior to stimulation; cells were washed three times with HBSS. Adherences in response to TNF-RM or TNF were then tested. The data are expressed as % of the control responses and presented as mean ± SEM of 3 experiments, each conducted with neutrophils from a different donor. The base and stimulated OD 570 nm values ± SEM for the controls were 0.08 ± 0.01 and 0.26 ± 0.05 and 0.07 ± 0.03 and 0.34 ± 0.05 respectively. Significance of difference between control and TNF-RM + NT8; control and TNF + NT8: *$p<0.01$ and **$p<0.001$ respectively (Tukey-Kramer multiple comparisons test).

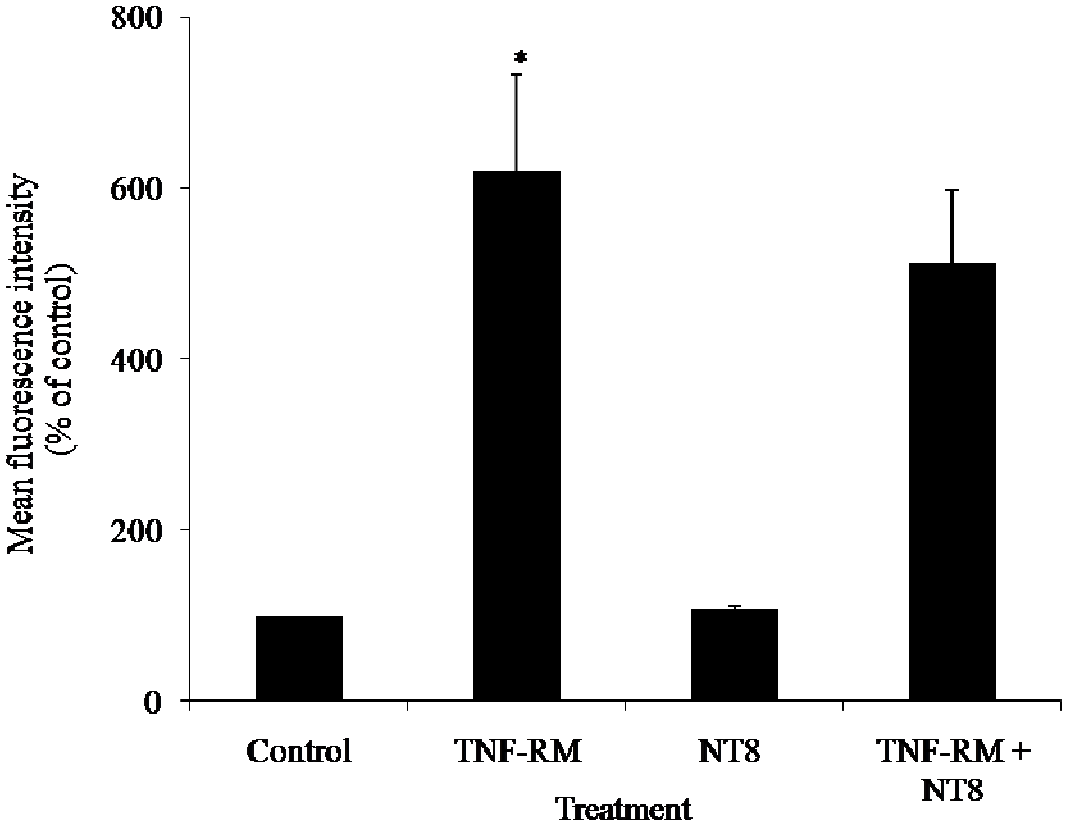


Figure 6.24 Effect of NT8 on TNF-RM-induced CD11b expression. Neutrophils (5 x 10^6/ml) pre-treated with NT8 (50 μM) for 1 h were then stimulated with TNF-RM (1 h). The cells were then stained with a PE-labelled mouse anti-human CD11b antibody and CD11b expression analyzed by flow cytometry. Data expressed as mean % of control ± SEM from 3 experiments, each conducted with neutrophils from a different donor. Significance of difference between control and TNF-RM: *$p<0.01$ (Tukey-Kramer multiple comparisons test).

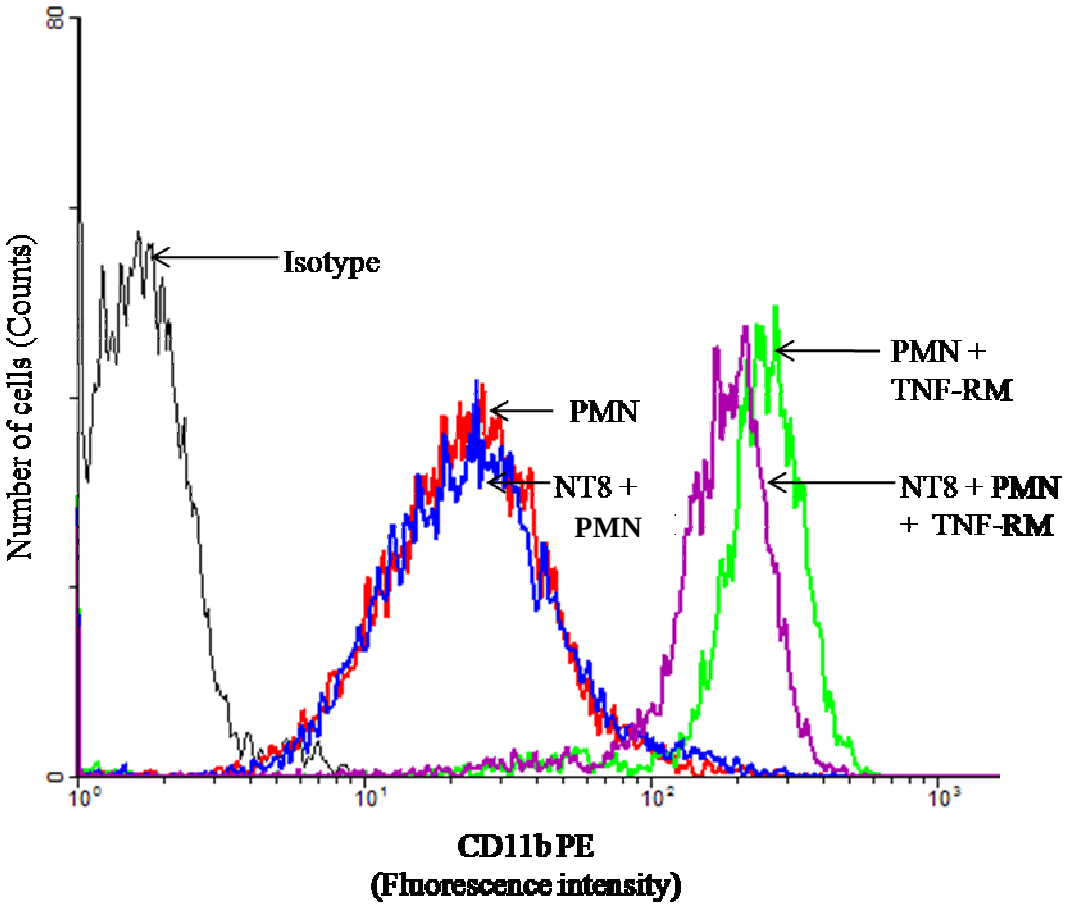


Figure 6.25 Histogram of the effects of NT8 on TNF-RM-induced increase in CD11b expression. Data is a representative experiment of results shown in Figure 6.24.

6.9 Effects on TNFR expression

In consideration of the data from Chapter 4, 5, 6 and from the above data generated, adherence induced by TNF was highly sensitive to the action of NT8. This response was inhibited at 20 µM. At this concentration the responses to agonists which bypass the surface receptor, PMA and A23187 were not affected. One possible action of NT8 on the TNF-induced response is to cause an alteration on the expression of TNF receptors. This was therefore investigated. Neutrophils were incubated with NT8 for 1 h and then examined for expression of TNFRI and TNFRII using anti-CD120a and anti-CD120b labeled monoclonal antibodies (Moghaddami *et al.*, 2003). The corresponding labeled IgG_1 isotype antibody was used as isotype control. The cells were then treated and analysed by flow cytometry.

The data presented in Figure 6.26 show that NT8 significantly decreased the expression of TNFRII but not TNFRI. This occurred at 20 µM, suggesting that the inhibition of the TNF-induced adherence response is likely to be due to a decreased in expression of this receptor. Representative histograms for this data have been presented in Figure 6.26 and 6.27A and B.

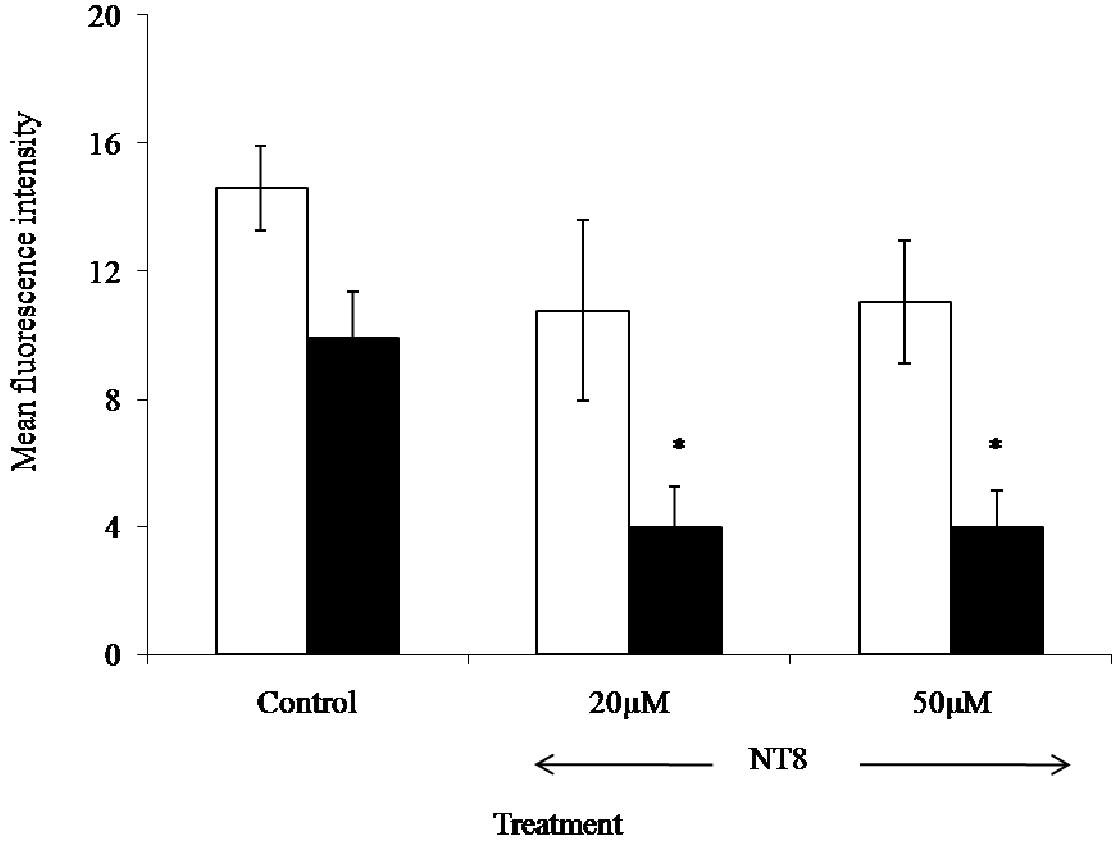


Figure 6.26 Effect of NT8 on neutrophil TNF receptor I and II expression. Neutrophils (5 x 10^6/ml) in HBSS were pre-treated with various concentration of NT8 (20 and 50 µM) and then stained with either anti-CD120a (□) or CD120b (■) for 30 min. An isotype control, mouse IgG_1, was also used. Then a goat anti-mouse FITC labeled immunoglobulin was added and the cells incubated for a further 30 min. The cells were washed and the levels of TNF receptor I and II are measured using a FACscan. Data expressed as mean fluorescence intensity minus the isotype control ± SEM of 4 experiments each done in duplicate with neutrophils from a different donor. Significance of difference in the CD120b group, between control and 20 µM and between control and 50 µM group: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

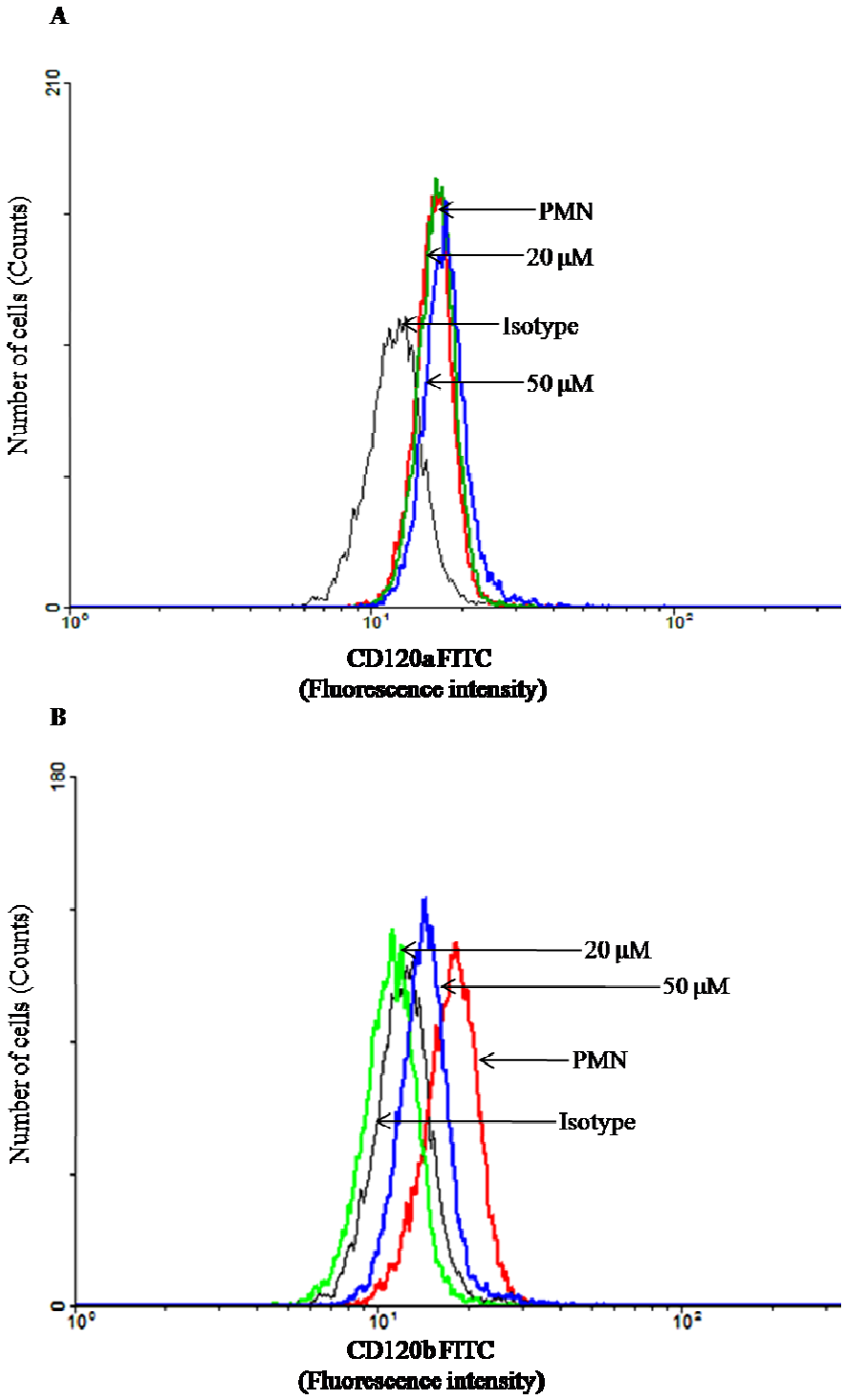


Figure 6.27 Histogram of the effect of NT8 on TNFRI (A) and TNFRII (B). The data is from a representative experiment of results presented in Figure 6.26.

6.10 Effects on sphingosine pathway

Since NT8 has a structure that is similar to 3-ketosphinganine (3KDS) ($C_{18}H_{37}NO_2$), one of the substrates crucial for ceramide formation, it is possible that NT8 affects 3KDS synthesis. A serine palmitoyl transferase assay was performed according to Williams *et al.*, (1984) and Holleran *et al.*, (1990) to measure the final yield product 3KDS as described in Chapte 2.13.3. A microsomal protein fraction was prepared from mouse liver. The reaction mix, containing all the initiating substrates such as pyridoxal phosphate, palmitoyl CoA, [^{3}H] serine, serine and microsomal protein was prepared. A modification was made to the experiment by adding NBPP, ATP or Mg^{2+} so as to increase the yield of the final product. NT8 (100 μM) was also added to the mixture to see if it inhibits the formation of 3KDS. The reaction was terminated by adding ammonium hydroxide and the extraction of the final product was affected by the addition of sphingosine base and washing with chloroform. The amount of 3KDS generated was then measured in a scintillation counter.

Due to the fact that this experiment is measuring the generation of 3KDS which depends highly on the availability of palmityl CoA enzyme, substrates/inhibitors such as NBPP, ATP and Mg^{2+} were incorporated into the experiment to aid in either the regeneration or the availability of this enzyme. Results showed that neither NBPP or ATP resulted in an increase of the yield for 3KDS (Figure 6.28). However when ATP was added along with Mg^{2+}, this resulted in an increase of 3KDS was yielded and under these conditions, NT8 at 100 μM did not result in the inhibition of the generation of 3KDS.

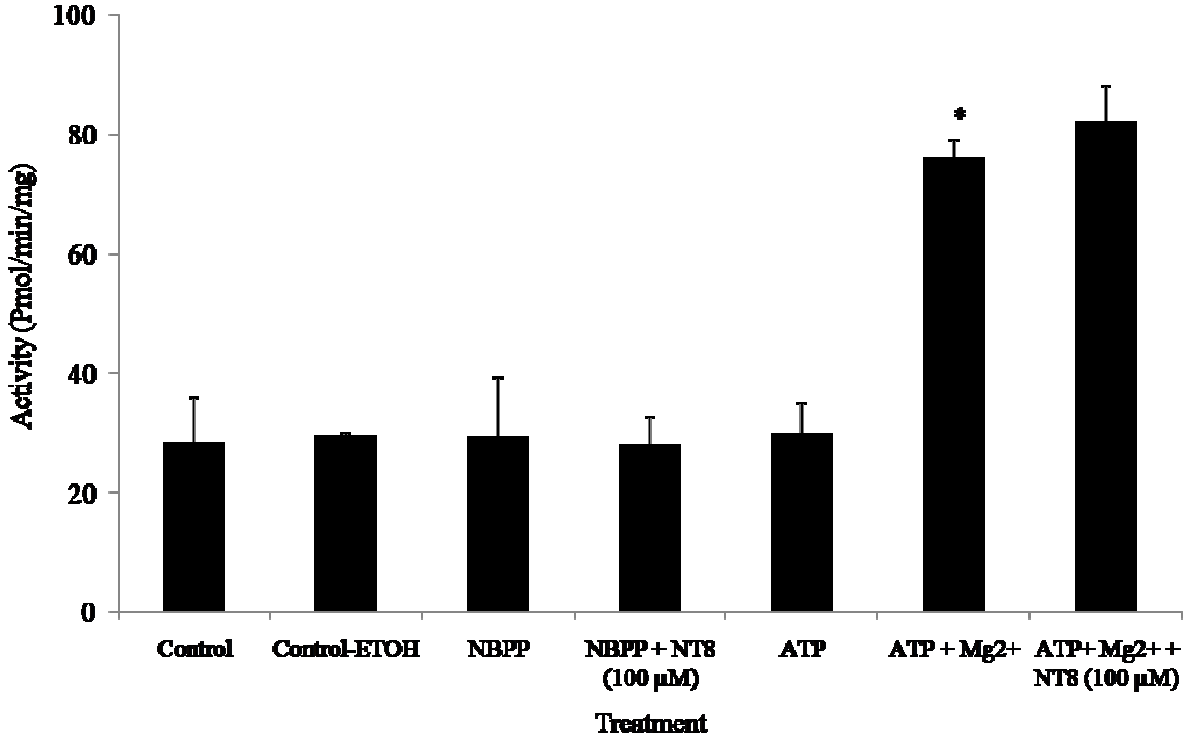


Figure 6.28 Effect of NT8 on serine palmitoyltransferase activity. Assays were conducted under different conditions so to increase the SPS activity. NBPP, ATP or a combination of ATP and Mg^{2+} was used along with NT8 and the resulting radioactivity was measured via a Beta counter. Data expressed as activity per pmol/min/mg produced ± SEM from 2 experiments. Significance of difference between control and ATP + Mg^{2+}: *$p<0.01$ (Tukey-Kramer multiple comparisons test).

6.11 Summary

It is evident from the data presented in this chapter that in comparison to several other cell surface receptor acting agonists NT8 preferential inhibits the neutrophil adherence response to TNF (Table 6.1). Of the six other agonists examined fMLP, LTB_4, GM-CSF, IL-8 C5a and LPS only the response to LTB_4 was affected to a similar degree to the TNF response (Tabe 6.1).

The effects on the TNF response were unrelated to a post receptor binding effect. At 20 µM neither the PMA nor the A23187 induced adherence response was affected. It was evident that NT8 down regulated the expression of TNFRII but not TNFRI on neutrophils, most likely accounting for the decreased response at this concentration. It was however interesting that the TNF-induced upregulation of CR3 expression was not affected by NT8.

As the concentration of NT8 increased to 50 µM, the selectivity of the TNF responses was lost as we saw an effect on adherence induced by all agonists tested. At this concentration substantial inhibition occurred for responses induced by PMA and A23187, suggesting that an effect at the post surface receptor level was a key to the mechanism of action. Attempts to define this further, met with limited success. The possibility that NT8 was affecting the sphingosine pathway was examined but this appeared not to be the case.

In chapter 5, data was presented which showed that inhibition of the respiratory burst response induced by TNF was restricted to 50 µM NT8. This also occurred for fMLP but not LTB_4 and GM-CSF. The results then suggest that NT8 has preference in inhibiting this response to TNF. This was further supported by the fact that the responses to AA and PMA

were not inhibited.

While TNF is not a chemoattractant for neutrophils it was of interest to see if NT8 altered other neutrophil functions such as chemotactic responsiveness to fMLP. No effect was observed. This again clearly supported our view that NT8 has selective effects on neutrophil responses.

Table 6.1 Summary of the effects of NT8 on neutrophil adherence.

Stimulus	NT8 (% of inhibition)		
	5 µM	**20 µM**	**50 µM**
TNF-RM	0	40[b]	85[c]
TNF	0	61[b]	85[c]
fMLP	0	0	81[c]
LTB_4	0	44[a]	71[b]
GM-CSF	0	6	76[a]
IL-8	0	25	55[b]
C5a	0	0	67[a]
LPS	0	11	52[a]
AA	0	0	77[b]
PMA	0	7	67[b]
A23187	0	1	79[b]

The values represent the % inhibition of the adherence response caused by the indicated amounts of NT8 from 3 experiments. Significance of difference from control: [a]$p<0.05$, [b]$p<0.01$ and [c]$p<0.001$ (Tukey-Kramer multiple comparisons test).

7.0 CHAPTER 7 THE EFFECTS OF NT COMPOUNDS ON MONONUCLEAR CELL RESPONSES

7.1 Introduction

The hallmark of chronic inflammatory diseases such as RA is the intense infiltration, activation and proliferation of macrophages and the subsequent release of cytokines which regulate lymphocytes *pe* se or other leukocytes such as macrophages at these inflammatory sites. Similarly, macrophage's action in this disease is also mediated through the release of cytokines such as TNF at these inflammed sites. These function in an *in vitro* setting provide a useful approach to assess the effects of NT8 on the lymphocyte and macrophage responses.

CQ and HCQ have been shown to inhibited superoxide anion release from monocytes as well as phospholipid methylation (Hurst *et al.*, 1986), down-regulated monocyte FcγR expression and depressed IL-1β, IL-2, TNF and IL-6 production (Goldring and Nemaorani, 1999; Jang *et al.*, 2006; Landewe *et al.*, 1995; Karres *et al.*, 1998). Moreover results from clinical trials showed that proliferation responses of lymphocytes from RA patients treated with chloroquine for a period of 3.6 years were inhibited compared to cells from controls (Panayi *et al.*, 1973). The presentation of *Listeria* antigens to T cells was also inhibited by CQ (Ziegler and Unanue, 1982). The anti-malarial drug was also found to disrupt calcium signals in T cells, and B cells (Goldman *et al.*, 2000). Another cell signal which CQ inhibited was the activation of ERK in PBMCs (Weber *et al.*, 2002).

Furthermore, HCQ has been found to inhibit macrophage accumulation in a peritoneal inflammation model (Ackerman *et al.*, 1980) and CQ resulted in a decreased papaya latex-induced rat paw inflammation (Gupta *et al.*, 1992). The effects on mononuclear leukocytes were manifested both as an inhibitor of cell proliferation, cytokine production,

signal transduction and antigen presentation. Since both T cells and macrophages are major players in the pathogenesis of chronic inflammatory diseases and CQ and HCQ have been found to be useful in treating conditions such as RA, we examined the effects of NT8 on T cell and macrophage function. It was therefore important to extent of the leukocyte inhibition properties of the NT compounds on mononuclear leukocyte functions. The aim of the present study was to exmine the effects of NT compounds on lymphocyte proliferation and cytokine production as well as effects on cytokine production by macrophages.

7.2 Effect of NT compounds on PHA-induced lymphocyte proliferation

Initial studies examined the effects of NT compounds on PHA-induced lymphocyte proliferation. Human PBMCs were pre-incubated with 50 µM of an NT compound or chloroquine for 1 h and then challenged with PHA. After 48 h of culture the degree of proliferation was assessed. The data presented in Table 7.1 demonstrate that none of the 13 NT compounds examined caused inhibition of T cell proliferation. However at the same concentration CQ caused a 50 % inhibition of the PHA-induced lymphoproliferation.

Table 7.1 Effect of NT compounds and CQ on PHA stimulated lymproliferation.

Compounds (NT)	**Proliferation (% of control responses)**
0	92 ± 1.53
1	90 ± 4.04
2	95 ± 6.01
3	97 ± 4.58
4	87 ± 2.73
5	88 ± 4.98
6	89 ± 2.52
7	99 ± 3.06
8	95 ± 2.52
9	95 ± 2.60
10	88 ± 4.04
11	93 ± 7.00
12	109 ± 11.06
13	96 ± 7.84
CQ	52 ± 0.58*

PBMCs were pre-treated with 50 μM of an NT compound or CQ for 1 h. Data are expressed as the mean % of methyl-[^{3}H]-thymidine incorporated in control group ± SEM from 3 experiments, each conducted with PBMCs from a different donor. Significance of difference between control and CQ: *$p<0.001$ (Dunnett compare all vs control).

7.3 Effect of NT8 and CQ on PHA-induced lymphocyte cytokine production

Although the above results showed that NT8 did not inhibit PHA-induced lymphoproliferation, measuring this function does not simply imply that other functions such as cytokine production are not affected. Further investigations were conducted to examine the effects of NT8 on the PHA-induced T cell production of IFN-γ, IL-2, LT and IL-10.

In these assays the PBMCs were pre-exposed to either 20 µM or 50 µM of NT8 for 1 h and then challenged with PHA. Other sets of PBMCs were treated with either lauric acid, NT0 or CQ. The cells were cultured for 48 h and pulsed with ^{3}H-TdR for further 6 h. Prior to harvesting the cells, cell-free culture supernatnats were collected for cytokine measurement.

The results are summerized in Table 7.2. The data showed that while CQ inhibited lymphoproliferation (Figure 7.1), NT8 had no effect and neither did lauric acid nor NT0. CQ at 50 µM significantly inhibited the production of IL-2 and IL-10 but not IFNγ and LT by PBMCs stimulated with PHA (Figure 7.2). The most affected was the production of IL-10. In compansion, NT8 treatment of PBMCs had no effect on the production of any of the four cytokines examined. The effects of CQ on lymphocyte proliferation did not follow a concentration related effect over 5-50 µM. Significance was seen at 5 and 50 µM but not at 20 µM.

Table 7.2 Effect of NT8 on lymphoproliferation and cytokine production in human mononuclear cell culture.

Stimulus	Function	NT8 (µM)		LA (µM)		NT0 (µM)	
		20	**50**	**20**	**50**	**20**	**50**
PHA	Proliferation	107 ± 12	109 ± 16	113 ± 21	121 ± 17	90 ± 6	102 ± 8
	IFN-γ	ND	156 ± 44	ND	92 ± 18	ND	87 ± 16
	IL-10	ND	97 ± 10	ND	102 ± 10	ND	98 ± 10
	IL-2	ND	177 ± 76	ND	151 ± 30	ND	180 ± 44
	LT	ND	161 ± 29	ND	124 ± 32	ND	133 ± 36
TT	Proliferation	98 ± 16	76 ± 24	88 ± 24	110 ± 22	88 ± 24	55 ± 17
	IFN-γ	ND	71 ± 17	ND	141 ± 22	ND	68 ± 16
	IL-10	ND	53 ± 13[a]	ND	90 ± 18	ND	40 ± 5[a]
	IL-2	ND	212 ± 63	ND	96 ± 9	ND	100 ± 34
	LT	ND	66 ± 25	ND	146 ± 34	ND	75 ± 16
S.aureus	Proliferation	85 ± 25	80 ± 17	87 ± 11	122 ± 37	95 ± 12	113 ± 51
LPS	TNF-α	ND	189 ± 62	ND	224 ± 5	ND	201 ± 118
	IL-1β	ND	125 ± 27	ND	174 ± 21	ND	88 ± 31
	IL-6	ND	117 ± 6	ND	118 ± 10	ND	87 ± 12

Data are expressed as the mean of % control values ± SEM from 3 experiments, each conducted with PBMCs from different donor.
Significance of difference from control: [a]$p<0.05$(Tukey-Kramer multiple comparisons test).
Abbreviations: LA-lauric acid, LT-lymphotoxin, TT-tetanus toxoid and ND- experiment not done.

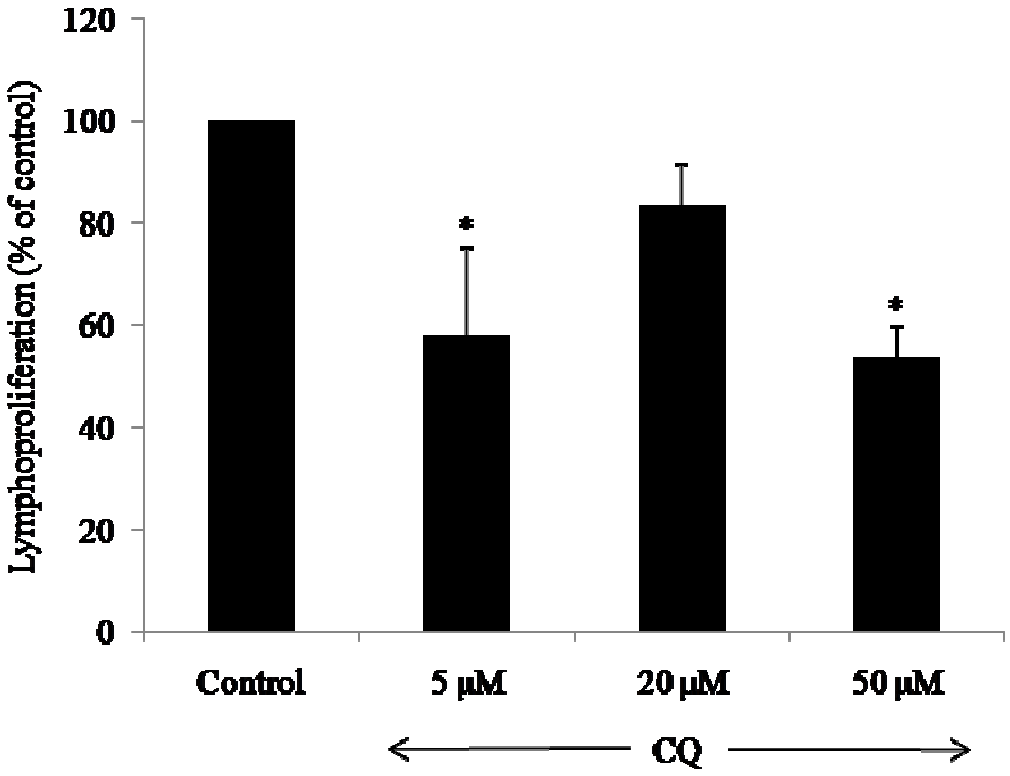


Figure 7.1 Effect of CQ on PHA- induced PBMC proliferation. PBMC (5 x 10^6/ml) were pre-treated with varying concentrations of CQ for 1 h and then stimulated by adding 100 µl of PHA (2 µg/ml) for 48 h 37 °C with 5 % CO_2. Six hours prior to harvesting, cells were pulsed with 1 µCi of methyl-[^{3}H]-thymidine and incorporated radioactivity measured. The data are expressed as % of the control and presented as mean ± SEM of 3 experiments, each conducted with PBMCs from a different donor. The basal and stimulated DPM values ± SEM were 1774 ± 529 and 185062 ± 73573 respectively. Significance of difference compared to control: *$p<0.05$ (Dunnett: compare all vs control).

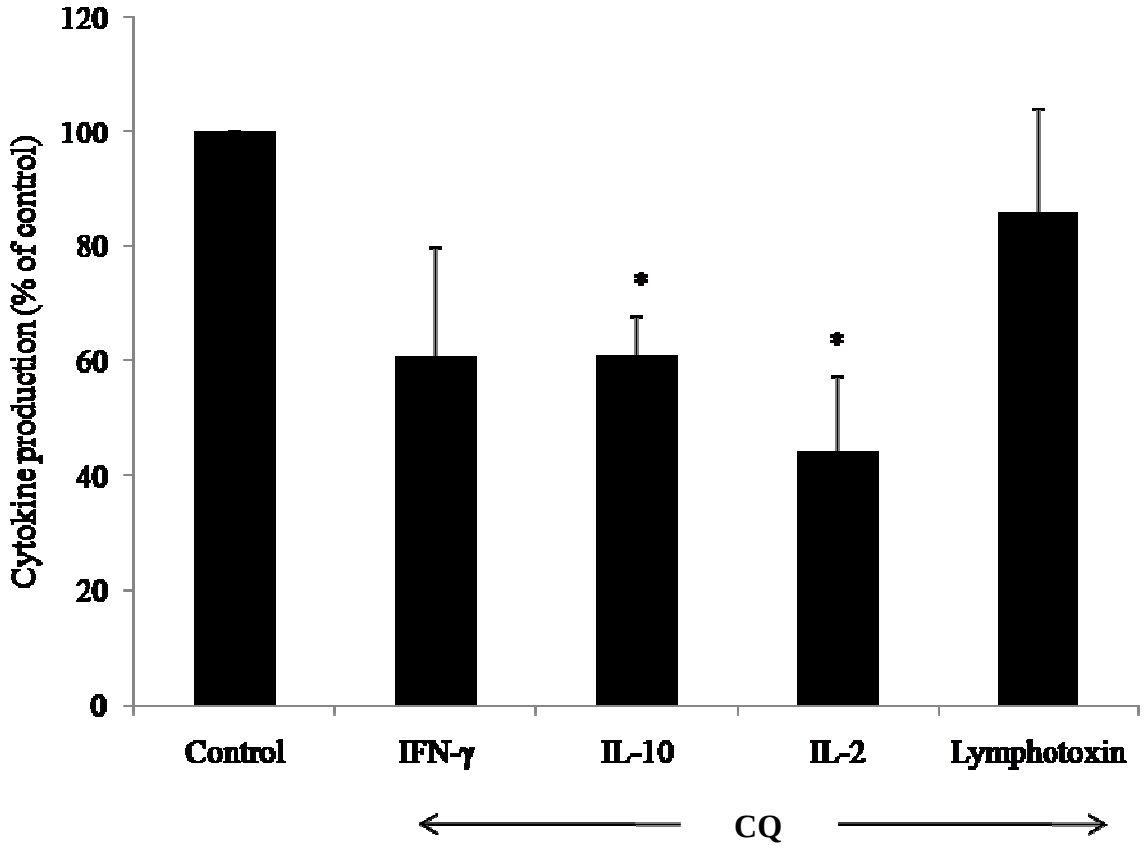


Figure 7.2 Effect of CQ on PHA-induced cytokine production. PBMCs (5 x 10^6/ml) were pre-treated with CQ (50 µM) for 1 h and then stimulated by adding 100 µl of PHA (2 µg/ml) for 48 h at 37 °C with 5 % CO_2. Cell culture fluids were harvested and cytokine levels measured by cytometric bead array. The data are expressed as % of the control and presented as mean ± SEM of 3 experiments, each conducted with PBMCs from a different donor. Significance of difference compared to control: *$p<0.05$ (Dunnett: compare all vs control).

7.4 Effect of NT8 and CQ on tetanus toxoid-induced lymphoproliferation and cytokine production

Further properties of NT8 on lymphocytes were examined in a system in which the responses are driven by an antigen, tetanus toxoid (TT). The PBMCs were pre-treated with 5, 20 or 50 µM NT8. The cells were then stimulated with TT and examined for lymphocyte proliferation and production of IFN-γ, IL-2, IL-10 and LT. Other treatments included NT0, lauric acid and CQ.

The results showed that as with the PHA-induced T cell responses, TT-induced lymphoproliferation were markedly inhibited by CQ (Table 7.2, Figure 7.3). The response was inhibited by > 95 % at 50 µM CQ. It was also evident that 5 µM CQ was effective in inhibiting the response (Figure 7.3). A comparison of the effects of 50 µM CQ on the production of the four cytokines showed that it significantly inhibits all four cytokines (Figure 7.4). IL-2, which is required for T cell proliferation was also inhibited by CQ. The most sensitive to CQ was LT and IFN-γ (Figure 7.4). This highlights some important properties of CQ which are likely to have relevance to its anti-inflammatory function.

In contrast to the effects of CQ, neither NT8 nor NT0 or lauric acid inhibited antigen-induced T cell proliferation and cytokine production (Table 7.2), although NT0 and NT8 both caused some inhibition to IL-10 production.

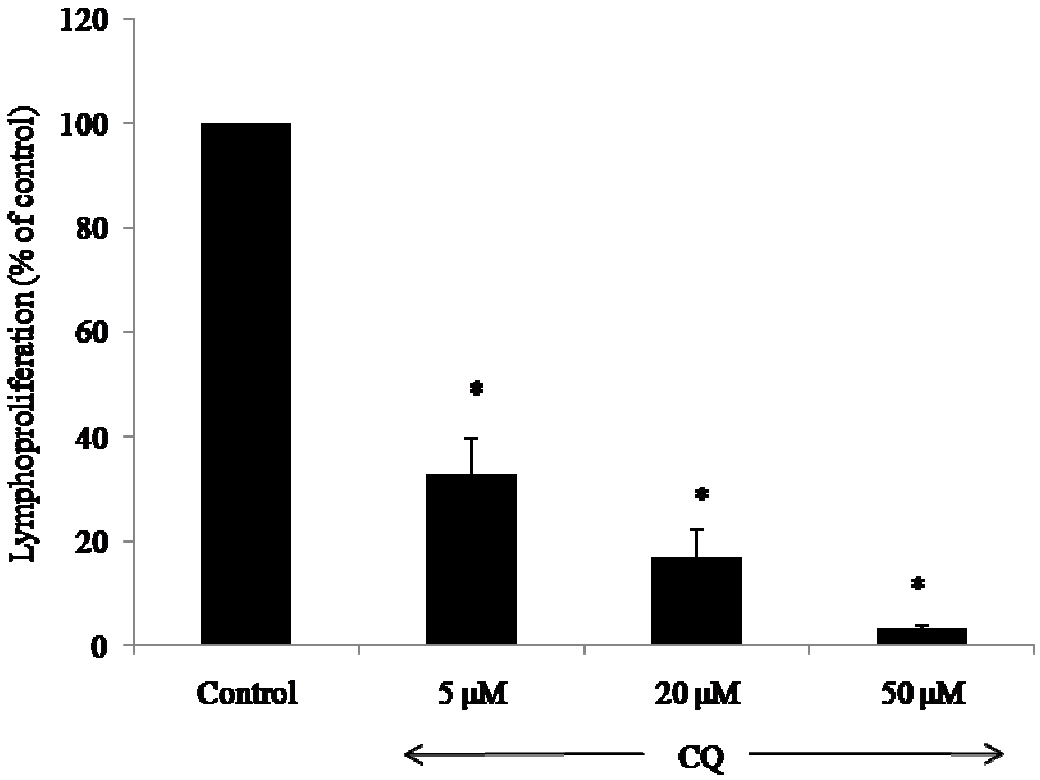


Figure 7.3 Effect of CQ on tetanus toxoid-induced lymphoproliferation. PBMCs (5 x 10^6/ml) were pre-treated with varying concentrations of CQ for 1 h and then stimulated by adding 100 µl of TT (1 µg/ml) for 5 days at 37 °C with 5 % CO_2. Six hours prior to harvesting, cells were pulsed with 1 µCi of methyl-[^{3}H]-thymidine and incorporated radioactivity measured. The data are expressed as % of the control and presented as mean ± SEM of 3 experiments, each conducted with PBMCs from a different donor. The basal and stimulated DPM values ± SEM were 3855 ± 1894 and 82069 ± 16946 respectively. Significance of difference compared to control: *$p<0.05$ (Dunnett: compare all vs control).

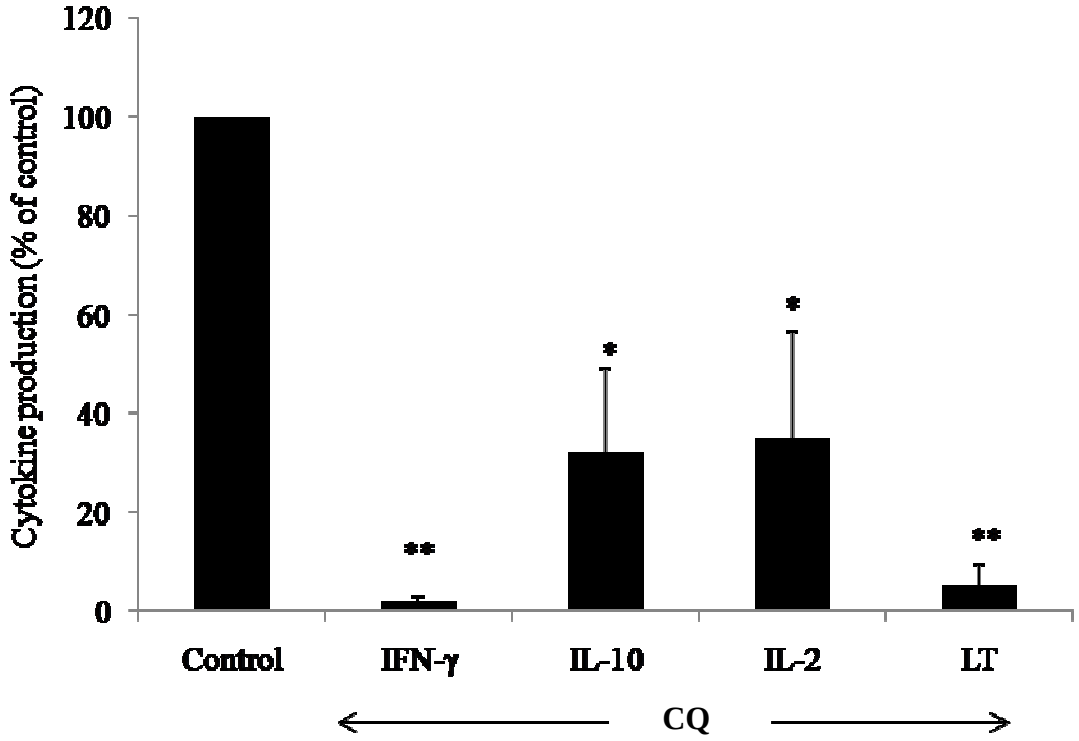


Figure 7.4 Effect of CQ on tetanus toxoid-induced cytokine production. PBMCs (5 x 10^6/ml) were pre-treated with CQ (50 µM) for 1 h and then stimulated by adding 100 µl of TT (1 µg/ml) for 5 days at 37 °C with 5 % CO_2. Cell culture fluids were harvested and cytokine levels measured by cytometric bead array. The data are expressed as % of the control and presented as mean ± SEM of 3 experiments. Significance of difference compared to control: *$p<0.05$ and **$p<0.01$ (Dunnett: compare all vs control).

7.5 Effect of NT8 and CQ on *S.aureus* –induced lymphoproliferation

Since NT8 has no effect on PHA mediated PBMC proliferation, the effect on *S.aureus,* a B cell mitogen, induced proliferation was examined (Forsgren *et al.*, 1976). PBMCs were first pre-treated with NT8, NT0, LA or CQ and then stimulated with *S.aureus*. Cell proliferation was assessed by measuring the degree of ^{3}H thymidine incorporation after 48 h of culture. Results showed that there was no effect on the *S.aureus* induced PBMC proliferation at 50 μM by NT8, lauric acid or NT0 (Table 7.2). However this response was significantly inhibited by CQ at 50 μM (Figure 7.5).

7.6 Effect of NT8 and CQ on LPS-induced cytokine product in PBMCs

LPS is a strong inducer of the pro-inflammatory cytokines TNF, IL-1β and IL-6 from macrophages (Xaus *et al.*, 2000; West *et al.*, 1997; Hirohashi and Morrison, 1996). These play important roles in the pathogenesis of chronic inflammatory diseases (Moller and Villiger, 2006; Feldmann and Maini, 2001). PBMCs were pre-treated with either NT8, NT0, LA or CQ and then stimulated with LPS. The results showed that NT8 had no effect on LPS-induced cytokine production (Table 7.2, Figure 7.6). At the same concentration CQ inhibited the production of these cytokines, although significance was only reached with IL-1β and IL-6. In view of the role of these cytokines in chronic inflammatory diseases such as RA, it is likely that this property of CQ contributes to its anti-inflammatory effects.

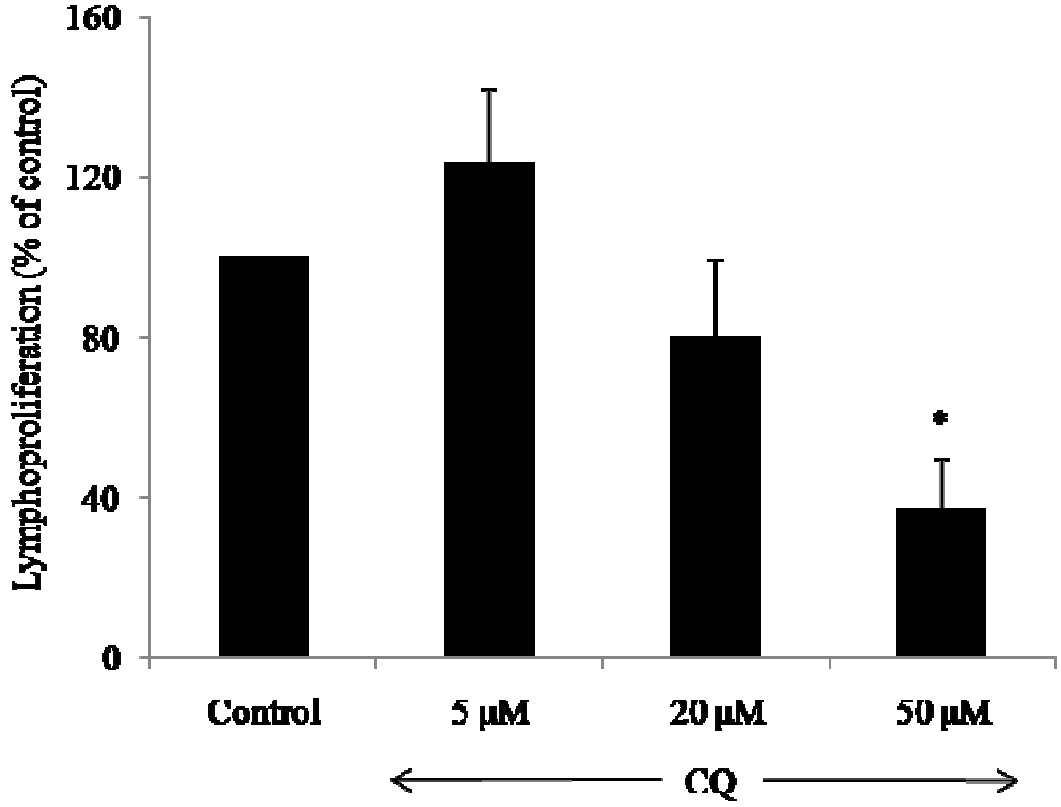


Figure 7.5 Effect of CQ on *S.aureus*-induced lymphoproliferation. PBMCs (5 x 10^6/ml) were pre-treated with varying concentrations of CQ for 1 h and then stimulated by adding 100 µl of *S.aureus* (1 x 10^6/ml) for 48 h at 37 °C with 5 % CO_2. Six hours prior to harvesting, cells were pulsed with 1 µCi of methyl-[^{3}H]-thymidine and incorporated radioactivity measured. The data are expressed as % of the control and presented as mean ± SEM of 3 experiments, each conducted with PBMCs from a different donor. The basal and stimulated DPM values ± SEM were 576 ± 89 and 4161 ± 2620 respectively. Significance of difference compared to control: *$p<0.05$ (Dunnett: compare all vs control).

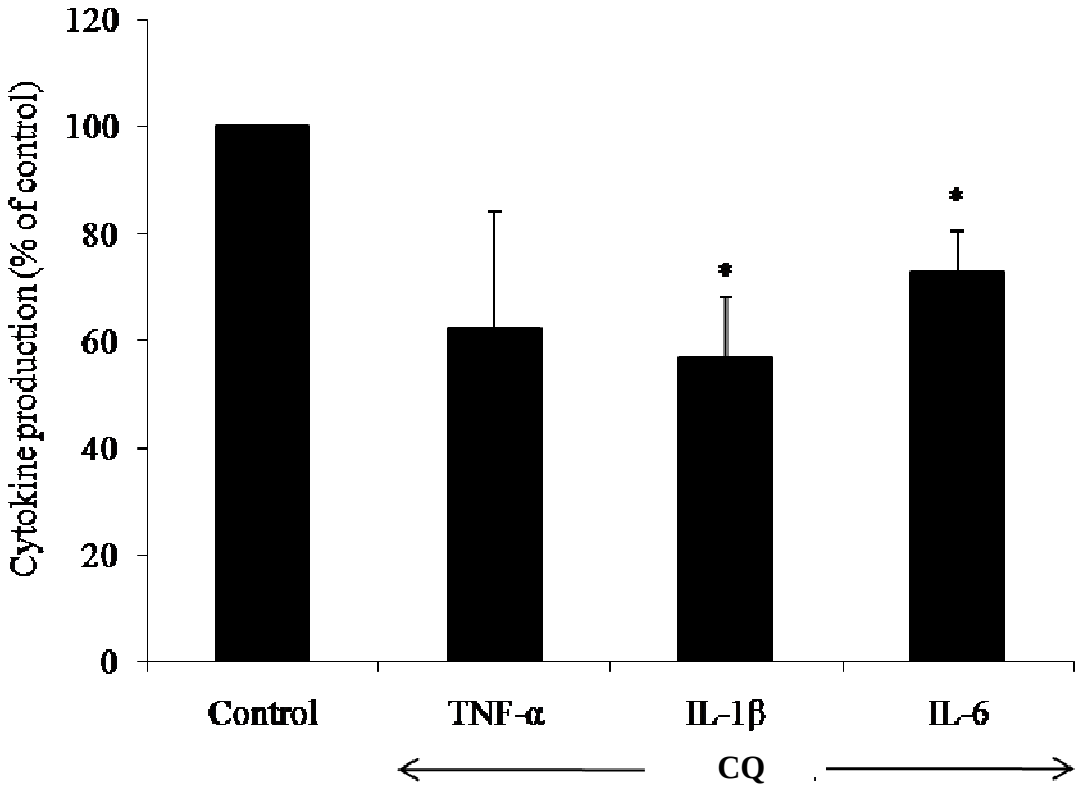


Figure 7.6 Effect of CQ on LPS-induced cytokine production. PBMCs (5 x 10^6/ml) were pre-treated with CQ (50 µM) for 1 h and then stimulated by adding 100 µl of LPS (10 ng/ml) for 48 h at 37 °C with 5 % CO_2. Cell culture fluids were harvested and cytokine levels measured by cytometric bead array. The data are expressed as % of the control and presented as mean ± SEM of 3 experiments, each conducted with PBMCs from a different donor. Significance of difference compared to control: *$p<0.05$ (Dunnett: compare all vs control).

7.7 Summary

The data showed that none of the NT compounds altered the lymphoproliferation response induced by PHA in human PBMC cultures. This is in contrast with their effects on neutrophil functions (Chapter 4).

Further studies with NT8 showed that the compound did not inhibit the cytokines, IFN-γ, LT, IL-2 and IL-10 in response to PHA. Similarly, very little effect was observed on the tetanus toxoid-induced lymphoproliferative response and cytokine production. The response of B cells to *S.aureus* was not affected as well. Furthermore, the response of macrophages in the PBMCs to LPS was also not affected, in terms of TNF, IL-1 and IL-6 production.

In contrast CQ inhibited lymphoproliferation and cytokine production induced by the mitogens, antigens and LPS.

8.0 CHAPTER 8 EXAMINATION OF THE ANTI-INFLAMMATORY PROPERTY OF NT8 *IN VIVO*

8.1 Introduction

While we still need to characterize the properties of NT8 more extensively, it is evident that NT8 has a preference for inhibiting neutrophil responses (Chapters 4 to 7), particularly sensitive was the cell adhesion response initiated by TNF and LTB_4. Because these are key mediators of the inflammatory responses and indeed both are targets for pharmaceuticals, we were interested to see if NT8 was anti-inflammatory *in vivo* ie. within the complexity of an inflammatory reaction (Montecucco *et al.*, 2008; Onnheim *et al.*, 2008; Richter *et al.*, 1995; Mathis *et al.*, 2007; Schultz *et al.*, 1991). The upregulation of neutrophil adhesion is an important early step in the inflammatory process and it would thus be expected that NT8's properties should be translated to the in vivo response.

The study employed a classical animal model being used widely, LPS induced peritoneal inflammation. Apart from being a well established *in vivo* model for acute inflammation, it can provide us with informations such as the type of cells as well as their infiltration rate that can be found in the inflammed cavity. Neutrophil, TNF and LTB_4 in particular, play an important role in this model (Canetti *et al.*, 2003; Cunha and Tamashiro, 1992; Cunha *et al.*, 2008; Mercer-Jones *et al.*, 1999; Zagryagskaya *et al.*, 2008). The aim of the present research was to examine the anti-inflammatory response of NT8 in the LPS-induced peritonitis model..

8.2 Effect of NT8 on LPS-induced inflammation

The effect of NT8 on the LPS-induced inflammatory response was examined. NT8 at 70 mM/kg was injected (ip) 1 h before a 50 µg LPS challenge (ip). The animals were sacrificed 24 h later and the peritoneal exude cells collected. The result showed that LPS induced a

significant recruitment of neutrophils to the peritoneal cavity, but not macrophages (Figure 8.1). Although pre-treatment of mice with NT8 before LPS caused a decrease in neutrophil influx, it did not reach significance. Further experiments were conducted with an increase in NT8 pre-treatment time.

In the subsequent experiments NT8 was injected 4 h prior to the LPS challenge. After 24 h peritoneal exude cells were collected and cells enumerated. While there was also a significant increase in neutrophil influx in the mice which received NT8 alone, there was a marked reduction in the number of neutrophils in the peritoneal cavity induced by LPS as a result of the NT8 treatment (Figure 8.2). No effect on macrophages number was observed.

In a second set of experiments, two doses; 40 mM and 80 mM/kg of NT8 were examined. The mice were treated with NT8 4 h prior to the LPS challenge. After 24 h the peritoneal exudates were collected and cellular infiltrate examined. The results showed that NT8 caused a signifcant decrease in neutrophil influx at 40 mM/kg body weight (Figure 8.3) but that this was markedly increased at 80 mM/kg (Figure 8.3). In contrast the effect of LPS on peritoneal macrophage number was not altered (Figure 8.4).

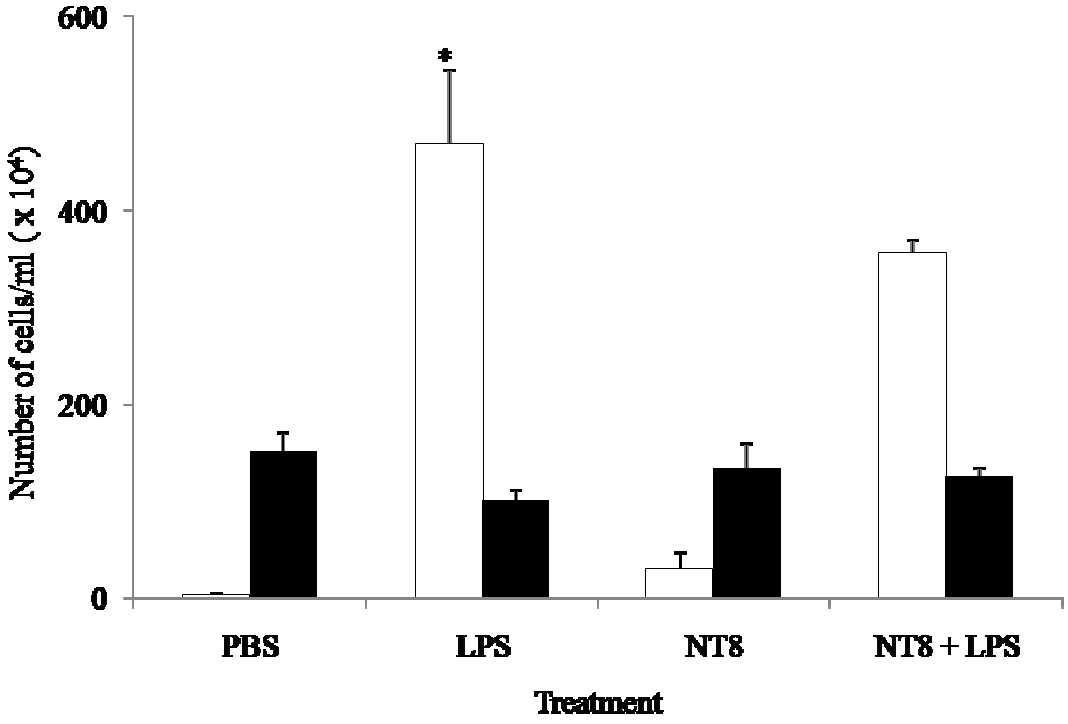


Figure 8.1 Effect of short (1 h) NT8 pre-treatment time on LPS-induced peritoneal cavity inflammation. Mice were injected intraperitoneally with NT8 (70 mM/kg) 1 h prior to PBS or LPS (50 μg) challenge. At 24 h, the animals were euthanized and number of neutrophils (□) and macrophages (■) in peritoneal cavity washouts enumerated. Data is expressed as the mean number of cells/ml ± SEM of 5 animals. Significance of difference in the number of neutrophils between PBS and LPS group: *$p<0.001$ (Tukey-Kramer multiple comparisons test).

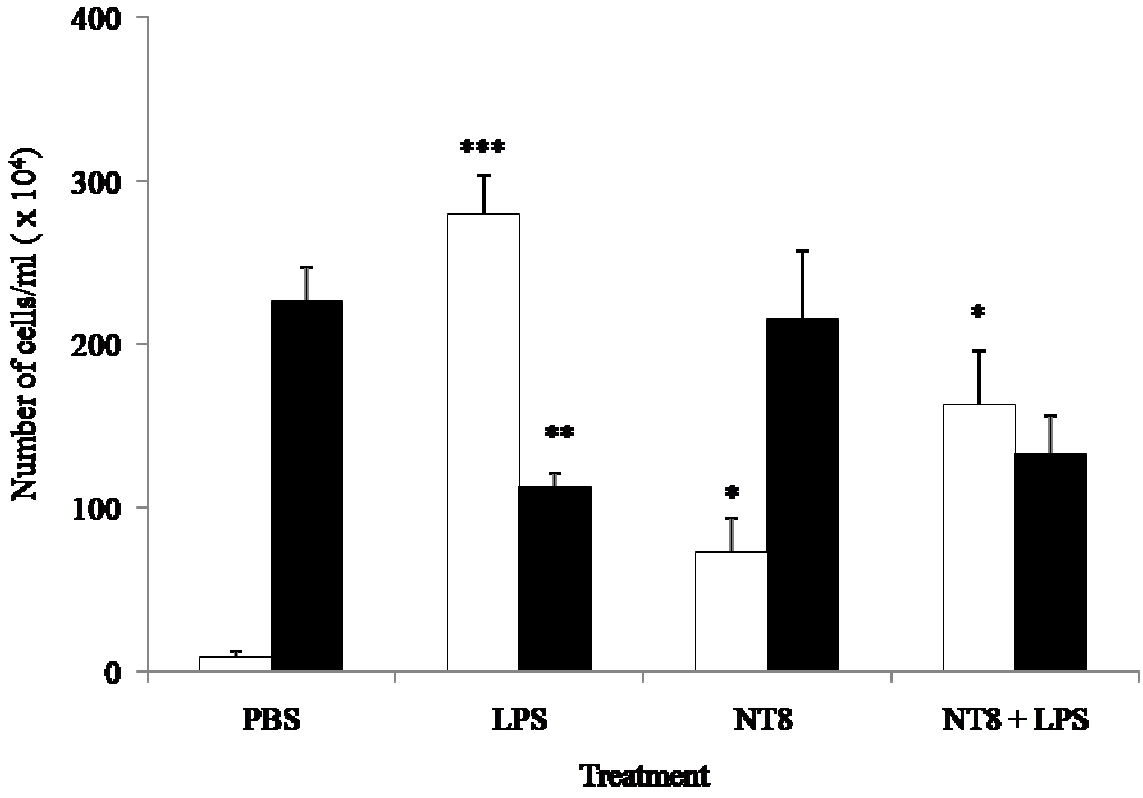


Figure 8.2 Effect of 4 h NT8 pre-treatment time on LPS-induced peritoneal cavity inflammation. Mice were injected intraperitoneally with NT8 (70 mM/kg) 4 h prior to PBS or LPS (50 μg) challenge. At 24 h, the animals were euthanized and number of neutrophils (□) and macrophages (■) in peritoneal cavity washouts were enumerated. Data is expressed as the mean number of cells/ml ± SEM of 6 animals. Significance of difference in the neutrophil group: between PBS and LPS; PBS and NT8; LPS and NT8 + LPS; and in the macrophage group: between PBS and LPS. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ (Tukey-Kramer multiple comparisons test).

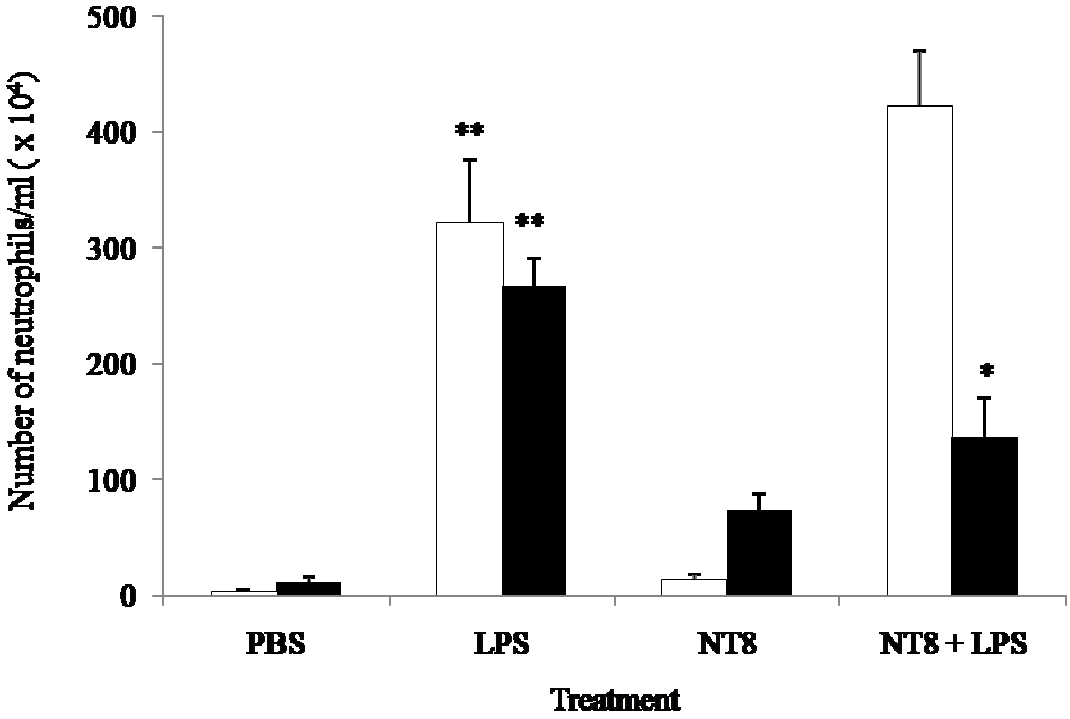


Figure 8.3 Dose dependent effect of NT8 on LPS-induced neutrophils influx. Mice were injected intraperitoneally with either 40 mM/kg (□) or 80 mM/kg (■). Four hours later, animals were then challenged (ip) with LPS (50 μg) or PBS. At 24 h, the animals were euthanized and number of neutrophils in peritoneal cavity washouts enumerated. Data are expressed as mean number of neutrophils/ml ± SEM of 6 animals per group. Significance of difference in 40 mM/kg group: between PBS and LPS; and 80 mM/kg group: between PBS and LPS, between LPS and NT8 + LPS: *$p < 0.05$ and **$p<0.01$ (Tukey-Kramer multiple comparisons test).

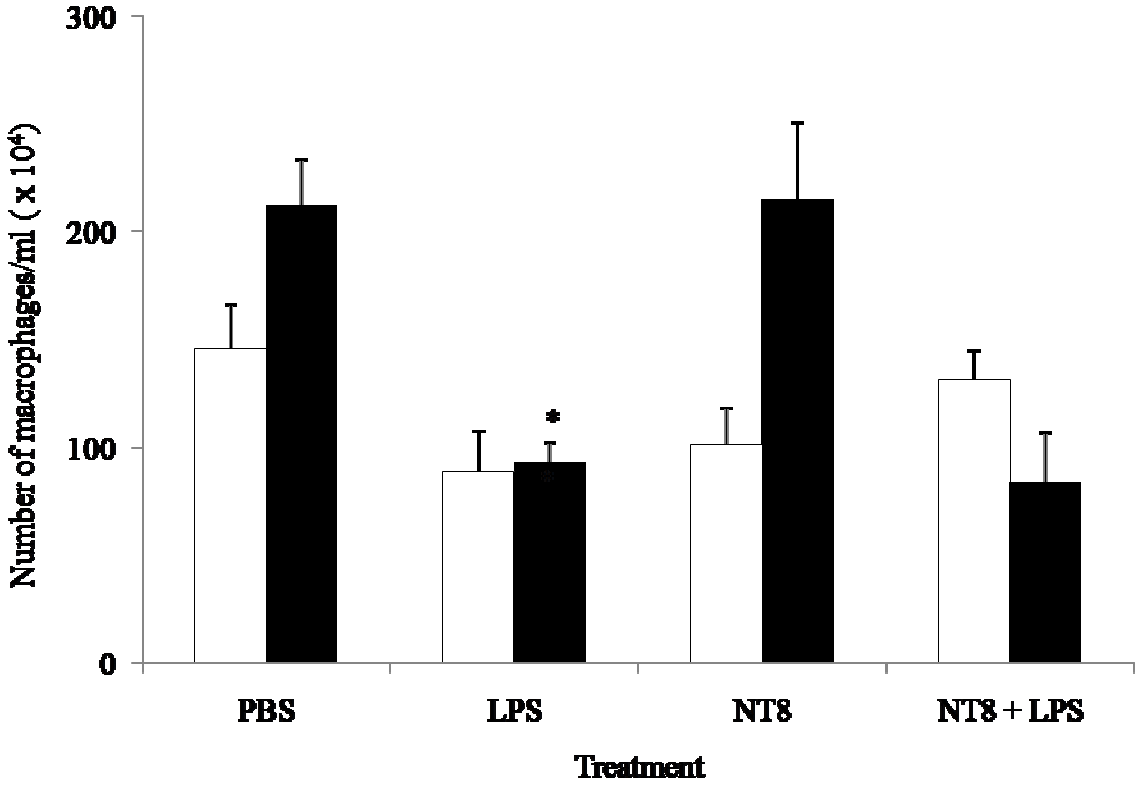


Figure 8.4 Dose dependent effect of NT8 on LPS-induced macrophages influx. Mice were injected intraperitoneally with either 40 mM/kg (□) or 80 mM/kg (■). Four hours later, animals were challenged (ip) with LPS (50 μg) or PBS. At 24 h, the animals were euthanized and number of macrophages in peritoneal cavity washouts enumerated. Data are expressed as the mean number of macrophages/ml ± SEM of 6 animals per group. Significance of difference in 80 mM/kg group: between PBS and LPS: *$p<0.05$ (Tukey-Kramer multiple comparisons test).

8.3 Summary

At optimal pre-treatment time and in a dose related manner NT8 inhibited the influx of neutrophils in the peritoneal cavity of mice injected with LPS. No effect on the number of macrophages in the peritoneal cavity occurred as a result of the treatment with NT8.

9.0 CHAPTER 9 DISCUSSION

Discussion

The research in this thesis has revealed the synthesis of a new structure, following the conjugation of quinoline with fatty acids, which gives rise to singular immunomodulatory properties (Table 9.1). These conjugates have selectivity for neutrophil responses, in particular adherence and cytokine production with essentially little effects on lymphocyte and macrophage function. Their effects contrast to those of CQ and HCQ. The comparative structures of the anti-malarials and NT8 are presented in Figure 9.1.

NT compounds: structure, physical and chemical properties

The immunomodulatory properties of anti-malarial drugs have been extensively studied and these agents have been widely used as model examples of improving drug action by structural modification, usually on the side chain and not the ring structure *per se*. Such modifications have been necessary to overcome the emergence of resistant strains of malarial parasites as well as the concerns in cell or organ toxicity reported in patients being given the drugs. Past studies aiming to generate the modified compounds have reported that replacing the 7-chloro group on the ring structure by electron donor and withdrawing groups such as NH_2, OCH_3 or NO_2 results in the reduction in anti-malarial activity (Egan, 2005), however, modification made to the side chain of 2-3 carbons or of medium chain length of 10-12 carbons still retained the anti-malarial property of the compound (Krogstad and Schlesinger, 1987; Ridley *et al.*, 1996). These modified compounds give rise to changes in their metabolism, efficacy *in vivo* against *plasmodium falciparum* and the chance of developing resistance towards the new compound (Ridley *et al.*, 1996).

The ability of short, medium and long chain fatty acids to regulate inflammation has been well documented. These fatty acids were successfully conjugated to the quinoline structure of chloroquine by adding the appropriate acid chloride (side chain of interest) to a solution of 4-amino-7-chloroquinoline to yield the new quinoline-fatty acid conjugate. The newly synthesized NT compounds were off white powders with one exception being NT6 which was an oil. The solids had melting point ranges from 87-150 °C except for NT13 which was 28-29 °C. The overall yield was 14- 58 % with the lowest being NT2 and highest was with NT0. The formation of NT2 had the lowest yield probably due to the lack of reactivity of the fully saturated long chain stearoyl chloride.

Synthetically, these compounds differ to the conventional preparations of chloroquine-like analogues, which typically involve taking 4,7-dichloroquinoline with neat excess amine and heating the solution to reflux to effect nucleophilic aromatic substitution. In our case we believed it was easier to start with an aminoquinoline and generate secondary amides rather than using long chain amines (branched or substituted) which has been well researched for decades.

The lead compound as determined from the biological results was NT8 (C12 carbon chain). In an effort to shed further light on its structure-activity relationship, its structure was energy minimized by use of the Spartan Computer Molecular Modeling program as shown in Figure 9.2. The model highlights the planarity of the aromatic rings and the linearity of the alkyl chain. To date we are unable to delineate as to why NT8 is the lead compound based on its structure. Future work will be conducted in our laboratory examining further aspects of altering structural elements of NT8.

Table 9.1 Summary effects of NT8 on neutrophil functions.

NT8 mediated responses						
Agonist	**Adherence**		**Chemotaxis**		**Chemiluminescence**	
	20 µM	**50 µM**	**20 µM**	**50 µM**	**20 µM**	**50 µM**
TNF-RM	√	√	NA	NA	X	√
TNF	√	√	NA	NA	X	√
fMLP	X	√	X	X	X	√
LTB_4	√	√	ND	ND	X	X
GM-CSF	X	√	NA	NA	X	√
IL-8	X	√	ND	ND	NA	NA
C5a	X	√	ND	ND	NA	NA
AA	X	√	NA	NA	X	X
PMA	X	√	NA	NA	X	X
A23187	X	√	NA	NA	X	X

Abbreviations: √- inhibition observed, x- no inhibition, ND- experiment not done and NA-not applicable.

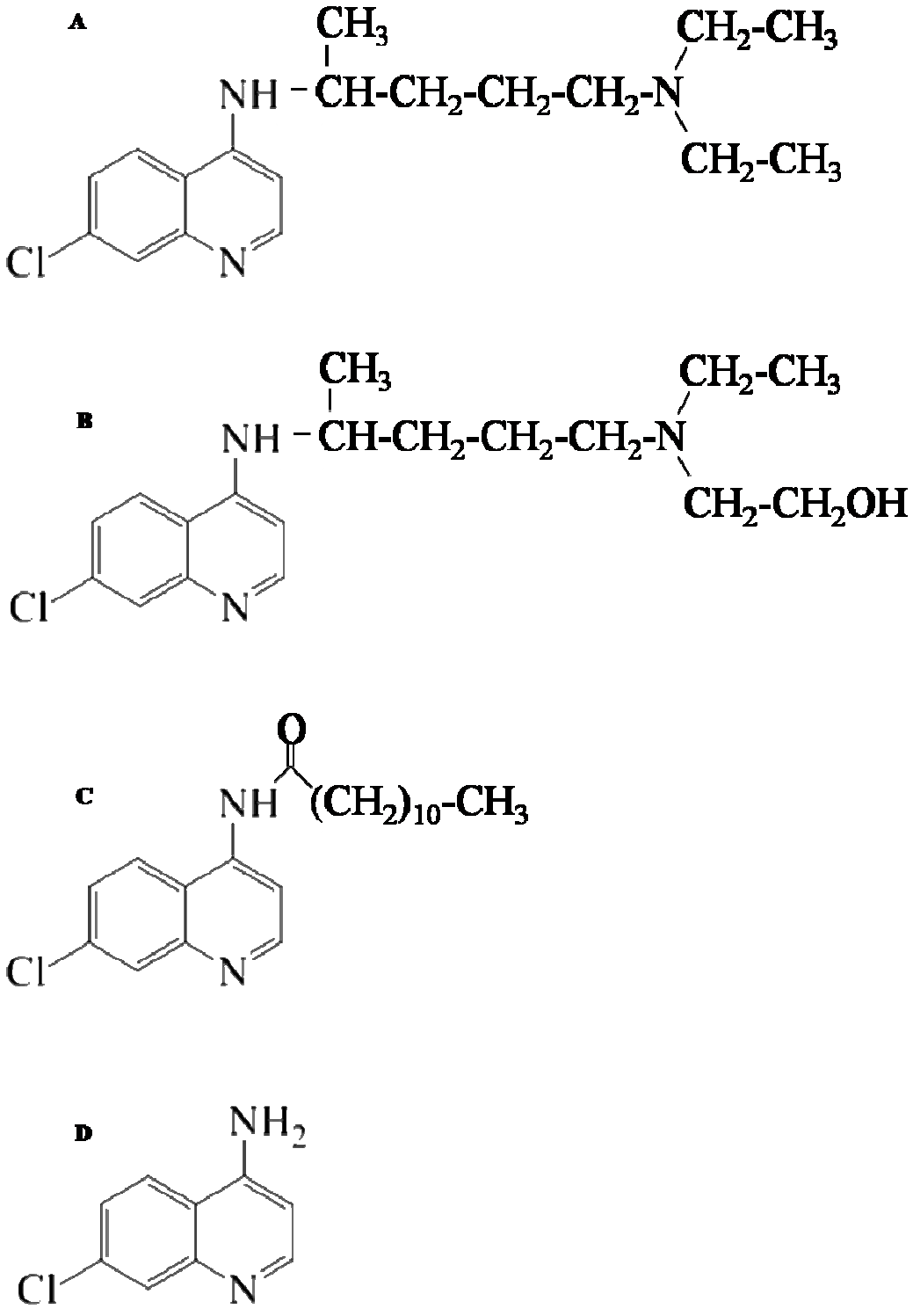


Figure 9.1 Structural comparison of CQ (A), HCQ (B), NT8 (C) and NT0 (D).

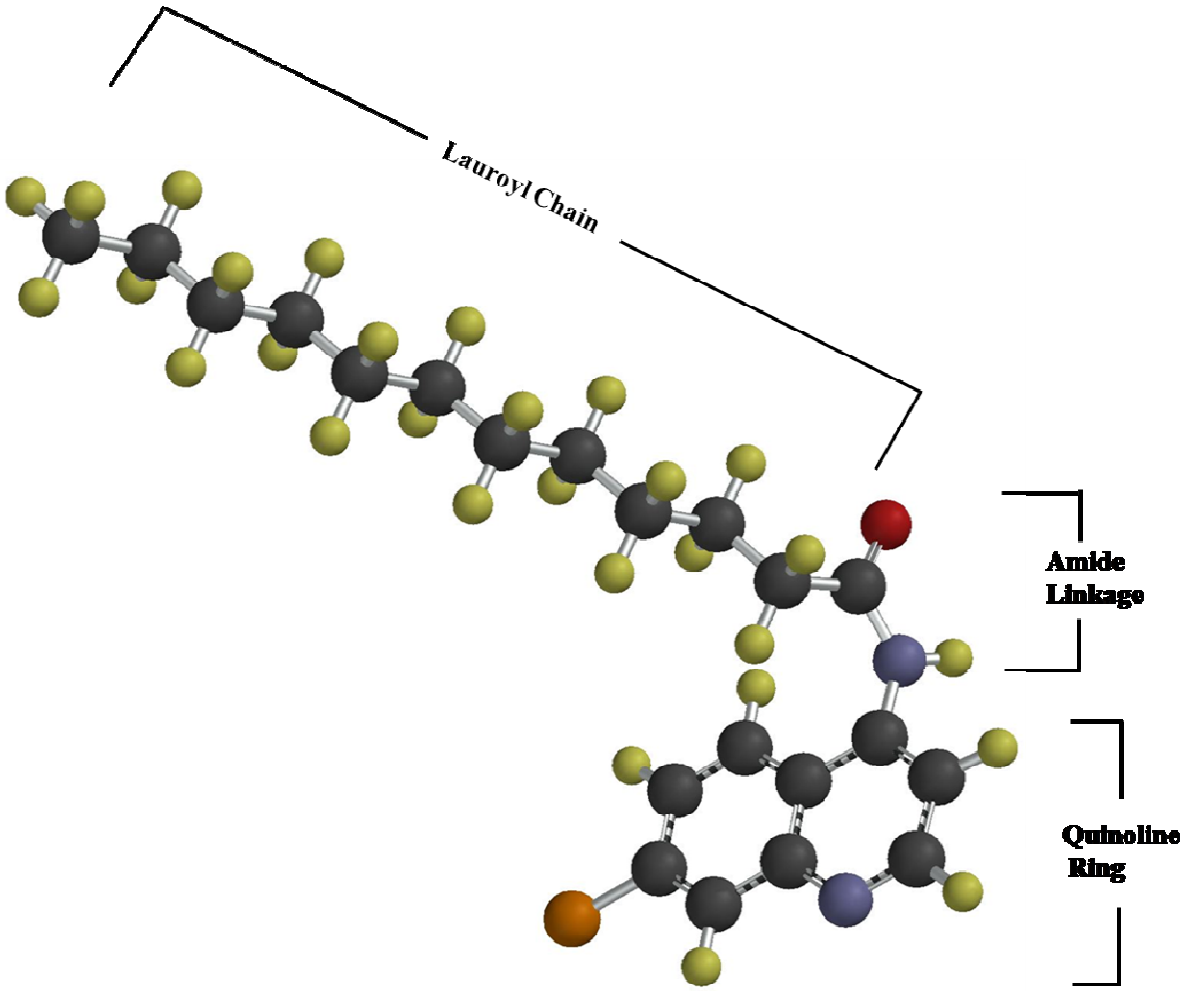


Figure 9.2 The three dimensional molecular structure of NT8 compound. Carbons are represented by grey molecules, hydrogens in green, oxygen in red, nitrogens in blue and chloride in orange.

Modulation of TNF-induced neutrophil adhesion

During inflammation, endogenously generated inflammatory mediators play diverse roles in bringing about the accumulation of neutophils to the site. This includes the stimulation of neutrophil adhesion to the endothelium and various tissue substrates by cytokines. Using both cytokine TNF rich medium and recombinant TNF to stimulate neutrophils, we have shown that several of the NT compounds were able to inhibit neutrophil adhesion. There was no simple relationshiop between fatty acid structure and the effects on these neutrophil functions. While the difference between NT1 and NT3 is that the latter has a double bond, both had similar inhibitory activity. NT8, which contained lauric acid (12 carbon saturated fatty acid) was the most effective of the 13 compounds tested. Both the basal compound NT0 and lauric acid had no effect on TNF-induced neutrophil adherence. This suggests that the synthesis has generated a molecule with a new function of an anti-neutrophil effect. This is somewhat suprising since previous studies have demonstrated that 6-12 carbon chain length fatty acids actually directly increase human neutrophil adhesion (Wanten *et al.*, 2002; Wanten *et al.*, 2000a).

Under these conditions the NT8 compound did not affect neutrophil adhesion induced by a number of other cell surface receptors acting agonists to a similar degree as the TNF-induced response. Thus at 20 µM concentration of NT8, there was significant inhibition of the TNF-induced response but not adhesion induced by fMLP, GM-CSF, IL-8, C5a, and AA. The only exception was LTB_4-induced adherence. Interestingly this concentration of NT8 caused a significant reduction in the expression of TNFRII. There was no significant effect on TNFRI expression. It is therefore likely that the effects that we are seeing at this concentration relates to a decrease in the expression of this receptor. The selectivity for

TNFRII was further strengthened by the finding that the TNF-induced increase of CR3 was not affected. It is however interesting that the response to LTB_4 was also affected.

From our results it is evident that TNFRII is essential for the neutrophil adhesion response to TNF. TNFRII has been reported to act as a ligand handling step for TNFRI (Tartaglia *et al.*, 1993; Porteu *et al.*, 1991) but others have suggested that TNFRII initiates signalling events responsible for effector function (Bigda *et al.*, 1994; Montecucco *et al.*, 2008; Weiss *et al.*, 1997). Our results suggested that TNF-induced adherence is likely to be regulated by TNFRII. The same concentration of NT8 which inhibited the TNF-induced adhesion, was unable to prevent adherence induced by PMA and A23187. Both of these agonists act at a post receptor binding level. Thus the data suggest an effect upstream of PKC and intracellular calcium mobilisation.

Furthermore our studies revealed that NT8 is unique in its action. Firstly it stimulated the cell directly to cause a decrease in TNFRII. Previously it has been reported that a range of endogenous and exogenous mediators cause the down-regulation of TNFR expression on neutrophils (Ferrante *et al.*, 1994; Moghaddami *et al.*, 2003; Schleiffenbaum and Fehr, 1990). This includes LPS, fMLP, C5a, LTB_4, PAF, GM-CSF, opsonised microbial pathogens and *n-3* polyunsaturated fatty acids (Moghaddami *et al.*, 2007) but in these cases, both TNFR were decreased on neutrophils. It has been reported that the decreased expression of the TNFR is due to the enzymatic cleavage of these (Dri *et al.*, 2000). The view is that a membrane-bound, non-matrix metalloproteinase, possibly ADAM-17 is involved for TNFRI (Walcheck *et al.*, 2006; Dri *et al.*, 2000) and a metalloproteinase and the serine protease, elastase for TNFRII (Dri *et al.*, 2000; Gasparini *et al.*, 2003). Unlike the above agents, NT8 did not appear to be a neutrophil stimulator. Whether or not NT8 affects these enzymes

differently to give rise to the selective action on TNFRII remain to be established and indeed is a challenging question.

We also found that the expression of CR3 on neutrophils was not decreased by treating neutrophils with NT8, possibly supporting some previous reports which suggested that upregulation of CR3 does not play a major role in mediating neutrophil adhesion (Schleiffenbaum *et al.*, 1989; Vedder and Harlan, 1988) and the finding that neutrophil from patients with a defect in CR3 can still adhere (Dobrina *et al.*, 1989).

The significance of the effects of NT8 on neutrophil adherence was suggested by *in vivo* studies in mice. Adherence to the endothelium of blood vessels is an essential step in the ability of neutrophil to move from the blood stream to be recruited in tissues. We found that the injection of NT8 in the peritoneum of mice caused a decreased accumulation of neutrophils in response to LPS. LPS is a strong inducer of TNF and it is likely to be an important cytokine in promoting neutrophil adhesion to the endothelium during inflammation.

Inhibition of neutrophil cytokine production

Interestingly the TNF-induced neutrophil production of IL-1β and IL-8 was significantly inhibited by NT8. This response was as sensitive to NT8 inhibition as was neutrophil adhesion. The data suggests also that the TNFRII is likely to play a critical role in the production of IL-1β and IL-8 in response to TNF. Expression of TNFRI alone seemed inadequate for this response.

Since a role for these cytokines in neutrophil activation and accumulation has been previously extablished, it is likey that NT8 wll inhibit the inflammatory response. In view of the

importance of neutrophils and these cytokines in inflammatory disorders such as rheumatoid arthritis (Feldmann and Maini, 2001) and chronic obstructive lung disease (Taggart *et al.*, 2005; Carroll *et al.*, 2005), a therapeutic based on NT8 may be useful.

Effects on TNF-induced cell migration inhibition and superoxide production

TNF has been recognised to cause the inhibition of neutrophil random migration and chemotactic migration (Ferrante *et al.*, 1988). However our studies demonstrated that NT8 treatment of neutrophils could not prevent the migration inhibition induced by TNF. Similarly there was no effect of NT8 on the TNF-induced oxygen radical production at the 20 μM concentration.

The findings indicated that the TNF-induced migration inhibition and the induction of the respiratory burst does not require TNFRII as both were not inhibited at concentrations of NT8 which significantly decreased the expression of TNFRII. This shows that NT8 selectively targets the adhesion response and cytokine production induced by TNF and highlights the importance of TNFRII in ths response. The results indicated that TNFRI is involved in stimulating neutrophil migration inhibition and superoxide production independent of TNFRII.

Effects on the responses induced by other surface membrane receptor agonists

Of several other surface receptors acting agonists studied, only the adherence response to LTB_4 was similarly affected. AT 20 µM NT8, there was significant inhibition of adherence induced by LTB_4. Again we cannot explain this at a level of PKC or intracellular calcium mobilisation since at this concentration NT8 failed to inhibit either the PMA or A23187 response, suggesting an upstream effect, possibly at the level of the LTB_4 receptor. In comparison, the LTB_4-induced oxygen-dependent respiratory burst was not affected by NT8 even at 50 µM. This further illustrates the relative selectivity of the effects of the compound for some neutrophil responses. Of the two LTB_4 receptors BLT1 and BLT2, the former plays an important role in neutrophil adhesion (Kim *et al.*, 2006; Mathis *et al.*, 2007; Schultz *et al.*, 1991). This in view of the effects of NT8, it is likely that the compound affects this LTB_4 receptor type.

Most interesting was the finding that a number of other surface receptor acting agonists had no effect at 20 µM of NT8 in relation to all neutrophil response measured. The agonist examined were fMLP, GM-CSF, IL-8 and C5a. At 50 µM NT8, all agonists significantly inhibited the adherence response (Table 9.1). Since the response induced by either PMA or A23187 was also inhibited at this concentration, it is likely that the effects are due to post surface receptor binding at this concentration of NT8. Interestingly, and in contrast to adherence, fMLP-induced chemotaxis was not affected at this concentration. This appears to dissociate pathways involved in neutrophil adhesion to those involved in chemotaxis and to some extend this is not surprisingly, as the two functions are promoted by quite different concentrations of fMLP (Hii *et al.*, 2004). The findings from our study showed that the fMLP-induced respiratory burst was inhibited by 50 µM NT8. We have previously

demonstrated that different signalling molecules are involved between the two different fMLP concentrations (Hii *et al.*, 2004), related to the low and high affinity fMLP receptors.

The selectivity of the action of NT8 on the neutrophil response was evident from the respiratory burst response of neutrophils induced by LTB_4, AA, PMA and A23187. The response to these agonists was not inhibited by NT8, thus apart from selectivity targeting specific neutrophil responses based on the concentration, we see selectivity at the 50 µM concentration.

Probing further into the mechanism of action of NT8 we were unable to demonstrate that the effects of the compound were due to the alteration in the synthesis of 3KDS, one of the substrates for ceramide formation (Hannun and Luberto, 2000; Williams *et al.*, 1984). This was suspected as the structure of NT8 is similar to 3KDS.

Comparison with effects of NT0 and lauric acid

In most of the experiments, the effects of NT8 were compared with any effects due to the base compounds NT0 and lauric acid. It was evident in all cases that at equivalent concentrations, these components had no effect on any of the neutrophil functions stimulated by any of the agonists. This illustrates that the synthesis has given rise to a novel structure in terms of its biological characteristics. Previous studies with medium chain fatty acids showed enhancement of neutrophil functions but this was attained at very high (mM) concentration (Wanten *et al.*, 2000a). NT0 like CQ and HCQ had no effect on neutrophil function at 50 µM.

Effects on lymphocyte and macrophage functions

Lymphocyte response in human PBMCs was examined by using the T cell mitogen, PHA and the B cell mitogen *S.aureus*. In terms of lymphoproliferation, there was no effect by exposing the lymphocytes to NT8. This was also reflected in the ability of the cells to produce the cytokines, IFN-γ, IL-2, LT and IL-10. Similarly there was no effect when the lymphoyctes were stimulated with antigen, TT.

To examine the effects on monocyte function, PBMCs were stimulated with LPS which induces TNF, IL-1 and IL-6 production. The results also demonstrated that NT8 did not affect the production of these cytokines. Hence this stands in contrast to the findings with neutrophils which were inhibited in their ability to be stimulated in key responses and further highlights the selectivity of NT8 for neutrophil functions.

Comparison with CQ

Although there have been previous reports that CQ and HCQ inhibited neutrophil functions (Ferrante *et al.*, 1986; French *et al.*, 1987; Labro and Babin-Chevaye, 1988; Raghoebar *et al.*, 1988; Hurst *et al.*, 1987, 1988), these effects were seen at high concentrations of the anti-malarials. When CQ and HCQ were examined in parallel with NT8, there was no effect on neutrophil functions at concentrations up to 50 μM. This was evident with adherence, chemotaxis and respiratory burst response which highlights the uniqueness of the compounds which we have generated.

The difference between NT8 and CQ/HCQ was also evident at the level of lymphocyte and macrophage function. CQ inhibited PHA-induced proliferation at 5-50 µM with a corresponding decrease in IL-2 and IL-10 but not LT. The decrease in IL-2 may explain the decrease in lymphoproliferation. The response to TT was even more sensitive to CQ. At 5 µM CQ there was an 80 % inhibition of the lymphoproliferative response. This marked inhibition was also seen in relation to cytokine production.

The B cell response was less sensitive and the inhibition of *S.aureus* induced proliferation was only evident at 50 µM of CQ. At 50 µM, monoycte production of cytokines was also inhibited by the anti-malarial but only about by 40 %, illustrating the preferrential effect on T cell function. Such effects on T cells are likely to contribute to the anti-inflammatory properties of CQ and its derivative HCQ.

It is interesting that while CQ and HCQ inhibited lymphocyte function and macrophage function, the NT0 did not have this property. This suggests that the additional groups on the side chain give rise to the anti-inflammatory effects (Figure 9.1).

Other studies in progress in our laboratory have been examining the effects of these NT compounds on *P. falciparum* intraerythrocytic forms. It is interesting that these have retained the anti-malarial activity to a similar degree as shown by CQ. Like CQ, NT8 is a weak base which would give the compound the ability to accumulate down the pH gradient to increase its concentration in the parasite compared to the red cell. The ability to accumulate at specific sites within cells may also promote its effects on neutrophils.

Effects of NT8 on experimental inflammation

Evidence of anti-inflammatory properties of NT8 was extended to an *in vivo* settings. Mice given single injections of NT8 showed reduced inflammatory reactions using a classical inflammatory model. In an LPS-induced peritonitis model, the neutrophil influx was significantly reduced by NT8. There was an indication that NT8 also affected the number of macrophages in the peritoneal cavity. LPS causes the apoptosis of peritoneal macrophages (Albina *et al.*, 1993). This response did not appear to be affected by treatment of mice with NT8. The ability of the compound to inhibit neutrophil accumulation was dependent on the period of exposure of mice to NT8 prior to the LPS challenge. When the injection was 4 h before challenge, a significant effect on neutrophil levels was seen but not with a 1 h pre-exposure time. This may be due to the time required for NT8 to be incorporated into neutrophils and prior to TNFR being engaged. LPS-induced influx of neutrophils and macrophages in the peritoneal cavity was found previously to be dependent on TNF (Yang *et al.*, 2002; Calkins *et al.*, 2001; Skerrett *et al.*, 1999; Mercer-Jones *et al.*, 1999) .

This finding would be consistant with the ability of NT8 to inhibit TNF-induced neutrophil adhesion. However, effects of the LTB_4 responses are also likely to be relevant since LTB_4-induced neutrophil adhesion occurred at the lower concentration in the *in vitro* studies (Schultz *et al.*, 1991). The present *in vivo* findings need to be expanded further with other inflammation models.

Significance of the findings and concluding remarks

In the course of conducting the research covered by this thesis, novel immunomodulators were identified which have the potential to be developed into immunosuppressive agents. In particular the compound NT8, quinoline-$CO(CH_2)_{10}CH_3$ was identified as a potential lead compound. By conjugating the quinoline to lauric acid, a structure was created which had unique immunosuppressive properties not present in either the quinolines or the fatty acids.

The compound showed selective inhibition of neutrophil function but not lymphocyte or macrophage responses. Indeed it was also selective with respect to the type of neutrophil function and the type of neutrophil agonist used. Neutrophil adherence was highly sensitive to the effects of NT8 and particularly interesting was the finding that its effects were mainly evident when TNF was used as the agonist, most likey related to its ability to cause selective decrease in expression of TNFRII. The work also highlighted some important biological function for TNFRII, perhaps not appreciated previously.

The inhibitory effects of NT8 were less evident with other neutrophil agonists, apart from LTB_4. This suggested that NT8 has an action on selective receptors which are key targets of anti-inflammatory agents. This was supported by proof of concept in a murine inflammatory model in which neutrophils, TNF and LTB_4 play important roles. Apart from the potential use of NT8 to treat acute inflammatory diseases, its usefulness in chronic inflammatory diseases is also likely to be of importance since neutrophils are known to play a role during the exacerbation stages of diseases e.g. in rheumatoid arthritis.

The relationship between the anti-malarial/anti-inflammatory agent CQ/HCQ and NT8 was also extensively examined. It was interesting that in contrast to CQ, NT8 inhibited neutrophil function but not lymphocyte and monocyte function. Interesting was the finding that T cell responses were most sensitive to CQ.

There are several areas in this thesis which require further investigations. Firstly, the selective action on neutrophil resposes versus macrophage responses should be more appropriately compared by examining the same functions eg. adherece and superoxide production. Another area of further studies relates to the finding that TNFRII may play key roles in neutrophil adhesion and cytokine production. The fact that NT8 down-regulates TNFRII and not TNFRI is also a very important finding. This may be related to the compound activating proteases that cleave the former receptor. Further investigation is likely to significantly increase our knowledge on how TNF receptor expression is regulated. The selective effects of NT8 on responses induced by a restricted number of agonists could be further examined. In this regard, its effect on the LTB_4 response and as to whether this relates to altering LTB_4 receptor expression needs further clarification. Embarking on the examination of the anti-inflammatory properties of NT8 in more complex inflammatory models relevant to specific diseases would verify the usefulness of the new compound. While we have learned aspects of the basis for its effects on neutrophil functions, there has been inadequate time to study these mechanisms of action in depth. Because of the interesting actions described above, further studies on the mechanisms by which NT8 alters the cellular functions and inflammatory response may lead to new approaches in targeting inflammatory pathways.

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