The Anti-inflammatory Action of Tetracyclines

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Summary

For six decades tetracyclines have been successfully used for their broad spectrum antibiotic effects. However, non-antibiotic effects of tetracyclines have been reported. The anti-inflammatory effects of tetracycline drugs have been investigated in the context of a range of inflammatory diseases including sepsis and a number of neurodegenerative diseases. This thesis investigates the effects of a range of clinically important tetracyclines (oxytetracycline, doxycycline, minocycline and tigecycline) on the ability of the J774.2 cell line to produce nitric oxide when stimulated with the bacterial cell wall component, LPS. The proteome of J774.2 cells was analysed in response to LPS stimulation (1μg/ml) with and without prior treatment with minocycline (50μg/ml), this allows the unbiased analysis of the cellular proteome in response to minocycline and LPS, protein spots of interest were excised and identified by nano-electrospray ionisation-linear ion trap mass spectroscopy. All of the tetracyclines that were investigated inhibited LPS-induced nitric oxide production in a dose dependent manner and this was due to the inhibition of inducible nitric oxide synthase expression. This is the first report to show that tigecycline inhibits inducible nitric oxide expression and nitric oxide production. Using two-dimensional gel electrophoresis and total protein staining eleven proteins were identified as being modulated by LPS. Of these eleven proteins; expression of some, but not all was modulated when the cells received a prior treatment with minocycline suggesting that minocycline does not completely block LPS-induced macrophage activation but probably specifically acts on particular inflammatory signaling pathways in macrophages. Three protein spots with a similar molecular weight but different pI values identified in this proteomic study were identified as ATP synthase β chain. These different protein spots probably correspond to different phosphorylation states of the protein, suggesting that minocycline affects the balance of protein kinase and protein phosphatase activity in the immune response.

Keywords: Nitric oxide, minocycline, lipopolysaccharide, proteomics

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Abbreviations

6-OHDA	6-hydroxydopamine
ALS	Amyotrophic lateral sclerosis
AP-1	Activator protein-1
AR	Aldose reductase
ARE	AU-rich element
AREBP	AU-rich element binding protein
BSA	Bovine serum albumin
CID	Collision induced dissociation
CMT	Chemically modified tetracycline
COX-2	Cyclo-oxygenase 2
C/EBP	CCAAT-enhancer-binding protein
CREB	cAMP response element binding
ERK	Extracellular regulated kinase
FBS	Foetal bovine serum
GPCR	G-protein coupled receptor
HD	Huntingdon's disease
HSP	Heat shock protein
IFN	Interferon
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
IRAK	interleukin-1 receptor associated kinase
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinase
LBP	LPS binding protein
LC	Liquid chromatography
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Mal	MyD88 adaptor-like protein
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium
MyD88	Myeloid differentiation factor
Nano-ESI	Nano-electrospray ionisation
NADPH	Nicotinamide adenine dinucleotide phosphate
NED	N-1-naphthylethylenediamine dihydrochloride
NF-κB	Nuclear factor κB
NO•	Nitric oxide
NOS	Nitric oxide synthase

$O_2^{-}\bullet$	Super oxide
PD	Parkinson's disease
PI3K	Phosphoinositol phosphate 3 kinase
PKC	Protein kinase C
PMIN	Pyrazolinominocycline
PTM	Post-translational modification
SARM	Sterile α and HEAT-Armadillo motifs-containing protein
SOD	Super oxide dismutase
TAB	TAK binding protein
TAK	Transforming growth factor β activated kinase
TBK	TANK binding kinase
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
TRAF	TNF receptor associated factor
TRAM	TRIF adaptor molecule
TRIF	TIR domain-containing adaptor including IFN-γ

Chapter 1 Introduction

Tetracyclines have been used successfully for six decades as broad spectrum antibiotics, however, more recently characterisation of other non-antimicrobial effects of tetracyclines has been documented, here the molecular mechanisms of the anti-inflammatory effects of a number of tetracycline derivatives are investigated with a view to further describe the molecular mechanisms underlying the anti-inflammatory properties that tetracyclines possess.

1.1 Tetracyclines

1.1.1 Historical Overview

In 1948 chlortetracycline and oxytetracycline were the first tetracycline compounds to be discovered by Duggar (Duggar, 1948) as natural fermentation products of the soil bacterium *Streptomyces aureofaciens* and in 1954 chlortetracycline was chemically purified and marketed for its anti-bacterial properties. Over the following years more tetracycline compounds were identified giving rise to a new group of effective anti-bacterial drugs. The natural production of tetracyclines by bacteria and the relatively easy procedures to isolate the tetracycline compounds provided very low production costs, and this, coupled with an anti-microbial effect over a broad spectrum of microbial organisms including Gram-positive and Gram-negative bacteria, the intracellular pathogens chlamydiae, mycoplasmas and rickettsiae and eukaryotic

protozoans and with a very low occurrence of adverse drug reactions lead to tetracyclines rapidly becoming the drugs of choice for many infectious diseases.

Common use of tetracycline compounds in anti-bacterial medicine and agriculture from the time of their discovery gave rise to bacterial resistance. In 1953, just 5 years after their discovery, a tetracycline resistant strain of *Shigella dysenteriae* was described closely followed by the observation of a multi-drug resistant strain of the same organism in 1955 (Watanabe, 1963). Development of semi-synthetic tetracycline compounds such as doxycycline and minocycline followed to try and improve pharmacokinetic properties of the drugs and to avoid tetracycline resistance mechanisms. Although these second generation tetracycline compounds did overcome some tetracycline resistant strains of bacteria some strains were still resistant to these compounds and this fact, coupled with the discovery of other anti-bacterial compounds lead to the subsequent decrease in their use. In 1994 a new group of tetracyclines was described, the glycylcyclines (Sum et al., 1994) and in 2005 one of these, tigecycline, was approved for clinical use by the United States Food and Drug Administration (FDA) claiming to be effective against bacteria that are resistant to other tetracycline compounds (Doan et al., 2006).

In 1996 the first reports of the anti-inflammatory action commonly possessed by tetracycline compounds were made (Amin et al., 1996) followed by descriptions of the beneficial therapeutic properties of tetracyclines in other diseases. The identification of these non-antibacterial properties of tetracyclines lead to the development of

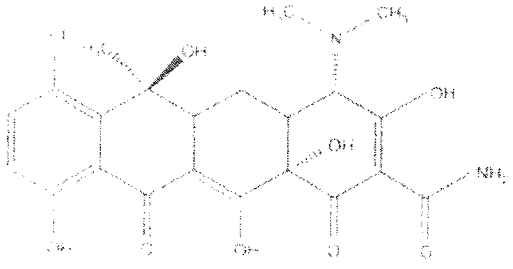
tetracycline compounds that do not have antibacterial properties while maintaining the anti-inflammatory actions (Patel et al., 1999). These so called chemically modified tetracyclines (CMT) along with the glycylcyclines have once again made a big impact on the use of tetracyclines in clinical medicine.

1.1.2 Chemical Structure

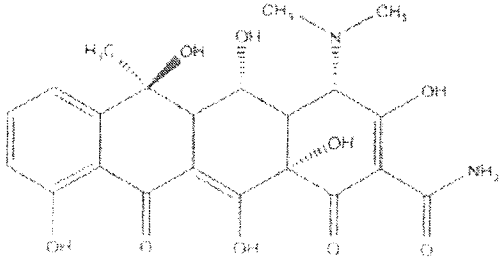
The tetracycline group of antibacterial compounds share a basic chemical structure which has three features that are required for them to be effective antibacterial agents (Zakeri and Wright, 2008) (Figure 1.1). Firstly, a tetracyclic napthacene carboxamide ring system, these rings must be fully composed of carbon and must be six-membered. This linear carbon structure is important for binding to the bacterial ribosome, and altering the chemical composition of the rings results in abolition of antibacterial action. One group however successfully replaced C6 with a sulphur atom and maintained an in vitro antibacterial action, albeit markedly reduced (Rasmussen et al., 1991). On further investigation the ability of the tetracycline to bind the bacterial ribosome had been lost but the compound was still able to associate with and disrupt the cell wall of the bacteria. This was not however selectively cytotoxic to bacteria and also killed mammalian cells in the same way.

Secondly, the compound must be able to bind divalent cations via the presence of a keto-enol group. Tetracyclines are thought to be able to bind any divalent cation but physiologically they are most probably complexed with either calcium or magnesium.

Natural Products

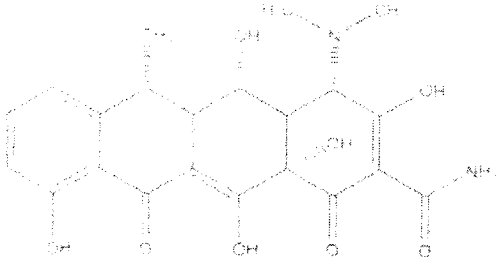


Chlortetracycline

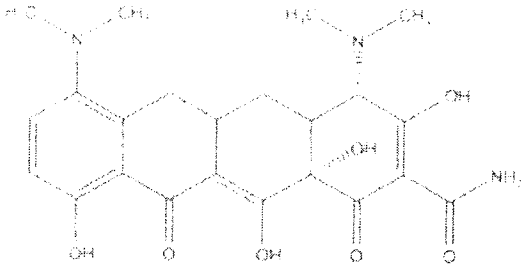


Oxytetracycline

Second Generation

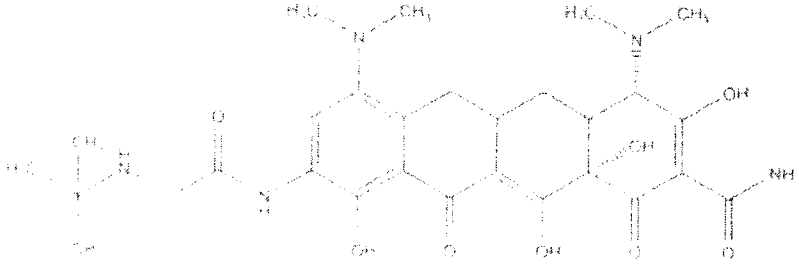


Doxycycline



Minocycline

Third Generation



Tigecycline

Figure 1.1 Chemical Structure of Tetracyclines. The chemical structure of naturally occurring tetracyclines, and second and third generation synthetically manufactured tetracycline derivatives. Adapted from (Zakeri and Wright, 2008)

In the stomach tetracyclines will bind to divalent cations of calcium, magnesium and zinc which are commonly found in dairy products or indigestion remedies. Absorption of these charged tetracycline-metal ion complexes is markedly reduced and therefore contraindicated. In the serum tetracyclines circulate as chelates of calcium and it is known that they transverse the cell wall of Gram-positive bacteria as calcium chelates via an active uptake mechanism. They then dissociate from the calcium atom and passively enter the cell through the second cell wall. Once in the cell they probably associate with a cation again and are complexed with magnesium when bound to the ribosome (Nikaido and Thanassi, 1993).

Thirdly, the tetracycline compound must have a dimethyl amino group at the C4 position. Crystal structures of various tetracycline compounds have identified this part of the compound as being essential in binding with high affinity to the ribosome. (Brodersen et al., 2000, Pioletti et al., 2001). Removal of the dimethyl amino group gives rise to a group of non-antibacterial tetracycline compounds (CMTs). The CMTs have similar bioavailabilities, absorption and distribution properties as the anti-bacterial tetracyclines, but are unable to bind to the bacterial ribosome with high affinity. The addition of a second dimethyl amino group at the C7 position in the case of minocycline and tigecycline appears to slightly increase their anti-bacterial properties, but it is unclear if this is due to further stabilisation of the tetracycline/ribosome complex or if it is related to improved accumulation/reduced efflux of the drugs (Agwuh and MacGowan, 2006).

1.1.3 Antibacterial Action

Tetracycline and tetracycline derivatives have been used as broad-spectrum antibiotics for over 50 years, tetracyclines bind to the ribosomal subunits in bacteria preventing the association of aminocylated-tRNA to the mRNA-ribosome complex and therefore preventing peptide formation.

Two crystal structures of tetracycline in complex with the 30S ribosomal subunit of *Thermus thermophilus* were independently published by Brodersen et al. (Brodersen et al., 2000) and Pioletti et al. (Pioletti et al., 2001) and is shown in figure 1.2, both publications identified one major binding site (Tet-1) of tetracycline which is situated near the A site of the ribosome, one and five secondary binding sites (Tet-2 to Tet-6) were also described respectively but is still unclear whether these secondary tetracycline binding sites have any impact on the anti-bacterial action of tetracycline antibiotics.

In the 30S ribosomal subunit of *Thermus thermophilus* six sites were described as tetracycline binding sites. Tet-1, the site with highest affinity for tetracycline measures 20Å by 7Å and was located between the residues 1054-1056 and 1196-1200 of the minor grove of H34 and residues 964-967 of the stem loop of H31, this location is very close in proximity to the A-site at which the aa-tRNA associates with the ribosome in the process of protein translation. Nucleotide bases 1196 and 1054 of H34 hold one tetracycline molecule with hydrophobic interactions. Meanwhile the hydrophilic region

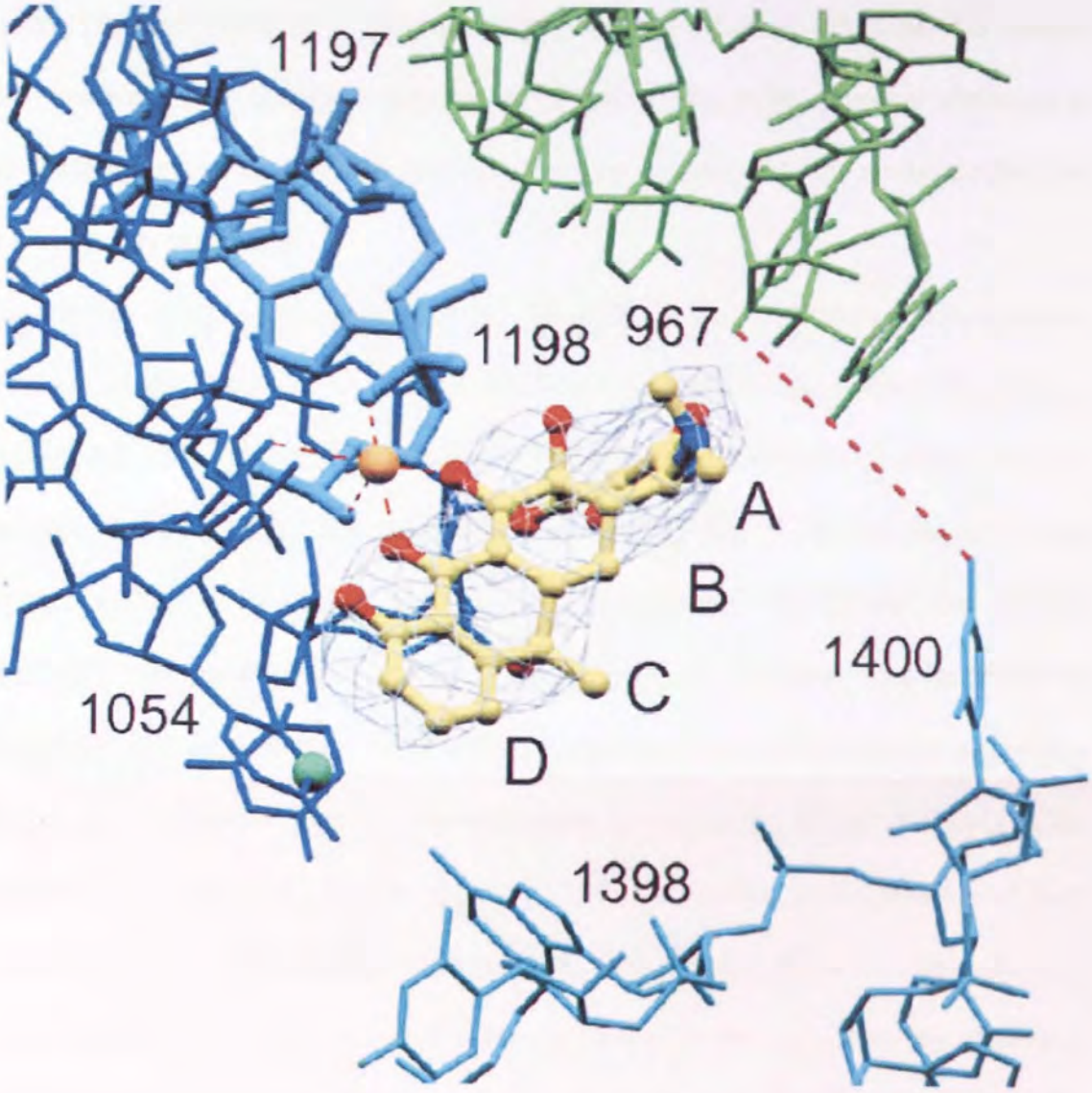


Figure 1.2 Tetracycline bound to the 30S ribosome of *Thermus thermophilus*. Crystal structure of tetracycline bound to the 30S ribosomal complex of *Thermus thermophilus*. The tetracycline molecule has associations with RNA residues and is in a complex with a magnesium ion (orange). Adapted from (Brodersen et al., 2000)

of the tetracycline molecule located on the lower peripheral area has interactions via a Mg^{2+} ion with the phosphate backbone of H34. Upon binding the distance between bases 1196 and 1054 is slightly increased but has minimal effect on the overall conformation of the ribosomal subunit and therefore this minor physical alteration in the conformation of the 30S subunit probably does not modulate the antibiotic function of tetracycline molecules.

The other tetracycline binding sites on the 30S subunit vary in position and no common chemical or physical motif was identified as being shared by these sites. The binding sites on the 30S ribosomal subunit that are important in conveying the bacteriostatic actions of the drugs is thought to be solely tet-1, this is close to the docking site of aa-tRNA. Studies of mutations in the ribosomal subunit that render the bacteria insusceptible to tetracycline also offer an insight into which binding sites are important for the tetracyclines mechanism of action. A guanine to cytosine mutation at position 1058 of the 16S subunit of rRNA prevents tetracycline binding at site tet-1 and confers resistance to tetracycline (Ross et al., 1998). This occurs due to the inability of base 1058 to bind to U1199, leading to a conformational change which abolishes the tet-1 site. TetM, TetO and TetS are tetracycline resistance proteins which act by binding to the A-site of the ribosome and may work by altering the local conformation and releasing the tetracycline molecule from tet-1, the efficiency of this is unclear and only works when low concentrations of tetracyclines are present.

It is unclear whether other tetracycline sites contribute to the antibacterial effects of tetracyclines. Although no definitive evidence has been presented, some groups have

suggested that sites tet-2, tet-4, tet-5 and tet-6 have a role in ribosomal formation due to their interactions with ribosomal binding proteins S4, S9, S17 and S7 respectively (Pioletti et al., 2001). The proteins S4 and S7 are involved in 30S ribosomal formation, therefore suggesting that these interactions inhibit the formation of the ribosome itself and are not directly involved in inhibition of the protein synthesis pathway itself as in the case of tet-1.

1.1.4 Bacterial Resistance to Tetracyclines

A significant decrease in the clinical use of tetracyclines has been observed since the early 1990s, this is due to bacteria developing resistance to tetracycline antibiotics, excaserbated by the major use of this group of drugs since their introduction in the 1950s. Other uses of the drug to promote cattle growth has also lead to widespread resistance by bacteria that are not pathogenic to humans. Inducing tetracycline resistance in bacteria that are not pathogenic to humans is problematic due to the ability of these bacteria to transfer resistance genes to human pathogens in the form of plasmids. Most genes coding for tetracycline resistance are found on plasmids rather than genomic DNA.

1.1.5 Efflux proteins

The first mechanism of tetracycline resistance and the best studied is the ability of bacteria to efficiently export the tetracycline compound out of the cell. This leads to

sub-inhibitory levels of tetracycline compounds in the bacteria and failure to inhibit protein synthesis and subsequent bacterial death. A number of *tet* genes have been identified as being involved in tetracycline export including *tetA, tetB, tetC, tetD, tetE, tetG, tetH, tetK, tetL, tetA*(P) and *otrB*, and are not exclusive to any particular species or group of bacteria being found in both Gram positive and Gram negative strains of bacteria.

Specificity of tetracycline efflux proteins to a specific member of the tetracycline family has also been described, where many members of the tetracycline efflux proteins will export tetracycline but not minocycline. This demonstrates that it is possible to overcome the tetracycline efflux systems by modifying residues on the tetracycline structure which may lead to them evading efflux mechanisms. To date, many efflux proteins have been described with specificity for tetracycline, with few efflux proteins with the ability to export minocycline having been identified and only the mexAB system in *Pseudomonas* species as able to remove tigecycline. Although all tetracycline molecules share a large amount of structural similarities both minocycline and tigecycline have different chemical groups on the C7 and C9 positions, both have a second dimethyl amino group on the carbon atom at position C7 and tigecycline has a glycyl side chain attached to the carbon at position C9. The dimethyl amino group located at position C4 is known to be essential in the antibacterial mechanism of action and possessing a second dimethylamino group at position C7 probably enhances the antibacterial action, the large glycyl side chain at position C9 is thought to stabilise the

tigecycline molecule in the bacterial ribosome but it also has an impact of the specificity that tetracycline efflux proteins have on the drug.

The genetic regulation of these genes has been well studied over recent years and much is now known. Expression of efflux pump proteins is regulated by the tetracycline repressor proteins (TetR). In the absence of tetracycline the repressor protein occurs as a homodimer and binds to the promoter region of genes for both the efflux protein and the repressor protein. Upon interaction with tetracycline in complex with a divalent magnesium ion a conformational alteration in the repressor protein is induced making the repressor homodimer no longer able to bind to the operator region and the genes are transcribed, this process is relatively quick upon the addition of tetracycline. Tetracycline repressor proteins have only been described in Gram negative bacteria and *tetK* and *tetL*, efflux pumps from Gram positive bacteria are regulated by attenuation of their mRNA transcripts. The mRNA transcript of the *tet* genes contains two ribosomal binding sequences, in the absence of tetracycline the mRNA binds at the first ribosomal binding site leading to a shorter peptide being translated. In the presence of tetracycline, a stem-loop structure is formed and leads to the mRNA binding to the second ribosomal binding site leading to the complete efflux protein being translated (Kisker et al., 1995).

1.1.6 Ribosomal protection

Another method of bacterial resistance to tetracyclines is ribosomal protection. *TetM, TetO, TetS, TetB(P), TetQ and OtrA* all code for genes that protect the bacterial

ribosome from the inhibitory actions of tetracycline, doxycycline and minocycline. These ribosomal protection proteins are approximately 72kDa in size and are located in the cytoplasm. Structural features common to all of these proteins are an N-terminal amino acid sequence which has a large amount of homology to the elongation factors Tu and G. The N-terminus contains a GTP-binding domain which has five areas of high structural homology between ribosomal protection proteins as well as elongation factors (Dantley et al., 1998). Homology between *tet* genes from micro-organisms that are distinctly different from one another is also very high, for example the *tetM* gene from the cell-wall free bacteria *Ureaplasma urealyticum* has between 95% and 99% conserved structural homology with *tetM* from *Staphylococcus aureus*, *Neisseria gonorrhoea* and *Neisseria meningitidis*.

The mechanism of action of the *tet* proteins involved in ribosomal protection is at present unclear but due to the close homology of the N-terminal to elongation factors it is possible that the tet proteins function as tetracycline resistant elongation factors. Another hypothesis for their mechanism of action is that they block the binding of the tetracycline molecule to the ribosomal proteins thereby affording the micro-organism protection from the inhibitory effects of the tetracycline drug. However, binding studies using a tritiated tetracycline show that the presence of TetO does not affect the ability of tetracycline to bind to the bacterial ribosome.

The level of protection that is afforded by ribosomal protection proteins is relatively low compared to that observed by tetracycline efflux proteins which typically protect micro-organisms from concentrations of the tetracycline that are 4-10 times higher.

The details of *tet* gene regulation are unclear but some interesting observations have been made in the *tetM* gene. A 400 base pair region immediately upstream of the *tetM* gene appears to be essential for the total expression of the gene. There have also been a number of reports that exposure of the bacteria to sub-inhibitory concentrations of tetracycline increase expression of TetM at both the protein level and at the mRNA level (Nesin et al., 1990). In contrast to the high level of homology in the DNA sequence upstream of the gene, sequence homology on the region of DNA downstream of the gene is markedly lower indicating that the upstream region of the gene is significantly more important in terms of gene regulation. There is limited evidence regarding the regulation of other *tet* genes but upstream regions of *tetO*, *tetS* and *tetQ* share ~70% structural homology with *tetM*, so it is likely that their regulation is also regulated although the details of this regulation are currently unclear.

1.1.7 Enzymatic inactivation

Enzymatic inactivation of tetracycline is the least described mechanism of tetracycline resistance and only one gene, *tetX*, has been described in two closely related strains of bacteria. The *tetX* gene product is a 44kDa cytosolic protein that is closely related to the rRNA methylase gene. TetX is able to chemically modify the tetracycline molecule in

the presence of molecular oxygen and NADPH. The requirement of NADPH led to the discovery of a large amount of amino acid sequence homology with NADPH oxidoreductases. TetX modifies oxytetracycline to 11a-hydroxy-oxytetracycline-6-12-hemiketal. Although to date tetX is the only Tet protein to be involved in chemical inactivation of tetracycline, another tetracycline resistance gene, *otrC*, has been described which does not function as either a tetracycline efflux protein or a ribosomal protection protein (Yang et al., 2004). Although there is currently no evidence to suggest that OtrC functions as a tetracycline inactivation protein it is possible that tet X is not alone in this group of tetracycline resistance proteins.

1.2 Inflammation

1.2.1 Macrophages

Inflammation is a response that a host organism will create in order to protect itself from invading pathogens or foreign objects, a physiological process initiated by the body in response to infection or other trauma or physiological stress. The inflammatory process is heavily regulated and, on initiation, causes vasodilation and increased blood flow to the site of inflammation, also the capillaries become more porous to allow immune cells and other inflammatory mediators to enter the site of inflammation (Janeway et al., 2005). This complex physiological process is mediated by a number of different cells, each with individual tasks, each working together to limit damage to

host tissue, remove the hazard from the host organism and repair any damage that has been done.

Most cells involved in the immune response are derived from pluripotent haemopoietic stem cells located in the bone marrow. These can differentiate into a number of different blood cells which can then settle in tissues to become resident macrophages, including a number of macrophage-like cells such as microglia, Kupffer cells and mesangial cells from the central nervous system, the liver and the kidney respectively.

Macrophages are a vital part of the innate immune response and act as professional antigen-presenting cells as well as phagocytic cells. In response to inflammatory signals, monocyctes will migrate to sites of inflammation along with other cells. Upon migration from the circulating blood into tissue monocytes differentiate into macrophages, first the cell will expand in size from a monocyte of approximately 10-18μm to a macrophage of more than 80μm. The cell will also alter the proportion of its intracellular organelles, increasing the number of lysosomes, mitochondria and components of the Golgi apparatus. The lifespan of the cells changes also, a monocyte may live for a few days whereas macrophages live for a few months. Resident macrophages will reside in tissues and act to clear up any cellular debris from normally occurring apoptosis by phagocytosis.

Upon activation of macrophages the cells change again, altering their ability to carry out immune functions, including the phagocytosis of invading pathogens or foreign

objects, and producing pro-inflammatory cytokines (such as TNF-α, IL-1β and IFNγ), pro-inflammatory proteins (such as iNOS, MMPs and COX-2), chemokines (such as RANTES and MIP) and cytotoxic reactive intermediates (such as NO• and O_2^{-}•).

1.2.2Nitric oxide

Nitric oxide (NO•) is one of the smallest physiological mediators with a molecular weight of just 30Da. It was first described as endothelium-derived relaxing factor, was found to cause vasodilatation in blood vessels and is released from vascular endothelial cells in response to acetylcholine from parasympathetic autonomic nervous innervation. The levels of NO• produced in the context of a vasodilatory action are relatively low and mammalian NO• production is much larger in the immune system as part of the innate immune response.

Nitric oxide is produced via an enzymatic process by nitric oxide synthase. L-arginine is the only naturally-occurring amino acid that can lead to nitric oxide in this process and is therefore used physiologically by nitric oxide synthase (NOS) as the substrate to produce nitric oxide. Nitric oxide contains one atom of nitrogen from the guanidino moiety of arginine and one atom of oxygen which is derived from molecular oxygen. The first step in the production of nitric oxide is the reaction of L-arginine with molecular oxygen using NADPH as a co-factor to produce N^{ω}-hydroxy-L-arginine. N^{ω}-hydroxy-L-arginine then reacts with another molecule of molecular oxygen using

NADPH again as a co-factor; the products of this second reaction are L-citrulline and nitric oxide (Figure 1.3) (Lincoln, 1997).

There are three isoforms of the NOS protein: neuronal nNOS, endothelial eNOS and inducible iNOS. nNOS and eNOS are primarily, although not exclusively, expressed constitutively in neuronal and endothelial cells respectively, they are dependent on levels of intracellular Ca^{2+} and produce low levels of nitric oxide (Forstermann et al., 1998). In contrast to nNOS and eNOS, iNOS is not constitutively expressed and is Ca^{2+} independent. iNOS is induced by an extensive range of cytokines and exogenous stimuli and produces relatively high levels of NO• (Taylor and Geller, 2000).

Production of NO• is associated with inflammatory pathology and if produced in inappropriately large amounts, as observed in sepsis (Tsukahara et al., 2001), rheumatoid arthritis (Cuzzocrea, 2006) and neurodegeneration (Brown, 2007), can lead to hypotension through the potent vasodilatory actions of NO• or can lead to extensive tissue damage due to the reactivity of the NO• molecule (Figure 1.4). NO• is a free radical species meaning that it has an unpaired electron; this makes the molecule very reactive and likely to react with macromolecules including lipids and proteins (Pacher et al., 2007). NO• does not itself react with DNA but can be converted to higher nitrogen oxides in physiological environments which will cause irreversible damage to DNA. Damage to DNA nucleotides or the phosphate backbone could lead to mismatch base pairing and subsequently cancer or cell death. Reactions of NO• with proteins can lead to nitration of amino acid residues; nitro-cysteine and nitro-tyrosine being the most

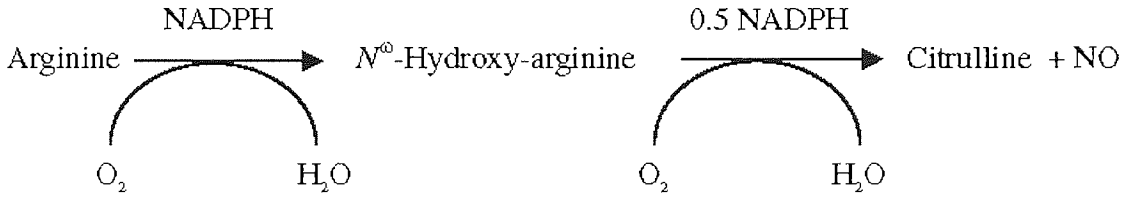


Figure 1.3. The production of NO from Arginine. Arginine is first converted to N^{ω}-hydroxy-L-arginine using molecular oxygen and NADPH as co-factors. This is then converted to citrulline and nitric oxide using NADPH and molecular oxygen.

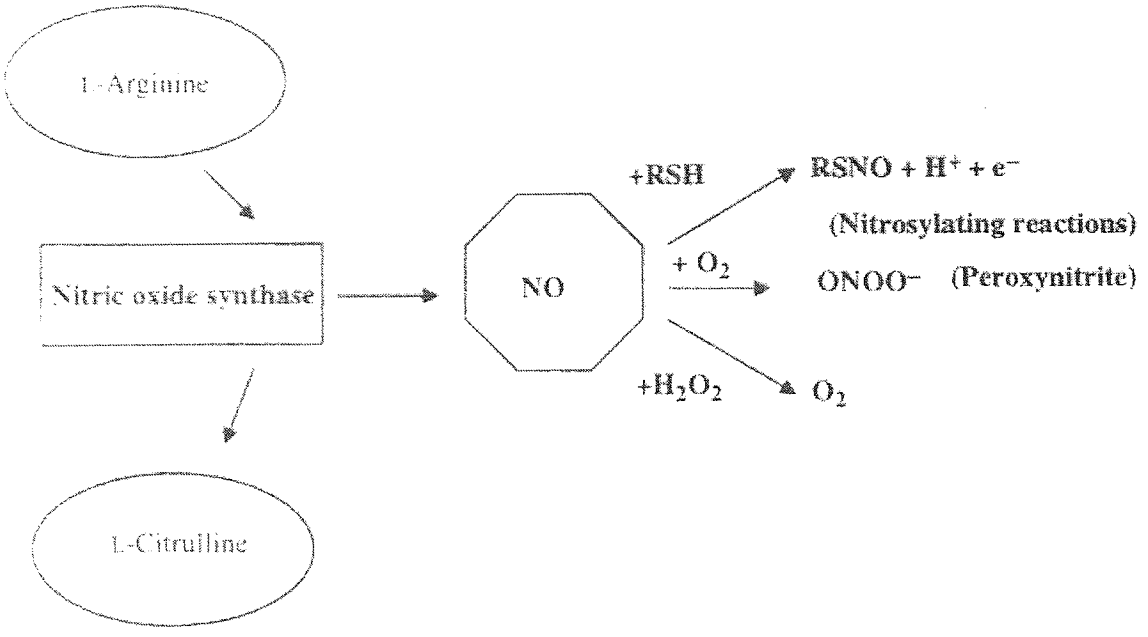


Figure 1.4 Toxic intermediates produced by nitric oxide. A number of toxic or potentially toxic intermediates produced by nitric oxide. Adapted from (Tripathi et al., 2007)

commonly seen NO• induced amino acid modifications. Peroxynitrite formation is also possible and is formed by the reaction of NO• radicals and superoxide (O_2^{-}) radicals, peroxynitrite can itself go on to react with macromolecules forming peroxynitryl residues. Both of these modifications of proteins can lead to alterations in the conformation of the proteins and therefore an alteration in their function. Nitric oxide will bind to and inactivate enzymes that use transition elements as co-factors such as cytochrome P450s and the reaction of nitric oxide or peroxynitrite with lipids can initiate lipid peroxoxidation, a chain-reaction of lipid degradation which can lead to severe loss of cellular lipid structures such as cell membranes.

1.3 LPS

1.3.1 LPS/LBP

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria and is a molecule that is recognized by the human innate immune system. On recognition of LPS by host cells through binding to its receptor, Toll-like receptor (TLR) 4, a cascade of events is triggered resulting in activation of the host innate immune system and production of cytokines including TNF-α, IL-1 and IL-6 and IFNγ and pro-inflammatory proteins including COX 2, iNOS and MMP in a coordinated attempt to activate other immune cells and to kill and remove the invading pathogen. LPS is a glycolipid composed of three distinct regions, a lipid A moiety which is conserved between different LPS molecules, a core polysaccharide and an O-antigen

and polysaccharide region which can differ between LPS produced by different strains of bacteria. Due to LPS being composed of lipid and sugar residues it is amphiphilic and in solution forms micellar aggregates. The structure and therefore biological function of these LPS aggregates is determined by the nature and arrangement of the fatty acid groups that are present on the individual LPS molecules, the resultant conformation of these LPS aggregates determines the activity of LPS from different bacterial species (Seydel et al., 2003).

First described in 1991, Toll-like receptors (TLR) are a relatively recent discovery in the transduction of an inflammatory stimulus and are involved in the activation of many aspects of the immune response including MHC expression, antibody production, and the expression of cytokines, chemokines and adhesion molecules. Currently, ten human TLRs have been described, all involved in activating an immune cascade but in response to different stimuli: TLR1 and TLR6 recognize mycobacteria, TLR2, TLR4 and TLR5 recognise bacterial components and TLR7, TLR8 and TLR9 recognise viral products.

The host innate immune system recognizes monomeric units of LPS and therefore requires a protein to disturb these LPS aggregates in order to present individual LPS molecules to TLR4. This protein is a 58 kDa glycoprotein called LPS binding protein (LBP) that is secreted from the liver and is found freely circulating in the plasma. LBP removes single LPS monomers from either circulating LPS aggregates or directly from the cell membrane from intact bacteria. The N-terminus of LBP binds LPS while the C-

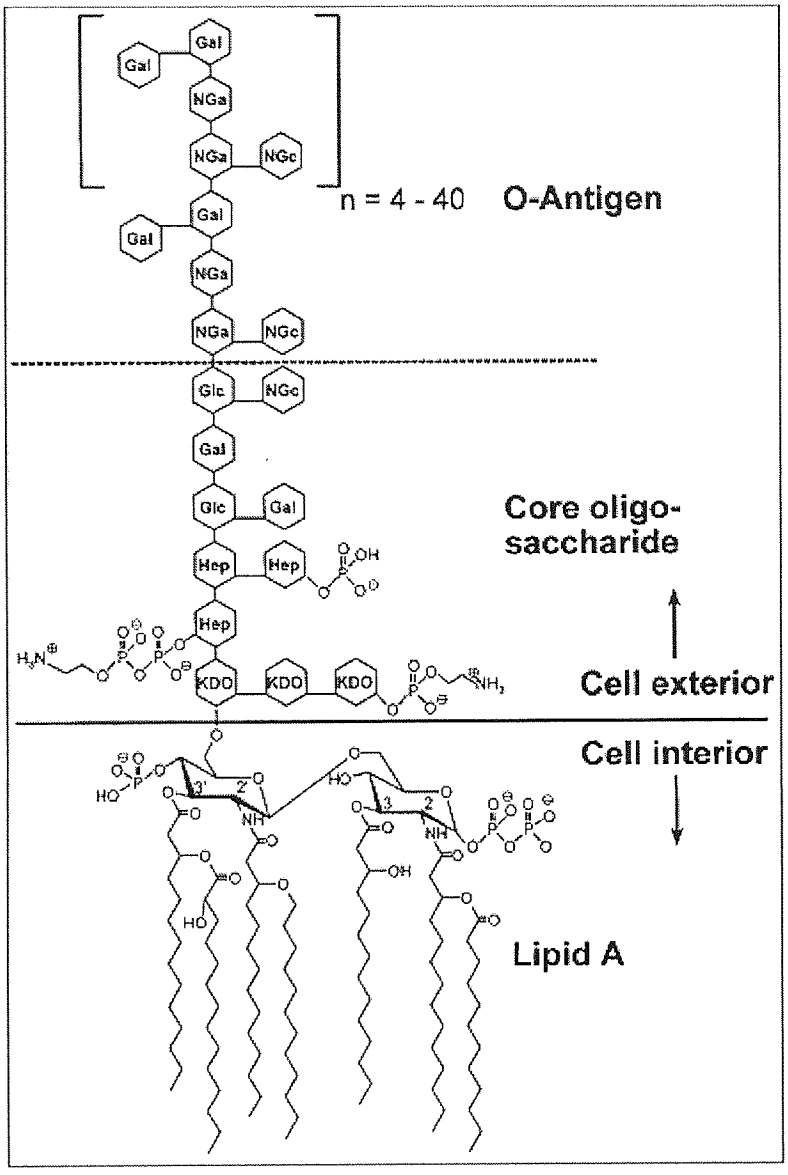


Figure 1.5. Chemical Structure of LPS. The chemical structure of the cell wall component of Gram negative bacteria. Showing the three distinct domains of LPS: Lipid A, Core oligosaccharide and the O-antigen (Magalhaes et al., 2007).

terminus of LBP has affinity for CD-14 which is found on the surface of cells involved in the activation of the innate immunity and is required for an immune response. Although involved in initiation of the innate immune response LBP can afford protection against an inappropriately large immune response and at high serum concentrations of LPS, LBP shuttles LPS to circulating lipoproteins making the LPS unavailable to initiate a response (Gutsmann et al., 2001). Serum levels of LBP are found to be elevated as a protective mechanism in sepsis (Kitchens and Thompson, 2003).

1.3.2 CD14/MD2/TLR4

CD-14 is important at the cell surface to aid LPS signaling, especially at low concentrations of LPS. CD-14 occurs as two forms, a soluble form (sCD-14) and a membrane bound form (mCD-14) attached by a glycosyl-phosphatidyinositol tail. Expression of mCD-14 is largely limited to cells actively involved in the innate immune response whereas sCD-14 helps LPS to signal in other cell types. CD-14 function is to present the LPS monomer to the TLR4 receptor complex. CD-14 however is not specific to LPS and has been found to enhance signaling of pathogenic ligands including LTA, peptidoglycans, and dsRNA through TLR2, TLR1, TLR6 and TLR3 (Bas et al., 2008, Lee et al., 2006).

TLR 4 is the transmembrane receptor for LPS; however TLR 4 does not bind directly to LPS, an adaptor molecule is required; this is myeloid differentiation 2 (MD-2; also

known as Ly96 and ESOP-1). MD-2 is a 143 amino acid glycoprotein which is the last LPS binding protein in the activation cascade. MD-2 contains many surface-exposed cysteine residues which are required for biological activity; MD-2 binds non-covalently to the N-terminus of TLR4 and acts as a chaperone modulating the correct glycosylation of the TLR4 protein (Ohnishi et al., 2003). MD-2 can form soluble oligomers, however it is thought that one monomer of MD-2 binds to one monomer of LPS (Viriyakosol et al., 2001).

Once an LPS monomer has been presented to CD14 by LBP and then transferred to MD-2 which is associated with TLR4, oligomerisation occurs. TLR4 homodimers are formed inducing activation of the intracellular portion of the TLR4 receptor complex. This is followed by recruitment of intracellular protein adaptors, four of which have been identified: myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor-like (Mal; also known as TIR domain-containing adaptor protein), TIR domain-containing adaptor including IFN-γ (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α and HEAT-Armadillo motifs-containing protein (SARM) (Carty et al., 2006). Different adaptor molecules determine the downstream signaling events that govern the cells response to LPS.

MyD88 is important in the expression of LPS-induced IL-1 and is also recruited by the IL-1 receptor, also via a TIR domain. MyD88 is a critical adaptor in the TLR4 response to LPS and MyD88 deficient mice are resistant to LPS induced endotoxemia (Kawai et al., 1999). Further studies with MyD88 deficient mice showed that they are protected

from LPS induced sepsis due to the inability to produce cytokines and pro-inflammatory proteins despite still being able to activate nuclear factor κB (NF-κB; see page 42 for description of NF-κB), albeit with altered kinetics of activation (Kawai and Akira, 2007b). Furthermore the ability of LPS to induce the expression of type-1 interferons is not affected by the absence of MyD88, this showed that the IL-1 responses of LPS are regulated in a manner distinct from that of the regulation of the type-1 interferon response activated by LPS, and that the distinction between the two different pathways is controlled at the receptor level and dependent on the specific recruitment of adaptor molecules to the TLR4 homodimer complex. The TIR domain containing adaptor has only recently been identified and its function has not been fully identified although it is thought to function to inhibit TRIF mediated signaling.

The two pathways are considered to be distinct from one another even though some down-stream elements in the signaling are common to both pathways, and are termed the MyD88-dependant pathway, using MyD88 and Mal, and the MyD88-independent pathway, using TRAM and TRIF. The kinetics of these two pathways are different from one another and it is thought that the MyD88 dependent pathway is involved in the immediate responses to a bacterial infection and includes the production of bacteriotoxic compounds such as NO• and pro-inflammatory cytokines such as TNF-α and that the MyD88-independent pathway is delayed and occurs after approximately 30 minutes from the original LPS stimulation.

MyD88 was originally observed to be a key adaptor molecule in the IL-1 and IL-18 signaling pathways and was then also shown to be vital in the response to ligands for TLR2, TLR4, TLR5, TLR7 and TLR9. Mal binds to TLR4 and forms a bridge to MyD88. Recruited and activated MyD88 then leads to the recruitment and activation of a complex of molecules comprising IL-1R-associated kinase 4 (IRAK4) and IRAK1, each associated with tumour necrosis receptor-associated factor 6 (TRAF6) and transforming growth factor β activated kinase 1 (TAK1) and TAK1 binding protein 2 (TAB2) (For a more detailed review of TLR4 signaling see (Kawai and Akira, 2007b).

1.3.3 MyD88 dependent signaling

MyD88 is an adaptor molecule that is used by all TLRs with the sole exception of TLR3. MyD88 dependent signaling leads to the activation of NF-κB and the mitogen activated protein kinases (MAPK) p38 MAPK and c-Jun N-terminal kinase which in turn activate AP-1 transcription factors (Figure 1.6). The signaling cascade between the activation of TLR4 and the recruitment of MyD88 to the activation of NF-κB and AP-1 transcriptions factors is complex and involves a number of different, individually regulated proteins.

Upon TLR4 homodimerisation MyD88 is recruited to the intracellular TIR domain of the TLR4 protein, along with mal, another TIR associating protein. More proteins are recruited to the complex, IL-1 associated receptor kinase (IRAK) 4 is recruited and is

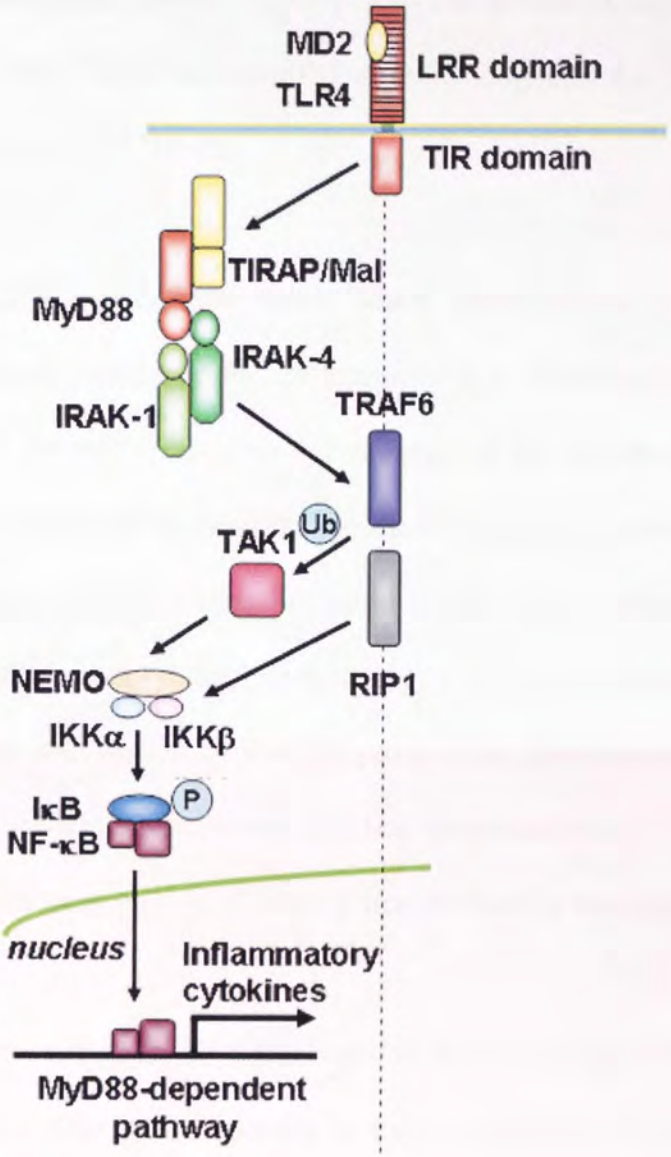


Figure 1.6 MyD88-dependent signaling. Diagram showing the adaptor molecules involved in MyD88-dependent TLR4 signaling (Adapted from (Kawai and Akira, 2007a)). LPS binds to TLR4 via MD-2 which activates the recruitment of Mal and MyD88 this complex then activates IRAK-4 and IRAK-1 and subsequently TRAF6. TRAF6 then activates TAK1 by phosphorylation which then activates the NF-κB Pathway.

required to activate IRAK-1 by phosphorylation. This allows the association with tumour necrosis factor (TNF) associated factor (TRAF) 6 which in turn then associates with transforming growth factor β activated kinase (TAK) 1. TAK-1 binding protein (TAB) 1 and TAB-2 are recruited and this completes the activation of TAK-1 leading to the activation of NF-κB.

NF-κB is a transcription factor which promotes the transcription of many pro-inflammatory proteins in the immune response. When inactive, NF-κB is located in the cytosol of the cell, associated with isoforms of the inhibitor of NF-κB (IκB). IκB binds to NF-κB and in doing so masks a nuclear localization signal. Upon activation of TAK-1, IκB kinase (IKK) is phosphorylated and activated leading to the phosphorylation of IκB. IκB phosphorylation targets the protein for ubiquitination and subsequent proteolytic degradation by the 26S proteosome, liberating NF-κB and un-masking the nuclear localization sequence. Nuclear translocation of NF-κB follows with DNA binding and transcription of many genes involved in the immune response.

TAK-1 also activates AP-1 transcription factors via the activation of p38 MAPK and JNK, which belong to the family of kinases called MAPK. MAPKs activated by dual phosphorylation comprise three families: extracellular regulated kinase 1/2 (ERK 1/2), c-jun N-terminal kinase (JNK) and p38 MAPK. MAPKs are activated via phosphorylation of a specific threonine-X-tyrosine motif of which the amino acid separating the two phosphorylation sites differs between the different families of MAPK and is glutamate, proline or glycine in ERK, JNK or p38 MAPK respectively.

Members of the MAPK family are phosphorylated and activated by proline directed dual specific MAPK kinases (MAPKK, MEK), which are themselves activated via phosphorylation reactions, which are mediated by MAPKK kinase (MAPKKK, MEKK) enzymes, TAK-1 is a MEKK. In the activation of p38 MAPK, TAK-1 activates MEK3/6 which in turn activates p38. TAK-1 mediated JNK activation is mediated via MEK7 (Reviewed in (Guha and Mackman, 2001).

This signaling cascade is complex and other proteins may be involved. Co-immunoprecipitation experiments have identified the novel protein kinase C (PKC) isoform, PKCδ and PKCε , as a mal binding protein; although the details of this interaction are unclear it appears to be important as it has been shown that PKCδ binds to Mal in TLR4 signaling (Kubo-Murai et al., 2007) and PKCε knock-out mice are unable to activate LPS-induced IKK, IκB and p38 activation (McGettrick et al., 2006).

1.3.4 MyD88 independent signaling

MyD88 knock-out mice show that the presence of MyD88 is not essential for a limited response to LPS; the expression of IFNγ is not affected by knocking down MyD88 from cells. On activation of the TLR4 complex the adaptor TRAM is recruited along with TRIF. This activates TANK binding kinase (TBK1). TBK-1 in turn phosphorylates TRAF3 and subsequently IFN regulatory factor (IRF) 3. IRF3 becomes phosphorylated and associates with another transcription factor, CREB binding protein

(CREBBP), this association causes nuclear translocation and transcription of IFNα and IFNβ (Figure 1.7).

Interestingly TLR4 is the only member of the TLR family of receptors expressed on the cell surface that leads to the expression of IFNγ. Other TLRs that cause IFNγ expression are located in the endosomal fraction of cells and respond to viral fragments. MyD88 independent signaling is delayed compared to MyD88 dependent signaling and may be due to re-localization of TLR4 to endosomes following initial activation (Kagan et al., 2008). The delayed kinetics of MyD88 independent signaling may be due to the requirement of interactions with other endosome related protein adaptors.

1.4 Tetracyclines and Inflammation

Tetracyclines are primarily regarded as anti-bacterial compounds with a bateriostatic effect but it has long been known that they possess other therapeutic properties such as anti-inflammatory, anti-histamine and pro-apoptotic activity. The earliest reported observation is that tetracyclines were beneficial in organ dysfunction associated with diabetes (Ryan et al., 1998), including diabetes associated nephropathy and diabetes associated oral pathologies. A number of experiments were subsequently carried out using drug induced diabetes as a model. To date a number of non-antibiotic properties of tetracycline compounds have been described. A number of studies with tetracyclines in sepsis have been conducted. Sepsis is characterised by severe inflammation caused by a systemic infection, usually a bacterial blood infection. Sepsis is a severe condition

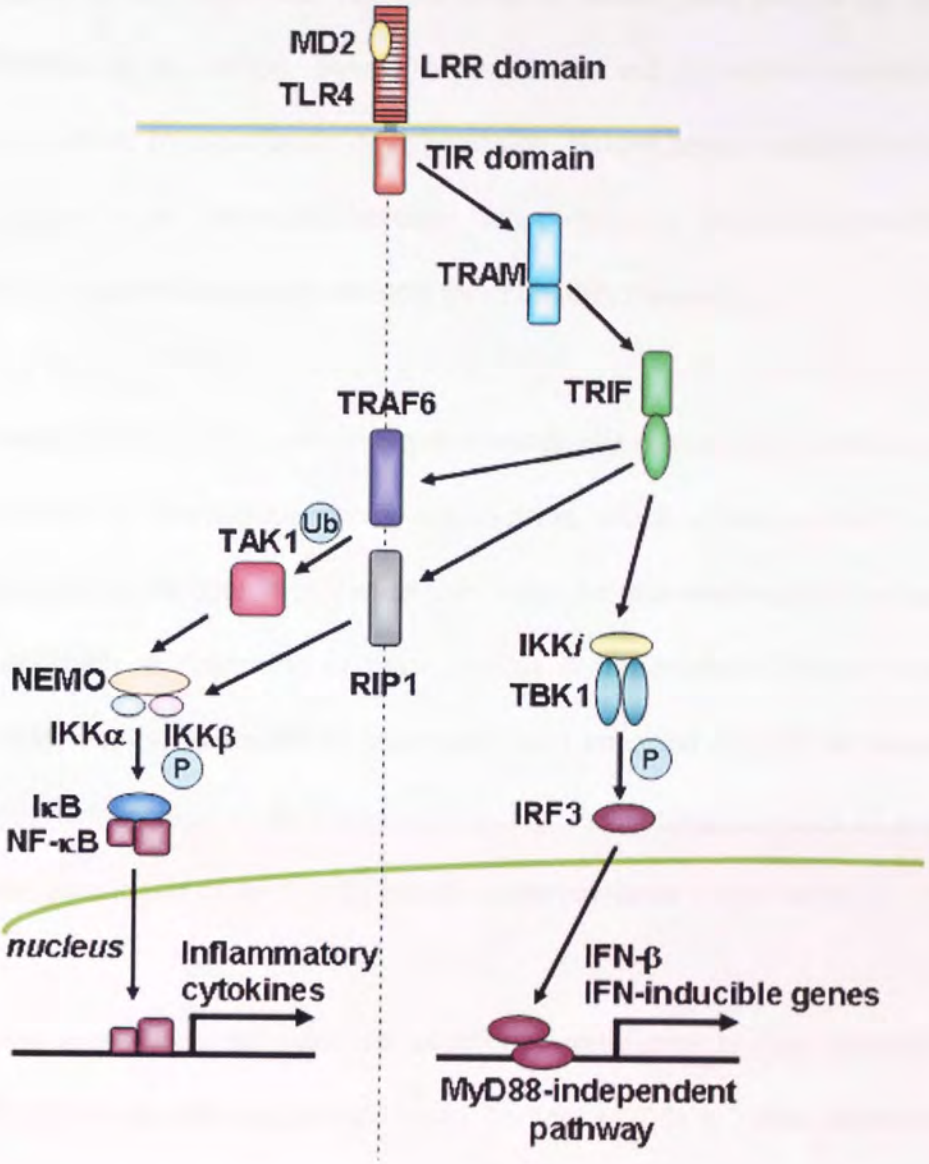


Figure 1.7. MyD88 independent pathway. Diagram showing the adaptor molecules involved in MyD88-independent TLR4 signaling (Adapted from (Kawai and Akira, 2007a)). LPS binds to TLR4 via MD-2 MyD88 recruitment is not essential for the activation of IFN-β or delayed activation of the NF-κB pathway.

which has a high fatality rate claiming more than 200,000 lives in the USA a year (Martin et al., 2003) and has increased in recent years due to an aging population (Watson et al., 2003). Severe sepsis is defined as sepsis associated with organ dysfunction, hypoperfusion or hypotension. Severe sepsis manifests when an immune response to an infection becomes inappropriately large due to dysregulation of endogenous inflammatory and anti-inflammatory mediators.

Treatments for sepsis are directed towards eliminating the invading pathogens and reversing or preventing severe hypotension which often is fatal in severe sepsis. Strategies to combat hypotension can either be pharmacological, by using vasoactive drugs such as dopamine or noradrenaline (norepinephrine) which work to increase cardiac output, by means of increasing heart rate, and also act as vasoconstricters, or non-pharmacological, such as fluid loading or the administration of packed red blood cells, transfusion of these cells greatly improves tissue oxygenation.

Rapid application and selection of relevant antibiotics is also essential in sepsis and although a specific antibiotic should be applied this is often impossible due to the identity of the bacteria being unknown and the administration of antibiotic therapy being required immediately. Therefore broad spectrum antibiotics are often used upon admission of a patient suffering from sepsis. Tetracyclines are a good choice, not only do they act to remove the pathogenic bacteria that are present, they also reduce the aberrant inflammatory response associated with this. The non-antibiotic effects of tetracyclines have been shown using germ-free animals or macrophage cell lines or

primary cultures. (Milano et al., 1997) showed that mice administered with tetracycline were protected against the lethal effects of LPS injection, and that doxycycline was even better at protecting against lethal endotoxemia.

NO• produced by cells involved in innate immunity in response to infection and inflammation and has been implicated in the pathophysiology of many inflammatory conditions, the use of tetracyclines to reduce the production of proteins such as iNOS and the subsequent production of NO• could provide a useful anti-inflammatory therapy for conditions such as sepsis, arthritis and neurodegenerative diseases including as Parkinson's disease and Huntingdon's disease.

Further mechanistic studies into the reasons behind the protective effects of tetracyclines in models of sepsis were conducted and many focused on nitric oxide production as this is the major reason behind inflammation mediated host tissue damage and aberrant vasodilation associated with sepsis, nitric oxide mediated damgage is very often fatal. (Amin et al., 1996) showed a reduction in the effects of nitric oxide in LPS activated RAW 264.7 murine macrophages, they described the reduction in the conversion of arginine to citrulline, reduction in the expression of iNOS protein, and showed that both minocycline and doxycycline were able to inhibit the accumulation of iNOS mRNA by RT-PCR and Northern blot analysis. This effect of tetracyclines was assessed by other groups and it was shown that doxycycline and also tetracycline had the effect of inhibiting nitric oxide production in another murine macrophage cell line, J774.2 (D'Agostino et al., 1998). Tetracycline causes reduced

production and release of nitric oxide as determined by the measurement of nitrite accumulation in the media of cultured cells in a dose-dependent manner; the reduction of iNOS protein expression without a reduction in total protein synthesis was also demonstrated, however a reduction in the levels of iNOS mRNA was not observed as previous studies had reported (Amin et al., 1996). One possible reason for conflicting iNOS mRNA accumulation data is that tetracyclines can affect the stability of the iNOS mRNA transcript, this coupled with differing techniques between laboratories could provide confusion over the modulation of iNOS mRNA in response to tetracyclines. Although data regarding the effect of tetracyclines on iNOS mRNA is inconsistent, the majority of studies have shown the inhibition of nitric oxide production in a wide variety of models. These include primary cultured cartilage from patients suffering from osteoarthritis which spontaneously release nitric oxide (Amin et al., 1996), serum levels of nitric oxide of mice administered with LPS (Milano et al., 1997), as well as microglia (Lai and Todd, 2006), and lung epithelia (Hoyt et al., 2006) stimulated with LPS. The inhibitory mechanisms of tetracyclines are not specific to the inhibition of iNOS and a number of other cytokine and pro-inflammatory proteins have been shown to be inhibited by tetracyclines including TNF-α, IL-1α, IL-1β, IL-6 (Lai and Todd, 2006), prostaglandin E2 (Kim et al., 2004), COX-2 (Chen et al., 1999) and MMPs (Kirkwood et al., 2004), also MHC II presentation in macrophages (Nikodemova et al., 2007) and immunoglobulin secretion from B-cells (Kuzin et al., 2001) and degranulation of mast cells (Eklund and Sorsa, 1999) was inhibited.

Activation of cells with compounds other than LPS that signal via other members of the TLR family, or by means other than TLR activation, are also are susceptible to tetracycline mediated inhibition of the production of pro-inflammatory cytokines and pro-inflammatory proteins. LTA and peptidoglycan activated macrophages can be inhibited by a range of tetracycline molecules as can endogenous pro-inflammatory compounds such as TNFα, TGFβ (Maitra et al., 2005)and cytomix (a combination of TNFα, IL-1β and IFNγ) (Hoyt et al., 2006), and other biological and chemical injury such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Du et al., 2001) and thrombin (Choi et al., 2005), through the inhibition of IKK phosphorylation, activation of p38 MAPK, ERK 1/2 and JNK (Nikodemova et al., 2006). Inhibition of TGFβ induced smad signaling (Maitra et al., 2005) and PKC activation (Nikodemova et al., 2007) has also been reported.

The wide range of actions of tetracyclines across a range of pathologies involving different cell and tissue types has led to research into the use of tetracycline compounds in a wide range of diseases. Rheumatoid arthritis, an autoimmune condition characterised by abnormal production of nitric oxide has been investigated with a tetracyclines efficiently inhibiting nitric oxide production and release from synovial tissue (Amin et al., 1996) and in phase II clinical trials (Tilley et al., 1995). Other conditions such as sarcoidosis have shown positive results clinically with minocycline showing a reduction in cutaneous lesions in 10 out of 12 patients (Bachelez et al., 2001). Diabetes too has offered positive data with tetracycline reducing diabetes

associated advanced glycated endproduct formation and reduced nephrotic dysfunction (Ryan et al., 1998).

The ability of tetracyclines to inhibit MMPs, which can cause a large amount of host tissue damage in inflammatory diseases, has led to the use of doxycycline at sub-microbial doses in dentistry to prevent MMP induced gum disease which is associated with periodontitis. Another role for the anti-MMP activity of tetracyclines is as an anti-metastatic compound in cancer and CMT-3 has recently been licensed by the FDA for use in cancer. CMT-3 is the first CMT to be licensed for use and has applications beyond its anti-metastatic properties (Bildt et al., 2007, Islam et al., 2003, Sandler et al., 2005, Trachtman et al., 1996).

One potential problem arising from the extended use of compounds which have anti-biotic properties such as tetracyclines is the emergence of anti-bacterial resistance (Roberts, 1996), although some bacteria show resistance to tetracycline compounds, tetracyclines are still important in many bacterial conditions. Rendering these drugs useless as antibiotics by increasing tetracycline resistant strains of bacteria would not be advantageous. The removal of the dimethyl amino group from position C4 of the tetracycline structure abolishes the bacteriostatic ability of tetracycline compounds (Greenwald and Golub, 2001). Some of these chemically modified tetracyclines still retain their anti-inflammatory properties and are able to inhibit LPS induced nitric oxide and TNF production (D'Agostino et al., 2001, Patel et al., 1999, Maitra et al., 2004).

Another area of medicine that has attracted considerable interest for potential non-antibiotic clinical applications of tetracyclines is neurodegeneration (Blum et al., 2004). Most of the studies into the use of tetracyclines in central nervous system medicine use minocycline due to its ability to penetrate the blood brain barrier compared with other tetracycline derivatives (Agwuh and MacGowan, 2006). There is a large amount of interest in new therapies for the prevention or reduced progression of neurodegeneration because of the current lack of effective treatments and the increasing incidence of these massively disabilitating conditions in the expanding ageing population. Despite considerable research being carried out in the field, there is still a distinct lack of understanding of the molecular events underpinning the pathology of the dementias seen today. Minocycline is a drug that offers a very good safety profile that has been used in medicine for many decades. Investigation of the potential of tetracyclines in neurodegeneration has progressed relatively quickly over recent years and there are currently a number of clinical investigations underway to establish the therapeutic uses in conditions such as these (Sapadin and Fleischmajer, 2006).

The use of minocycline in Huntington's disease (HD) has been investigated using various animal models of the disease as well as in vitro and in vivo chemically induced models of the disease. A commonly used cell culture based model of HD is by mutating the Huntingtin gene. This model was used by Mievis et al (Mievis et al., 2007) and on administration of minocycline a reduction in cytotoxic factors such as IL-1, cytochrome c and Apoptosis Inducing Factor that are associated with the disease was observed.

Inconsistencies in reports of the effectiveness of minocycline in animal models of HD have emerged and could be due to the use of different models used, or as suggested by some of the authors of these reports that the route of administration of the drug is important. Minocycline in combination with other therapies may lead to greater improvement as shown by co-administration with pyruvate (Ryu et al., 2006) and Co-enzyme Q10 (Stack et al., 2006). The ability of minocycline to reduce progression of HD has been independently shown by a number of groups now (Huntington Study, 2004, Chen et al., 2000, Lin et al., 2001) however minocycline does not appear to be able to reverse the effects of HD once the damage to the brain has been done, this means that the potential of minocycline in HD would be greater when initial administration of the drug is in the early stages of the disease.

Parkinson's disease (PD) is a neurodegenerative condition characterised by loss of dopaminergic neurons in the substantia nigra section of the brain. Minocycline has shown beneficial results in animal models of PD chemically induced by MPTP (Du et al., 2001) and 6-hydroxydopamine (6-OHDA) (He et al., 2001). Minocycline administered intra-peritoneally showed a reduction in cell death and microgliosis in the substantia nigra, and oral minocycline reduced MPTP induced loss of dopamine and dompaminergic neurons, minocycline also showed a reduction in the formation of nitrotyrosine residues indicating that the protective effects of minocycline are at least in part due to the inhibition of formation of nitric oxide.

Amyotrophic lateral sclerosis (ALS) is a debilitating neurodegenerative disease resulting from the loss of motor neurons. There are no current treatments available to reverse the disease by improving motor neuron function. After a mutation in the gene encoding super oxide dismutase (SOD) 1 was identified in many cases of familial ALS a number of mouse strains with SOD mutations (SOD1G39A and SOD1G37R) were developed and used as a model for research into ALS. Minocycline has shown positive effects in mouse models of ALS (Zhu et al., 2002), however clinical benefits were not observed in a subsequent large clinical study (Gordon et al., 2007).

The effects of minocycline in stroke and other models of ischemia/reperfusion, pathologies that are not related to microbial or autoimmune insult or from an endogenous genetic malfunction, have also been investigated. Minocycline is protective against both focal ischemia (Liu et al., 2007, Yrjanheikki et al., 1999) and global neurological ischemia (Yrjanheikki et al., 1998) with similarly positive results from hypoxic-ischemic neonatal brain (Carty et al., 2008) and an embolic stroke model (Wang et al., 2003). These protective results correlate with cellular studies on microglial cells which show a decrease in activation markers and a reduction in the production and release of inflammatory and toxic intermediates such as iNOS, MMPs, nitric oxide and IL-1. Other reports have suggested use for tetracyclines in neuropsychological disorders including schizophrenia (Levkovitz et al., 2007, Hashimoto, 2008) and depression (Pae et al., 2008, Molina-Hernandez et al., 2008).

The chemical basis for tetracycline mediated inhibition of activation of the immune system is currently unclear. A number of different tetracycline compounds exist, differing from each other by minor modifications to the basic tetracycline structure. It is apparent that all tetracycline compounds possess this anti-inflammatory effect to differing degrees whether minor modifications to the chemical structure of tetracyclines modulate the pharmacological anti-inflammatory effect or influence the cellular uptake/efflux of the drug thereby resulting in an apparent pharmacological effect is unclear.

Clinical studies into the use of tetracyclines in inflammatory disease have been carried out with a differing amount of success. Clinical observations of the use of tetracyclines in sepsis have been positive beyond that of the clearance of the pathogenic bacteria. A small clinical study into the use of tetracyclines in the treatment of sarcoidosis was very successful (Bachelez et al., 2001), as was a relatively small study in patients with multiple sclerosis (Zabad et al., 2007). Most phase II clinical studies are set up to evaluate the ability for a group of patients to tolerate the drug regime and to assess doses that could be used in a larger, more comprehensive study into the pharmacological benefits of tetracyclines in various inflammatory diseases. Tetracyclines are generally very well tolerated at doses selected by clinicians.

Phase III studies looking at placebo-controlled clinical improvement or comparative pharmacological outcomes are usually less successful than phase II studies whose primary outcome is tolerability and safety pharmacology. A study with minocycline in

Huntington's disease showed improvement in disease progression (Huntington Study, 2004), however this report was also accompanied by other reports that minocycline had no effect of disease progression in Huntington's disease (Thomas et al., 2004, Blum et al., 2004). Conflicting reports as to the clinical effectiveness of tetracyclines in diseases suggests that there is a place for tetracyclines in anti-inflammatory medicine but that more investigation needs to be undertaken regarding the molecular mechanisms underlying particular diseases and more in depth investigation into the action of tetracycline at a cellular level. Success in artificially produced animal or cell culture models will not necessarily translate into clinical success in which the diseases may be more complex and whose pathology may be multifactorial.

Another aspect of a clinical vs. a laboratory study is often the point of administration of the drug compared to that of the pathogenic stimuli. Most studies will dose with the drug before the pathological insult, this is not the case in clinical practice, and the pharmacological treatments are not administered until after the onset of the disease. This discrepancy in procedures, although commonplace in experimentation, could be one possibility for the lack of clinical success despite the massive amounts of therapeutic benefit from cell and animal based models. This point has however been addressed by a number of groups, D'Agostino et al. (D'Agostino et al., 1998) showed that both tetracycline and doxycycline do retain their inhibitory effects on NO• production when administered at the same time as the LPS insult or at 6 hours post LPS insult, although with reduced efficacy. Administration of tetracycline or doxycycline 12 hours after LPS treatment showed relatively little inhibition of nitric oxide production.

The same group assessed the effects of some chemically modified tetracyclines in the same manner and found a similar result (D'Agostino et al., 2001), administration of CMT-1 or CMT-8 at 6 hours after LPS activation had comparable effects on nitric oxide inhibition as administration of the CMTs at the same time as LPS or 6 hours prior. The time point of CMT-3 dosing was investigated in relation to sepsis induced by cecal ligation and puncture (Halter et al., 2006), showing that when administered at 6 or 12 hours after induction of sepsis a reduction in fatality associated with an improvement of pulmonary pathology was observed whereas administration of CMT-3 24 hours after sepsis was induced failed to protect against fatality in rats. Minocycline had therapeutic properties when administered 24 hours after a 4 hour middle cerebral artery occlusion. These data are from animal and cell culture based models but show that tetracyclines are active post-insult and that the duration of effectiveness of tetracyclines post-insult may depend on the insult and associated pathologies.

1.5 Aims

The aims of this study are to investigate the inhibitory effect of a number of tetracycline compounds on NO• production from the J774.2 murine macrophage cell line. A proteomic approach will be used to investigate the global alterations in protein expression in response to LPS and how minocycline affects these changes in protein expression.

Chapter 2 Materials and Methods

2.1 Reagents

2.1.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

2.1.2 Tetracycline Drugs

Oxytetracycline hydrochloride, doxycycline hyclate and minocycline hydrochloride were purchased from Sigma-Aldrich. An intravenous preparation of tigecycline was purchased from AAH pharmaceuticals (Coventry, UK). Oxytetracycline, doxycycline and minocycline were stored at 4°C and protected from moisture and light as this could lead to oxidation of the tetracycline compounds. Tigecycline was stored at room temperature in the absence of moisture and light.

2.1.3 Antibodies

An anti-NOS2 antibody (cat no sc-650) was purchased from Santa-Cruz Biotechnology (Santa-Cruz, USA). The anti-iNOS antibody was developed against the C-terminal of the mouse iNOS protein and raised in a rabbit. The β-actin antibody (cat no. ab-6276)

was purchased from AbCam (Cambridge, UK). The anti-β-actin antibody was developed against a synthetic peptide corresponding to amino acids 1-14 of the N-terminal of β-actin from Xenopus laevis and raised in mouse. Anti-rabbit IgG and anti-mouse IgG secondary antibodies were purchased from Sigma-Aldrich.

2.2 Cell Culture

2.2.1 Cell Line

The J774.2 murine macrophage cell line was obtained from the European Collection of Cell Cultures (ECACC) and purchased from Sigma-Aldrich. The J774.2 cell line was recloned from J774A.1 cell line originally derived from a tumour in an adult female BALB/c mouse.

2.2.2 Cell Passage

Cells were cultured in 75cm^2 flanks (Griener Bio-one; Stonehouse, UK) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2mM glutamine (Invitrogen) and streptomycin 100μg/ml and penicillin 100U/ml (Invitrogen; Paisley, UK) and 10% heat inactivated foetal bovine serum (Lonza) and maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. Cells were passaged when the cell population was approximately 90% confluent, cells were washed with 5ml phosphate buffered saline (PBS) and then detached by scraping into fresh media; cell clumps were dissociated by

repeated pipetting of the cell suspension. An aliquot of this cell suspension was then re-seeded in a fresh flask with fresh DMEM media.

2.2.3 Cell Counting

Cells were washed with 5ml PBS and detached by scraping into fresh DMEM media. Cell clumps were dissociated by repeated pipetting as described in section 2.2.2. A 20ul aliquot was mixed with an equal volume of trypan blue; counting was performed using a haemocytometer.

2.2.4 Cryopreservation

Cells were grown to confluence in a $75cm^2$ flask, media was aspirated and cells were washed with 5ml PBS. Cells were then scraped into fresh media and centrifuged at 1400 rcf for 5 minutes. The supernatant was aspirated and discarded and the cell pellet was resuspended in heat inactivated FBS supplemented with 10% dimethyl sulphoxide (DMSO). Cells were frozen at -80°C for 24 hours before being transferred to liquid nitrogen for long term storage.

2.2.5 Cell Dosing

For cell based experiments the cells were counted and seeded at $8x10^5$ cells per well (35mm diameter) in 3ml of DMEM media and left to attach overnight, this cell number

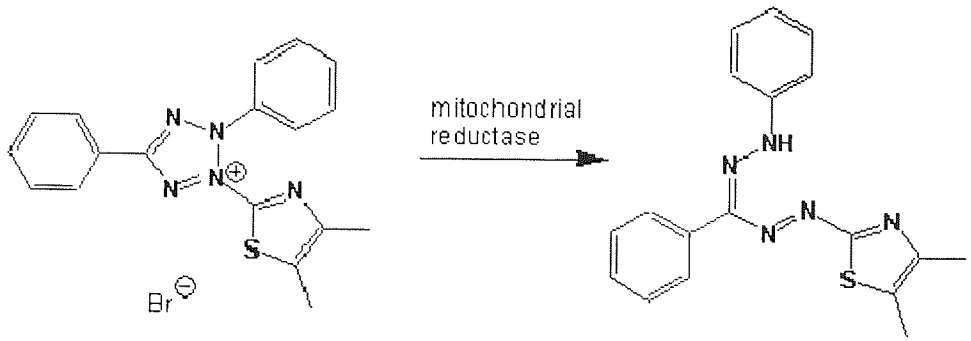
was determined by investigating the growth of cells over 72 hours. The medium was aspirated and replaced with 1ml fresh media and cells were left to equilibrate for at least 2 hours. Tetracycline drug solutions were made fresh immediately before use; tetracyclines were made at a concentration of 5mg/ml in sterile milliQ-water (Millipore) and then further diluted with sterile milliQ-water to the desired concentrations. LPS was suspended in sterile PBS at a concentration of 5mg/ml and stored at -20°C in 50µl aliquots. Immediately before use a 50µl aliquot of LPS was thawed and diluted with PBS until the desired concentration was achieved.

2.3 Cytotoxicity

2.3.1 MTT

Cytotoxicity was assessed by the cells' ability to reduce 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) to a formazan product (Figure 2.1A) first described by Mosmann (Mosmann, 1983). J774 cells were plated in a 96-well plate in 100µl DMEM and stimulated for 24 hours with or without LPS and in the presence or absence of a 1 hour pre-treatment with oxytetracycline, doxycycline, minocycline or tigecycline. After 24 hours 25µl of MTT solution (5 mg/ml in PBS) was added to the wells and incubated at 37°C for 4 hours, the cells were then lysed with lysis buffer (20% w/v SDS in 50% DMF and 2.5% acetic acid) and the plate was incubated at 37°C for 16 hours. After the incubation with lysis buffer the absorbance of the wells was measured at 570 nm.

(A)



(B)

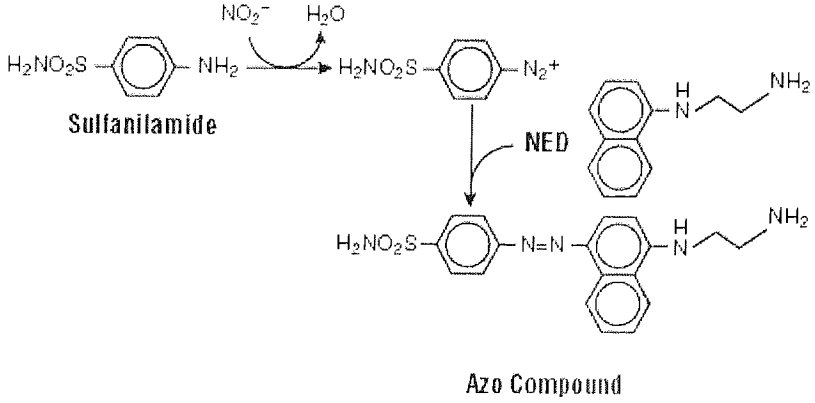


Figure 2.1 Chemical reactions of the MTT and Griess assays. (A) Chemical reaction of MTT in viable cells produces a colourimetric product quantifiable by measuring absorbance at 570nm. (B) Chemical reaction of nitrite with sulphanilamide and subsequently N-1-naphthylethylenediamine dihydrochloride (NED) in the Griess assay to obtain a measurable colourimetric product.

2.4 Nitrite determination

2.4.1 Sample Collection

Cell culture media was collected following an experiment and centrifuged at 15000 rcf for 5 minutes to remove any debris from the sample. The supernatant was aspirated and transferred to a fresh tube and stored at -20°C until analysed.

2.4.2 Griess Assay

To assess the amount of nitric oxide released by the cells a stable breakdown product of nitric oxide, nitrite, was measured using the Griess assay (Figure 2.1B). Nitrite can be oxidised further to nitrate, however measurement of nitrite has been shown to be proportional to the total amount of nitric oxide produced. Briefly, 50µl of cell culture media were applied in triplicate in a 96-well microtiter plate. To each sample and standard 50µl of sulphanilamide (1% sulphanilamide in 5% phosphoric acid) was added and the plate was incubated at room temperature in the absence of light for 10 minutes, 50µl of N-1-naphthylethylenediamine dihydrochloride (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, NED) was then added to each of the samples and standards and the plate was then incubated for a further 10 minutes in the absence of light. This chemical reaction between nitrite and sulphanilamide and subsequently NED produces a purple product that is measurable by determining the absorbance at 550nm with an Anthos 2001 plate reader. The unknown concentrations of nitrite were

calculated by constructing a standard curve using known concentrations of sodium nitrite.

2.5 Western blot

2.5.1 Sample collection

Cell culture media was aspirated and cells were washed with 5ml PBS. Protein samples were collected by adding 250µl of hot Laemlli sample denaturing buffer (10% (v/v) glycerol, 2% (w/v) SDS, 0.007 (w/v) bromophenol blue, 63mM Tris-HCl, 2mM sodium pyrophosphate, 5mM EDTA, 50mM dithiothreitol, DTT) to each well of a 6-well cell culture plate and cells were scrapped into this buffer. Protein samples were then drawn up and down a 21G needle to sheer genomic DNA. Samples were heated to 95°C for 5 minutes, transferred to fresh tubes and stored at -20°C, a 40µl aliquot was kept for protein determination.

2.5.2 Protein determination

Due to the high concentrations of detergents and reducing agents in the buffer in which the proteins were collected, protein determination was carried out using the RcDc protein assay (BioRad) which is compatible with reducing agents and detergents in a 96-well plate format according to the manufacturer's instructions. Full details of assay and protocol can be found in appendix.

2.5.3 SDS-PAGE

Separation of proteins was by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were cast using the mini Protean system (BioRad). Proteins were resolved on a 7.5% gel with the presence of a 4% stacking gel, the composition of these gels is described in table 2.1. Equal amounts of protein (20μg) was loaded to each well in the stacking gel and electrophoresed at 115V for 105 minutes in electrophoresis buffer (20mM Tris-base, 192mM glycine, 0.1% (w/v) SDS).

2.5.4 Western Blotting

Proteins were electro-transferred from the acrylamide gel to Hybond nitrocellulose membrane (GE Healthcare, Amersham, UK) using a Transblot apparatus (BioRad). The electro-transfer was conducted at 115V for 105 minutes in transfer buffer (20mM Tris-base, 192mM glycine, 20% methanol). Following the electro-transfer the nitrocellulose was blocked in 3% BSA in Tris-buffered saline for 2 hours, following this blocking step the 3% BSA in tris-buffered saline with 0.1% Tween20 (TTBS) solution was aspirated and replaced with the primary antibody in TTBS with 0.2% BSA overnight at room temperature. Following the primary antibody incubation the nitrocellulose membrane was washed 8 times for 15 minutes each. The secondary antibody was suspended in TTBS with 0.2% BSA and placed onto the membrane for 90 minutes followed by 8 x 15 minute washes. The nitrocellulose membrane was then developed using ECL reagent (GE Healthcare) and Hyperfilm (GE Healthcare).

Table 2.1 Chemical composition of SDS-PAGE gels.

Resolving gel:	15%	11%		7.5%
	18ml	12ml	48ml	12ml
H2O (ml)	3	4.4	17.6	5.8
Buffer 1 (1.5M Tris (pH8.4), 0.4% (w/v) SDS) (ml)	3	3	12	3
Acrylamide solution (ml)	6	4.4	17.6	3
10%APS (μl)	60	45	180	45
TEMED (μl)	6	4	16	4

Stacking gel:	7.5ml
H_2O (ml)	4.87
Buffer2 (0.5M Tris (pH 6.8), 0.4% (w/v) SDS) (ml)	1.87
Acrylamide solution (ml)	0.75
10%APS (μl)	75
TEMED (μl)	10

2.6 Proteomics

2.6.1 Sample preparation

Experiments were conducted in triplicate. Samples were scraped directly into Rehydration Buffer (8M urea, 2M thiourea, 2% (w/v) CHAPS, 2% (w/v) 3-(Decyldimethylammonio)-propane-sulfonate inner salt (SB 3-10)). Samples of the sample treatments were then pooled together by mixing equal volumes of the samples. A flow diagram describing the processing of the samples is shown in figure 2.3.

2.6.2 First Dimension Protein Separation

The pooled sample (50µg) was loaded into the rehydration tray and the IPG ReadyStrips (BioRad).were passively rehydrated overnight at a constant temperature of 20°C. After rehydration the IPG strips were transferred to the focusing tray and the proteins were focused using the following programme: 0-500V over 500Vhours, 500-3500V over 3500Vhours, and 3500V for 90000Vhours at 20°C. Strips were removed from the focusing tray and stored at -80C until second dimension separation.

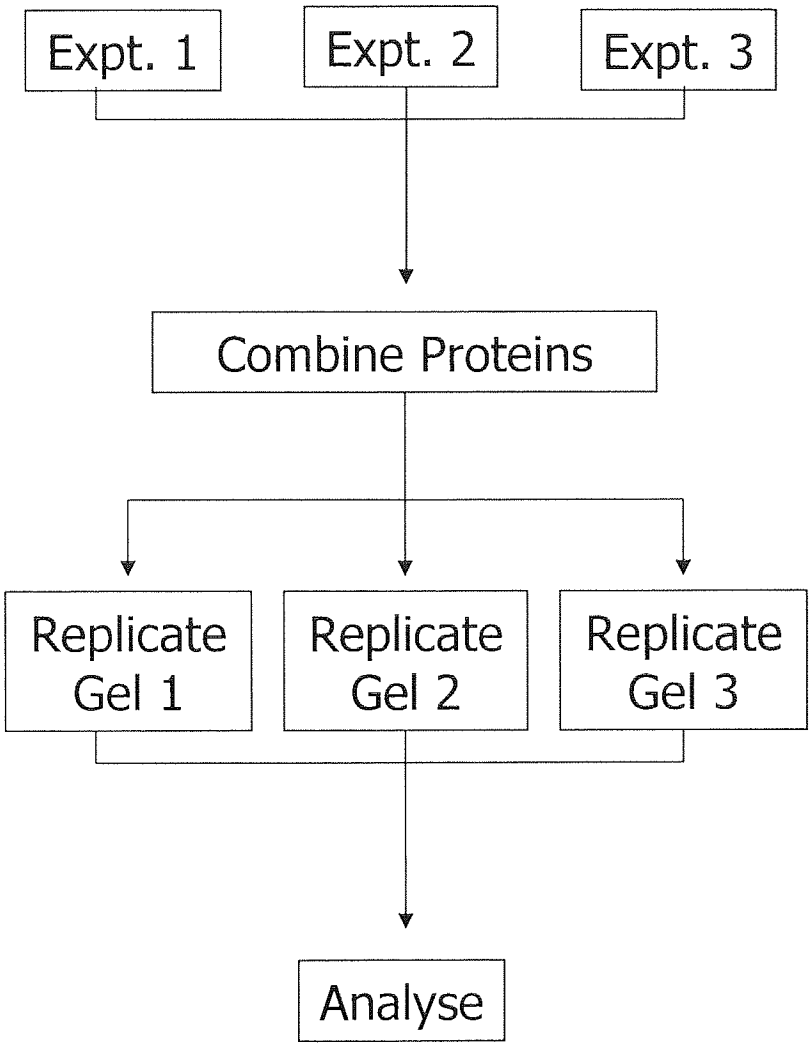


Figure 2.2 Diagram of the processing of samples used in proteomic studies. Independent experiments were carried out, and equal volumes of each sample were combined reduceing inter-experimental differences. This pooled sample was then run independently three times to assess robustness in the two-dimensional gel electrophoresis protocol. Replicate gels were analysed collectively by PDQuest software.

2.6.3 Second Dimension Separation

Focused IPG strips were removed from frozen storage and allowed to thaw at room temperature. IPG strips were incubated in equilibration buffer (8M Urea, 2M SDS, 5mM Tributylphosphine (TBP), 40mM Tris) for 20 minutes with rotation. IPG strips were placed on to a 4-20% Tris-Glycine Precast Protean gel and overlayed with 0.1% agarose. Proteins were separated in the second dimension by electrophoresis at 90V for 2 hours.

2.6.4 Gel Staining

Proteins that were resolved by 2D gel electrophoresis were stained with Flamingo total protein stain (BioRad). Flamingo total protein stain is more sensitive than coomassie blue or silver. Comparison between Flamingo total protein stain and the commonly used silver staining technique showed Flamingo to be 1000 times more sensitive. Not only was Flamingo better because of increased sensitivity but also due to its specificity for proteins, other staining techniques detect polysaccharides, nucleic acids and contaminant halide ions as well as proteins. This can causes streaking on the gel images leading to difficulty in analysing images.

Following electrophoresis, gels were fixed for 16 hours in fix buffer (40% ethanol, 10% acetic acid) in a glass container. Gels were then transferred to Flamingo stain solution,

10x Flamingo stain was diluted to a 1x working concentration with milli-Q water. Gels were incubated in Flamingo stain for 8 hours.

2.6.5 Gel Imaging

Gel images were scanned using a Pharos FX Plus fluorescent scanner (BioRad). Gels were imaged using Quantity One Software (BioRad) with an excitation wavelength of 532nm and an emission wavelength of 605nm; images were obtained with a PMT voltage of 45%. Complete gel images were cropped for proteomic analysis.

2.6.6 PDQuest Analysis

Cropped gel images were analyzed using PDQuest software (BioRad). Spots were identified and matched between gels; spot densities were calculated and normalized to total number of pixels in each image. Spots whose pixel density altered by 2-fold were deemed to be significant.

2.7 LC/MS

2.7.1 Spot Excision

Protein spots of interest were identified using PDQuest analysis, spots of interest were located on the original gels using a UV light box and excised using 200µl disposable

pipette tips, the end of which was cut to the diameter of the spot of interest. The excised gel plug was stored in a LoBind microcentrifuge tube (Eppendorff) at -80°C.

2.7.2 Proteolysis of protein spots

Gel plugs were washed twice with 100µl TTBS to remove any contaminant material on the surface of the gel piece, and then washed twice with milli-Q water to remove any residual TTBS. The gel piece was then washed with 100µl of 200mM ammonium bicarbonate; the ammonium bicarbonate was aspirated from the gel piece and discarded. The gel was then washed with milli-Q water. The gel piece was then dehydrated with acetonitrile changing the acetonitrile solution three times. Trypsin Gold (Promega) was reconstituted as described in the manufacturer's instruction to a concentration of 1µg/ml in 200mM ammonium bicarbonate. The dehydrated gel piece was incubated in 200µl of acetonitrile containing 1µl of the reconstituted trypsin solution and incubated at 37°C for 16 hours. After the incubation with trypsin the acetonitrile/trypsin solution was aspirated and transferred to a fresh LoBind microcentrifuge tube. Tryptic peptides were extracted from the gel by the addition of 100µl of milli-Q water, vortexing for 10 minutes and then sonicating for 5 minutes, the water was removed and pooled with the acetonitrile/trypsin solution collected previously. 50µl of extraction solution (10% v/v formic acid, 0.1% v/v acetonitrile in water) was added to the gel plug and vortexed for 10 minutes and the sonicated for 5 minutes, solution was aspirated and pooled with the other pooled material. This extraction step was repeated twice more and then the pooled samples were vacuum

dried. The vacuum dried protein pellet was resuspended in milli-Q water containing 0.1% v/v formic acid; the sample was centrifuged at 13,000 rcf to remove any particulate matter from the sample.

2.7.3 Capillary Liquid Chromatography

The tryptic peptide samples were loaded into Autosampler vials, vial inserts were used to raise the physical level of these samples, this allowed analysis of less volume of sample in the LC/MS and therefore higher concentrations of the protein samples. Tryptic peptides were loaded onto a C-18 peptide captrap (Presearch, UK) over a 5 minute period by flowing the sample through the capillary C18 biobasics liquid chromatography column with an internal diameter of 75μm (Integrafrit; Presearch, UK) with 100% Buffer A (99.9% water with 0.1% formic acid) a rhyedine valve was used to control the direction of flow (figure 2.6), To load the tryptic peptide samples onto the captrap, split 1 was positioned so that the flow was directed through the peptide trap due to the tubing being blocked in the other direction and the flow at the second split was freely flowing to the waste, there was limited flow through the LC column due to the high resistance there compared to the tube of 75μm internal diameter, after 5 minutes the flow through the system was altered to allow the flow to now flow through the LC column, the valve was switched so that at split 2 the flow could no longer flow to the waste, the flow at split 1 was now flowing to the waste however the tubing here has some resistance due to a smaller internal diameter of 50μm, this causes a small amount of the flow to be directed through the peptide trap and subsequently down the

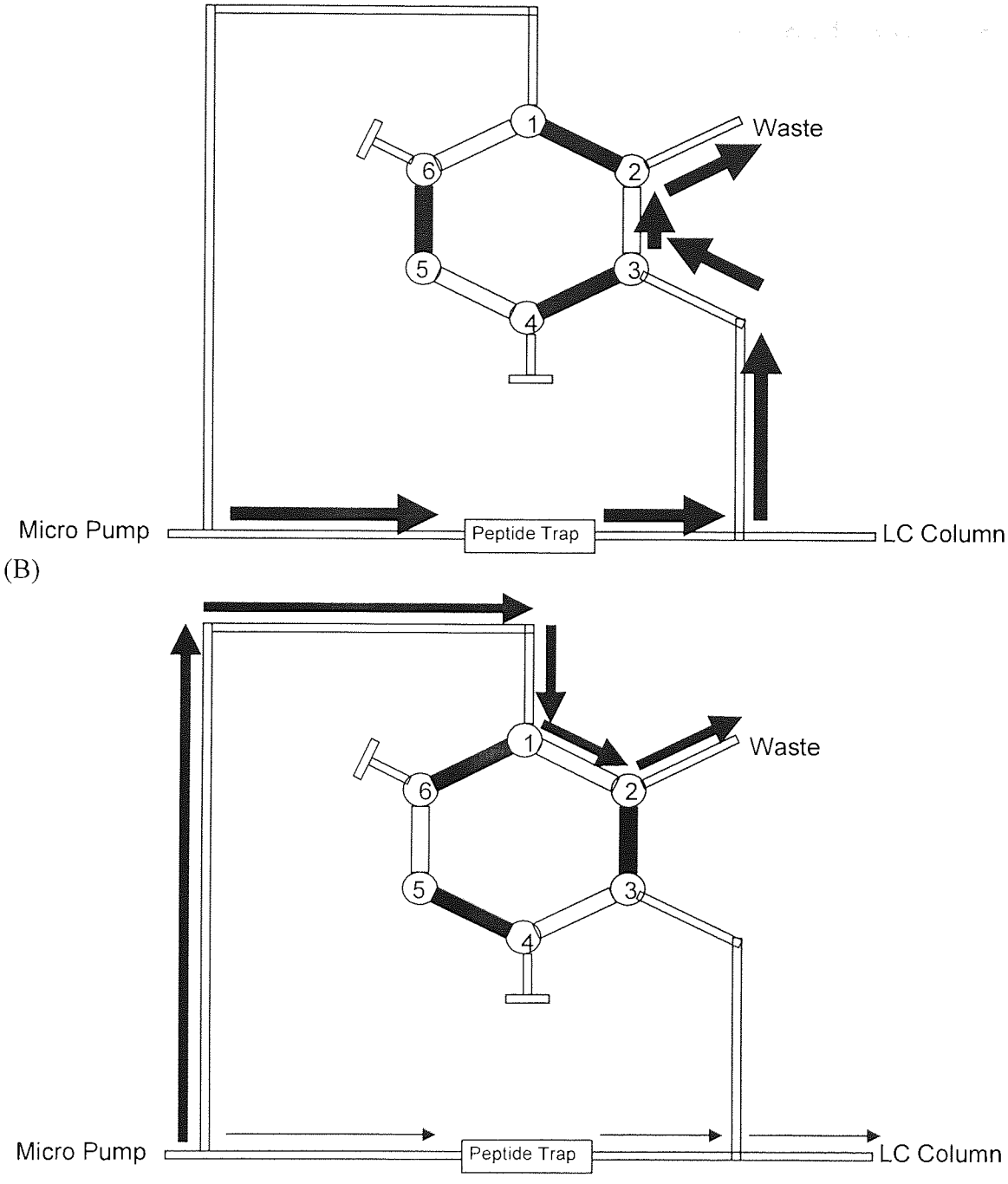


Figure 2.3 Control of solvent flow in the liquid chromatography system. Diagram of the flow of solvent through the capillary liquid chromatography system. (A) shows the flow though the system to allow loading of the tryptic peptides onto the C-18 peptide trap. (B) shows the flow through the system to direct the flow through the LC column and mass spectrometry detector.

LC column. This split of the flow is required to reduce the flow rate of solvent through the LC column and was assessed to be approximately 300nl/min. Peptides were eluted from the peptide trap and subject to liquid chromatographic separation using flow through the system was altered to 95% buffer A and 5 % buffer B (99.9% acetonitrile, 0.1% formic acid) for 5 minutes, then steadily increased to 60% buffer A, 40% buffer B over 45 (A)minutes, peptides will elute from the column during this period. The flow was then altered to 40% buffer A, 40% buffer B for five minutes, this phase will elute any other compounds that are on the column. The flow was then altered to 95% buffer A, 5% buffer B for 5 minutes, this phase re-equilibrates the column ready for another cycle.

2.7.4 Linear Ion Trap

The Finnigan LXQ linear ion trap (Thermo Scientific, UK) was set to complete three different scan events. The first scan event was a full mass spectrum (full MS), this identified all of the mass fragments present in the sample which were being eluted from the LC column at that time and scanned a mass/charge range of 465-1600. The subsequent scan events carried out were dependent on the full MS scan and were triggered if the mass peaks above a threshold value of 500 counts. If these data dependent scan events 2 and 3 were initiated they would go on to analyse the largest 3 peaks in the full MS.

Scan event 2 was a Zoom scan; this scan determined the mass/charge of the fragment of interest and identified different masses of the same peptide resulting from the presence of different isotopes of carbon (C12 and C13). Neighbouring peaks of the same peptide were spaced by multiples of 1Da, a difference between the peaks of 1 Da/z means that the charge of the peptide is 1 (from equation 2.1). This allows the calculation of the actual mass of the peptide for example a mass/z of 1000 with a charge of 1 represents a peptic fragment of 1000Da, a mass/z of 1000 with a charge of 2 represents a peptic fragment of 2000Da.

Scan event 3 is a MS^2 scan which isolated the fragment identified in scan event 1 and fragmented it further with a normalized collision-induced dissociation energy of 30%. The mass fragments obtained from this were analysed to derive amino acid sequence. In gently fragmenting the peptic fragment further a number of different fragments were obtained some of which relate to b^+ and y^+ ions of the peptide (figure 2.4). The mass difference between successive b ions and successive y ions represents the mass of individual amino acids, and using the data regarding b^+ ion and y^+ ions together the amino acid sequence could be determined.

Successive scan events were only triggered on the three largest fragments from the initial full MS scan, if no fragments reached the threshold value set then the full MS scan would continue until a fragment was identified. If many fragments of the full MS scan were above the threshold value then only the largest three fragments were analysed by other scans. Therefore the maximum number of scan events between two.

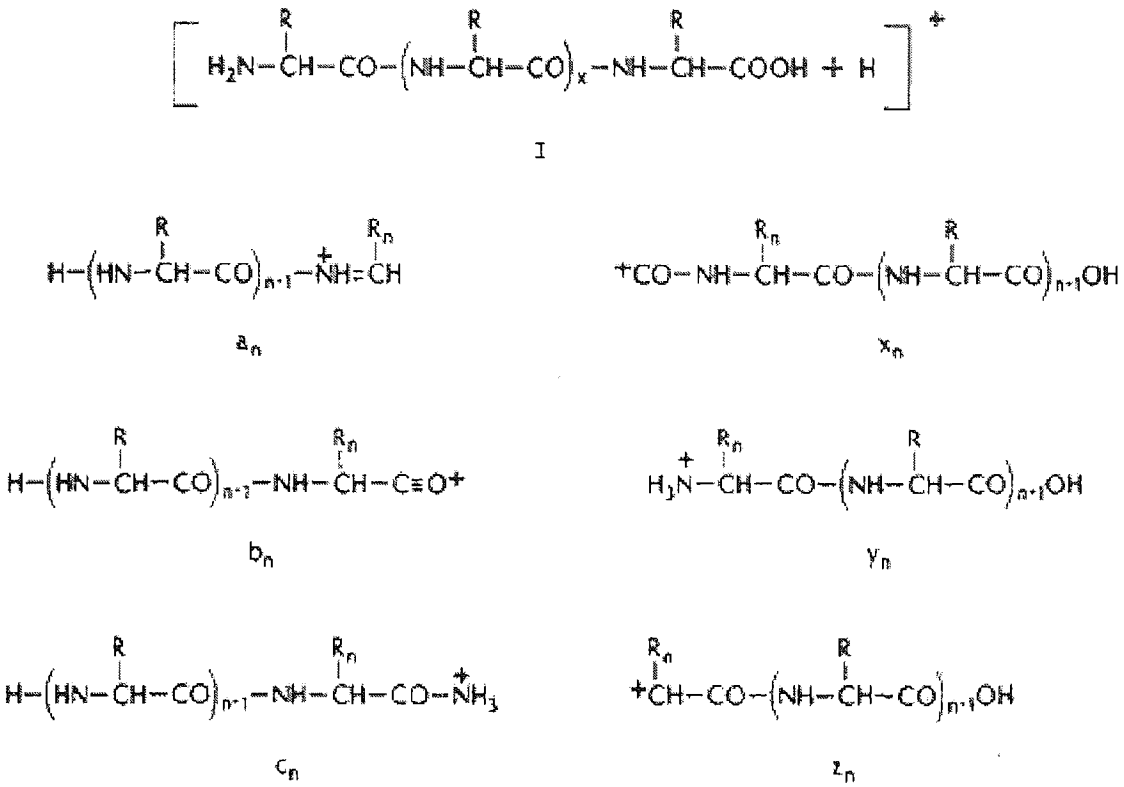


Figure 2.4 Formation of ions from collision induced dissociation. Diagram showing the formation of different chemical species (a/x ions, b/y ions and c/z ions) from the fragmentation of peptides from collision induced dissociation (adapted from (Johnson et al., 1987). The ions that are most commonly formed and used for amino acid determination are the b/y ions. Data from the mass of different b/y ions is used to calculate the amino acid sequence of a peptide.

full MS scans was 6 scans. A prescan was carried out before each scan event; the ion trap opened for 1ms to count the number of ions in the trap, the trap then calculates the time it needs to open to allow entry of the optimal number of ions in for analysis. The maximum injection time for a full MS scan was limited to 10ms and 50ms for Zoom and MS^2 scan events

2.7.5 BioWorks

BioWorks 3.0 software (ThermoFisher Scientific) was used for the analysis of mass spectrometry data. The software converted raw data files acquired from the mass spectrometer and converted into .srf files. A selected data base of proteins was then virtually digested with a selected digestion enzyme and the proteins were identified and allocated a probability score calculated from the peptides identified compared to the virtual peptides that have been created, a significant match is a protein identified with a probability score of <0.05.

$$\frac{\Delta \text{Mass}}{\Delta \text{Mass/Charge}} = \text{Charge of peptide}$$

Example: (A) $\frac{1\text{Da}}{1\text{Da/z}} = 1\text{z}$

(B) $\frac{1\text{Da}}{0.5\text{Da/z}} = 2\text{z}$

Equation 2.1 Equation used to calculate the mass of a peptic fragment from data from of a Zoom scan.

Chapter 3 The Effect of Tetracyclines on Nitric Oxide Production

3.1 Introduction

NOS is the protein responsible for the physiological production of nitric oxide (NO•). There are three isoforms of the NOS protein: endothelial (e)NOS, inducible (i)NOS and neuronal (n)NOS also termed NOS1, NOS2 and NOS3 respectively. nNOS and eNOS are primarily, although not exclusively, expressed constitutively in neuronal and endothelial tissues respectively, they are dependent on levels of intracellular Ca^{2+} and produce low levels of NO•. In contrast to nNOS and eNOS, iNOS is not constitutively expressed and is Ca^{2+} independent, iNOS is induced by an extensive range of cytokines and exogenous stimuli and produces relatively high levels of NO•.

Transcriptional regulation of the iNOS gene is the primary mechanism of regulation and is the most well defined method of regulation of iNOS protein expression (Aktan, 2004). The iNOS gene has a number of transcription factor binding sites in its promoter region including binding sites for activating protein 1 (AP-1), CCAAT-enhancer box binding protein (C/EBP), cAMP-responsive element binding protein (CREB) and a number of nuclear factor-κ B (NF-κB) response elements (Taylor and Geller, 2000). In a resting cell the basal level of iNOS is undetectable but is induced by stimulation with LPS and cytokines such as TNFα, IL-1β and IFNγ.

The relationship between NF-κB and AP-1 transcription factors in relation to iNOS expression is complex and for complete expression of iNOS activation of both NF-κB and AP-1 transcription factors are required. Only partial expression of iNOS is observed with only the NF-κB or the AP-1 binding motif, partial expression is also observed following the removal of one of the two AP-1 binding motifs in the iNOS promoter region.

Post-transcriptional regulation of iNOS function does occur via the modulation of mRNA stability. Many mRNA transcripts associated with the inflammatory response have AU-rich elements (ARE); these can be composed of a single AUUUA motif or of AUUUA repeats in the 3' region of the mRNA transcript. Following transcription of mRNA containing an ARE, the transcript is able to associate with ARE binding proteins (AREBP). Some AREBP are involved in stabilizing mRNA transcripts, this means that transcripts are available for translation for longer and therefore result in increased protein levels (Akashi et al., 1994). There are also AREBP that bind to the ARE of mRNA transcripts and act to destabilize mRNA, therefore reducing proteins levels. Although this regulation of iNOS is not well defined it does appear that iNOS mRNA stability is reduced when p38 MAPK is inhibited.

NO• is a major contributing factor in inflammatory pathogenesis, NO• produced erroneously in conditions such as rheumatoid arthritis, neurodegeneration and sepsis can lead to excessive host tissue damage. Also in sepsis, inappropriately large levels of NO• can lead to severe hypotension which is often fatal. The ability to reduce levels of

NO• in diseases such as these is an important strategy in inflammatory medicine. The identification of drugs with good safety profiles that can reduce NO• is important.

In this chapter the effect of a range of clinically important tetracyclines (oxytetracycline, doxycycline, minocycline and tigecycline) upon on the production of NO• in macrophages was investigated. The ability of a drug to reduce the production of a cytotoxic compound such as NO• would be beneficial in diseases that are associated with excessive or inappropriate production of nitric oxide. Reducing the production of nitric oxide in inflammation would reduce host tissue damage that results from aberrant NO• production.

3.2 Results

3.2.1 Cell Growth Analysis

Cells were seeded in 6-well plates at two different densities, $4x10^5$ and $6x10^5$, and a well was harvested and counted at 24, 48 and 72 hours after plating the cells. Trypan Blue was used during counting to identify viable cells only. J774 cell growth in 6-well plates was confluent at approximately $1.5x10^6$ cells after plating the cells at two different cell densities (figure 3.1) and subsequent cell loss associated with overgrowing was not observed for up to 48 hours after confluence had been reached. Cell based assays were therefore plated at $8x10^5$ and left overnight to allow them to attach to the plate, further stimulations were conducted for no more than 25 hours following this.

3.2.2 J774.2 macrophages produce nitric oxide in response to LPS

The J774.2 murine macrophage cell line stimulated with LPS was used to investigate the effect of tetracyclines on the inflammatory response. J774.2 cells were exposed to different concentrations of LPS ranging from 0.01μg/ml - 100μg/ml for 24 hours. After a 24 hour incubation period the cell culture media was collected and the nitrite levels were measured. Figure 3.4(A) shows a typical dose response curve.

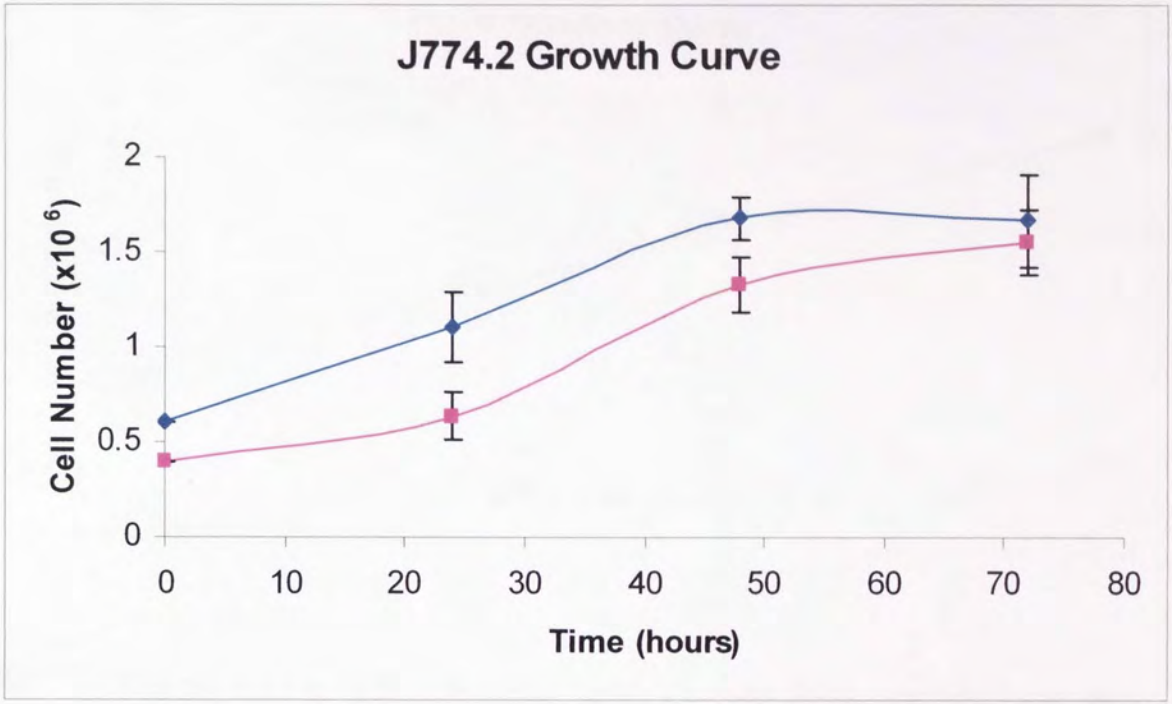


Figure 3.1 Characterization of J774.2 growth. Cells were seeded at $4x10^5$ and $6x10^5$ respectively. Cell counts were performed at 24, 48 and 72 hours after cell seeding. Only viable cells were included in the cell count assessed by trypan blue exclusion.

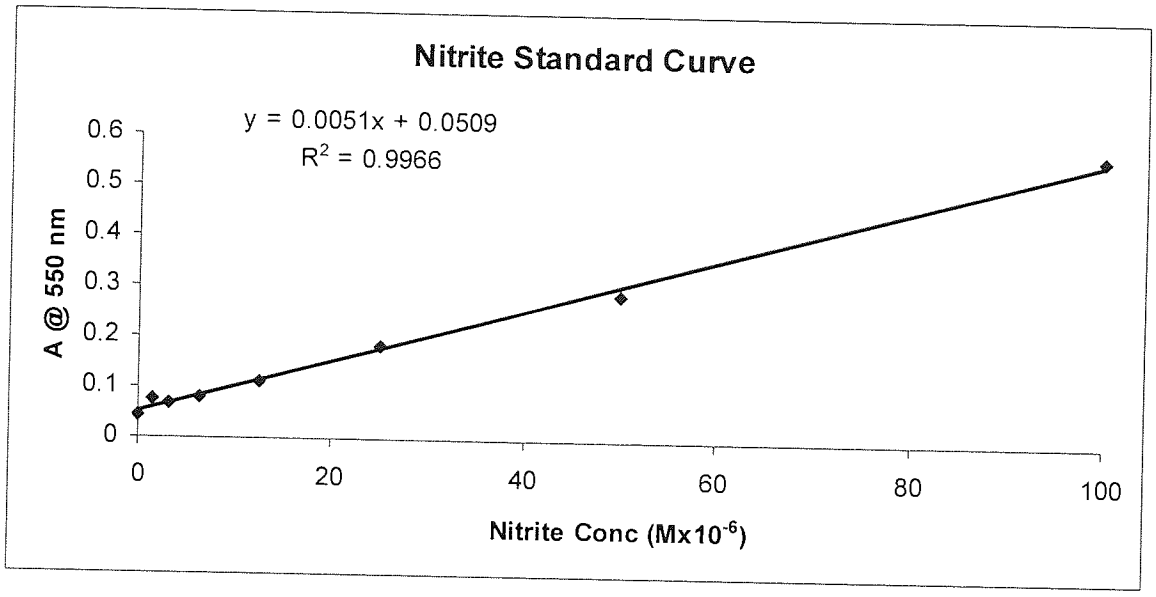


Figure 3.2 Standard curve for the Griess assay. Sodium nitrite standard curve used to assess unknown nitrite levels in cell culture media using the Griess Assay. This is a representative sodium nitrite standard curve, one was produced at the time of each assay.

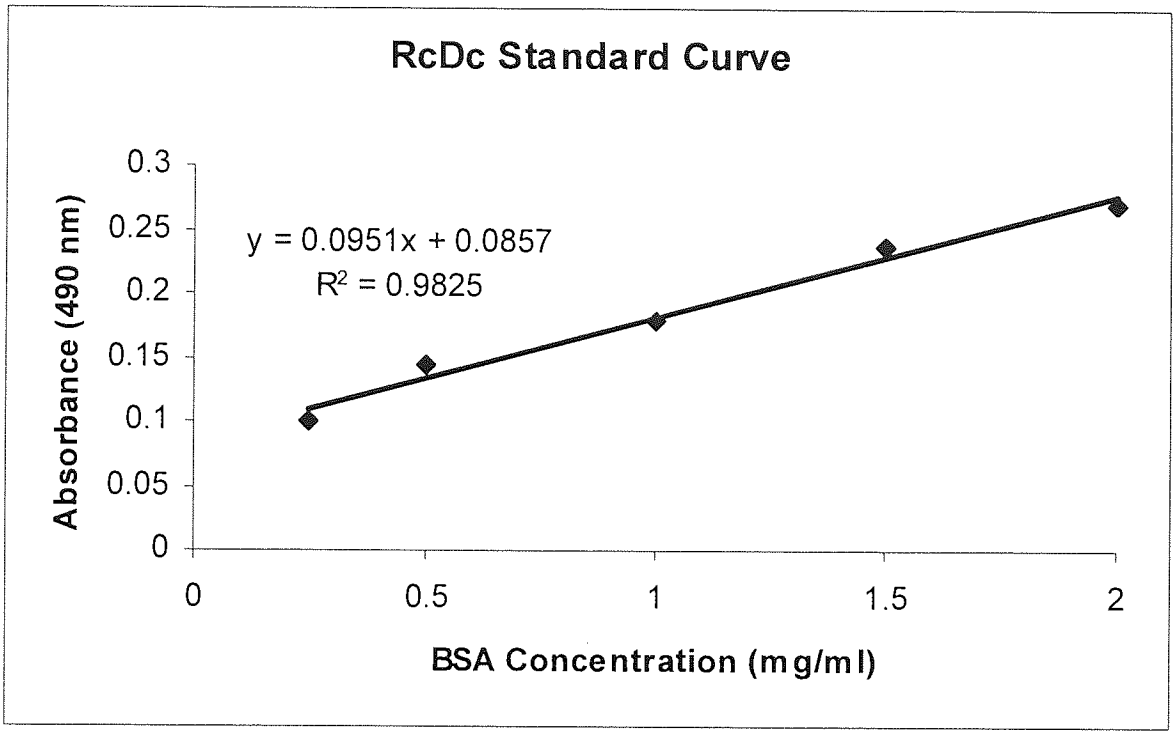
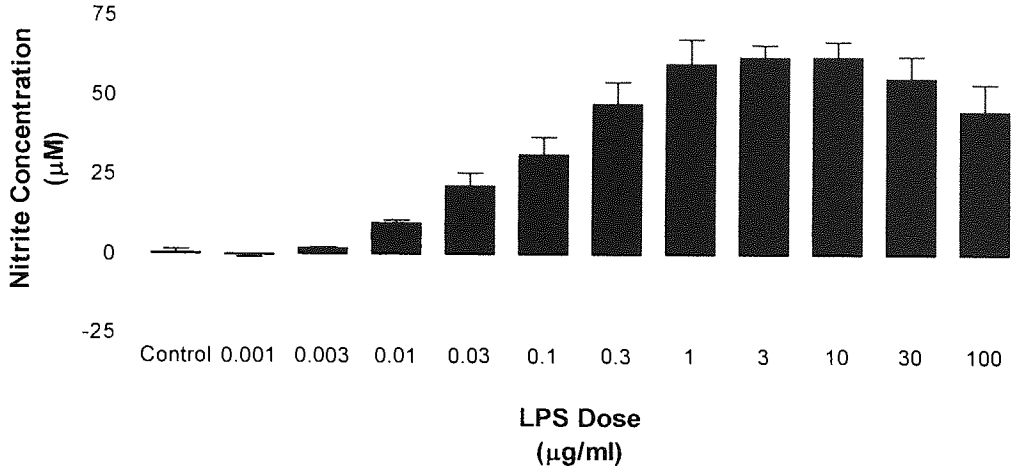


Figure 3.3 BSA Standard Curve used for protein concentration determination.

(A)



(B)

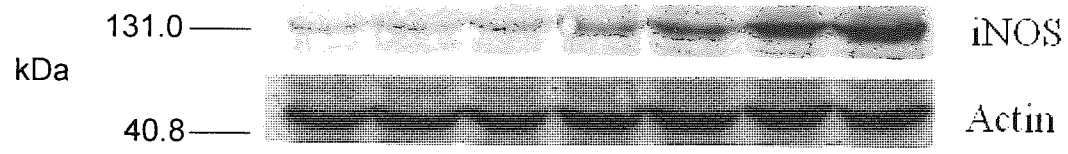


Figure 3.4 Effect of LPS on the iNOS induced production of nitric oxide. (A) The accumulation of nitrite as assessed by the Griess assay. Free nitrite levels were measured by their conversion by sulphanilamide and NED to a colourimetric azo compound. Unknown concentrations of nitrite were calculated using a standard curve constructed from known concentrations of sodium nitrite. Data expressed as mean of three independent experiments +/- the s.e.m. (n=3). (B) The expression of iNOS increases with increasing concentrations of LPS as assessed by western blot analysis. Relative expression on iNOS was assessed using β-actin as a loading control. This is a representative western blot (n=3).

No detectable nitrite was observed in untreated cells whereas significant levels were produced when the cells were stimulated with 0.01μg/ml for 24 hours. The amount of nitrite produced increased as the dose of LPS increased with a maximal level of nitrite produced after a 24 hour stimulation with 1μg/ml. The amount of nitrite detected in the cell culture media after 24 hour incubation with doses of LPS higher than 1μg/ml showed no further increase. The maximal level of nitric oxide production as assessed by measurement of nitrite levels in the cell culture media after a 24 hour incubation with LPS was at a dose of 1μg/ml of LPS, after which nitrite levels did not.

3.2.3 J774.2 macrophages increase iNOS expression in response to LPS

The main regulation of NO• produced by iNOS is regulated by gene regulation whereas nitric oxide produced by both eNOS and nNOS is dependent on post-translational modulation of enzyme and the presence of co-factors such as calcium. There are other suggestions that iNOS is in part regulated by post-translational events although the details of these post-translational events involved in iNOS regulation are currently unclear as conflicting data has been published (Amin et al., 1996, D'Agostino et al., 1998). In order to further characterise the effect of LPS on nitric oxide production the expression of iNOS protein was assessed by western blot analysis after 24 hour stimulation with varying concentrations of LPS ranging from 0.01μg/ml to 100μg/ml.

iNOS protein expression was not detectable by western blot analysis in resting cells but its expression was induced by LPS at a concentration of 0.03μg/ml (Figure 3.4(B)).

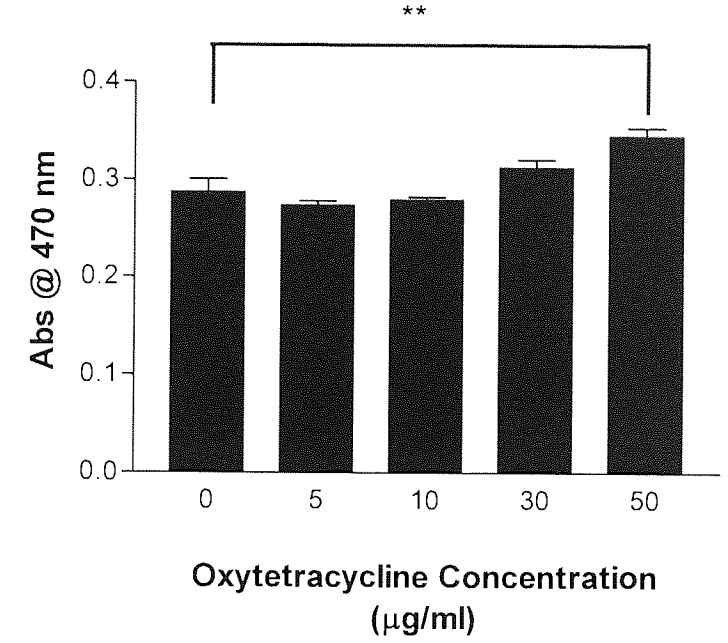
Protein expression of iNOS increased as the concentration of LPS was increased and the maximum level of iNOS protein was detected at a concentration of 1μg/ml LPS. Concentrations of LPS higher than 1μg/ml did result in a higher level of iNOS protein being expressed compared to the level of iNOS protein expressed in response to 1μg/ml LPS.

3.2.4 The effect of Tetracyclines on cell viability

To establish a range of doses of the tetracycline compounds that could be used in this study of their pharmacological activity the cytotoxic potential of the tetracycline compounds was determined. It was important to show that the pharmacological effects described in the study were not in any part due to a reduction in the viability of the cell line model used here and that any anti-inflammatory effects of tetracycline compounds shown and investigated here are true pharmacological effects. The ability of the J774.2 cell line to convert MTT to a formazan compound was determined. This chemical reaction is catalysed by mitochondrial reductase enzymes in the mitochondria of the cells. Mitochondrial dysfunction is an early stage of cell death and measuring mitochondrial function is a good indicator of cell loss due to both apoptosis and necrosis.

The tetracyclines investigated did not decrease cell viability over the concentration range 5μg/ml to 50μg/ml (Figures 3.5-3.8). None of the doses of tetracycline investigated were

(A)



(B)

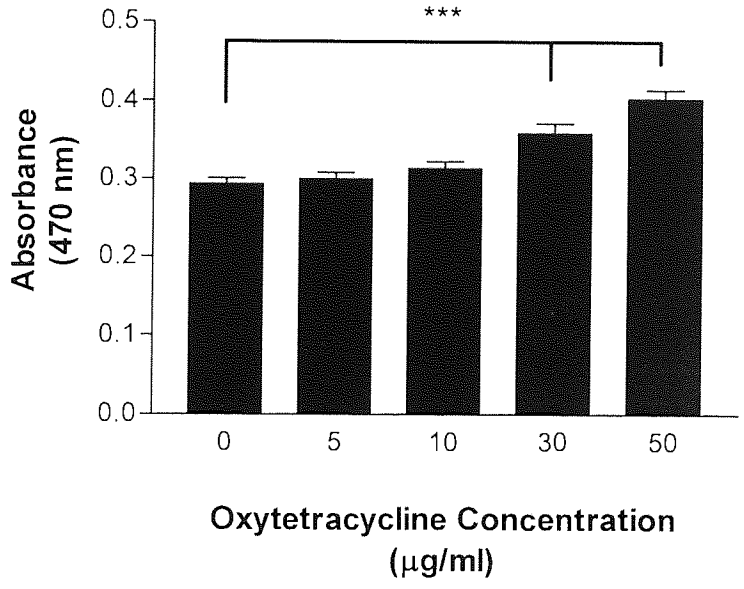
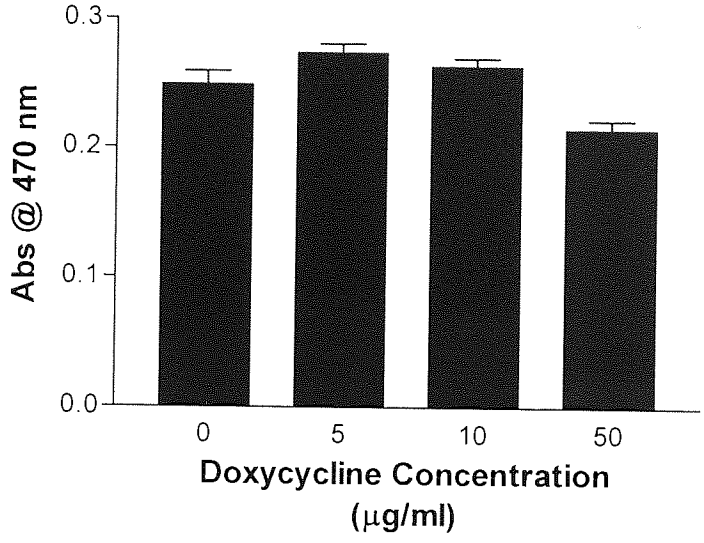


Figure 3.5 Effect of oxytetracycline and LPS on viability of J774.2 cells. Cells were treated with various concentrations of oxytetracycline for 25 hours with or without LPS treatment after which MTT substrate was added and formazan conversion was then measured. (A) Cells were treated with oxytetracycline (3μg/ml-50μg/ml) for 25 hours (B) cells were treated with various concentrations of oxytetracycline for 1 hour followed by the addition of LPS (1μg/ml) for 24 hours. Data expressed as average of 3 independent experiments +/- s.e.m. (n=3), statistical significance calculated by one-way Analysis of Variance (ANOVA) with a Tukey's post-test (**=$p<0.01$, ***=$p<0.001$).

(A)



(B)

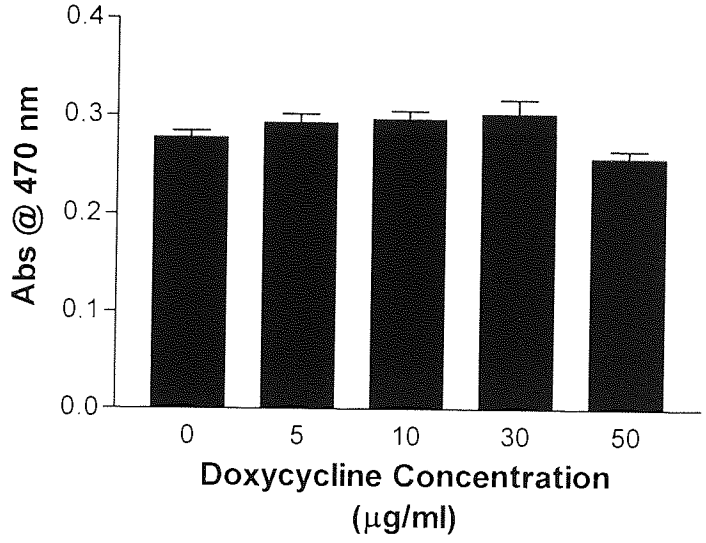
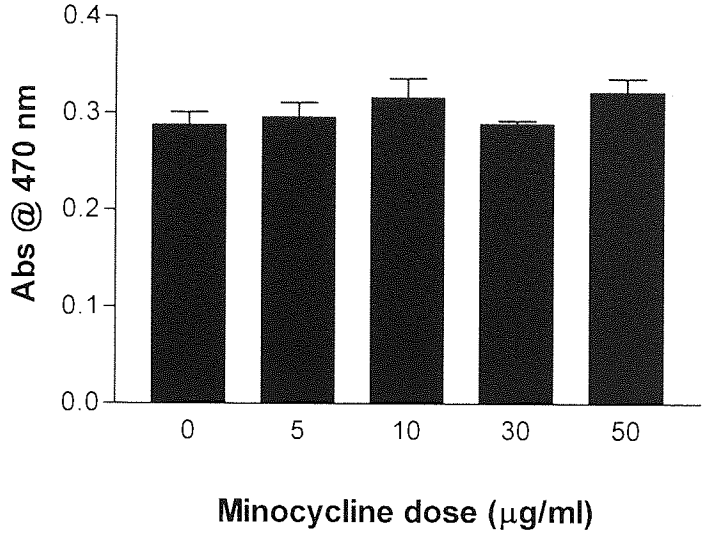


Figure 3.6 Effect of doxycycline and LPS on viability of J774.2 cells. Cells were treated with various concentrations of doxycycline for 25 hours with or without LPS treatment after which MTT substrate was added and formazan conversion was then measured. (A) Cells were treated with doxycycline (3μg/ml-50μg/ml) for 25 hours (B) cells were treated with various concentrations of doxcycline for 1 hour followed by the addition of LPS (1μg/ml) for 24 hours. Data expressed as average of 3 independent experiments +/- s.e.m. (n=18), statistical significance calculated by one-way ANOVA with a Tukey's post-test. There were no statistically significant differences.

(A)



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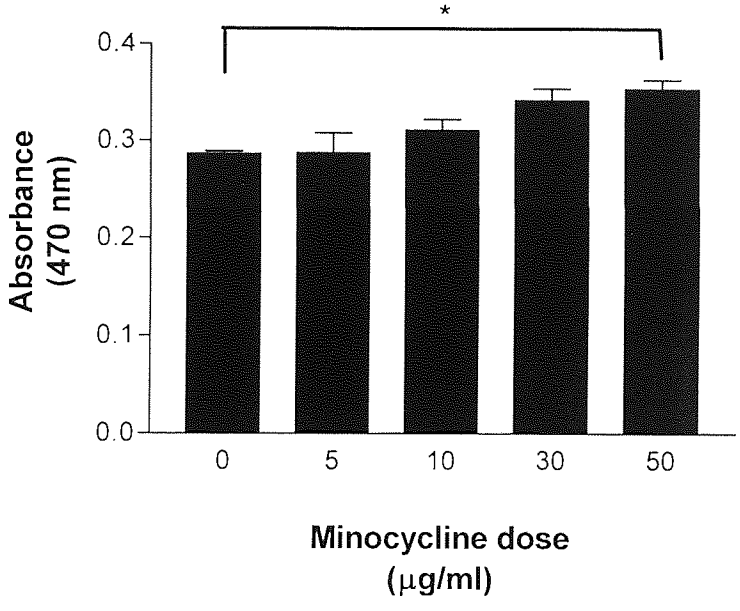
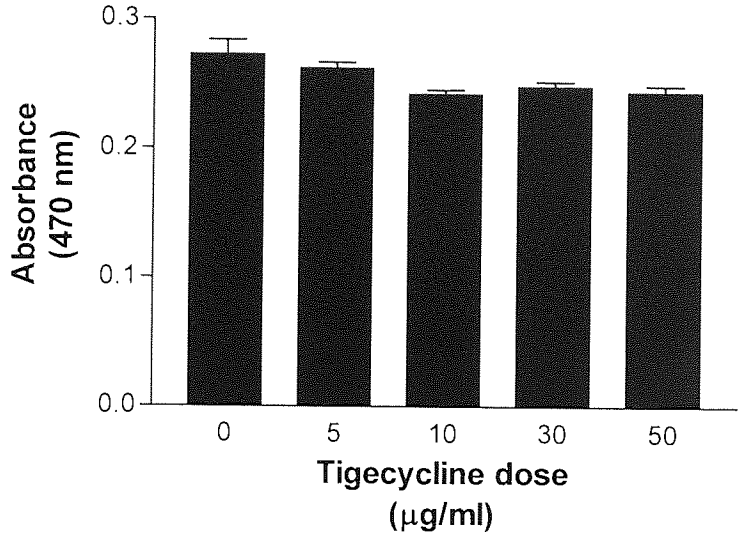


Figure 3.7 Effect of minocycline and LPS on viability of J774.2 cells. Cells were treated with various concentrations of minocycline for 25 hours with or without LPS treatment after which MTT substrate was added and formazan conversion was then measured. (A) Cells were treated with minocycline (3μg/ml-50μg/ml) for 25 hours (B) cells were treated with various concentrations of minocycline for 1 hour followed by the addition of LPS (1μg/ml) for 24 hours. Data expressed as average of 3 independent experiments +/- s.e.m. (n=18), statistical significance calculated by one-way ANOVA (*=$p<0.05$) with a Tukey's post-test.

(A)



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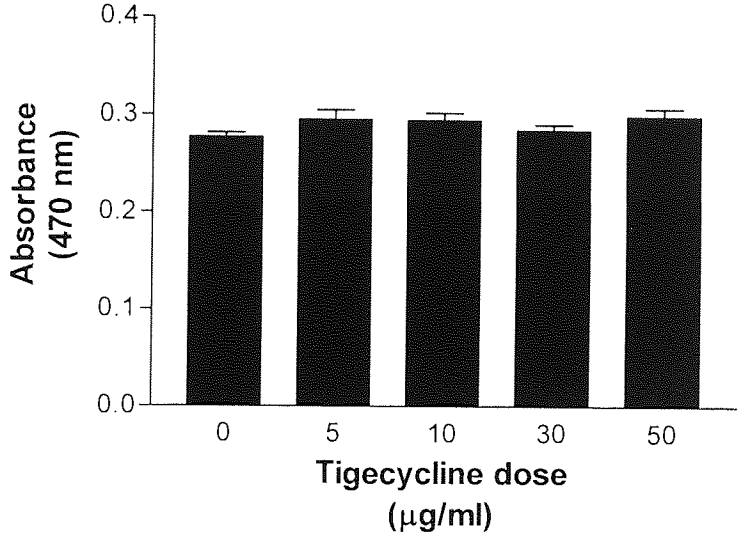


Figure 3.8 Effect of tigecycline and LPS on viability of J774.2 cells. Cells were treated with various concentrations of tigecycline for 25 hours with or without LPS treatment after which MTT substrate was added and formazan conversion was then measured. (A) Cells were treated with tigecycline (3μg/ml-50μg/ml) for 25 hours (B) cells were treated with various concentrations of tigecycline for 1 hour followed by the addition of LPS (1μg/ml) for 24 hours. Data expressed as average of 3 independent experiments +/- s.e.m. (n=18), statistical significance calculated by one-way ANOVA with a Tukey's post-test. There were no statistically significant differences.

cytotoxic over these concentrations. The highest levels of oxytetracycline and minocycline used actually increased the number of viable cells; this was observed in both cells treated with and without LPS and oxytetracycline and cells treated with LPS and minocycline.

Oxytetracycline increased the viability of cells treated with 50μg/ml of the drug and when the cells were treated with LPS then this increased viability was observed at both 50μg/ml and 30 μg/ml of oxytetracycline. Minocycline at a dose of 50μg/ml failed to increase the viability of cells that had not been treated with LPS but did increase the viability of cells treated with LPS. Concentrations of the drugs investigated here lower than 30μg/ml did not increase cellular viability, and cellular viability was unchanged in response to doxycycline or tigecycline.

Due to these findings the dose of 50μg/ml of any of the tetracyclines was not exceeded in further experiments.

3.2.5 The effect of tetracyclines on LPS induced nitric oxide production

NO• was measured by assessing the accumulation of nitrite in the cell culture media of cells treated with LPS for 24 hours with or without a one hour pre-treatment with either oxytetracycline, doxycycline, minocycline or tigecycline. Figures 3.6-3.9 show that no detectable NO• was produced by cells without stimulation with LPS, also upon treatment

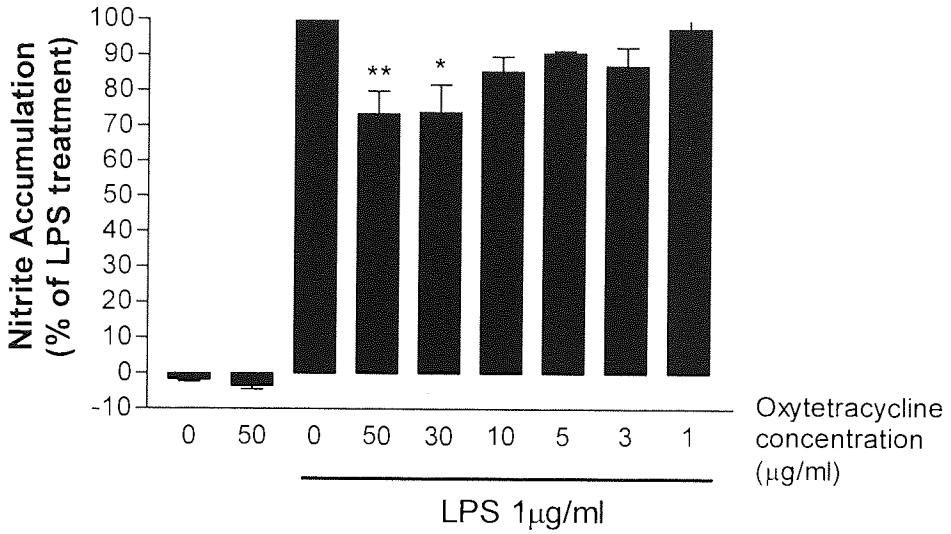


Figure 3.9 Effect of oxytetracycline on LPS induced nitric oxide production. Nitric oxide production was assessed by nitrite accumulation in cell culture media. Free nitrite levels were measured by their conversion by sulphanilamide and NED to a colourimetric azo compound. Unknown concentrations of nitrite were calculated using a standard curve constructed from known concentrations of sodium nitrite. Data expressed as mean of three independent experiments +/- the s.e.m. (n=3). Statistical significance calculated by one-way ANOVA with a Tukey's post-test (*=$p<0.05$, **=$p<0.01$).

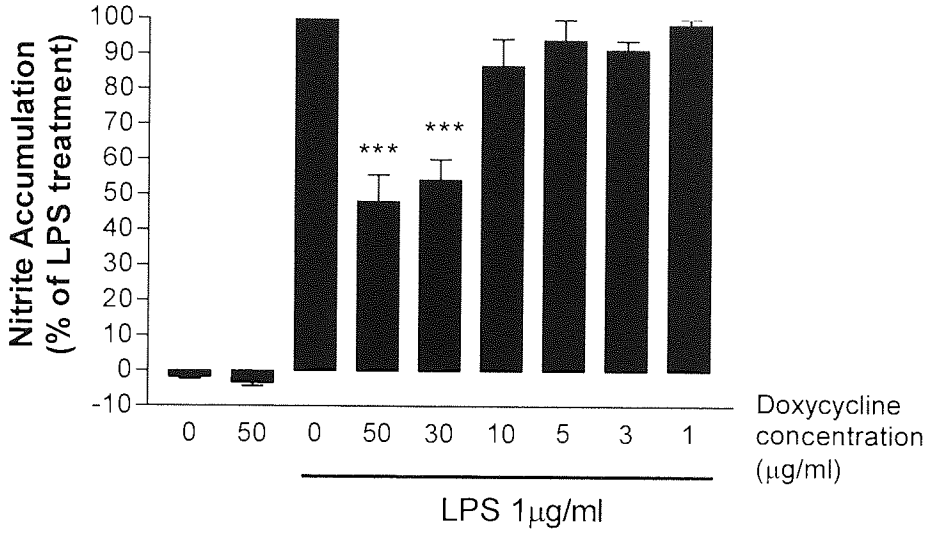


Figure 3.10 Effect of doxycycline on LPS induced nitric oxide production. Nitric oxide production was assessed by nitrite accumulation in cell culture media. Free nitrite levels were measured by their conversion by sulphanilamide and NED to a colourimetric azo compound. Unknown concentrations of nitrite were calculated using a standard curve constructed from known concentrations of sodium nitrite. Data expressed as mean of three independent experiments +/- the s.e.m. (n=3). Statistical significance calculated by one-way ANOVA with a Tukey's post-test (***=$p<0.001$).

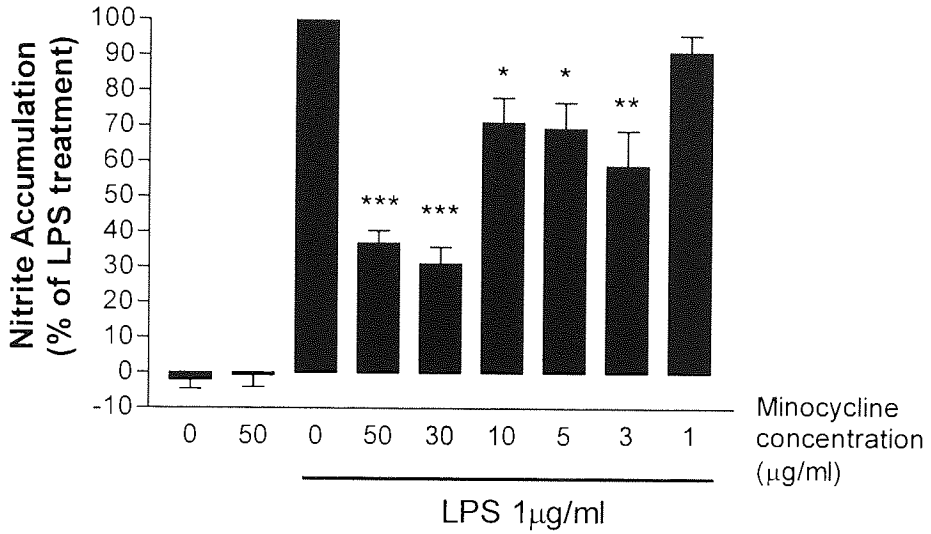


Figure 3.11 Effect of minocycline on LPS induced nitric oxide production. Nitric oxide production was assessed by nitrite accumulation in cell culture media. Free nitrite levels were measured by their conversion by sulphanilamide and NED to a colourimetric azo compound. Unknown concentrations of nitrite were calculated using a standard curve constructed from known concentrations of sodium nitrite. Data expressed as mean of three independent experiments +/- the s.e.m. (n=3). Statistical significance calculated by one-way ANOVA with a Tukey's post-test (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$).

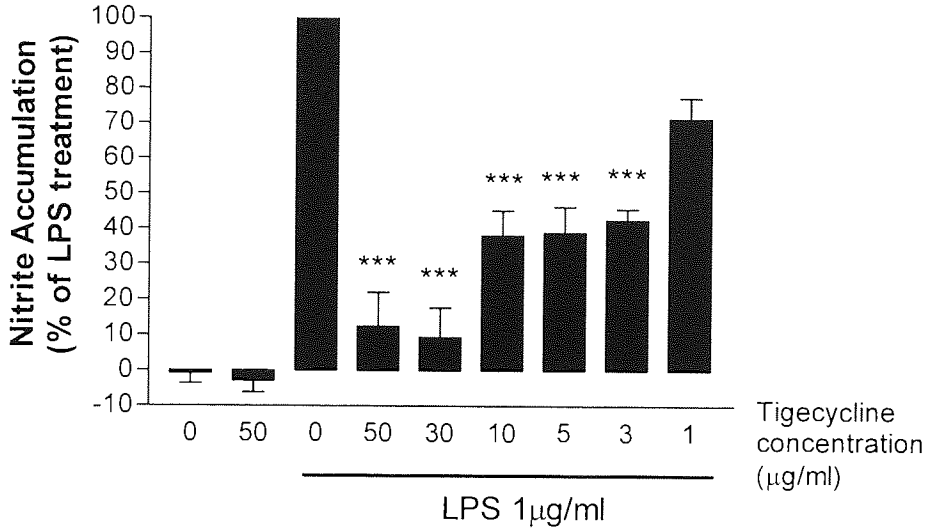


Figure 3.12 Effect of tigecycline on LPS induced nitric oxide production. Nitric oxide production was assessed by nitrite accumulation in cell culture media. Free nitrite levels were measured by their conversion by sulphanilamide and NED to a colourimetric azo compound. Unknown concentrations of nitrite were calculated using a standard curve constructed from known concentrations of sodium nitrite. Data expressed as mean of three independent experiments +/- the s.e.m. (n=3). Statistical significance calculated by one-way ANOVA with a Tukey's post-test (***=$p<0.001$).

Table 3.1. IC_{50} values of tetracyclines on LPS induced nitric oxide production. The concentration of tetracycline that caused a 50 percent inhibition of LPS-induced nitric oxide production was calculated.

1μg/ml LPS	
Tetracycline drug	IC_{50} (μg/ml)
Oxytetracycline	>50
Doxycycline	42.9
Minocycline	28.0
Tigecycline	10.0

with each of the tetracycline compounds cells NO• production was below detectable levels.

Each of the tetracycline drugs investigated here caused a dose dependent inhibition of LPS-induced NO• production. Figure 3.6 shows that oxytetracycline initially caused a statistically significant inhibition of LPS induced NO• production at a dose of 30μg/ml causing a decrease in the production of NO• of 26.03% inhibition. The maximal inhibition of nitric oxide was observed at the highest dose of oxytetracycline used here, 50μg/ml, and saw a maximal inhibition of LPS induced nitric oxide production of 26.55%. Oxytetracycline had an IC_{50} of >50μg/ml.

Doxycycline initially caused inhibition of LPS induced nitric oxide at a dose of 30μg/ml (Figure 3.7), this reduced the amount of nitric oxide produced by 45.75%. This inhibitory effect of doxycycline continued in a dose dependent manner and was found to be maximally inhibitory at a dose of 50μg/ml which inhibited LPS induced NO• production by 51.97% and had an IC50 of 42.9μg/ml.

NO• production was also reduced in a dose dependent manner by minocycline (figure 3.8). The inhibitory effect of minocycline was initially observed at a concentration of 3μg/ml (41.1% inhibition) and was maximal at a dose of 30μg/ml (68.91% inhibition), This level of inhibition was maintained at a dose of 50μg/ml (63.11% inhibition). Minocycline had an IC_{50} of 28.0μg/ml.

Tigecycline also reduced NO• production in a dose dependent manner. A dose of 3μg/ml caused an inhibition of 57.54%. This inhibition continued in a dose-dependent manner with a maximal inhibition at a tigecycline dose of 30μg/ml (90.58% inhibition). This maximal inhibition continued at a dose of 50μg/ml (87.47% inhibition). The IC_{50} for tigecycline was 10.0μg/ml.

3.2.6 The effect of tetracyclines on LPS induced iNOS expression

To further investigate the effect of tetracyclines on the production of LPS induced NO• the protein that is responsible for the production of NO• in macrophages; iNOS was analysed. The levels of iNOS protein were assessed using the same cells from the nitrite analysis experiments in oxytetracycline, doxycycline and minocycline treated cells. The basal level of iNOS expression was undetectable by western blot analysis, and each of the tetracycline treatments did not induce any expression of iNOS. LPS induced iNOS protein expression which was subsequently decreased upon pre-treatment of the cells with oxytetracycline (figure 3.13 (A)), doxycycline (figure 3.13 (B)) and minocycline (figure (C)) in a dose dependent manner.

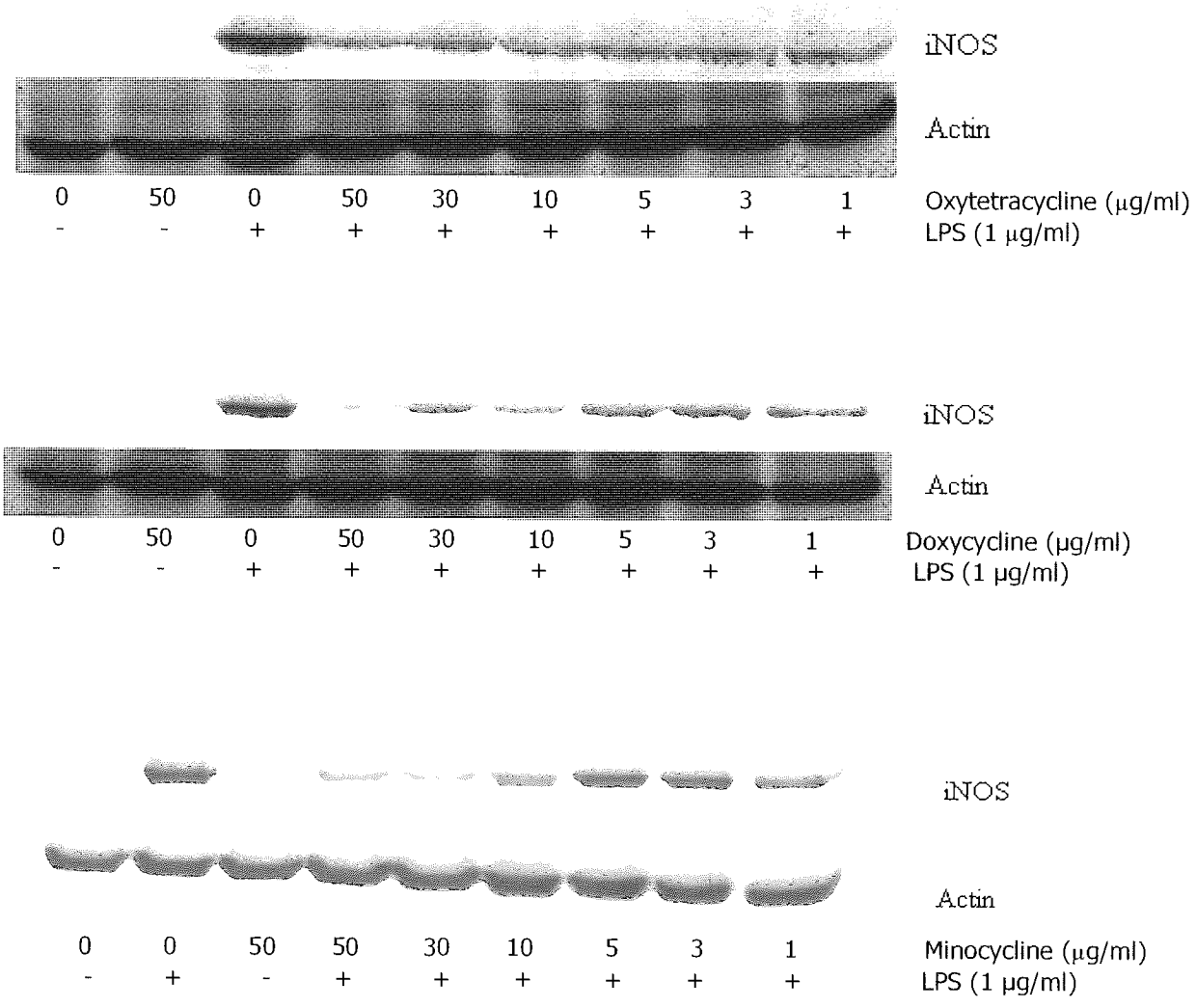


Figure 3.13 Effect of tetracyclines on LPS induced iNOS expression. iNOS protein expression was analysed at 24 hours after stimulation with LPS. The ability of different tetracycline derivatives to inhibit this expression was assessed by treating the cells with the relevant tetracycline one hour prior to LPS treatment. Each western blot is representative of three similar results.

3.3 Discussion

NO• is a very reactive compound produced by the body as part of normal physiological processes, NO• is produced to control vascular tone and also as a cytotoxic compound in the innate immune system. Mis-regulation of iNOS and aberrant production of NO• is associated with a range of pathologies including sepsis, rheumatoid arthritis and neurodegeneration. The ability to pharmacologically modulate the production of NO• would be a valuable clinical tool in inflammatory medicine.

The J774.2 cell line responded to LPS by producing NO• in a dose dependent manner (Figure 3.1(A)), this was associated with a dose dependent increase in iNOS protein expression (Figure 3.1(B)), these data correlated well. A significant level of nitrite was first observed at an LPS dose of 0.01μg/ml. This was not associated with an increase in the expression of iNOS, this may have been due to the level of iNOS expression being below the level of detection by western blot analysis, or it could have been due to the kinetics of iNOS expression. If the iNOS protein is expressed in response to 0.01μg/ml LPS its expression may have increased and then subsequently decreased within the 24 hour experimental period. This would have resulted in a small amount of NO• being produced (which then degraded to nitrite), following the 24 hour incubation period with LPS, iNOS may have reverted back to basal expression levels whereas the small amount of NO• produced during the period would have remained in the cell culture media in the form of nitrite.

The levels of nitrite accumulation increased with LPS doses of 0.03μg/ml to 1μg/ml which was associated with a dose dependent increase in the expression of the iNOS protein. Maximal expression of iNOS occurred at 3μg/ml LPS, however maximal cumulative levels of nitrite were at 1μg/ml LPS after which nitrite levels were constant. Although no higher doses of LPS were analysed by western blot analysis, nitrite accumulation analysis continued and also reached maximal levels at 10μg/ml LPS. Higher doses of LPS that were investigated (30μg/ml and 100μg/ml) showed lower cumulative levels of nitrite in the cell culture media. Although not investigated, this may be due to LPS induced cytotoxicity at high concentration resulting in fewer cells and therefore reduced maximal cumulative levels of nitrite present in the cell culture.

It was important to investigate the possible cytotoxic effects of tetracycline drugs on the J774.2 cell line to select a non-toxic range of doses for investigating the therapeutic effects of tetracyclines. Figure 3.2 to figure 3.5 show that none of the tetracyclines investigated reduced cell viability at doses up to 50μg/ml. Six doses ranging from 1μg/ml to 50μg/ml were selected for investigation into the effect of tetracyclines on the production of NO•.

The doses that are used in this study were doses that are pharmacologically achievable. There is variability between different tetracycline compounds regarding their bioavailability and peak serum concentrations and there is relatively little data on their volume of distribution it is therefore difficult to accurately estimate tissue and cellular concentrations that are achievable clinically. A number of pharmacokinetic studies have

been carried out on tetracycline and tetracycline derivatives and found that tetracyclines have a very high absorption from the gastrointestinal tract (>99%) and are absorbed in the stomach. A single orally administered dose of 200mg is absorbed from the stomach the peak plasma concentrations are achieved after approximately 1-2 hours and maximum concentrations in the plasma (C_{max}) is approximately 2μg/ml (Agwuh and MacGowan, 2006). It is possible to reach higher plasma concentrations in animal models, with a dose of 50mg/kg in mice a C_{max} of 5μg/ml (Hersch et al., 2003) was achieved with no increase in the occurrence of adverse drug reactions suggesting that higher doses would result in higher C_{max} in humans without associated adverse drug reactions.

The IC_{50} data obtained for each of the tetracycline derivatives here are greater than the expected C_{max} values. Although the C_{max} is relatively low, tissue accumulation is quite large in comparison (Agwuh and MacGowan, 2006); tissue concentrations are, in some cases, higher, tetracycline concentrations as high as 30μg/ml have been observed in lung tissue (Agwuh and MacGowan, 2006). The relatively low plasma concentrations of tetracyclines may due to the high affinity of serum albumin to bind minocycline and other tetracyclines, it is also possible that circulating blood cells actively accumulate tetracycline derivatives. Walters et al have demonstrated that neutrophils accumulate minocycline and doxycycline to 80 and 40 fold higher than that of the plasma concentrations respectively (Walters, 2006). Gingival fibroblasts have also been shown to actively accumulate tetracyclines (Walters et al., 2005).

An increase in cell viability was observed in response to 50μg/ml oxytetracycline (figure 3.2(A)), and 30μg/ml and 50μg/ml in cells treated with LPS (figure 3.2(B)). Minocycline treatment also resulted in an increase in viability in cells treated with LPS. The effect of tetracycline on the increase in viability in cells treated with LPS is at least in part due to the ability to inhibit the production of cytotoxic intermediates such as NO• as shown in figure 3.6 and figure 3.8. The role of oxytetracycline in the increased viability of cells that had not been treated with LPS may be due to the anti-apoptotic actions of tetracyclines. Both minocycline and doxycycline have been shown to inhibit caspase activation in response to a number of cellular insults such as LPS, MPTP and hypoxia-reoxygenation (Lai and Todd, 2006). The ability to inhibit apoptotic pathways such as the caspase pathway is a possible mechanism for an increase in viability, when no apoptotic stimuli such as LPS is applied to the cells it is still possible that oxytetracycline is inhibiting a basal turnover of cells.

Interestingly, this effect is only observed with oxytetracycline, which in the NO• inhibition analyses it had the highest IC_{50}. This suggests that the anti-apoptotic effects of tetracyclines and the iNOS inhibitory effects of tetracyclines may be due to different mechanisms.

The mechanism of action of tetracyclines in bacteria is to inhibit protein synthesis by disrupting the association between aminocylated-tRNA with mRNA at the ribosome, although there is no evidence to show that tetracyclines are able to associate with the eukaryotic ribosome to inhibit protein synthesis. Although this study does not present

direct evidence that tetracyclines do not bind to the murine ribosomal complex, figure 3.13 does show that tetracyclines inhibit the expression of iNOS but do not affect the expression of β-actin. This means that this inhibitory effect on the iNOS protein is not due to a decrease in total protein synthesis and that tetracyclines are able to specifically inhibit iNOS protein expression.

Figures 3.9-3.12 shows that all tetracycline compounds assessed here inhibited LPS induced NO• production; figure 3.13 shows that this is due to an inhibition of iNOS expression, the protein responsible for producing NO•. This suggests that this inhibitory effect is therefore due to inhibition at a transcriptional level and that tetracyclines have no effect on the activity of the iNOS protein.

All of the tetracycline compounds investigated here inhibited the production of NO• in a dose dependent manner; this suggests that the ability to inhibit NO• production is a common feature of all tetracycline compounds. The differences in efficacy and potency observed between the different compounds could by due to different affinities to the macromolecule or macromolecules that are responsible for this action. In terms of anti-microbial efficacy it has been shown that the tetracycline molecules do have different levels of affinity to the ribosomal subunits of bacteria resulting in altered therapeutic efficacy.

Similarly the differences in efficacy and potency could be due to the ability of the tetracycline molecules to enter the cells. Tetracycline cellular entry in unclear and could

vary between cell type, it may be due to active accumulation or to passive diffusion. If it is the case that tetracyclines are actively accumulated within mammalian macrophages then it is possible that this process would favour some tetracycline derivatives over others as observed with minocycline and doxycycline accumulation in neutrophils. Equally minor structural differences may effect the rate at which the drug is exported from the cell, this differs between different tetracycline drugs in bacteria. All tetracyclines investigated here exert an inhibitory effect on $NO^{\bullet}$ production although with different efficacies and potencies. However, it is not clear whether this difference in ability to inhibit $NO^{\bullet}$ production is due to the pharmacological activity of the different drugs or whether it is due to cell's ability to accumulate intracellular levels of the drugs.

Tigecycline had the lowest IC_{50} value and caused the largest amount of $NO^{\bullet}$ inhibition of the tetracycline compounds investigated here. However, due to there being relatively little published data regarding this relatively novel drug minocycline was chosen as the tetracycline compound to continue the investigations as it was not possible to complete proteomic studies on all of the tetracycline compounds.

Chapter 4 Proteomics

4.1 Introduction

Genomics, transcriptomics and proteomics are important tools in modern biology, used to employ non-hypothesis driven research. The human genome project offered a vast amount of data regarding human genes and was the first 'omics' technology to be investigated on such a large and comprehensive scale. The genomes of other organisms have also been sequenced and now offer a large amount of detail regarding the genetic material of many microbes, animals and humans. However, looking at genes is merely the origin of diversity between organisms, and although genetic polymorphisms can be identified at this level, genomics does not offer information regarding expression of these genes and it is difficult to derive information on the phenotypic consequences of genomic data. Transcriptomics is involved with the investigation of mRNA transcripts and offers data that in one sense is less complex after essentially removing non-coding regions of DNA, but adds another layer of complexity owing to gene splicing and the ability to produce many different mRNA transcripts from a single gene. Also, different cells and tissues have a different transcriptome although sharing an identical genome. Transcriptomics is therefore a function of which transcription factors, gene repressors and gene promoters are active at a particular time or in response to a particular stimulus.

Proteomics is the next step after transcriptomics. It also filters out some aspects of complexity such as transcripts that are subject to mRNA stabilization or destabilization, and non-translated mRNA transcripts such as microRNA. This results in only macromolecules that have an impact on cell function being identified.

Proteins are regulated at a post-translational level and are subject to modifications such as: phosphorylation, acetylation, glycosylation, nitrosylation, oxidation and ubiquitination. This is important in analysis of cellular functions as post-translational modifications (PTMs) can cause either activate or deactivate of functional proteins, can cause sub-cellular translocation or can even lead to degradation of proteins or protein complexes.

Historically proteomics is based on the separation of proteins using two-dimensional gel electrophoresis. The first step is to separate denatured proteins according to their pI value; the proteins are separated on a gel containing an immobilised pH gradient. Proteins migrate along the gel until they reach a net neutral zwitterionic state (their isoelectric point), which is a function of partial charges associated with each amino acid in the protein sequence. The same protein with different post-translation modifications will have a different pI value and therefore will separate from one another in the first dimension of two-dimensional separation. The second dimension of protein separation is SDS-PAGE. SDS molecules in the sample buffer coat each denatured protein in SDS, and the negatively charged SDS molecules are responsible for propagating

electrophoretic movement and therefore separate proteins according to their molecular weight.

Proteomics is a powerful tool in biological investigations allowing us to observe the entire protein profile of a section of tissue, a population of cells, or clinical samples such as blood plasma or urine. The use of proteomic techniques has grown dramatically in recent years due to its ability to identify new targets of disease that were not previously identified by knowledge-directed research. A large amount of proteomics studies are involved in biomarker discovery that can then be exploited as a diagnostic biomarker or a target for treatment.

Proteomics has two major advantages over traditional techniques. Firstly, it allows us to ask the question "what proteins are modulated by a particular treatment or stimulus?" rather than "is this particular protein modulated by a particular treatment or stimulus?". Secondly, due to us subjecting our protein sample to a two dimensional separation technique, information can be generated regarding post-translational modifications of proteins as well as just alterations in translational expression.

4.2 Results

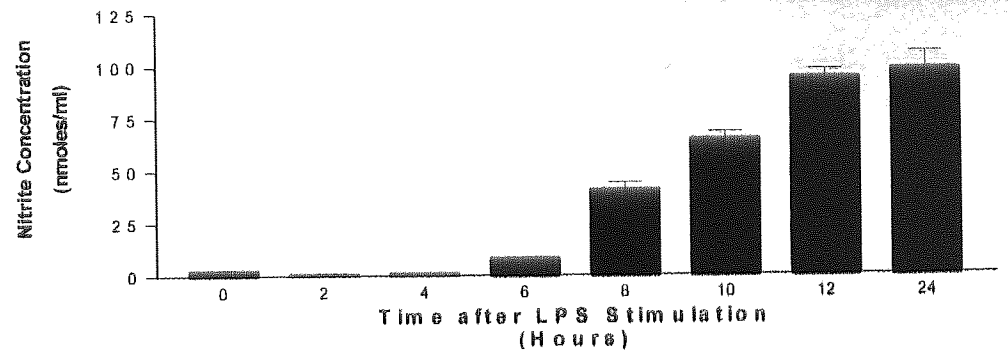
4.2.1 The Expression of iNOS over time in response to LPS

To select a timepoint to analyse the proteome of the J774.2 cell line the expression of a protein known to be expressed in response to LPS was investigated. The kinetics of iNOS, the expression of which has also been shown to be up regulated by LPS and subsequently inhibited by minocycline (Figure 3.10) was selected for this study.

Cells were plated into 6-well plates and left to attach overnight, after which the media was changed and the cells were left to equilibrate in fresh media for at least 2 hours. Cells were then stimulated with LPS (1μg/ml) and samples were collected as described in section 2.5.1 at the relevant time points and analysed for nitrite accumulation and iNOS expression.

Figure 4.1(A) shows the accumulation of nitrite in the cell culture media which was proportional to the levels of nitric oxide produced by the cells. Nitrite levels were significantly detected 6 hours after LPS stimulation, and after which the accumulation of nitrite increased over the 24 hour period that was assessed and was maximal at 24 hours. The expression of the enzyme responsible for the production of nitric oxide was also analysed and was shown in figure 4.1(B and C). Without stimulation with LPS, the iNOS protein is not detectable in the J774.2 cell line. Expression of iNOS was first detected 4 hours after LPS stimulation, and increased to a maximal level of expression

(A)



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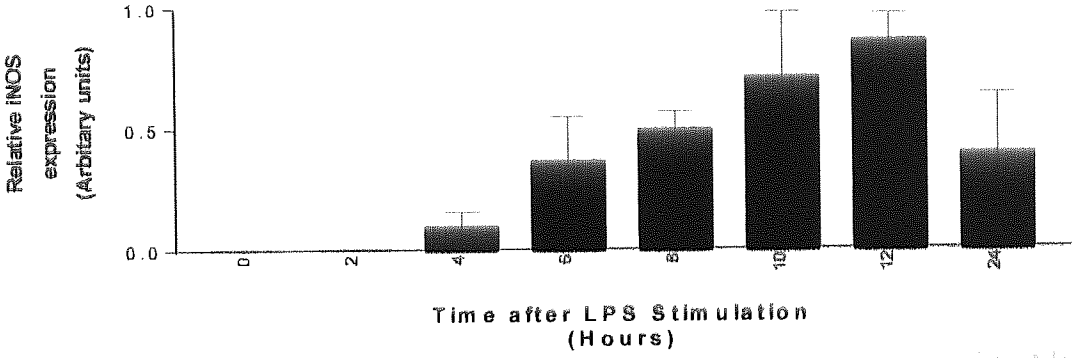


(C)

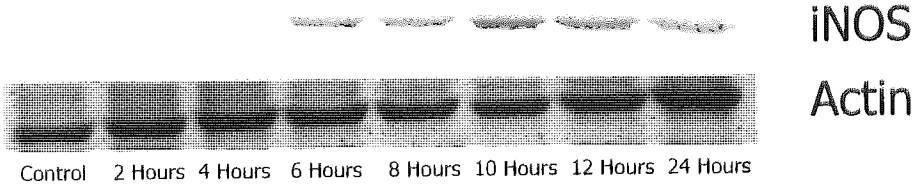


Figure 4.1. Expression of nitrite production and iNOS over a time. Relative expression of iNOS normalised to β-actin expression as assessed by western blot analysis. (A) Nitrite levels detected in the cell culture media following LPS stimulation as assessed by the Griess assay. (B) Densitometery of western blot data showing expression of iNOS relative to the expression of β-actin at different timepoints following stimulation with 1μg/ml LPS. (C) A representative western blot showing the expression of iNOS and corresponding β-actin expression.

at 10 hours; this level of expression was maintained at 12 hours and had decreased at 24 hours after LPS stimulation. It was therefore decided to analyse the proteome of the J774.2 cell line at 10 hours after stimulation with LPS as this was the earliest time point at which maximal expression of iNOS was accomplished.

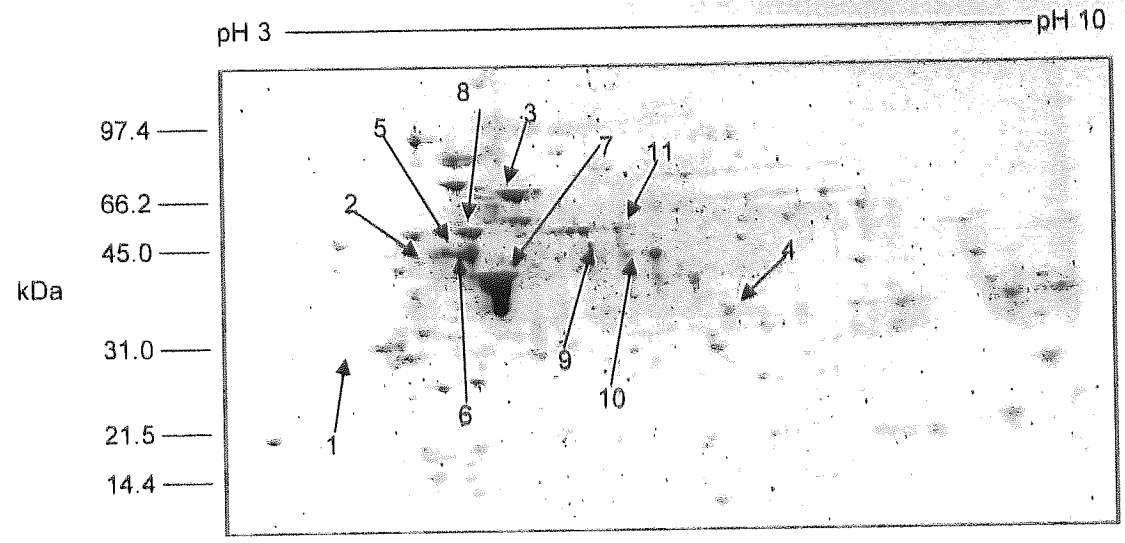
4.2.2 Effect of LPS on the J774.2 proteome

In order to investigate the effect of minocycline on the J774.2 LPS-stimulated proteome it was important to first identify proteomic changes that occurred as a result of LPS stimulation. Cells were incubated with or without LPS for 10 hours. Figure 4.2 shows two-dimensional gel separation of the un-stimulated J774.2 proteome (figure 4.2(A)) and the LPS-stimulated J774.2 proteome (figure 4.2(B)). Analysis of the J774.2 proteome identified 138 protein spots. Of the 138 protein spots identified the densities of 4 of these protein spots (spots 1-4; figure 4.2 and table 4.1) were increased by at least 2-fold in the LPS treated cells compared to the un-stimulated J774.2 proteome. LPS stimulation also resulted in 7 protein spots being reduced in density by 2-fold (spots 5-11; figure 4.2 and table 4.1).

4.2.3 Effect of minocycline on the LPS activated J774.2 proteome

The proteome of minocycline treated J774.2 cells is shown in figure 4.3. The effect of minocycline on the proteome of LPS-stimulated J774.2 cells was to increase the density of 7 protein spots (spots 5, 7-9 and 12-14; figure 4.3 and table 4.2) by at least 2-fold.

(A)



(B)

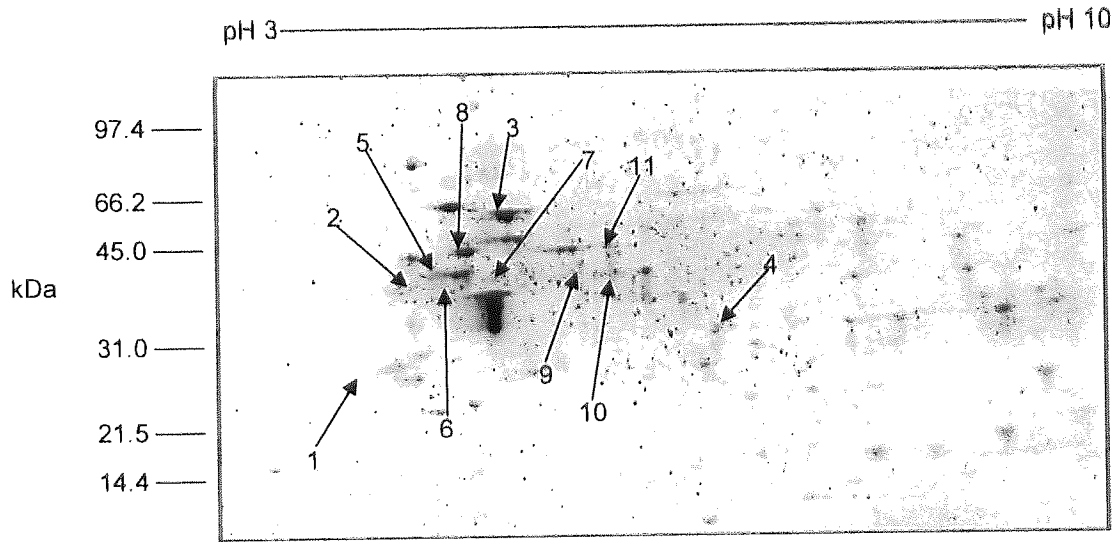
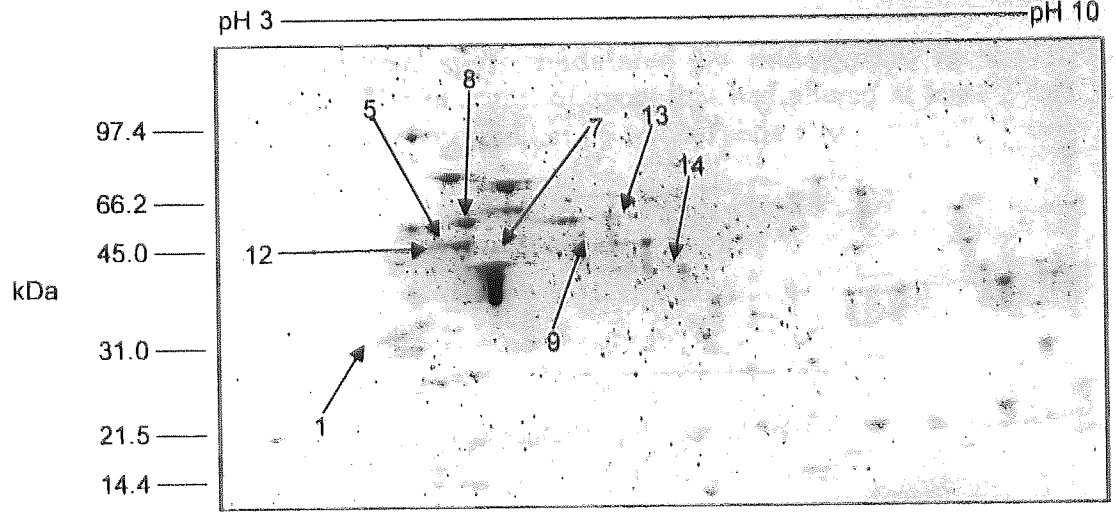


Figure 4.2 Effect of LPS on the J774.2 Proteome (A) Unstimulated cells, (B) LPS treated cells. Proteins were separated in two-dimensions and stained with a fluorescent total protein stain. Densities of protein spots were analysed by PDQuest analysis. Numbered spots are spots whose density was altered at least 2-fold in response to LPS stimulation compared to unstimulated cells.

Table 4.1 Densities of protein spots modulated by LPS. The densities of spots that had altered at least 2-fold in LPS treated cells compared to unstimulated cells.

Protein Spot	Density (compared to un-stimulated cells)
1	5.80
2	4.08
3	3.68
4	2.33
5	0.40
6	0.43
7	0.44
8	0.24
9	0.35
10	0.34
11	0.50

(A)



(B)

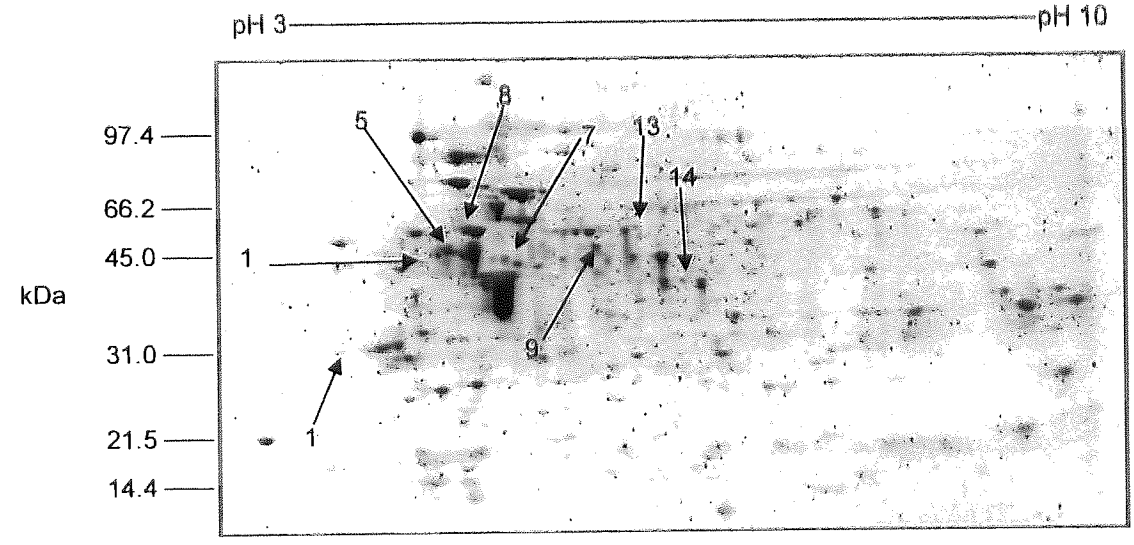


Figure 4.3 Effect of minocycline on the LPS activated J774.2 proteome (A) LPS treated cells, (B) LPS treated cells with a 1 hour minocycline pre-treatment. Proteins were separated in two-dimensions and stained with a fluorescent total protein stain. Densities of protein spots were analysed by PDQuest analysis. Numbered spots are spots whose density was altered at least 2-fold in response to LPS stimulation compared to unstimulated cells.

Table 4.2 Densities of protein spots modulated by minocycline in the LPS-activated J774.2 proteome. The densities of spots that had altered at least 2-fold in minocycline + LPS treated cells compared to LPS treated cells.

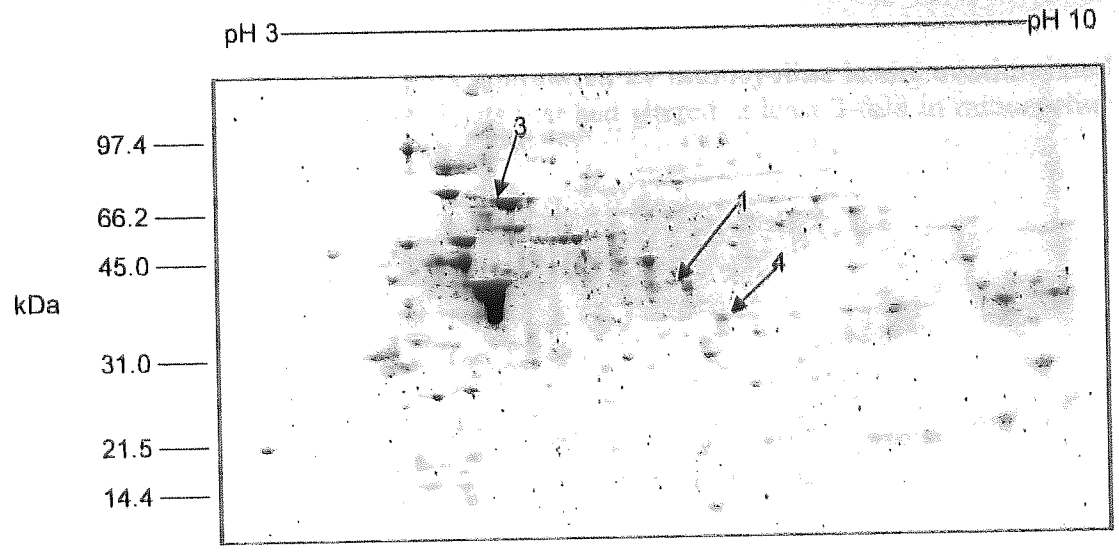
Protein Spot	Density (compared to LPS-stimulated cells)
5	2.30
7	5.22
8	2.90
9	2.48
12	2.12
13	Not expressed in LPS treatment
14	Not expressed in LPS treatment
1	0.25

This was accompanied by the reduction in density of 1 protein spot (spot 1;figure 4.3 and table 4.2) in cells treated with LPS with a 1 hour pre-treatment with minocycline compared to treatment with LPS alone. Interestingly, 5 of the protein spots identified as being modulated by minocycline + LPS compared to LPS stimulation alone were modulated by LPS treatment compared to unstimulated cells.

4.2.4 Effect of minocycline on the unstimulated J774.2 proteome

To fully investigate the effects of minocycline its effects up on the unstimulated J774.2 proteome were also investigated. Minocycline was found to modulate the expression of some proteins in the J774.2 macrophage cell line. Of the 138 spots identified, 3 protein spots (3, 4 and 14; figure 4.6 and table 4.3) were increased in response to minocycline alone. Of these protein spots identified as having been modulated by minocycline, 2 were increased by LPS treatment (3 and 4) and 1 was modulated by minocycline in the presence and the absence of LPS.

(A)



(B)

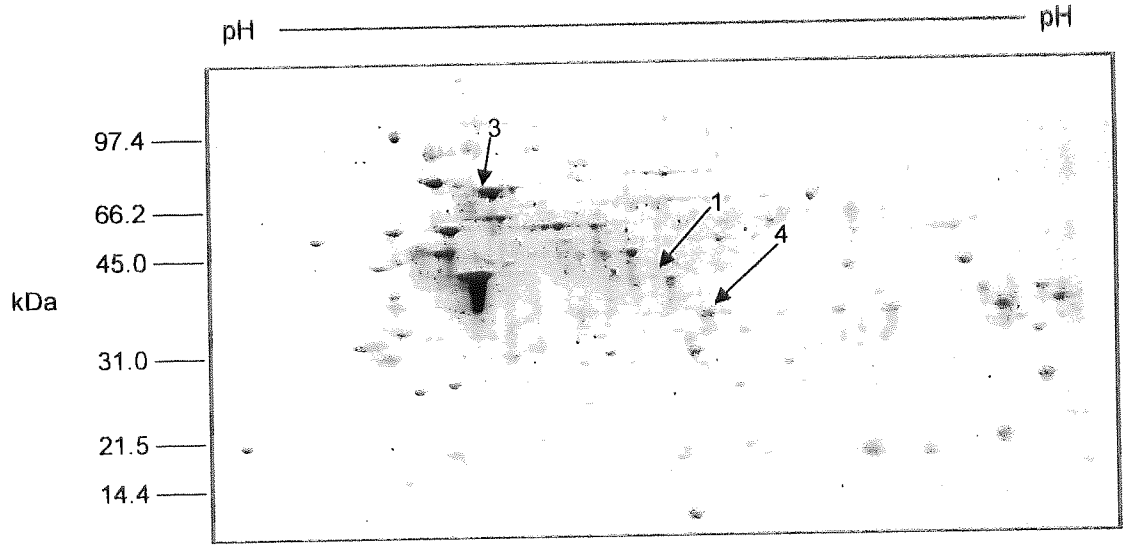


Figure 4.4 Effect of minocycline on the J774.2 proteome (A) untreated cells, (B) minocycline treated cells. Proteins were separated in two-dimensions and stained with a fluorescent total protein stain. Densities of protein spots were analysed by PDQuest analysis. Numbered spots are spots whose density was altered at least 2-fold in response to minocycline treatment compared to unstimulated cells.

Table 4.3 Densities of protein spots modulated by minocycline in the unstimulated J774.2 proteome. The densities of spots that had altered at least 2-fold in minocycline treated cells compared to unstimulated cells

Protein Spot	Density (compared to LPS-stimulated cells)
3	3.53
4	2.98
14	Not expressed in control

4.3 Discussion

In this study a proteomic approach was used to explore the effects of minocycline on LPS activated J774.2 murine macrophages. After investigating the kinetics of the expression of iNOS, a protein investigated in chapter 3, reported to be induced by LPS and also shown in this report to be inhibited by minocycline (Figure 3.10), 10 hours was selected as the optimal time after LPS stimulation for proteome analysis. This was selected as the point at which expression of the iNOS protein reached its maximal level following LPS stimulation.

It was shown here that LPS induced the production of nitric oxide via the measurement of nitrite levels in the cell culture media, and the expression of the iNOS protein in a time dependent manner. The iNOS protein was first detectable 4 hours after LPS stimulation and maximal expression occurred at 10 hours. Maximal expression was maintained at 12 hours, subsequently decreased and was approximately 50% of maximal expression 24 hours after LPS expression. The maximal expression of iNOS at 10 hours and the maximal nitrite levels found in cell culture media occurred at 24 hours. This is because western blot analysis of iNOS protein expression describes the amount of iNOS protein present at that particular time point whereas the analysis of nitrite measures the cumulative levels of nitrite in the cell culture media over a period of time from the initial stimulation and throughout the experimental procedure.

Flamingo total protein stain is more sensitive than coomassie blue or silver staining. Comparison between Flamingo total protein stain and the commonly used silver staining technique found flamingo to be 1000 times more sensitive. Not only does Flamingo have increased sensitivity for the detection of proteins but it also has increased specificity. Other staining techniques stain macromolecules other than proteins such as polysaccharides, nucleic acids and halide ions. This often causes streaking on the gel images leading to difficulty in analysis.

Proteomic analysis in this study identified 138 protein spots that were common to all of the gels. Of these 138 spots, 11 protein spots were modulated (at least 2-fold) in response to LPS (1μg/ml) stimulation. A number of proteins are known to be modulated by LPS stimulation, including proteins involved in a cellular response to a bacterial infection and other inflammatory pathways. Proteins involved in metabolism and respiration within the cell are also modulated by LPS to divert energy away from routine cellular tasks in order to initiate the immune response required.

It was important to investigate the effects of minocycline on unstimulated cells in order to determine whether the effects of minocycline were due to a direct effect upon the cells or were in response to the effects of LPS stimulation. Although no effect on cellular function in response to minocycline treatment alone in relation to nitric oxide production was observed, it is possible that minocycline exerted a different effect upon cell metabolism or function. It was found that three protein spots were modulated by minocycline alone compared to unstimulated cells. Interestingly, two of these spots

(protein spots 3 and 4) were increased with treatment of LPS, suggesting that minocycline activates some pathways that are involved in LPS stimulation without activating a complete immune response.

Of the four proteins found to be up-regulated in response to LPS, one (spot 1) is down-regulated by prior addition of minocycline. Similarly, of the seven proteins down-regulated by LPS, four (spots 5, 7, 8 and 9) were up-regulated on the prior addition of minocycline. This suggests that although minocycline restores some of the proteomic changes due to LPS it did not completely reverse the effects of LPS in the murine macrophage. Minocycline must therefore have specific effects on some but not all of the processes involved in LPS activation.

It is important to note that the alterations in the proteome of the J774.2 cell line were not necessarily due to transcriptional alterations. Two-dimensional protein separation as used here will separate the same proteins that have different post-translational modifications (PTMs), therefore an increase in a protein spot in this analysis may be due to an increase in transcription level of this protein, but could also but due to an increase in this particular PTM status of this protein.

Macrophage activation is a complex procedure, activated and regulated by multiple proteins in a complex, highly co-ordinated and highly regulated fashion. Proteins whose expression is increased in response to LPS and proteins whose expression is decreased by LPS may have different expression kinetics. The two-dimensional

separation technique will only identify proteins that are either up- or down- regulated or otherwise modulated within this time frame and will not identify proteins whose expression has been increased and decreased back to basal level (or vice versa) within the 10 hour period of stimulation. Whilst recognising this restriction, the 10 hour time point for proteomic analysis was selected since it gave maximum expression of the iNOS protein.

Using a proteomics approach, any bias from the results is avoided by not directing investigations in any particular way. By examining the entire complement of proteins expressed by the model cell system and observing any changes that occur as a result of treatment with LPS and minocycline independently of each other or in combination an objective assessment of the effects can be made.

Chapter 5 Protein Identification

5.1 Introduction

Techniques in protein identification that have been greatly developed in recent years have become commonplace in proteomics. It is of great importance to be able to accurately identify proteins quickly and in a high throughput manner. Developments in systems containing liquid chromatography coupled with mass spectrometry are used to accurately identify protein sequences. The use of capillary liquid chromatography coupled with nano-ESI linear ion trap mass spectrometry here allow the use of small amounts of protein sample and are therefore ideal for proteomics where the proteins are resolved on two-dimensional gels and subsequently excised. Tandem mass spectrometry, where two mass spectrometers are aligned, or linear ion trap technology, where data dependent exclusion of ions from the ion trap is possible, allow fragmentation of tryptic peptides and therefore amino-acid sequence data.

A number of genes and proteins are known to be regulated by LPS by a number of transcription factors activated via a number of different signaling pathways. Macrophage activation by LPS is a very complex sequence of events tightly regulated by a number of pathways. Proteins involved in energy metabolism and maintenance of protein structure and function have been shown to be modulated by LPS. LPS also activates proteins that are involved in both apoptotic pathways and cell survival pathways, cytokines and chemokines and pro-inflammatory proteins.

5.2 Results

5.2.1 BSA Identification

To assess the specificity and reliability of the data obtained from the linear ion trap, a sample of BSA was digested with trypsin (as described in section 2.7.2). BSA was dissolved in milliQ water and diluted by serial dilutions to a concentration of 2µg/ml. This sample was then subject to a tryptic digest and applied to the capillary liquid chromatography and nano-ESI linear ion trap mass spectrometry as described above. Data was analysed using BioWorks 3.0 software. Figures 5.1-5.5 show that BSA was identified and allocated a p value of $3.2x10^{-11}$, 9 peptides of BSA were identified accounting for 96 amino acids which is 15.44% by mass (15.82% by amino acids) of the complete protein.

5.2.2 Protein identification

Proteins that were identified by PDQuest analysis in chapter 4 as being proteins that has been significantly modulated either by LPS compared to untreated cells or by minocycline compared to either LPS treated cells or to untreated cells were subject to capillary liquid chromatography and nano-ESI linear ion trap mass spectrometry. Of the 14 protein spots of interest it was only possible to identify 10 of these figures 5.6-5.15, the proteins that were successfully identified are summarised in table 5.1.

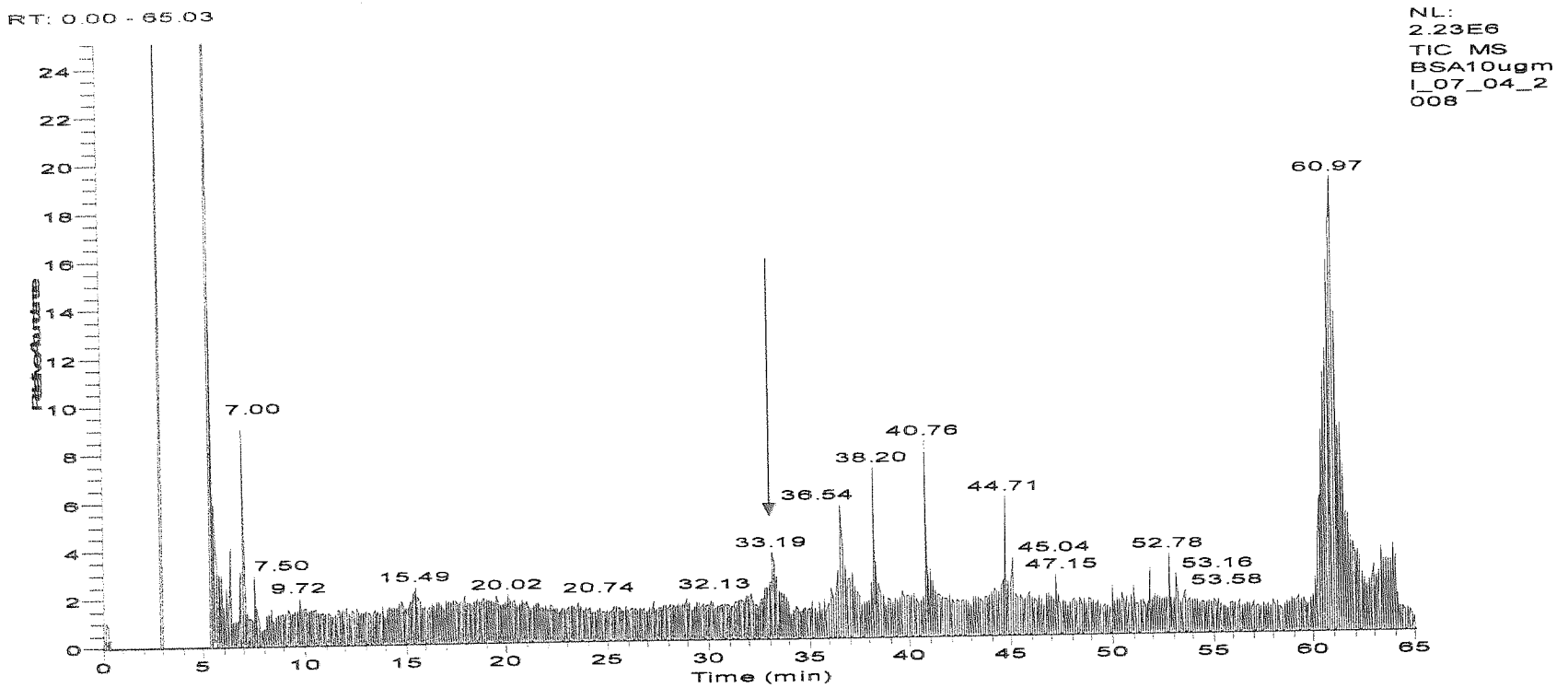


Figure 5.1 A representative liquid chromatogram of BSA. A Liquid chromatogram of a 10µg/ml solution of BSA subjected to proteolytic digestion with trypsin and capillary liquid chromatography. Peptides were loaded onto a C-18 peptide trap in the first 5 minutes, following this peptides were eluted from the peptide trap and separated by liquid chromatography with a gradient buffer. At 5 minutes the mobile phase consisted of 5% acetonitrile with 0.1% formic acid. At 10 minutes the mobile phase was altered and the content of acetonitrile was increased to 40% over 40 minutes in a linear fashion. At 50 minutes the acetonitrile content was increased to 60% to elute any remaining material from the column. At 60 minutes, the buffer composition returned to 5% acetonitrile to re-equilibrate the column for the next sample. The peak identified by a red arrow was analysed by nano-ESI linear ion trap mass spectrometry (figure 5.2).

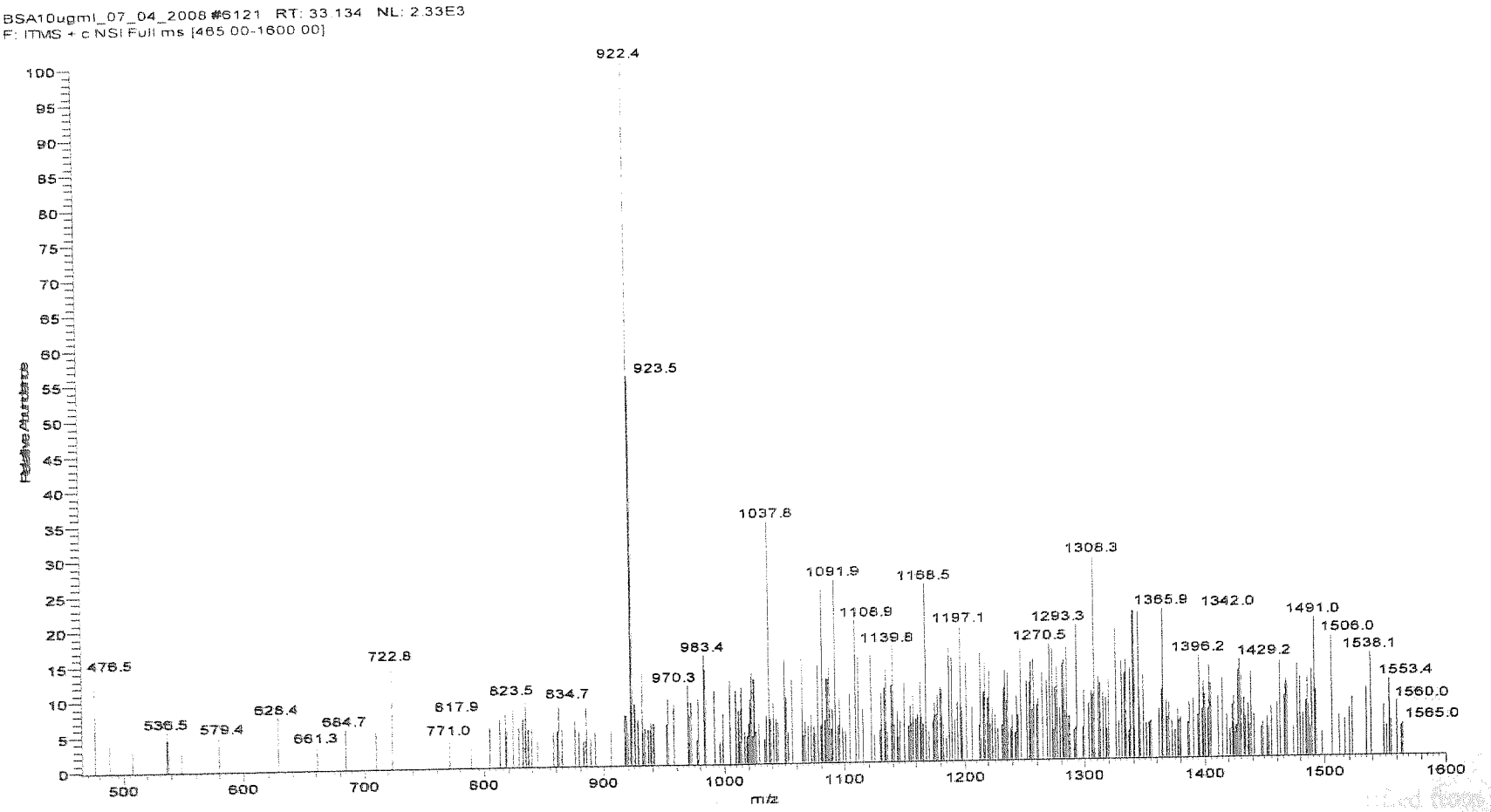


Figure 5.2 A representative full mass spectrum of BSA. A full mass spectrum of the peak with a retention time of 33.1 minutes. A full spectrum of all of the masses of compounds eluted from the liquid chromatography column at 33.1.

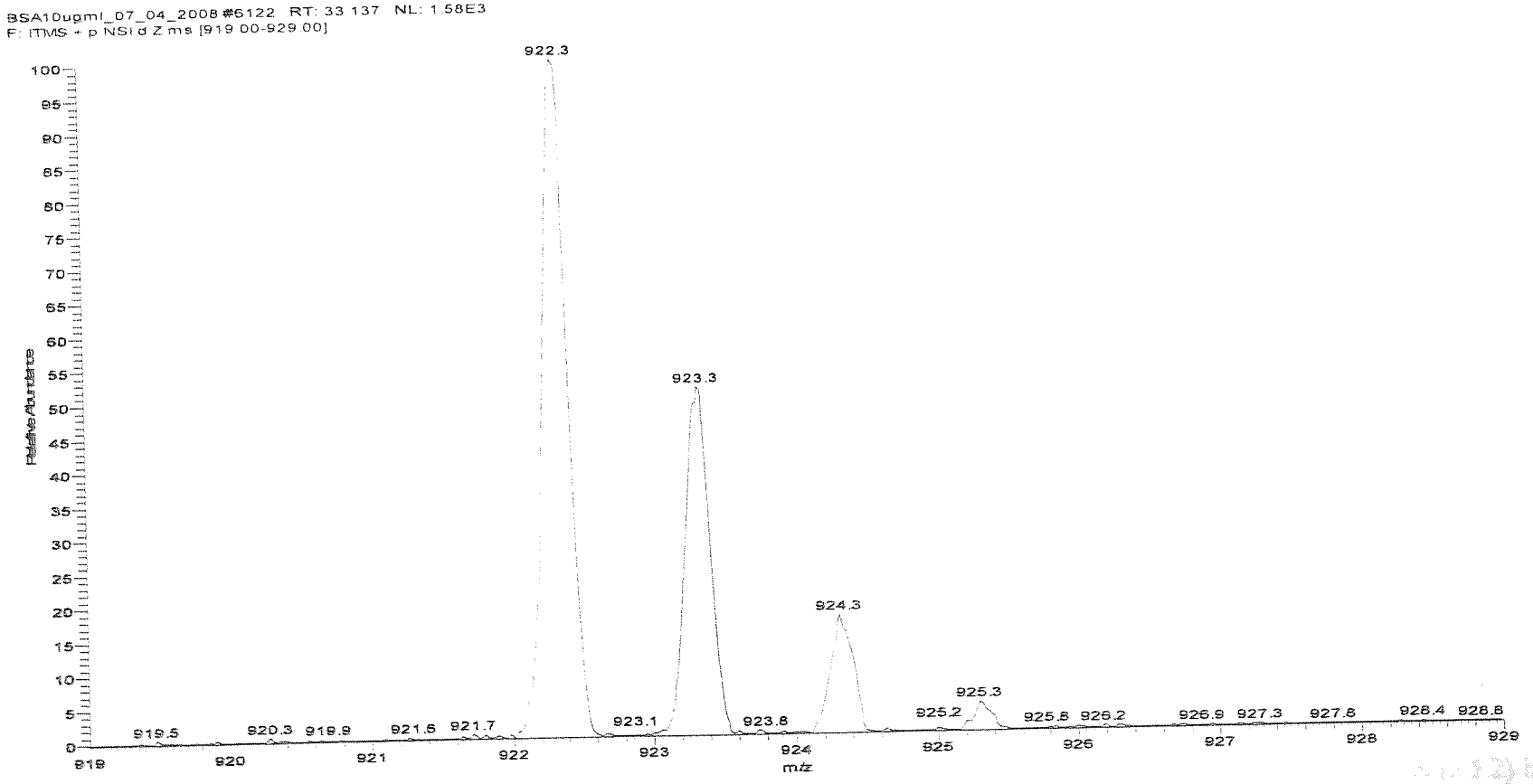


Figure 5.3 An MS zoom spectra of the precursor ion m/z=922.4 of a BSA peptide. A zoom scan of the largest peak identified from the Full MS scan (figure 5.2). This scan allows us to identify the charge of the peptide and therefore calculate its actual mass from m/z data. These peaks are formed from the peptides with varying amounts of carbon isotopes being present, the peaks are therefore 1Da apart. These peaks are at 922.3Da/z, 923.3Da/z, 924.3Da/z and 925.3Da/z and are each 1Da/z apart, therefore according to equation 2.1 the charge is +1. The actual mass of the peptide is therefore 922Da.

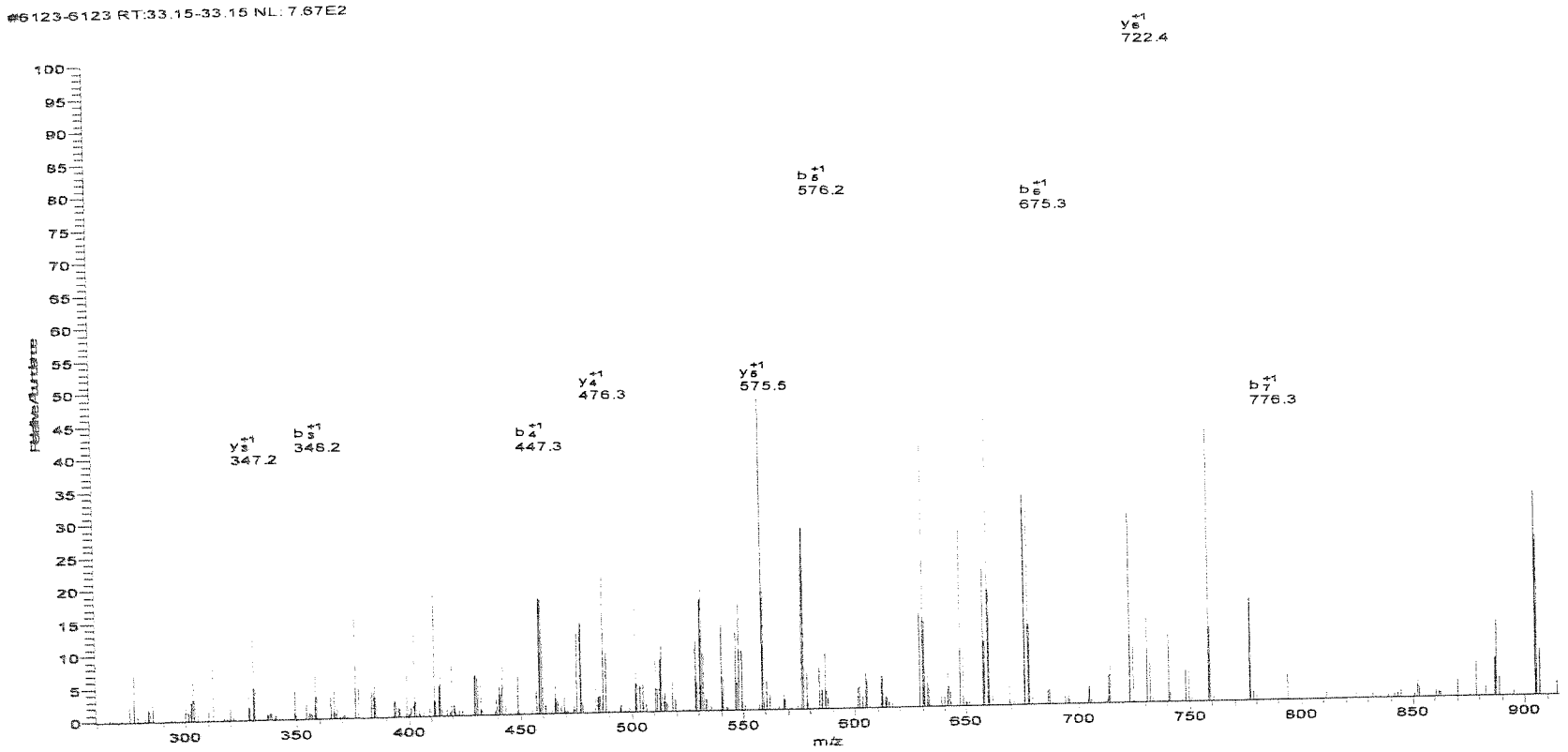


Figure 5.4 MS^2 spectra of the precursor ion m/z=922.4 of a BSA peptide. The largest peak from the Full MS scan (figure 5.2) is fragmented by normalised collision induced dissociation (CID). The peptide fragments at various positions along its chemical structure but most commonly at the amino bond between amino acids giving rise to b^+ and y^+ ion (as described in figure 2.6). The distance between successive b^+ ions and successive y^+ ions respectively represents the molecular weight of the peptides constituent amino acids.

(A)

Amino Acid Sequence				
A	b_1	72.04	-	
E	b_2	201.09	851.45	y_1
F	b_3	348.16	722.41	y_2
V	b_4	447.22	575.34	y_3
E	b_5	576.27	476.27	y_4
V	b_6	675.33	347.23	y_5
T	b_7	776.38	248.16	y_6
K		-	147.11	y_7

(B)

Sequence	MH+	% by Mass	Position	% by AA's
LVNELTEFAK	1163.63	1.68	66 - 75	1.65
YLYEIAR	927.49	1.34	161 - 167	1.15
AEFVEVTK	922.49	1.33	249 - 256	1.32
LVTDLTK	789.47	1.14	257 - 263	1.15
LGEYGFQNALIVR	1479.80	2.14	421 - 433	2.14
KVPQVSTPTLVEVSR	1639.94	2.37	437 - 451	2.47
VPQVSTPTLVEVSR	1511.84	2.18	438 - 451	2.31
ATEEQLKTVMENFVAFVDKCCAADDK	2905.34	4.19	562 - 587	4.28
LVVSTQTALA	1002.58	1.45	598 - 607	1.65

(C)

MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVKL
VNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYGMADCCEKQEPERNECFLSHKDD
SPDLPKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQECCQAED
KGACLLPKIETMREKVLTSSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLT
KVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLPPL
TADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHAC
YSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVG
TRCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKA
FDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEA
CFAVEGPKLVVSTQTALA

Figure 5.5 Peptides identified from BSA. (A) the masses of b^+ ion and y^+ ions identified in figure 5.4 is aligned, the mass difference between b^+ ions and y^+ ions gave sequence of amino acids is identified. (B) A table of all of the peptides that were identified from the capillary liquid chromatography and nano-ESI linear ion trap mass spectrometry of a sample of BSA proteolytically digested with trypsin. (C) The sequence of BSA with the peptides that had been identified highlighted in yellow.

Table 5.1 proteins identified by nano-ESI linear ion trap mass spectrometry. A summary of proteins that were identified by nano-ESI linear ion trap; the spot ID refers to the spot number in chapter 4.

Spot ID	Protein
1	Unidentified
2	Unidentified
3	Heat Shock Protein 70
4	Aldose Reductase
5	ATP Synthase β-chain
6	ATP Synthase β-chain
7	Unidentified
8	Vimentin
9	Probable phospholipid Transporting ATPase VA
10	α-Enolase
11	Heat Shock Protein 60
12	ATP Synthase β-chain
13	Unidentified
14	Olfactory Receptor Olfr1204

Spot ID	3
Protein	Heat Shock Protein 70
Accession number	IPI00323357.3
Molecular weight pI	70.8 5.24
p-value	$1.1x10^{-5}$
LC-MS derived peptide sequences.	TVTNAVVTVPAYFNDSQR DAGTIAGLNVLR ARFEELNADLFR FEELNADLFR LLQDFFNGK

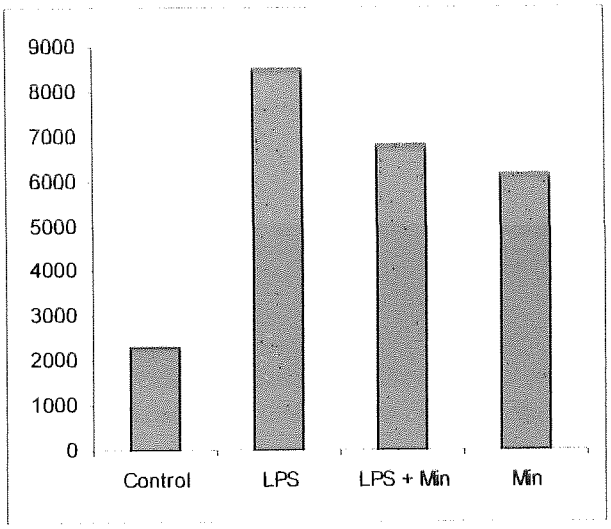


Table 5.2 Protein identification of Protein Spot 3. Data obtained from capillary liquid chromatography and nano ESI LIT mass spectrometry on spot 3. Graph shows densitometry of protein spots from different treatments.

Spot ID	4
Protein	Aldose Reductase
Accession number	IPI00223757.3
Molecular weight	35.5
pI	6.79
p-value	$1.8X10^{-8}$
Peptides Identified	TIGVSNFNPLQIER

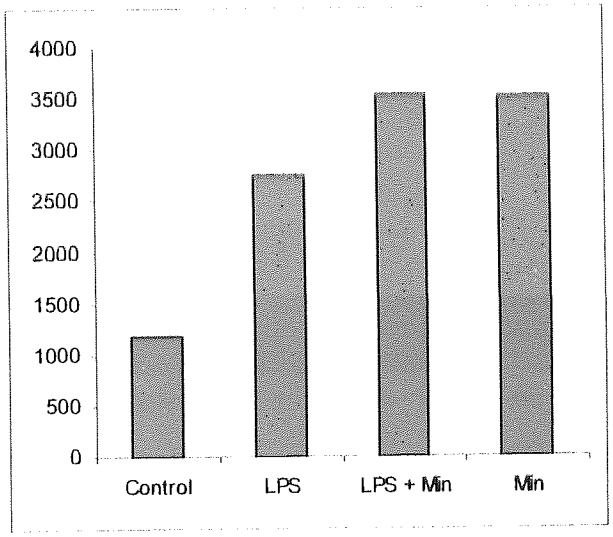


Table 5.3 Protein Indentification of protein spot 4. Data obtained from capillary liquid chromatography and nano ESI LIT mass spectrometry on spot 4. Graph shows densitometry of protein spots from different treatments.

Spot ID	5
Protein	ATP Synthase β-chain
Accession number	IPI00468481.2
Molecular weight pI	56.3 5.07
p-value	$1.7X10^{-6}$
Peptides Identified	FTQAGSEVSALLGR

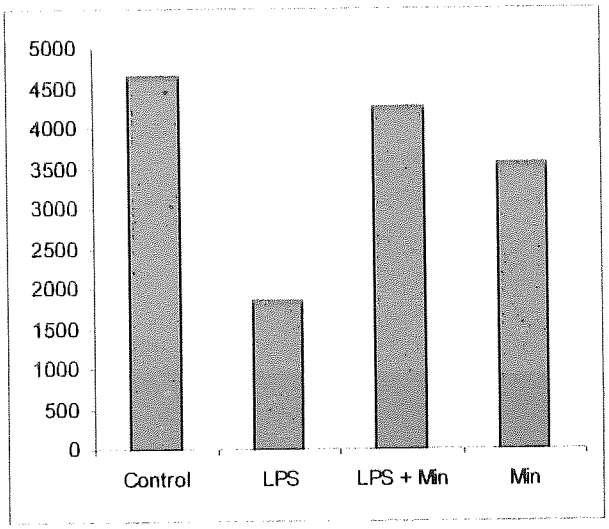


Table 5.3 Protein Identification of Protien Spot 5. Data obtained from capillary liquid chromatography and nano ESI LIT mass spectrometry on spot 5. Graph shows densitometry of protein spots from different treatments.

Spot ID	6
Protein	ATP Synthase β-chain
Accession number	IPI00468481.2
Molecular weight pI	56.3 5.07
p-value	$1.5X10^{-8}$
Peptides Identified	LVLEVAQHLGESTVR VLDSGAPIKIPVGPETLGR AIAELGIYPAVDPLDSTSR

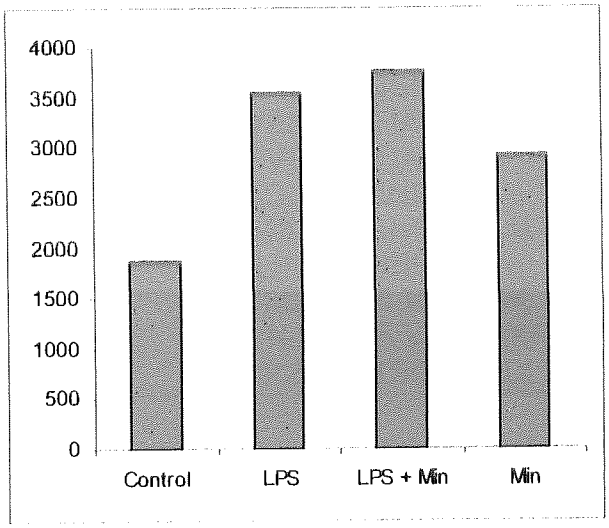


Table 5.4 Protein Identification of Protein Spot 6. Data obtained from capillary liquid chromatography and nano ESI LIT mass spectrometry on spot 6. Graph shows densitometry of protein spots from different treatments.

Spot ID	8
Protein	Vimentin
Accession number	IPI00227299.5
Molecular weight pI	53.5 4.91
p-value	$7.3X10^{-13}$
Peptides Identified	TYSLGSALRPSTSR SLYSSSPGGAYVTR LLQDSVDFSLADAINTEFK FANYIDK ILLAELELQLK ILLAELELQLKGQGK EEAESTLQSFRQDVDNASLAR KVESLQEEIAFLK KEVSLQEEIAFLKK FADLSEAANR FADLSEAANRNNDALR ISLPLPTFSSLNLR ETNLESLPLVDTHSK ETNLESLPLVDTHSKR DGQVINETSQHHDDLE

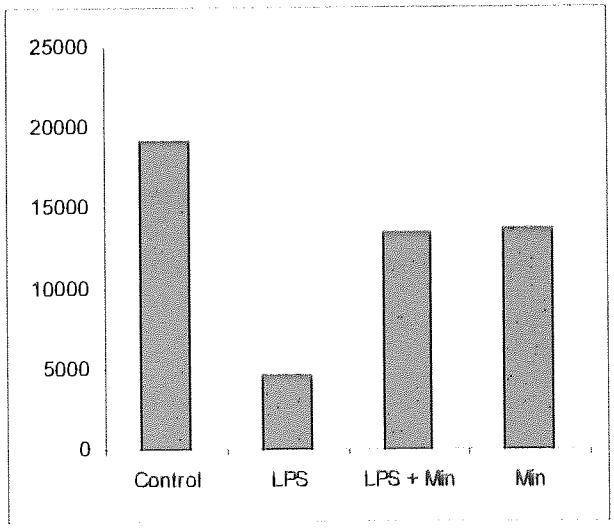


Table 5.6 Protein Identification of Protein Spot 8. Data obtained from capillary liquid chromatography and nano ESI LIT mass spectrometry on spot 8. Graph shows densitometry of protein spots from different treatments.

Spot ID	9
Protein	Phospholipid Transporting ATPase
Accession number	IPI00338618.5
Molecular weight	168.7
pI	6.22
p-value	$4.7X10^{-5}$
Peptides Identified	FRGYIMHSNGEKAGLHK

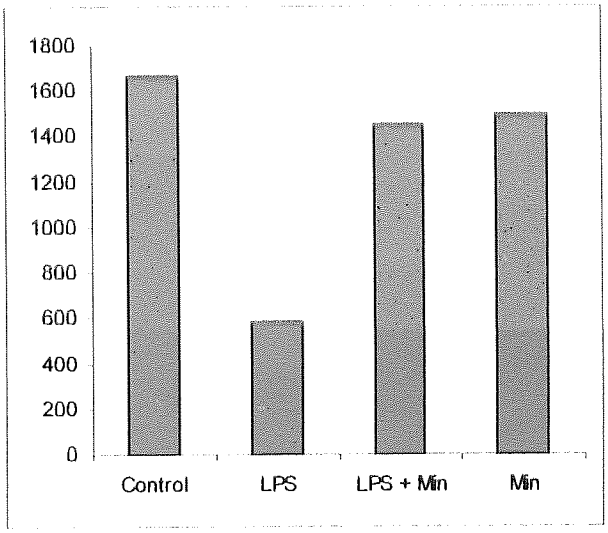


Table 5.7 Protein Identification of Protein Spot 9. Data obtained from capillary liquid chromatography and nano ESI LIT mass spectrometry on spot 9. Graph shows densitometry of protein spots from different treatments.

Spot ID	10
Protein	α-Enolase
Accession number	IPI00462072.2
Molecular weight pI	47.0 6.38
p-value	$1.9X10^{-5}$
Peptides Identified	YITPDQLADLYK

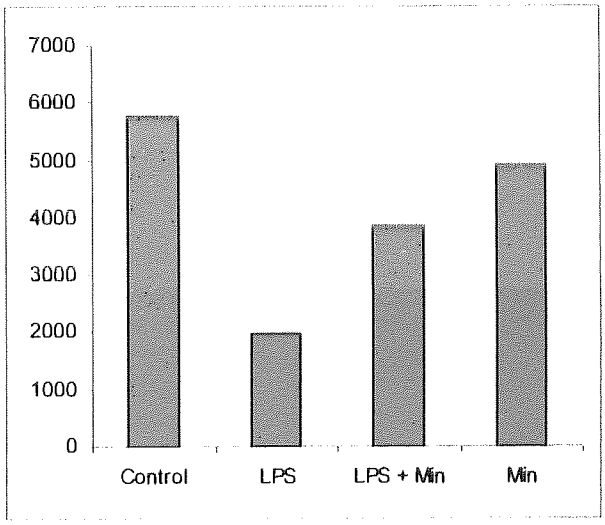


Table 5.8 Protein Identification of Protein Spot 10. Data obtained from capillary liquid chromatography and nano ESI LIT mass spectrometry on spot 10. Graph shows densitometry of protein spots from different treatments.

Spot ID	11
Protein	Heat Shock Protein 60
Accession number	IPI00308885.5
Molecular weight	60.9
pI	5.83
p-value	$7.9x10^{-10}$
Peptides Identified	LVQDVANNTNEEAGDGTTTATVLAR VGLQVVAVK LSDGVALK

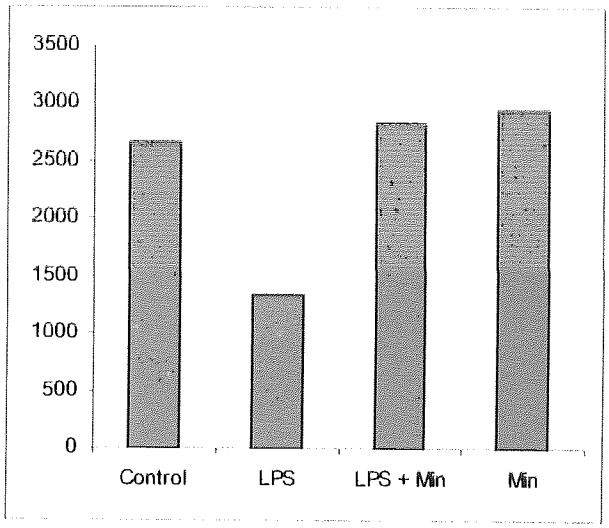


Table 5.9 Protein Identification of Protein Spot 11. Data obtained from capillary liquid chromatography and nano ESI LIT mass spectrometry on spot 11. Graph shows densitometry of protein spots from different treatments.

Spot ID	12
Protein	ATP Synthase β-chain
Accession number	IPI00468481.2
Molecular weight	56.3
pI	5.07
p-value	$2.3X10^{-8}$
Peptides Identified	LVLEVAQHLGESSTVR FTQAGSEVSALLGR LVPLKETIK

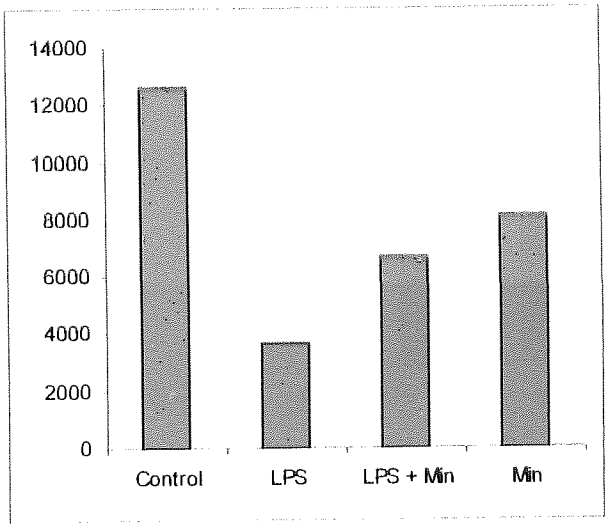


Table 5.10 Protein Identification of Protein Spot 12. Data obtained from capillary liquid chromatography and nano ESI LIT mass spectrometry on spot 12. Graph shows densitometry of protein spots from different treatments.

Spot ID	14
Protein	Olfactory Receptor Olfr1204
Accession number	IPI00353314.5
Molecular weight	34.7
pI	8.4
p-value	$3.8x10^{-4}$
Peptides Identified	PAATLPIDKAVALFYTMITPMLNPLIYTLR

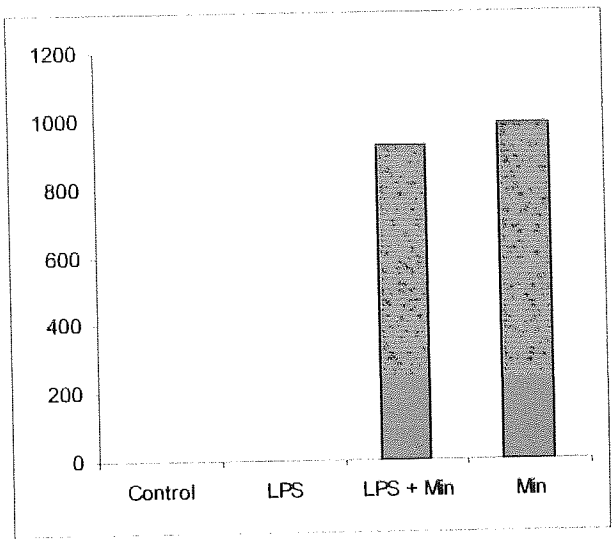


Table 5.11 Protien Identification of Protein Spot 14. Data obtained from capillary liquid chromatography and nano ESI LIT mass spectrometry on spot 14. Graph shows densitometry of protein spots from different treatments.

5.3 Discussion

During this proteomic analysis Flamingo stain, a fluorescent total protein stain, was used because it is more sensitive and more specific than other available techniques for identifying proteins. Although this allowed us to visualize to a greater degree the amount of modulation it led to the densitometry analysis being conducted on lower amounts of protein in the gel. This led to a large number of the protein spots excised from the gel being unidentifiable by the LC-nanoESI techniques employed here.

The timepoint at which the proteome of the J774.2 cell line was investigated was selected from analyzing the kinetics of LPS induced iNOS expression (figure 4.1). Although expression of iNOS was shown to be induced from an undetectable basal expression level it was not identified in the proteomic analysis. It is possible that although expression of iNOS was largely increased from the basal level it is still not abundant enough in terms of proportion of the total cellular proteome and therefore may be below the level of detection on a total protein stain.

Below, each of the proteins identified in this proteomic study are discussed in terms of their known function, mechanisms of regulation and their known or hypothesized potential role in LPS induce inflammation or in minocycline mediated inhibition of LPS induced inflammation.

5.3.1 Aldose Reductase

Aldose reductase (AR) is an enzyme involved the metabolism of glucose and the activity of AR has previously been associated with secondary complications with diabetes. AR is an enzyme involved in the catalysis of the polyol pathway in glucose metabolism.

The increased expression of AR in response to LPS stimulation could be associated with a role in protection. As well as metabolising components of the glucose pathway AR can also reduce 4-hydroxy-trans2-noneal, a toxic product of lipid peroxidation, and although the mechanism is unclear AR appears to reduce the level glucose induced oxidative stress (Rittner et al., 1999).

AR also has an impact on the production of NO• and inhibiting AR expression or activity caused a reduction in LPS induced NO• production and subsequent death (Kaiserova et al., 2008). However this was not observed here, and nitric oxide was produced despite AR also being expressed.

During an inflammatory response it is the job of macrophages to kill invading pathogens and remove toxic or potentially toxic intermediates. The increased expression of AR may be a protective mechanism of macrophages to survive the toxic intermediates they produce and release to kill pathogens. Inevitably the nitric oxide and superoxide radicals that macrophages produce are damaging to surrounding host tissue

and themselves, the expression of aldose reductase is at least in part able to detoxify products of lipid peroxidation.

The exact mechanisms underlying the regulation of aldose reductase are currently unclear but may involve the production of reactive oxygen species and PI3K and Akt pathways (Kang et al., 2007). LPS is known to activate the PI3K and Akt pathways and the production of reactive oxygen species and reactive nitrogen species is well established in LPS stimulation from production of superoxide by NADPH oxidase, extracellular superoxide is then dismutated with superoxide dismutase (SOD) into hydrogen peroxide as a result of dismutating the negatively charged superoxide radical in to the uncharged hydrogen peroxide molecule allows the passive diffusion across the cell membrane. Once inside the cell the unstable hydrogen peroxide molecule can react with intracellular metal ions such as iron (Fe^{2+}) and be converted into a hydroxyl radical and one hydroxyl ion via fenton chemistry and could therefore be the source of intracellular reactive oxygen species.

5.3.2 ATP Synthase β chain

ATP synthase is a protein composed of 16 subunits encoded by both nuclear and mitochondrial DNA and is involved in maintaining the energy requirements of the cell. The regulation of expression of the multi-subunit complex of ATP synthase is currently unclear and different subunits of the complex are regulated by different mechanisms,

the expression of some of the subunits are controlled at the level of transcription whereas other subunits are controlled at a post-transcriptional level.

The subunit identified here is the beta subunit produced from a mitochondrial precursor mRNA. The beta subunit is an important component of the ATP synthase protein and is responsible for binding cofactors involved in ATP synthesis. Expression of the beta subunit is thought to be regulated at a post-transcriptional level due to discrepancies observed between mRNA and protein levels. The beta subunit of ATP syntheses does contain an AU rich element (ARE) which can be involved in stabilizing mRNA transcripts thereby increasing protein levels without changing mRNA levels (Reyes and Izquierdo, 2007).

Three protein spots that were described as altering in response to LPS and/or minocycline were identified by nano-LC-ESI-MS as the beta subunit of ATP synthase. These spots that were all identified as the beta chain of ATP synthase were at similar molecular weights but had separated with different pI values, probably indicating that they have different post-translational modifications. A number of post translational modifications have been described including phosphorylation (Nakamoto et al., 2008) and nitrosylation (Sultana et al., 2007).

5.3.3 Heat Shock Proteins

Heat shock proteins (HSPs) are molecular chaperones in the cell and are involved in maintaining the structural integrity of proteins (Saibil, 2008). Molecular chaperones, of which HSPs are a major class, are found in various organelles: the cytosol, mitochondria and the endoplasmic reticulum. Molecular chaperones direct the folding and unfolding of proteins in cellular processes and are involved in sensing cellular stress from the environmental or pathological stimuli, upon sensing serious misfolding of proteins or protein aggregation molecular chaperones can initiate apoptosis.

The increased expression and in some cases cellular release of HSPs in response to LPS stimulation has been observed (Saba et al., 2007). HSPs act as cytokines and can function to immunologically activate neighbouring cells and cause apoptosis. HSP60 has been shown to act as a danger signal for the immune system and to initiate a strong proinflammatory response in the immune system of both humans and mice.

The up-regulation of both HSP60 and HSP70 in response to LPS has been well documented; however the two HSPs identified here show different expression kinetics from one another. HSP60 is down regulated in response to LPS whereas HSP70 is up-regulated in response to LPS. The difference in modulation of these proteins is interesting as they are regulated in a similar manner.

Investigation into the expression of HSP60 and HSP70 in LPS stimulated rat lung pericytes found that although after 18 hours exposure both HSP60 and HSP70 expression was increased, after 4 hours exposure to LPS expression of both HSP60 and HSP70 was decreased compared to basal expression levels (Edelman et al., 2007). This study observed a similar response from both HSP60 and HSP70, the expression of both was initially reduced (4 hours) and then increased (18 hours), and this is not the case in my study. Differences between the studies are numerous, a different species and model were used and analysis occurred at a different time point. It may be the case in our model that both HSP60 and HSP70 do both initially decrease in expression followed by an increase in expression however the kinetics of this pattern of expression may be different between the two HSPs and it may be the case that at 10 hours after LPS stimulation that HSP70 has decreased and then subsequently increased in expression whereas HSP60 has not yet seen the subsequent increase in expression. A suggested reason for the observed initial decrease in expression of HSP60 and HSP70 is that they are released from the cell upon a toxic or pathological stress, this occurs immediately resulting in an apparent decrease in HSP protein levels in the cell and it is not until a later time point that the cell has replenished and increased the cellular levels of HSPs.

Another possible reason for the differential modulation of HSPs in response to LPS is due to the fact that the production of $NO^{\bullet}$ can result in decreased expression of HSP60 in the rat brain following ischemia (Kim and Lee, 2007). It was shown that upon stimulation with LPS and the iNOS inhibitor, aminoguanidine, HSP60 expression was greater than that when stimulated with LPS alone. It is shown here that $NO^{\bullet}$ levels are

increased in response to LPS in this model, it is therefore possible that this increased level of iNOS and NO• results in a decrease in HSP60 expression. HSP70 has not been shown to be modulated by iNOS and NO• in this way.

Another point of interest is that minocycline attenuates the effects of LPS on HSP60 levels of expression but fails to revert expression of HSP70 back to basal levels. This further supports the case that HSP60 is modulated by NO• as when LPS induced NO• production is reduced by treating with minocycline HSP60 expression is not changed. In contrast, minocycline does not reduce the LPS induced increase in expression of HSP70 suggesting either that HSP70 is not subject to NO• induced modulation such as HSP60, or that the mechanisms of regulation of both of these HSPs are distinct from one another.

5.3.4 Vimentin

Vimentin is an intermediate filament protein and is associated with actin and tubulin and involved with movement of the cytoskeleton of the macrophage. Vimentin has a role in the differentiation of macrophages and in macrophage activation. Experiments using silencing mRNA techniques have shown that the absence of vimentin prevents the differentiation of macrophages (Benes et al., 2006).

Previous proteomics studies investigating proteins regulated by LPS have shown vimentin to up-regulated by LPS (Saba et al., 2007). This however is not what is

observed here. Vimentin is capable of being phosphorylated on sixteen different residues (Ando et al., 1989, Ando et al., 1991) to activate its function (Li et al., 2006). It is possible that the protein spot in this study corresponds to a functionally inactive form of vimentin which upon LPS stimulation is phosphorylated leading to a decrease in the amount of inactive vimentin present in the cells and a related increase in the functionally active form of the protein.

5.3.5 α-Enolase

α-Enolase is a multi-functional protein with glycolytic properties, which is involved in the removal of a water molecule from 2-phosphoglycerate to yield the high energy compound phosphoenol pyruvate. A reduction in α-enolase activity has been associated with neurodegenerative diseases. An PTM of enolase has been described in mild cognitive impairment (Sultana et al., 2007). Enolase is subject to S-nitrosylation mediated by iNOS and NO production in response to LPS. It has been shown that minocycline inhibits iNOS and NO production and therefore an increase in the non-S-nitrosylated form would be present.

Enolase is also a substrate for caspase-1 in septic shock patients (Shao et al., 2007). Enolase is cleaved upon activation of caspase-1 leading to reduced levels of α-enolase. Minocycline has been shown to inhibit caspase activation by LPS and other stimuli such as MPTP and, hypoxia-reoxygenation in macrophages and other tissues. The inhibition of LPS induced caspase activation by minocycline would lead to the increase

in α-enolase expression in cells treated with both minocycline and LPS compared to cells treated with LPS alone.

5.3.6 Olfactory Receptor

Olfactory receptors are the first step in the signal transduction pathway that leads to the perception of smell. The mouse genome consists of over 1400 olfactory receptors, making this one of the largest groups of receptors in the mammalian genome (Crasto et al., 2001). The olfactory receptors are G-protein coupled receptors (GPCR), with 7 transmembrane domains. Originally olfactory receptors were expressed exclusively in olfactory neurons, however other tissues have since been found to express receptors from this family of GPCRs including tissues involved in taste, erythroid cells, notochord tissue, prostate tissue and the testis (Vanderhaeghen et al., 1997).

With olfactory receptors being expressed in such a range of tissues it has become apparent that they have functions other than the propagation of the sense of smell. Research into the non-olfactory role of olfactory receptors has shown than they have an important role in chemotaxis is sperm (Fukuda et al., 2004). Although expression of olfactory receptors has not been described in macrophages their recognized role in chemotaxis is a likely function for a macrophage expressed olfactory receptor.

This protein is not expressed in untreated cells neither is it expressed in response to LPS stimulation. Its expression is only increased in the presence of minocycline, and this is the case whether LPS is present or not.

Chapter 6 General Discussion

Tetracyclines were originally discovered and clinically exploited in 1948; 60 years on, tetracyclines still play an important role in the control of infectious disease. Continuous development of the basic tetracycline structure has led to many tetracycline derivatives being identified and used therapeutically the latest of which is tigecycline which was licensed for clinical use in 2005 (Doan et al., 2006). A large amount of information is known about the anti-microbial actions of tetracyclines. The tetracycline compound associates with the 30S ribosomal subunit thereby disturbing the association between aminoacylated-tRNA and mRNA (Brodersen et al., 2000, Pioletti et al., 2001), resulting in the disruption of bacterial protein synthesis.

There is a large amount of research showing that a number of tetracycline derivatives possess therapeutic actions other than the well-defined bacteriostatic actions. These novel therapeutic actions include anti-metastatic (Saikali and Singh, 2003), anti-apoptotic (Castanares et al., 2005, Kelly et al., 2004) and anti-inflammatory actions(Amin et al., 1996, D'Agostino et al., 1998, D'Agostino et al., 2001). Although it has been shown by a number of groups that the use of tetracyclines in inflammatory pathology would be clinically beneficial, the underlying molecular mechanisms remain unclear.

Four clinically important tetracycline derivatives were selected for investigation in this thesis; oxytetracycline, doxycycline, minocycline and tigecycline. The ability of these

tetracycline derivatives to inhibit the production of nitric oxide in an LPS-stimulated macrophage-like cell line was assessed. It was also shown that each of the tetracycline compounds selected here inhibited the LPS induced production of nitric oxide in a dose dependent manner (figures 3.9-3.12) albeit with different efficacies (tigecycline > minocycline > doxycycline > oxytetracycline). Although the ability of oxytetracycine, doxycycline and minocycline to inhibit LPS induced nitric oxide production and iNOS protein expression have already been observed this is the first report to show that tigecycline is able to inhibit LPS induced NO• production and that tigecycline mediated inhibition of LPS induced NO• production is the most potent and most efficacious of all of the different tetracycline derivatives investigated here. Also presented here is data showing that this tetracycline mediated inhibition of nitric oxide production is as a result of the inhibition of LPS induced iNOS expression (figure 3.10).

To investigate the cellular responses to minocycline mediated inhibition of NO• production the non-hypothesis driven approach of two-dimensional separation of proteins and proteomic analysis was undertaken. A number of proteins (11) were found to be modulated by LPS. This is a similar number of proteins shown to be modulated by LPS from other proteomic reports. There is little consistency between this report and others regarding the identity of the proteins that have been modulated in response to LPS. This is probably due to a number of reasons, firstly the cell models vary between studies and although the cells are either monocyte or macrophage cell lines or primary cells the protein profile could vary substantially. Secondly, the implementation of two-dimensional protein separation protocols can vary between laboratories and the choice

of separation parameters are important such as the immobilized pH range over which the proteins are separated in the first dimension. Choosing a pH range which is too narrow will discriminate against proteins with either high or low pI values; however, selecting a pH range which is too large may lead to poor resolution of spots with similar pI and molecular weights. There is a similar effect with the second dimension separation where using a high percentage acrylamide gel, this leads to good separation of low molecular weight proteins and poor separation of high molecular weight proteins, again in an opposite fashion a gel with a low percentage of acrylamide will separate low molecular weight proteins poorly and high molecular weight proteins well. The separation parameters used here are broad to enable us to view as much of the proteome as possible. Here, a pH range of 3-10 was used in the first dimension and a gel with a gradient of acrylamide composition (4%-20%) was used allowing us to observe good separation of both high and low molecular weight proteins. Other proteomics studies with LPS have used acrylamide gels ranging from 10%-15% (Saba et al., 2007), and this therefore biases the data towards lower molecular weight proteins (~<70kDa).

Previous proteomic studies have reported data that at first appears to conflict with each other regarding the effect of LPS on the expression of ATP synthase β chain. Pabst et al (Pabst et al., 2008) reported a LPS induced decrease in the expression of ATP synthase β chain whereas Saba et al (Saba et al., 2007) described an LPS induced increase in ATP synthase β chain expression. This report describes three protein spots that were identified as ATP synthase β chain, one which increased and two which decreased in

expression. The three different spots that correspond to ATP synthase β chain have the same molecular weight but different pI values, suggesting that they have altered post-translational modification states, thereby explaining the apparently conflicting observations of Pabst et al. (2008) and Saba et al. (2007).

Proteins identified as being modulated by LPS in other published studies are related to cellular processes such as metabolism and the immune response. Saba et al. (2007) identified heat shock protein 70 and ATP synthase β chain as being up regulated by LPS from *Porphyromonas gingivalis*, this report also identified modulation of α-enolase, heat shock protein 60 and vimentin, however these latter observations were in the opposite trend to their modulation in this report. Reasons for this discrepancy between the different data are the different time points used (10 hours in this report, 24 in Saba et al), the different source of LPS (*E. coli* used in this report, *P. gingivalis* in Saba et al. (2007) and different cells used (J774.2 murine cell line used here, THP-1 human cell line used in Saba et al. (2007)). Also worth noting is the different modulation of heat shock protein 70 that was observed in response to LPS from *P. gingivalis* compared to live *P. gingivalis* cultures. In short, care should be taken when comparing proteomic data between studies and the limitation of the conclusions drawn from each study should be considered and analyzed in the context of other studies before deciding that data from one report conflicts with another. A proteomic study into the effects of LPS on a murine macrophage cell line identified a total of 11 proteins that had altered expression in response to LPS (Zhang et al., 2006), again there was no consistency between their study and the data presented here .

Some data here are different to other proteomic studies, for example here a decrease in α-enolase and vimentin was observed whereas previous studies have reported an increase in the level of expression of this protein (Pabst et al., 2008). I suspect that this is due to post-translational modifications and in other reports they have observed an increase in one post-translational modification state of the proteins whereas this report has identified a decrease in the basal post-translational modification state of the protein. To examine this further and to identify if this is truly the case the proteins could be separated by two-dimensional electrophoresis as in this report but instead of using a stain that will detect total protein, western blot analysis could be used to identify if any other protein spots correspond to this protein.

A number of gene array studies have been carried out on LPS stimulated macrophages, yet only the mRNA transcripts of heat shock protein 60 and heat shock protein 70 were identified of the proteins that were identified in this report. I have discussed previously that some of the observations in changes to the proteome may have been due to post-translational modifications and therefore may not have had an impact on the levels of the mRNA transcripts. Also gene array technology is often directed towards a cluster of genes that are known to be regulated by a particular transcription factor and therefore potentially missing an unexpected result. Gene array experiments have identified a lot of cytokines and proinflammatory proteins to be up- regulated in response to LPS (Lund et al., 2006, Guha and Mackman, 2001), often these cytokines are exported from the cells to act in an autocrine or paracrine fashion and for this reason are not detected

in the proteome of the cells although their transcriptional regulation is greatly enhanced. The levels of expression of proteins can also be modulated by mechanisms other than an increase or decrease in the transcription of the gene. The degradation of a protein can be enhanced or diminished thereby increasing or decreasing the protein levels respectively without altering the rate of transcription.

All tetracyclines investigated here exhibited inhibitory effects on LPS induced NO• production and iNOS protein expression, a number of other tetracycline derivatives not investigated here have also demonstrated these inhibitory effects on LPS-induced NO• production and iNOS expression. This ability to reduce the amount of NO•, which contributes to host tissue damage in inflammatory disease, is therefore most likely due to some part of the tetracycline chemical structure that is shared by all tetracyclines.

The ability of minocycline to bind divalent cations, in particular calcium, has been discussed previously and is known to be important in cellular accumulation of tetracyclines. The chelating properties of tetracyclines may be important for their anti-inflammatory actions. The inhibition of the catalytic properties of certain enzymes may be affected by the removal of free calcium via chelation by tetracyclines. This is the case with regard to matrix metalloproteinases which require divalent ions of calcium or zinc to function correctly. Tetracyclines not only decrease the expression of MMPs but also act to decrease their functional activity by limiting the amount of free calcium or zinc present. Tetracyclines have been shown to inhibit MMPs at the level of protein

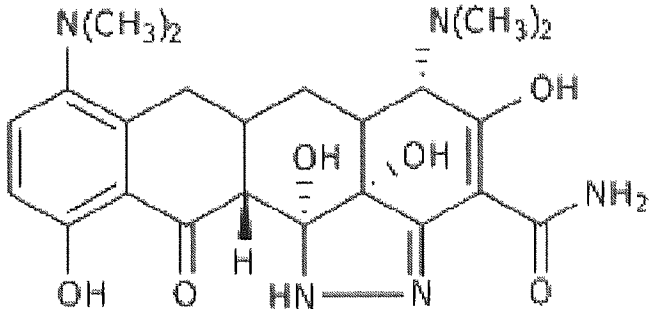
expression and mRNA expression, so although tetracyclines do have a direct effect on protein function they also reduce the amount of protein expressed.

Calcium is important in cellular signaling processes and is released by intracellular stores as well as entering via calcium channels from the extracellular space in response to certain stimuli. TLR activation by LPS is one of many stimuli that result in an increase in intracellular calcium levels. This increase in intracellular calcium results in the activation of proteins and transcription factors. Tetracyclines could be working to decrease the effects of an increase in intracellular calcium by chelating free calcium making it unable to activate cellular processes.

PMIN is a minocycline molecule that has been modified to include a hydroxypyrazoline group between carbons at position 12 and 1 (figure 6.1 (A)). This prevents the tetracycline compound from binding calcium or any other divalent cations (Bastos LF et al., 2008). This minocycline derivative although unable to bind calcium still exerted some clinical benefits in the form of pain relief and reduction of oedema in a mouse model (Bastos LF et al., 2008). No biochemical data were acquired in this study and only physiological outcomes were noted. This does however show that although the ability to chelate calcium may be important it is not the only mechanism of action of tetracyclines.

Another suggested mechanism of action is the anti-oxidant capacity of tetracyclines. It was shown through in vitro assays that minocycline has an anti-oxidant effect similar o.

(A)



(B)

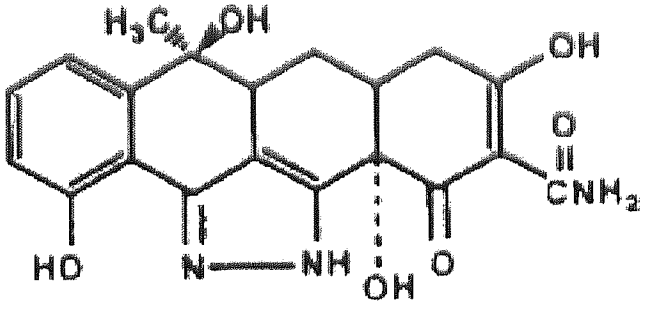


Figure 6.1. Chemical structures of pyrazolinominocycline and chemically modified tetracycline 5. (A) The chemical structure of 12S hydroxy-1,12-pyrazolinominocycline. (Adapted from (Bastos LF et al., 2008). (B) The chemical structure of chemically modified tetracycline (CMT)-5. (Adapted from (D'Agostino et al., 2003).

that of α-tocopherol (Kraus et al., 2005). Other tetracyclines also exerted an anti-oxidant effect. Tetracyclines donate an electron to the free radical species and in turn become free radicals themselves located on the D-ring of the basic tetracycline structure; the tetracycline free radical is chemically more stable than the original free radical species. The co-treatment of minocycline with an anti-oxidant such as α-tocopherol on LPS stimulated cells would allow us to observe which, if any, are minocycline mediated effects on LPS-stimulated cells that are due to the anti-oxidant capacity of minocycline tetracycline free radical is chemically more stable than the original free radical species. The co-treatment of minocycline with an anti-oxidant such as α-tocopherol on LPS stimulated cells would allow us to observe which, if any, are minocycline mediated effects on LPS-stimulated cells that are due to the anti-oxidant capacity of minocycline.

The effect of tetracyclines on the phosphorylation status of proteins should also be considered. New proteomic analysis presented in this thesis (Chapter 5) shows that LPS initiates post-translational modifications on ATP synthase β chain and that minocycline reverses some, but not all of these LPS induced changes in ATP synthase β chain modification. Minocycline also modulated the post-translational modifications of ATP synthase in cells that had not been stimulated with LPS. Protein phosphorylation is dependent on the balance between the action of protein kinases and protein phosphatases. An increase in phosphorylation could be due to the increase in expression or activation of a protein kinase or a decrease in expression or activation of a protein phosphatase.

The inhibition of MAPK has been suggested to be important in the anti-inflammatory actions of tetracyclines. P38 MAPK, JNK and ERK 1/2 have all been demonstrated to be inhibited by tetracyclines (Maitra et al., 2004). MAPK activation is an important aspect of the inflammatory process and is involved in the transcriptional regulation of pro-inflammatory cytokines and pro-inflammatory proteins including iNOS, MAPK activation leads to activation of AP-1 transcription factors. The proteins identified in the proteomic section of this study as being up-regulated by LPS have not been shown to be regulated by AP-1 transcription factors, however iNOS discussed in chapter 3 is regulated by AP-1 transcriptions factors.

Although the inhibition of MAPK by tetracyclines has been shown by a number of groups, the mechanism is not known and could be due to the inhibition of loci up-stream of the MAPK family of proteins. Tetracycline inhibition of MAPK has been shown in LPS activation as well as other inflammatory and non-inflammatory but toxic stimuli. This suggests that tetracyclines affect some part of the MAPK pathway but that their action is not specific to TLR activation of MAPK. MAPK activation is dependent on activation via phosphorylation which is mediated by MAPK kinase (MAPKK) proteins which in turn are activated via phosphorylation by MAPKK kinase (MAPKKK) proteins. Deactivation of MAPK proteins is mediated by MAPK phosphatase (MKP) proteins, just as a decrease in activity of MAPKK or MAPKKK would result in decreased activation of MAPK proteins so would an increase in MKP proteins. Therefore more work needs to be conducted regarding the mechanism by which tetracyclines inhibit MAPK activation.

The NF-κB pathway is important in inflammatory signaling. NF-κB is a protein complex which under basal conditions is located in the cytosol of the cell and is associated with an inhibitory factor (IκB). Upon inflammatory stimulation IκB is phosphorylated by IκB kinase (IKK) which subsequently leads to the degradation of IκB unmasking a nuclear localization sequence and causing nuclear accumulation of NF-κB. Investigations into the inhibitory effects of doxycycline on NF-κB in lung epithelial cells showed that doxycycline was unable to reduce cytomix (LPS, IFNγ, TNFα) induced NF-κB activity as assessed by a reporter gene assay (Hoyt et al., 2006). Interestingly data from microglial cells show that minocycline is able to inhibit the LPS induced phosphorylation and subsequent degradation of IκB (Nikodemova et al., 2006). NF-κB is activated further by post-translational modifications and interactions with other proteins; this may explain the disparate results.

NF-κB activation is dependent on IκB dissociation but nuclear translocation may not be enough to cause full NF-κB activation. NF-κB is phosphorylated when bound to the NF-κB response element and although this phosphorylation is not essential for NF-κB activation it does enhance the transcriptional abilities of NF-κB (Zhong et al., 1998, Vermeulen et al., 2003). The phosphorylation of the NF-κB molecule is mediated by MSK-1 (Vermeulen et al., 2003), which is activated by MAPK activated kinase 2 (MAPKAP2) which in turn is activated by p38 MAPK. Therefore the inhibition of p38 MAPK could result in decreased NF-κB activity without disrupting NF-κB nuclear translocation. If this tetracycline-mediated inhibition of NF-κB activity is occurring at the post-translational level and does not involve direct inhibition of NF-κB binding to NF-κB

response elements by tetracyclines then this could result in only partial inhibition of NF-κB resulting in the conflicting data that has been published regarding the effects of tetracyclines on NF-κB activation.

A number of possible mechanisms have been suggested above as an explanation of tetracycline-mediated anti-inflammatory actions. The range of effects exerted by tetracyclines suggests that the exact mechanism of action of tetracyclines is a combination of these suggestions. Tetracycline mediated chelation of calcium is an important function of the tetracycline molecule, it is involved in cellular accumulation and when removed as in the case of CMT-5 (Figure 6.1 (B))all anti-inflammatory actions appear to be lost. Although this aspect of tetracycline chemistry cannot explain all of the actions associated with the anti-inflammatory action of tetracyclines it is probably partly responsible. Similarly, tetracyclines acting as anti-oxidants could explain some of the anti-inflammatory properties of tetracyclines but would probably not cause the distinct transcriptional changes observed here. An effect on MAPK has been demonstrated by a number of groups and can explain a number of transcriptional changes observed in tetracycline treated cells suggesting that at some level tetracyclines are able to interfere with LPS-mediated kinase or phosphatase activity.

Studies in this thesis have described the effect that a range of tetracycline compounds have on the production of nitric oxide and the expression of inducible nitric oxide synthase. By means of non-hypothesis driven proteomics experimentation a number of LPS-modulated proteins and a number of minocycline-modulated proteins have been

identified. Although this provides more details of the molecular mechanisms involved in minocycline-mediated immunomodulatory actions, there are still some currently unanswered questions regarding the anti-inflammatory mechanism of action of tetracyclines. Further work in this area needs to be undertaken, including information regarding chemico-physical interactions, if any, between tetracyclines and cellular macromolecules, and also the effect of tetracyclines on inflammatory signaling pathways.

6.1 Future Work

Identifying cellular macromolecules that bind to tetracyclines would provide information on specific cellular compartments that tetracyclines need to penetrate and accumulate in order to efficiently exert their anti-inflammatory action. In order to investigate this it would be possible to construct a tetracycline-affinity column and pass a whole cell lysate through the column to retain a sub fraction of tetracycline binding molecules. Previous studies have used this technique to identify proteins that bind to their drug of interest. Godl et al (Godl et al., 2005) were interested in the interactions of the angiogenesis inhibitor SU6668; they attached the molecule to EAH sepharose 4B using carbodiimide chemistry. They separated the protein fraction that was retained by the SU6668 affinity column on a (16BAC)/SDS-PAGE and were able to identify 28 proteins that had an affinity to their drug of interest. A similar technique could be employed to identify any proteins that have an affinity for tetracycline compounds. One potential problem with this technique is that the conformation by which the molecule of interest (minocycline in our case) is anchored to the column, if this part of the molecule is important in any

tetracycline-protein interaction this result may be missed. The benefit of using minocycline in this technique would allow multiple residues of the compound to bind to the sepharose support.

Since it is unknown whether these interacting molecules are proteins or nucleic acids or other molecules the methods of detection used when analysing the subfraction of tetracycline binding molecules would need to differ dependent on whether the macromolecules of interest are proteins, nucleic acids or other macromolecules.

To investigate proteins that have an affinity for tetracyclines then a similar technique to the one used here could be used, whereby the protein sample could be resolved by either a 1- or a 2-D gel electrophoresis and then the gel stained in a fluorescent dye such as Flamingo total protein stain. The benefits of using fluorescent stains instead of more traditional protein staining techniques such as coomassie or silver staining procedures are two fold: specificity and sensitivity. Flamingo stain and other commercially available fluorescent total protein stains are very specific to proteins whereas commonly used silver staining procedures will detect other macromolecules including nucleic acids and polysaccharides. Sensitivity is also of concern as the protein of interest that is of most important may be expressed at a relatively low levels and therefore being able to detect low abundance proteins is essential. The ability to investigate macromolecules other than proteins in this context is also important and it would be worth investigating any parts of DNA or RNA that have affinity to tetracyclines. To exert their anti-bacterial action the tetracyclines bind to the 30s ribosome in bacteria, in fact the physical interactions of the

tetracycline molecule is not with any part of the ribosomal protein but in fact with the mRNA molecule in complex with the ribosome, it is possible that the mechanism of action of tetracyclines is to bind to mRNA and effecting mRNA stability. Another possible mechanism of action is via binding to microRNA. There is at present little information about the short single strands of RNA termed microRNA but they are gene products that are never intended to become proteins and are thought to be involved in the regulation of protein expression probably by regulating mRNA stability, and as there is also a lot of evidence of tetracyclines binding to RNA (Brodersen et al., 2000, Pioletti et al., 2001) then this is a very real possibility for explaining the action of tetracyclines.

Some work has been carried out into the signaling mechanisms involved and these studies have mainly looked at components of the MAPK family of proteins that are involved in the inflammatory process. Although it has been well established that activation of MAPK proteins (Maitra et al., 2004) is inhibited by tetracyclines the molecular mechanisms underlying this mechanism of inhibition is currently unclear and even the impact of down stream event of MAPK is unknown. A proteomic approach to this could be taken and similar protein separation techniques as to the ones used in this thesis could be used and analysed using a phosphor-specific fluorescent stain such as Diamond ProQ instead of the flamingo total protein stain here. Global alterations in the phosphoproteome could be analysed, indicating which parts of the MAPK pathway are affected and if any other inflammatory pathways are modulated by tetracyclines. Phosphorylation is a very transient and dynamic process and the selection of time points analysed would need to be carefully selected.

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RC DC Protein Assay

Instruction Manual

Catalog # 500-0119
500-0120
500-0121
500-0122

For Technical Service
Call Your Local Bio-Rad Office or
in the U.S. Call **1-800-4BIORAD**
(1-800-424-6723)

Section 1 Introduction

The *RC DC* Protein Assay is a colorimetric assay for protein quantitation with all the functionality of the original *DC* Protein Assay. This assay is based on the Lowry[1] assay but has been modified to be reducing agent compatible (*RC*) as well as detergent compatible (*DC*).

Section 2 Product Description

RC Reagents Package, includes

- *RC* Reagent I (250 ml)
- *RC* Reagent II (250 ml)

(Sufficient for 500 standard assays or 2,000 microfuge tube assays)

RC Reagent I contains UPPA-I
RC Reagent II contains UPPA-II
UPPA is a trademark of Geno Technology, Inc.

Section 3 Reagent Compatibility

The listed reagents were tested and found to be compatible with the *RC DC* Protein Assay. The presence of one or more of these substances may change the response of the protein to the assay reagents. Thus the protein standard should always be prepared in the same buffer as the protein sample.

Reagents	One Wash	Two Washes (Optional)
Dithiothreitol (DTT)	100 mM	350 mM
Tributylphosphine (TBP)	2 mM	-
β-mercaptoethanol	5%	10%
Sequential Extraction Buffer 2♦	Not Compatible	Full Strength
Sequential Extraction Buffer 3♦♦	Not Compatible	Full Strength
Laemmli Buffer (with 5% β-mercaptoethanol)	Full Strength	-
CHAPS	2%	-
Tween 20*	2%	-
Triton X-100**	2%	-
EDTA	100 mM	-
Imidazole	500 mM	-
Tris, pH 8.4	500 mM	-
NaOH	2.5 M	-

*Tween is a registered trademark of ICI Americas, Inc.
**Triton is a registered trademark of Rohm and Haas.
♦ 40 mM Tris, 8 M urea, 4% (w/v) CHAPS, 0.2% (w/v) Bio-Lyte 3/10 ampholyte, 2 mM TBP (Catalog #163-2103)
♦♦ 40 mM Tris, 5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) SB 3-10, 0.2% (w/v) Bio-Lyte 3/10 ampholyte, 2 mM TBP (Catalog #163-2104)

Section 4 Assay Instructions

Standard Assay Protocol (5 ml)

1. Add 20 µl of *DC* Reagent S to each 1 ml of *DC* Reagent A that will be needed for the run. This solution is referred to as Reagent A'. Each standard or sample assayed will require 510 µl of Reagent A'.
   (Reagent A' is stable for one week even though precipitate will form after one day. If precipitate forms, warm the solution and vortex. Do not pipet the undissolved precipitate as this will likely plug the tip of the pipet and alter the volume of Reagent A' added to the sample.)
2. Prepare 3-5 dilutions of a protein standard from 0.2 mg/ml to 1.5 mg/ml protein. A standard curve should be prepared each time the assay is performed.
   (For best results, the standards should always be prepared in the same buffer as the sample.)
3. Pipet 100 µl of standards and samples into clean, dry test tubes.
4. Add 500 µl *RC* Reagent I into each tube, vortex. Incubate the tubes for 1 minute at room temperature.
5. Add 500 µl *RC* Reagent II into each tube, vortex. Centrifuge the tubes at 15,000xg for 3-5 minutes.
6. Discard the supernatant by inverting the tubes on clean, absorbent tissue paper. Allow the liquid to drain completely from the tubes.
7. Add 510 µl Reagent A' to each tube, vortex. Incubate tubes at room temperature for 5 minutes, or until precipitate is completely dissolved. Vortex before proceeding to the next step.
8. Add 4 ml of *DC* Reagent B to each tube and vortex immediately. Incubate at room ternperature for 15 minutes.
9. After the 15 minutes incubation, absorbances can be read at 750 nm. The absorbances will be stable for at least 1 hour.

Microfuge Tube Assay Protocol (1.5 ml)

1. Add 5 µl of *DC* Reagent S to each 250 µl of *DC* Reagent A that will be needed for the run. This solution is referred to as Reagent A'. Each standard or sample assayed will require 127 µl of Reagent A'.
   (Reagent A' is stable for one week even though precipitate will form after one day. If precipitate forms, warm the solution and vortex. Do not pipet the undissolved precipitate as this will likely plug the tip of the pipet and alter the volume of Reagent A' added to the sample.)
2. Prepare 3-5 dilutions of a protein standard from 0.2 mg/ml to 1.5 mg/ml protein. A standard curve should be prepared each time the assay is preformed.
   (For best results, the standards should always be prepared in the same buffer as the sample.)

3 Pipet 25 µl of standards and samples into clean, dry microfuge tubes.

4 Add 125 µl *RC* Reagent I into each tube, vortex. Incubate the tubes for 1 minute at room temperature.

5 Add 125 µl *RC* Reagent II into each tube, vortex. Centrifuge the tubes at 15,000xg for 3-5 minutes.

6 Discard the supernatant by inverting the tubes on clean, absorbent tissue paper. Allow the liquid to drain completely from the tubes.

7 Add 127 µl Reagent A' to each microfuge tube, vortex. Incubate tubes at room temperature for 5 minutes, or until precipitate is completely dissolved. Vortex before proceeding to the next step.

8 Add 1 ml of *DC* Reagent B to each tube and vortex immediately. Incubate at room temperature for 15 minutes.

9 After the 15 minutes incubation, absorbances can be read at 750 nm. The absorbances will be stable for at least 1 hour.

Section 5 Storage

RC Reagent I and *RC* Reagent II should be stored at room temperature (20°C to 30°C) away from direct sunlight. Both reagents have a shelf life of 12 months. (Also see *DC* Protein Assay Instruction Manual for storage conditions for *DC* Protein Assay Reagent A, Reagent B and Reagent S.)

Section 6 Troubleshooting Guide

Questions	Recommendations
1 May I use a wavelength other than 750 nm?	Yes, absorbance can be measured at 650-750 nm.
2 What should I do if the protein pellet is still soft after centrifugation for 3 to 5 minutes?	Increase the centrifugation duration to 6-10 minutes. Protein pellet may take longer to dissolve after the addition of Reagent A'.
3 What can I do to minimize interference from supernatant carry-over?	**Option #1:** A second wash can be performed as follows: **Standard Assay** After step #6, repeat step #4 with 500 µl of Reagent I, repeat step #5 with 160 µl of Reagent II, repeat step #6 before going on to step #7. **Microfuge Tube Assay** After step #6, repeat step #4 with 125 µl of Reagent I, repeat step #5 with 40 µl of Reagent II, repeat step #6 before going on to step #7. **Option #2:** At step #6: to maximize supernatant removal, discard supernatant by aspiration before going on to step #7. **Option #3:** After step #6, dry tubes under vacuum to reduce residual supernatant before going on to step #7.

Note: Also see *DC* Protein Assay Instruction Manual for additional troubleshooting recommendations.

Section 7 References

1 *Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., "Protein Measurement with the Folin Phenol Reagent," Journal of Biological Chemistry, 193 (1951):265-275.*

Section 8 Order Information

Catalog #	Items	RC Reagent I	RC Reagent II	DC Reagent A	DC Reagent B	DC Reagent S	IgG Standard	BSA Standard
500-0121	RC DC Protein Assay Kit I, includes contents of RC DC Protein Assay Reagents Package and Bovine Gamma Globulin Standard	X	X	X	X	X	X	
500-0122	RC DC Protein Assay Kit II, includes contents of RC DC Protein Assay Reagents Package and Bovine Serum Albumin Standard	X	X	X	X	X		X
500-0120	RC DC Protein Assay Reagents Package, includes RC Reagent I (250 ml), RC Reagent II (250 ml), DC Reagent A (250 ml), DC Reagent B (2 L), and DC Reagent S (5 ml)	X	X	X	X	X		
500-0119	RC Reagents Package, includes RC Reagent I (250 ml) and RC Reagent II (250 ml)	X	X					
500-0117	RC Reagent I, 250 ml	X						
500-0118	RC Reagent II, 250 ml		X					
500-0111	Bio-Rad DC Protein Assay Kit I, includes contents of Bio-Rad DC Protein Assay Reagents Package and Bovine Gamma Globulin Standard			X	X	X	X	
500-0112	Bio-Rad DC Protein Assay Kit II, includes contents of Bio-Rad DC Protein Assay Reagents Package and Bovine Serum Albumin Standard			X	X	X		X
500-0116	Bio-Rad DC Protein Assay Reagents Package, includes Reagent A (250 ml), Reagent B (2 L) and Reagent S (5 ml)			X	X	X		

Related Materials

Catalog #	*Product Description*
500-0001	Bio-Rad Protein Assay Kit I, includes Dye Reagent Concentrate (450 ml) and Bovine Gamma Globulin Standard
500-0002	Bio-Rad Protein Assay Kit II, includes Dye Reagent Concentrate (450 ml) and Bovine Serum Albumin Standard
500-0006	Bio-Rad Protein Assay Dye Reagent Concentrate, 450 ml
500-0005	Protein Standard I, Bovine Gamma Globulin
500-0007	Protein Standard II, Bovine Serum Albumin
223-9950	Disposable Polystyrene Cuvettes, 3.5 ml, 100
223-9955	Disposable Polystyrene Cuvettes, 1.5 ml, 100

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