Analysis of Hsp90 in nematodes and its role in drug resistance

Nik Ahmad Irwan Izzauddin Nik Him
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Faculty of Veterinary Medicine
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DEDICATION

This thesis is dedicated to my parents, Nik Him Nik Mat and Nik Jah Nik Yaacob and my family members, who offered me unconditional love and support throughout the years I have been here. Thanks for everything.

ABSTRACT

The exposure of cells to environmental stresses, including heat shock, heavy metals, alcohols or oxidative stress results in the cellular accumulation of a number of chaperones, commonly known as heat shock proteins (Hsps). Hsps maintains the correct folding and conformation of proteins and are vital in regulating the stability between protein synthesis and degradation. Hsps are not only fundamental in the cellular stress response but are also important in regulating numerous important normal functions, such as cell proliferation and apoptosis. The results presented in the thesis focus on Hsp90 in nematodes, its possible roles in acquired drug resistance and further characterisation of *Brugia pahangi* Hsp90.

Hsp90 plays a key role in the cellular stress response by interacting with proteins after their native conformation has been altered by stress. Hsp90 is essential in all eukaryotes and plays a central role in multiple cellular processes. Since knock-out of *hsp90* is lethal to most eukaryotes, inhibitors of Hsp90 have been widely used to study its function. The most widely used inhibitor is geldanamycin (GA). GA binds to the N-terminal/ATP binding site of Hsp90 which results in conformational changes and the degradation of client proteins. Although the gene encoding *hsp90* has been characterized, little is known regarding its function in parasitic nematodes. Previous studies have shown that *Caenorhabditis elegans* Hsp90 is unique amongst eukaryotes because it fails to binds GA. This was suggested as an example of 'adaptive evolution' because *C. elegans* inhabits the same ecological niche of the soil as the *Streptomyces* species which produces GA. In contrast *B. pahangi* Hsp90 does bind to GA. The first objective of this project was to determine whether the resistance of *C. elegans* Hsp90 to GA is the norm or the exception amongst nematodes and to determine whether GA binding is associated with particular clades of nematodes. The results showed that the ability of Hsp90 to bind GA is associated with the life-cycle of the nematode. Free-living species or those that have a free-living larval stage in the soil do not bind GA, while those species which are obligate parasites (*Trichinella* and the filarial worms), or which are enclosed within a protective egg shell while in the environment (Ascarids), possess an Hsp90 that binds GA. These data support the adaptive evolution hypothesis.

Further characterisation of *B. pahangi* Hsp90 showed that it was expressed in all life-cycle stages but in the pull-down assay, only adult *B. pahangi* Hsp90 bound to GA, not Hsp90 from L3 or Mf. The inability to detect L3 or Mf Hsp90 binding to GA beads could perhaps reflect a lack of sensitivity of the assay, or alternatively may suggest that Hsp90 from these stages is processed in such way that it fails to bind to GA. In addition, only a small amount (6.3%) of *B. pahangi* Hsp90 bound to GA after a series of pull-down assays. Whether these data represents an accurate reflection of the amount of Hsp90 that can bind to GA is not known, but if correct, the results suggest that it might be necessary to inhibit only a small percentage of total Hsp90 to kill the worm.

Attempts were make to identify proteins that associate with Hsp90 by comparing the proteome of GA-treated adult *B. pahangi* with control worms. In this analysis, several proteins with structural functions were identified. These proteins were down-regulated after exposure to GA at 1.0 μM. Comparison of Hsp90 levels in *B. pahangi* and *C. elegans* showed that Hsp90 was expressed at relatively higher levels in *B. pahangi* ($p<0.05$) but there was no significant difference in the affinity of *B. pahangi* or *C. elegans* for Hsp90 binding to ATP beads. Results in Chapter 4 showed that the binding of Hsp90 to GA beads was competed by free GA or ATP. In addition, novobiocin, another Hsp90 inhibitor which binds at C-terminal domain of Hsp90 also inhibited binding of *B. pahangi* Hsp90 to GA beads, suggesting an interaction between the N-terminal and C-terminal of Hsp90.

Hsp90 has also been shown to be involved in the acquisition of drug resistance in fungi. A previous study on fungi showed that the emergence of fluconazole resistance depended on high levels of Hsp90 and was abolished when Hsp90 expression was reduced. So, given the conservation of Hsp90 it is possible that Hsp90 may be involved in the acquired resistance of nematodes to anthelmintic. The analysis carried out did not produce any correlation between expression levels of Hsp90 and drug resistance in nematodes. RNAi experiments were carried out to reduce Hsp90 levels in *C. elegans* and to investigate whether Hsp90 may play a role in the tolerance of ivermectin-resistant *C. elegans* to drug. Results showed that reducing Hps90 levels altered the sensitivity of ivermectin-resistant *C. elegans* to drug.

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DECLARATION

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged. No part of this work has been submitted for any other degree but has been reported in part in the following publication:

Gillan, V., Maitland, K., McCormack, G., Him, N.A., Devaney, E. (2009). Functional genomics of hsp-90 in parasitic and free-living nematodes. *International Journal for Parasitology* **39,** 1071-1081.

Him, N.A., Gillan, V., Emes, R.D., Maitland, K., Devaney, E. (2009). Hsp-90 and the biology of nematodes. *BMC Evolutionary Biology* **9,** 254.

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LIST OF ABBREVIATIONS

AAA	ATPase associated protein family
Aha1	activator of Hsp90 ATPase
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
Cdc37	cell division cycle 37 homologue
Cdk4	cyclin-dependent kinase 4
Cdk6	cyclin-dependent kinase 6
CGC	*C. elegans* Genetics Centre
CK-II	casein kinase II
CO_2	carbon dioxide
CR	charged region
ddH_2O	double distilled water
DDT	dichlorodiphenyltrichloroethane
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
ErbB2	erythroblastic leukemia viral oncogene homolog 2
Erg11	lanosterol 14-alpha-demethylase
ES	excreted/secreted
ESI-MS	ionisation/multi-stage mass spectrometry
FCS	fetal calf serum
g	gram
GA	Geldanamycin
GHKL	gyrase-Hsp90-histidine kinase-MutL family

GluCl	glutamate-gated chloride channel
Grp94	94 kDa glucose-regulated protein
h	hour
HBSS	Hanks balanced salt solution
Hch1	high copy Hsp90 suppressor
HCl	hydrochloric acid
HIP	Hsp70-interacting protein
HOP	Hsp70/Hsp90-organising protein
HSE	heat shock element
HSF	heat shock factor
Hsp	heat shock protein
Hsp100	heat shock protein 100
Hsp90	heat shock protein 90
Hsp70	heat shock protein 70
Hsp60	heat shock protein 60
Hsp40	heat shock protein 40
Hsp16.2	heat shock protein 16.2
Hsp10	heat shock protein 10
HTPG	high temperature protein G
IDV	integrated density value
IF	isoelectric focusing
IgG	immunoglobulin G
IPG	immobilised pH gradient
IPTG	isopropyl ß-D-1-thiogalactopyranoside
J2	second stage juvenile
kDa	kilodalton
KH_2PO_4	potassium dihydrogen phosphate

L1, L2, L3, L4	first, second, third and fourth stage larvae
LC	liquid chromatography-electrospray
M	molar
Mf	microfilaria
mg	milligram
min	minute
ml	millilitre
mM	millimolar
MON	monocilin 1
mRNA	messenger RNA
nACH	nicotinic acetylcholine
NaCl	sodium chloride
NAOH	sodium hydroxide
NCP	nitrocellulose paper
ng	nanogram
NGM	normal growth medium
^{o}C	degrees centigrade
OD	optical density
p53	tumour protein 53
PBS	phosphate buffered saline
P-gp	p-glycoprotein
PI-3K	phosphatidylinositol 3-kinase
PLK-1	polo-like kinase 1
rHsp90α	recombinant human Hsp90α
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute

SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sHsp	small heat shock protein
SkBr3	breast cancer cell line
TRAP	tumour necrosis factor receptor-associated protein
TRD	tomato root diffusate
TPR	tetratricopeptide repeat
Vhs	volt per hour
WHO	World Health Organisation
µAMP	microampere
µg	microgram
µl	microlitre
µM	micromolar
17-AAG	17-allylamino-17-demethoxygeldanamycin
17-DMAG	17-demethoxy-17-N,N-dimethylamino-ethylamino-geldanamycin

Chapter 1

Introduction

1 Introduction

1.1 Heat shock proteins

In 1962, Ritossa reported the effects of evelated temperature on the puffing patterns of the salivary gland chromosomes of *Drosophila busckii* (Ritossa, 1962). An increase in incubation temperature resulted in the development of a defined set of new transcription loci on the polytene chromosomes. Similar effects were also observed after treatment with 2,4-dinitrophenol and sodium salicylate in *Drosophila melanogaster* larvae (Ritossa, 1963). These observations indicated that elevated temperatures and chemical compounds induced the expression of certain genes. However, the term 'heat shock protein' only emerged in 1974 when Tissieres and co-workers identified the first gene products representing this protein group in the salivary glands of *D. melanogaster*. These proteins not only appeared in salivary glands of *D. melanogaster* but also in the brain, Malphighian tubules and wing imaginal disc, indicating that the response was not tissue specific. Besides that, a similar effect of ecdysterone treatment was also observed in salivary glands but the induction was much less compared to that following heat shock. These unique proteins were referred to as heat shock proteins because of the nature of the stimulus that caused their induction. The remarkable discovery of heat shock proteins (Hsps) led to numerous subsequent studies in which Hsps were shown to be present in all species tested and to be well conserved (as reviewed by Lindquist & Craig, 1988).

As their name implies, Hsps respond to an increase in temperature but they are also induced when a cell undergoes various types of other stresses. Almost any abrupt change in cellular environment will induce the synthesis of heat

shock, or stress, proteins (Csermely *et al.*, 1998). Some Hsp members are constitutively expressed while some are only expressed after stress (Samali & Orrenius, 1998). There are a number of conditions that can induce the heat shock response in cells including environmental stresses, toxins, pathophysiological and disease states. In addition, Hsps can be induced under non-stress conditions such as cell growth and development and at particular phases of the cell cycle etc. (Morimoto, 1998)(Figure 1.1).

A consequence of exposure to stress is the accumulation of misfolded or denatured proteins. Hsps bind to damaged polypeptides and either help to refold them or facilitate their degradation by the ubiquitin-proteasome pathway. Hsp induction requires the activation of heat shock factor (HSF)(Lis & Wu, 1993; Morimoto, 1993; Morimoto, 1998). HSF is a transcription factor that is responsible for regulating Hsp expression (Yamamoto *et al.*, 2009). Up to now four HSFs have been characterised from animals namely, HSF-1, HSF-2, HSF-3 and HSF-4 (Morimoto, 1998). HSF-3 was shown to be only present in avian species while, HSF-1, HSF-2 and HSF-4 were shown to be present in human, mouse and chicken. There are multiple HSFs in plants. HSF-1 is the major regulator of Hsps (Sreedhar & Csermely, 2004). Following activation, HSF-1 binds to heat shock elements (HSEs), stress-responsive promoter elements essential for heat shock inducibility. These contain multiple adjacent inverted arrays of the binding site (5'-nGAAn-3')(Fernandes *et al.*, 1994), and binding of HSF-1 activates the transcription of Hsps (Wu, 1995; Morimoto, 1998). The induction of Hsps helps to repair damaged proteins and assists in the recovery of the cells. Then, when the stress is removed, Hsp levels drop back to normal. This adaptation is vital for cells to restore homeostasis and improves survival.

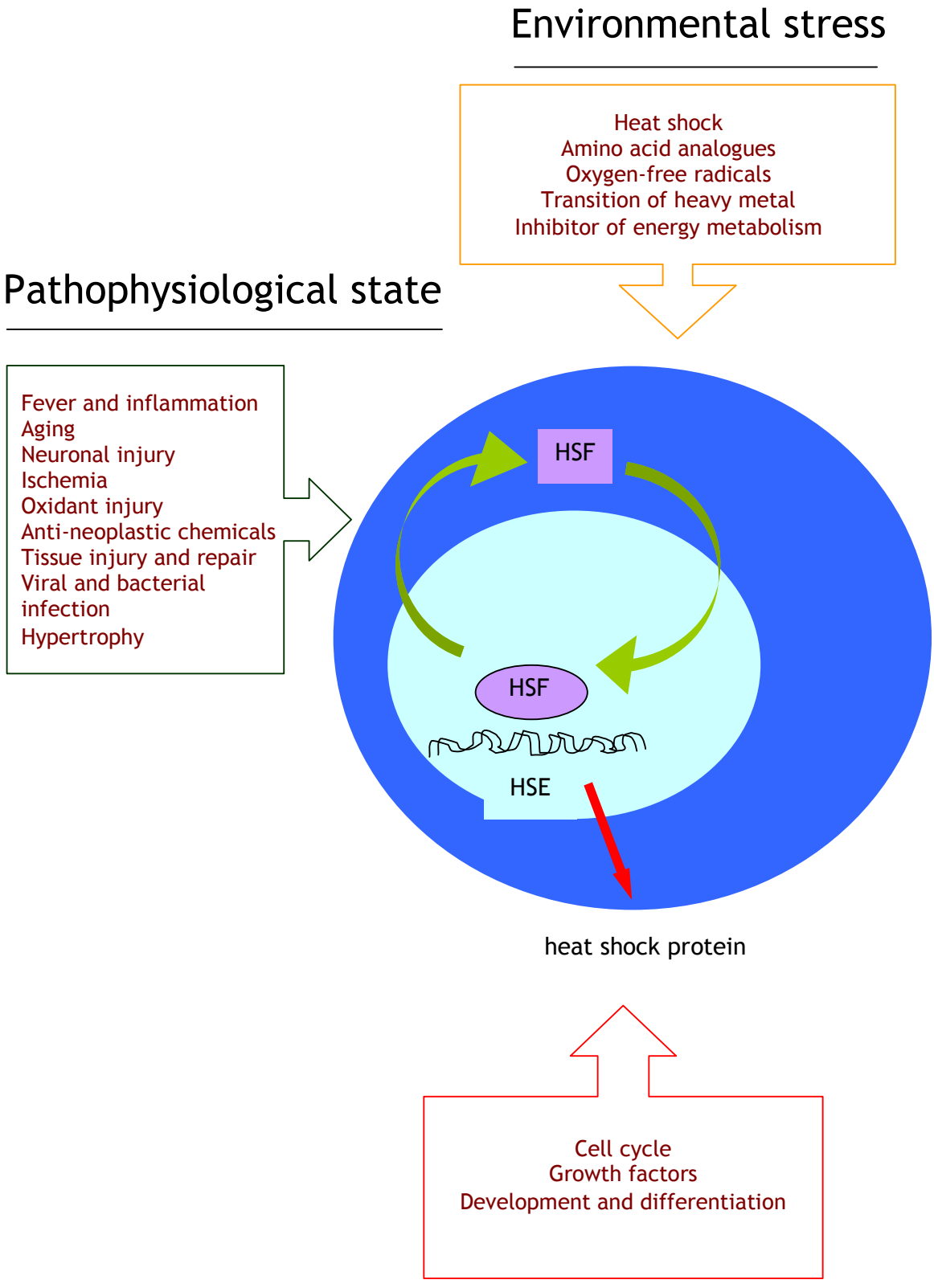


Figure 1.1: Conditions that induce the heat shock response (after Morimoto, 1998).

Hsps respond to environmental stress, pathophysiological stress and non-stress conditions following activation of heat shock factor (HSF) that binds to heat shock elements (HSEs) and activates transcription of Hsps.

1.1.1 The heat shock protein family and their functions

The major function of all Hsps is in molecular chaperoning. According to Hartl (1996), molecular chaperones can be defined as 'proteins that bind to and stabilize an otherwise unstable conformer of another protein-and by controlled binding and release, facilitate its correct fate *in vivo*: be it folding, oligomeric assembly, transport to a particular sub-cellular compartment, or disposal by degradation'. Molecular chaperones assist or mediate the correct assembly of other polypeptides but are not a component of the final fold (Ellis & Hemmingsen, 1989). As molecular chaperones, Hsps help in protein folding, refolding and in the stabilization of unstable proteins. After cells are exposed to stresses such as heat shock, proteins become less stable and may become locally unfolded or misfolded. In response to the stress, transcription of Hsps is induced to protect cells from further damage. Hsps facilitate the unfolded/misfolded proteins to acquire their native state. This mechanism helps to maintain the protein levels in response to stresses. Hsps are also involved in the translocation of protein across intracellular membranes (Phillips & Silhavy, 1990; Hannavy *et al.*, 1993). The other important aspect of Hsps as molecular chaperones is their function to prevent the aggregation of proteins. This function is vital to ensure proper folding of polypeptides especially for newly synthesised polypeptides.

Hsps are classified into different families, based on their molecular weight, structure and function. Those families include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small heat shock protein family (sHsp) (reviewed by Johnson & Craig, 1997). Table 1.1 shows the major families of heat shock proteins and their functions. Hsp100 is a member of the AAA (ATPase associated with various cellular activities) superfamily (Neuwald *et al.*, 1999). The AAA

superfamily is a diverse family of enzymes that act on other macromolecules and catalyze processes (Maurizi & Xia, 2004). Hsp90 is a member of the gyrase-Hsp90-histidine kinase-MutL (GHKL) family of ATPases (Ban *et al.*, 1999). It is essential in eukaryotes and involved in multiple cellular processes especially in maturation of substrate or client proteins engaged in signalling pathways (reviewed by Pearl & Prodromou, 2006). Hsp90 is discussed further in Section 1.2.

Hsp70 has various functions in cells. It assists in protein folding, refolding aggregated proteins, assembly of newly synthesized proteins, and is involved in the transportation of proteins across membranes (Fujihara & Nedler, 1999). In the free-living nematode, *Caenorhabditis elegans*, *hsp70* constitutes a multi-gene family (Heschl & Baillie, 1989) with different temporal and spatial expression patterns. In addition, some Hsp70s have been characterised from various parasitic nematodes, including *Brugia malayi* (Selkirk *et al.*, 1989) and *Parastrongyloides trichosuri* (Newton-Howes *et al.*, 2006). However, the exact function of the *hsp70* gene family in parasitic nematodes remains uncertain.

Hsp60 is considered to be a typical mitochondrial chaperone (also known as Cpn60) and in eukaryotes, can be found in mitochondria, the cytosol and at the cell surface. In plants, Hsp60 is localised to the chloroplast (Cappello *et al.*, 2008). Hsp60 plays an essential role in the transportation and maintenance of mitochondrial proteins (Koll *et al.*, 1992) as well as the transmission and replication of mitochondrial DNA (Smiley *et al.*, 1992) The intracellular Hsp60, which resides in mitochondria and chloroplasts, was shown to interact with Hsp10 (or Cpn10) to assist newly synthesised proteins to achieve their native conformation (Cheng *et al.*, 1989; Richardson *et al.*, 2001). Besides that, Hsp60

was shown to be present on the surface of normal (Soltys & Gupta, 1997) and tumour cells (Piselli *et al.*, 2000; Feng *et al.*, 2002; Shin *et al.*, 2003) and has been suggested to be a danger signal for the immune system for the activation of anti-tumour T-cell responses (Feng *et a*l., 2002; Osterloh *et al.*, 2004). In addition, mutations in GroEL (a homologue of Hsp60 in bacteria) blocked the export of some proteins (Kusukawa *et al.*, 1989), which suggested a role for Hsp60 in protein transportation. In the parasitic nematode, *Trichinella spiralis*, the induction of Hsp60 was suggested to be involved in maintenance of tolerance to the toxic oxidant H_2O_2 (Martinez *et al.*, 2002).

Hsp40 is categorized based on homology to the founding member in *Escherichia coli*, DnaJ (Ohtsuka *et al.*, 1990; Hattori *et al.*, 1993; Ohtsuka, 1993). The key factor of Hsp40 function resides in the J-domain. This well conserved domain is thought to promote the function of Hsp40 by recruiting an Hsp70 (Sugito *et al.*, 1995; Minami *et al.*, 1996).

Small Hsps (sHsps) are a varied group and widely distributed in both prokaryotes and eukaryotes. sHsps range in size from 12 to 43 kDa with the majority between 14 and 27 kDa (Garofalo *et al.*, 2009). Different organisms have different numbers of sHsps, ranging from one in *Saccharomyces cerevisiae* (Petko & Lindquist, 1986) to 30 in higher plants (Mansfield & Key, 1987). In plants, the sHsps are localized to the chloroplast, cytoplasm, nucleus, endoplasmic reticulum and mitochondria (Garofalo *et al.*, 2009). sHsps are involved in stabilization of misfolded proteins and thermotolerance. Furthermore, a well conserved domain in sHsps, known as the α-crystallin domain is an important component of the eye lens which functions for the lifespan of an organism and is essential for vision (Ingolia & Craig, 1982). *hsp16*

is a multi-gene family in the free-living nematode *C. elegans*. Over-expression of *hsp16.2,* the most-stress inducible of the family, resulted in increased longevity and resistance to a variety of stresses (Walker & Lithgow, 2003). According to Halaschek-Wiener *et al* (2005), genes of the *hsp16* family were shown to be very highly up-regulated in long-lived *daf-2* mutants.

Table 1.1: Hsp families and their major functions

Hsp families	Major functions
Hsp100	Protein disaggregation, thermotolerance
Hsp90	Regulatory interactions with signaling proteins, stabilization of misfolded proteins
Hsp70	Protein folding, membrane transport of proteins
Hsp60	Protein folding (limited substrates in eukaryotic cytoplasm)
Hsp40	Protein folding, co-chaperone for Hsp70
Small Hsp	Stabilization of misfolded proteins, thermotolerance, eye lens structural proteins

1.2 Hsp90

The 90 kDa heat shock protein (Hsp90) is one of the most abundant proteins in the cytosol of both eukaryotic and prokaryotic cells. It can constitute up to 1-2% of the cellular protein under normal conditions (Welch & Feramisco, 1982). The concentration of Hsp90 increases even more upon sudden temperature upshifts. Hsp90 is a unique molecular chaperone that is involved in cellular regulatory processes. To date members of the Hsp90 family have been found in the cytosol, the endoplasmic reticulum and chloroplasts (Rebbe *et al.*, 1987; Felts *et al.*, 2000; Krishna & Gloor, 2001). Hsp90 is essential in all cells because of the nature of the proteins which it chaperones. Unlike other Hsps, Hsp90 chaperones specific client proteins that associate together for folding and degradation. Hsp90 keeps proteins on the correct folding pathway and at the same time prevents possible non-specific interactions with other proteins. Because of the importance of Hsp90 in cells it has been extensively studied, but much of the information on Hsp90 interactions comes from studies on mammalian cell lines and yeast.

1.2.1 Hsp90 structure and conformation

Hsp90 is a hydrophobic protein and its hydrophobicity further increases after heat shock (Iwasaki *et al.*, 1989; Yamamoto *et al.*, 1991). The first report of the crystallization of the full-length yeast Hsp90 was published nearly 14 years ago by Prodromou *et al* (1996). Based on proteolysis data (Nemoto *et al.*, 1997; Young *et al.*, 1997), Hsp90 consists of three domains: a 25 kDa N-terminal, a 35 kDa middle domain (M) and a 12 kDa C-terminal domain (see Figure 1.3 for outline drawing representing the Hsp90 structural domains). The N-terminal domain is linked to the M-domain by a charged region (CR). The 25 kDa N-

terminal domain of Hsp90 is the binding site for ATP (Prodromou *et al.*, 1997) and geldanamycin (GA)(Whitesell *et al.*, 1994) and is highly conserved across all species. GA is a specific inhibitor of Hsp90 (Prodromou *et al.*, 1997; Whitesell *et al.*, 1994; Roe *et al.*, 1999)(Section 1.3.1). The N-terminus is the binding site for another Hsp90 inhibitor, radicicol (Soga *et al.*, 1998). The N-terminal domain contains an unusual adenine-nucleotide binding pocket known as a Bergerat fold (Dutta & Inouye, 2000). It is unusual because it has no similarity to ATP-binding domains found in other kinases or the chaperone Hsp70 (Whitesell & Lindquist, 2005) even though it belongs to the GHKL superfamily. The middle domain (M-domain) of yeast Hsp90 (amino acids 272-617) was found to mediate the interaction with Aha1 and Hch1 (Lotz *et al.*, 2003). Aha1 and Hch1 are two Hsp90-associated proteins required by Hsp90 for full activation. The M-domain is a major site for client protein interactions (Fontana *et al.*, 2002). The C-terminus functions as a dimerisation domain (Minami *et al.*, 1994). The C-terminal domain is also supposed to possess an alternative ATP-binding site, which becomes accessible when the N-terminal pocket is occupied (Marcu *et al.*, 2000b; Soti *et al.*, 2002).

CR refers to the charged region which serves as a flexible linker between the N-terminal and M-domains (see Figure 1.2). All cytosolic eukaryotic Hsp90 proteins have a charged region of varying size. The CR is possibly involved in regulatory function and participates in the association of the protein with steroid receptors (Dao-Phan *et al.*, 1997) and with the protein kinase CK-II (Miyata & Yahara, 1995). However, genetic studies show that the CR is not fundamental for the life-sustaining functions of Hsp90 in yeast, because deletion of the CR did not demonstrate any noticeable phenotypes *in vivo* (Louvion *et al.*, 1996). However, it may be involved in some “back-up” or regulatory functions

(Csermely *et al.*, 1998). Figure 1.3 shows the dimer of Hsp90 and its biochemical functions.

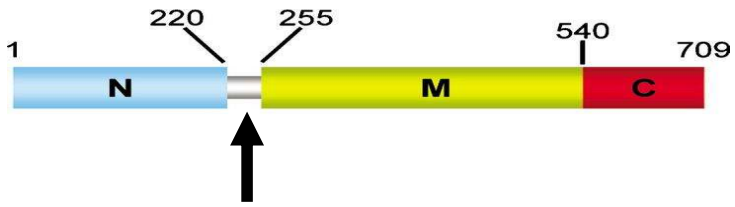


Figure 1.2: Structural domains of yeast Hsp90 (after Pearl & Prodromou, 2006)

N = The N-terminal domain

M = The middle domain

C = The C-terminal domain

= The charged region links the N-terminal domain to the M-domain

Numbers denote the amino acid positions of different domains of yeast Hsp90.

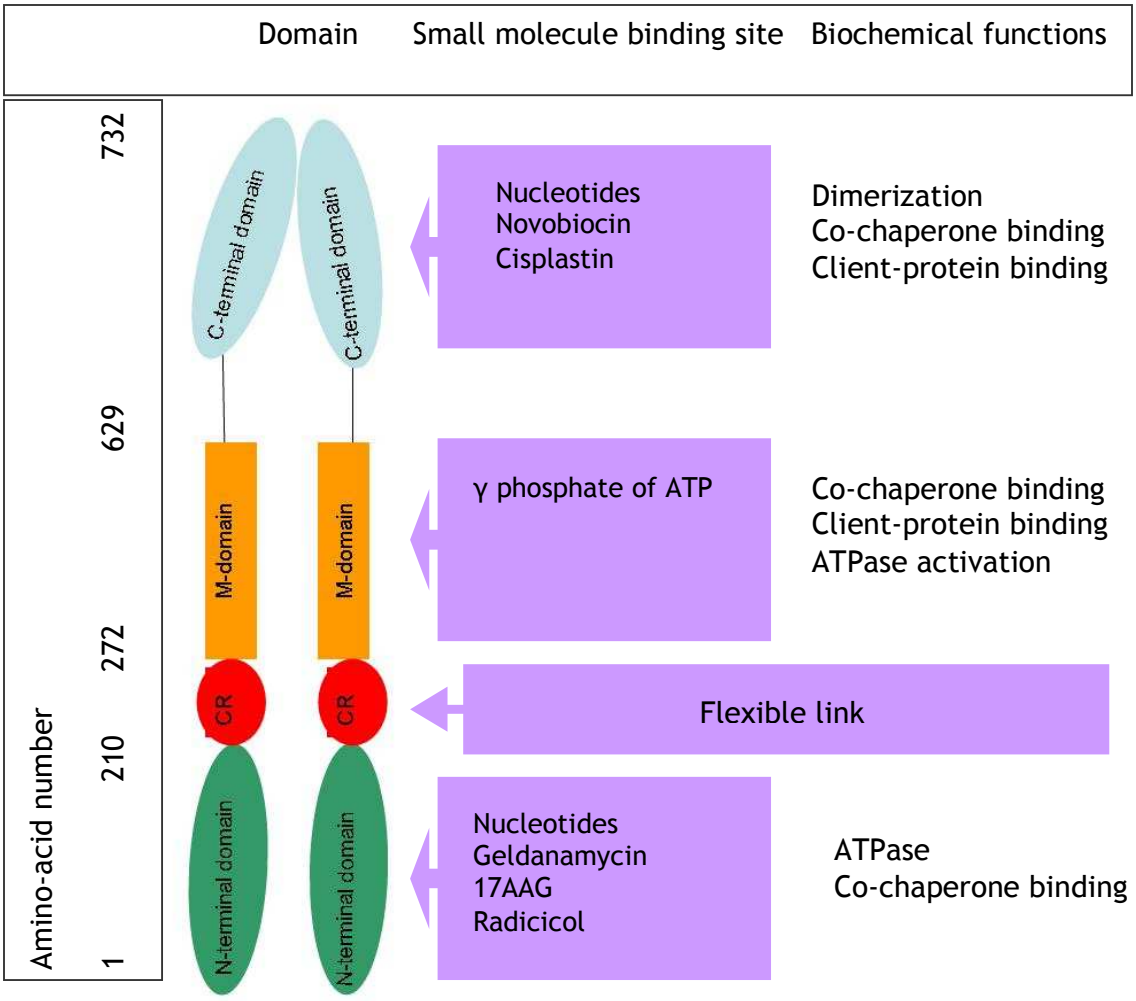


Figure 1.3: Structure of Hsp90 dimer and its biochemical functions

The N-terminal domain of Hsp90 is a binding site for ATP/GA. The M-domain functions as a binding site for co-chaperones and client proteins. It is also required for the ATPase activation. The C-domain is vital for the dimerization of Hsp90. The CR links the N-domain to the M-domain (after Whitesell & Lindquist, 2005).

1.2.2 Classification of Hsp90

Hsp90 exists in all organisms but is absent in the Archaebacteria (Tanaka & Nakamoto, 1999; Stechmann & Cavalier-Smith, 2004). Chen *et al* (2006) divided the Hsp90 gene family into five sub-families, namely HSP90A, HSP90B, HSP90C, TRAP and HTPG, which differ in their cellular localisation. The Hsp90 homologue, HTPG (high temperature protein G) is the shortest in the Hsp90 family, with a molecular mass between 66.7–78.0 kDa and it exists in the Eubacteria but not in the Archaebacteria. Deletion of HTPG in Eubacteria is not lethal but results in a slight growth disadvantage (Bardwell & Craig, 1988). HSP90A, which exists in all the eukaryotic kingdoms, can be further divided into two different classes in vertebrates, Hsp90-alpha (Hsp90α) and Hsp90-beta (Hsp90ß). These both exist in the cytosol where Hsp90α is the inducible form and Hsp90ß, the constitutive form (Rebbe *et al*., 1987; Hoffmann & Hovemann 1988). HSP90B exists in all species of Protista, Plantae and Animalia, and some species of Fungi. HSP90B is an Hsp90 homologue which resides in the endoplasm reticulum and is also known as Grp94 (94 kDa glucose-regulated protein). HSP90C is an Hsp90 homologue which can be found in chloroplasts and is only present in Plantae. HSP90C has duplicated into Hsp90C1 and Hsp90C2 in higher plants. TRAP (tumor necrosis factor receptor-associated protein) is an Hsp90 homologue which resides in the mitochondria and is most closely related to HTPG (Stechmann & Cavalier-Smith, 2004). Refer to Table 1.2 for details of the Hsp90 family.

Table 1.2: Members of the Hsp90 molecular chaperone family

Name	Subgroup	Characteristic localization	Kingdom
HTPG	HtpG-A HtpG-B HtpG-C	Cytoplasm	Bacteria but absent in Archaebacteria
HSP90A	Hsp90α Hsp90β	Cytoplasm	Exists in all eukaryotic kingdom
HSP90B	Also known as Grp94	Endoplasmic reticulum	Exists in all eukaryotic kingdom
HSP90C	Hsp90C1 Hsp90C2	Chloroplast	Plantae
TRAP		Mitochondrial	Animalia and some species in Protista (Alveolata, Englenozoa)

(Adapted from Chen *et al*., 2006).

1.2.3 Hsp90 functions

As its name implies, Hsp90 responds to environmental changes such as elevated temperature, oxidation, pH etc. This response is important for maintaining a favourable environment which is suitable for cells to cope with environmental changes. Hsp90 plays a fundamental role in all cells. It has been suggested to play a central role in the assembly (Jackson *et al.*, 2004) and disassembly machine (Wegele *et al.*, 2004). This unique chaperone works together with Hsp40, Hsp70 and other co-chaperones such as the TPR proteins etc. in activation or maturation of a wide range of client proteins and substrates, including some important protein kinases and transcription factors. Because of the nature of its client proteins, it is involved in cell cycle, growth, apoptosis, endocrine function, plant immunity and also in evolution (Figure 1.4).

In vitro Hsp90 alone can prevent protein aggregation and promote refolding (Welch *et al.*, 1992) but *in vivo* Hsp90 functions with associated proteins known as co-chaperones (see Table 1.3). For example the v-Src tyrosine kinase has been reported to form stable complexes with Hsp90 (Blankenship & Matsumura, 1997), where Hsp90 maintains the kinase in an inactive form and helps in its membrane recruitment. However, this requires association with other co-chaperones to complete the whole processes. Table 1.3 shows the important components of the Hsp90 chaperone machine (adapted from Whitesell & Lindquist, 2005).

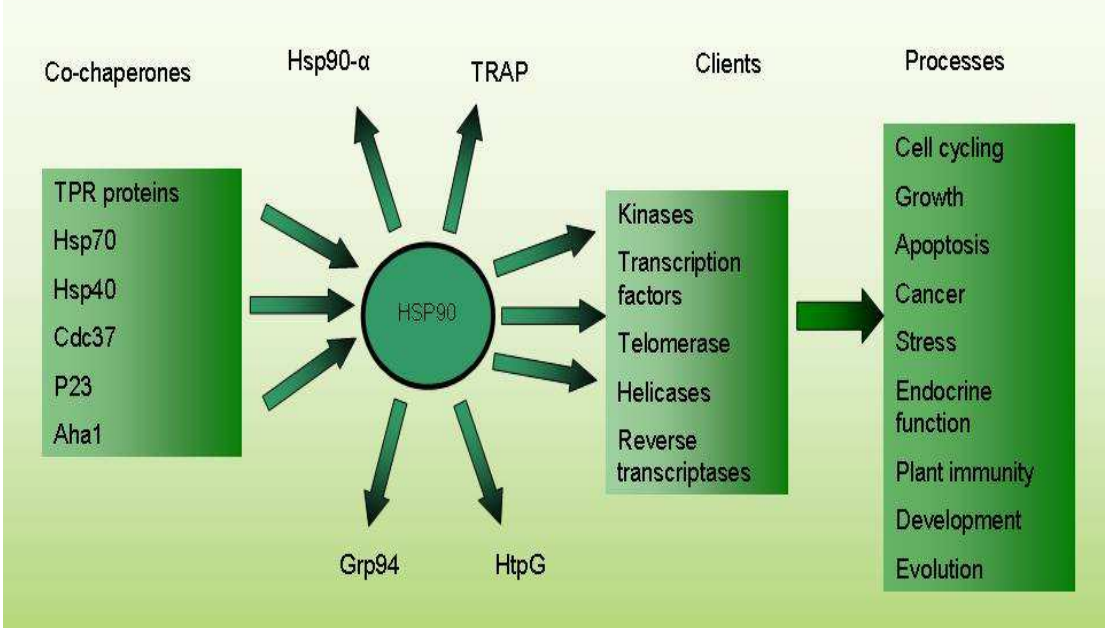


Figure 1.4: Hsp90 has a central role in the assembly of various proteins involved in many cellular processes.

Cytosolic Hsp90 and its homologues, Grp94, HtpG, Hsp90-α and TRAP along with co-chaperones form the cellular assembly machine. This assembly machine acts on a range of client proteins for their maturation and activation, thereby controlling multiple cellular processes e.g. cell cycle, apoptosis and stress recovery (after Jackson *et al*., 2004).

Table 1.3: Hsp90 and the important components of the chaperone machinery

Protein Family	Classification	Function
Hsp90	Chaperone	Supports meta-stable protein conformations, especially in signal transducers
Hsp70	Chaperone	Helps fold nascent polypeptide chains; participates in assembly of multiprotein complexes
Hsp40	Co-chaperone	Stimulates Hsp70 ATPase activity
HIP, HOP	Adapters	Mediates interaction of Hsp90 and Hsp70
Cdc37/p50	Co-chaperone	Modulates interactions with kinase
AHA1	Co-chaperone	Stimulates Hsp90 ATPase activity
p23	Co-chaperone	Stabilizes Hsp90 association with clients
Immunophilin	Prolyl isomerase	Modulates interactions with hormone receptors

HOP – Hsp70/Hsp90-organizing protein, HIP – Hsp70-interacting protein, Cdc37 – cell division cycle 37 homologue, AHA1 – activator of Hsp90 ATPase homologue.

(Adapted from Whitesell & Lindquist, 2005)

In the process of protein folding by Hsp90, Hsp70-Hip will initiate the binding to an unstructured target protein. This process is aided by the Hsp70 co-chaperone, Hsp40. Hsp90 then forms a structure with Hop which is responsible for linking Hsp70 to Hsp90. Then, immunophilins and p23 are added to the conformation and parallel with this, Hsp70-Hip and Hop are released from the mature complex (Figure 1.5). p23 binding is an important process because it allows the completion of folding (Dittmar *et al*., 1997**)**. According to Ali *et al* (2006), p23 is required to stabilise Hsp90 in the ATP-bound form. Furthermore, p23 binding is vital to extend the time Hsp90 is in the appropriate conformation for client protein activation. Finally the mature target protein is released to complete the whole process. Any disruption by Hsp90 inhibitors leads to multiple physiologic defects in live cells. For example, for protein kinases, Hsp90 is thought to stabilize the exposed catalytic domains before assembly of the kinase into the final signalling complex (Xu & Lindquist, 1993; Alique *et al*., 1994; Stepanova *et al*., 1996), and the interruption of the conformation with Hsp90 inhibitors will lead to failure of the final fold.

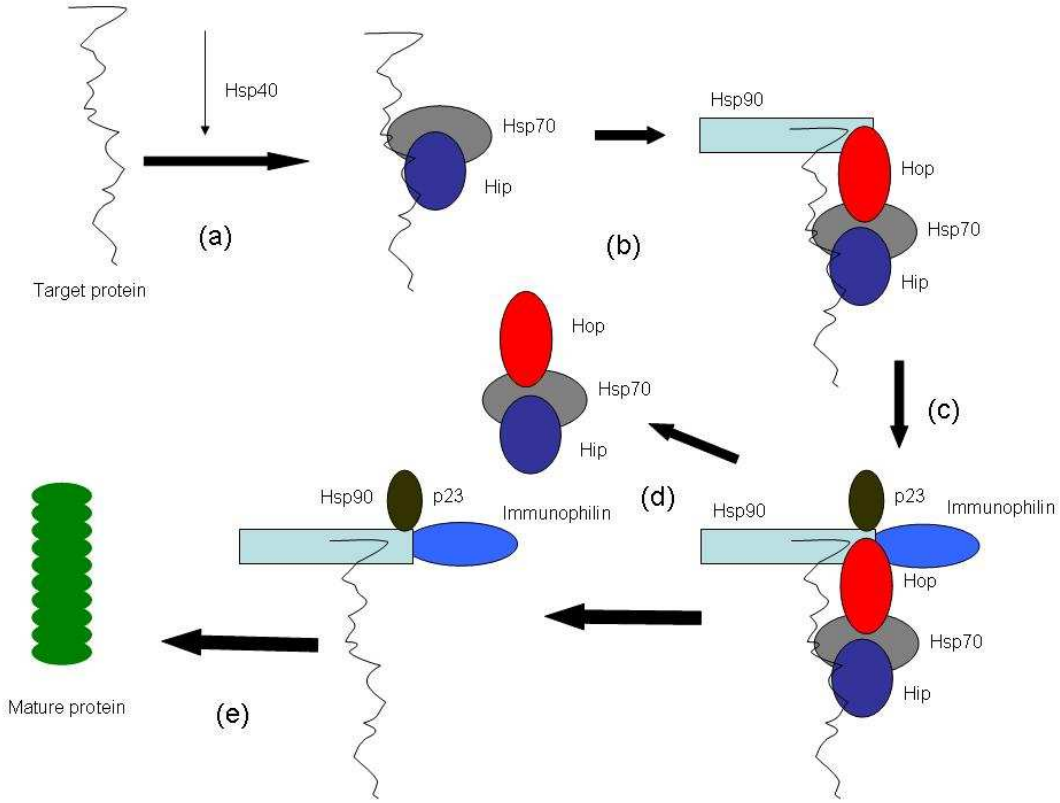


Figure 1.5: Hsp90 in the folding process.

(a) Unfolded target protein. Hsp70-Hip initiates the binding to the target protein aided by Hsp40.

(b) Hsp90-Hop conformation binds to the Hsp70-Hip-target protein.

(c) The binding of immunophilin aids the maturation of the complex. The binding of p23 to the complex allows the completion of the folding.

(d) Hip-Hsp70-Hop is released from the mature complex.

(e) Target protein is released in its mature and stable conformation

(Adapted from Csermely *et al*., 1998).

1.3 Hsp90 inhibitors

The realisation that Hsp90 chaperones proteins required for cellular viability opened up a new direction in the development of drugs that alter Hsp90 function. GA, the first Hsp90 inhibitor that was recognised, belongs to the family of benzoquinone ansamycins. GA binds to the N-terminal domain of Hsp90, while other inhibitors such as novobiocin bind to the C-terminal domain. The binding of inhibitor to the N-terminus or C-terminus of Hsp90 alters the conformation of the molecule and at the same time prevents association with client proteins that may be required for viability. The use of Hsp90 specific inhibitors results in a decline in client protein activity *in vivo* and in the degradation of vital signalling proteins that are involved in cell proliferation, cell cycle regulation and apoptosis (reviewed by Maloney & Workman, 2002). As an outcome of this, several Hsp90 inhibitors have been developed and some of them are already in clinical trials. Table 1.4 shows some of the Hsp90 inhibitors (reviewed by Sreedhar *et al.*, 2004).

Table 1.4: Hsp90 inhibitors

Domain targeted	Inhibitors
N-terminal	Geldanamycin and analogues (17-AAG, 17-DMAG)
	Radicicol and its analogues (aigialomycin D, pochonin D)
	Purine-scaffold Hsp90 binders, PU3
C-terminal	Cisplatin
	Novobiocin

(Adapted from Sreedhar *et al*., 2004)

1.3.1 Geldanamycin

GA, which belongs to the family of benzoquinone ansamycins, is an antibiotic that specifically inhibit the cytosolic chaperone Hsp90 (Pratt, 1998; Neckers, *et al.*, 1999) GA was first purified from the broth of *Streptomyces hygroscopicus* (DeBoer *et al.*, 1970), a soil bacterium. GA is soluble in alcohols, aliphatic chlorinated solvents, acetone, benzene and ethyl acetate but poorly soluble in water (DeBeor *et al.*, 1970). GA consists of a quinone ring and hydrophobic ansa bridge (Rinehart & Shield, 1976) (see Figure 1.6 for the chemical structural of GA). At first GA was thought to be an inhibitor of tyrosine kinases (Uehara *et al.*, 1989), as it was shown that GA abolishes Src kinase activity in whole cell assays (Uehara *et al.*, 1988). However, in other studies, GA was shown to be unable to directly inhibit the kinase activity of the purified recombinant protein (Whitesell *et al.*, 1992). It was then suggested that GA indirectly inhibits Src kinase activity by binding to unrecognised Src associated proteins. Subsequently by an affinity isolation method using GA immobilised on agarose beads, it was shown that GA specifically binds to Hsp90 (Whitesell *et al.*, 1994**).** In addition, it was observed that levels of src-Hsp90 were reduced as the GA concentrations were increased. This early experiment demonstrated that GA not only binds to Hsp90 but also interrupted the chaperone function of Hsp90 and its associated kinase proteins.

The first co-crystal structure of Hsp90 bound to GA was published in 1997 (Prodromou *et al.*, 1997). Subsequent affinity chromatography experiments together with co-crystal structures provided evidence that GA inhibits Hsp90 by binding to its N-terminal ATP binding site (Whitesell *et al.*, 1994; Prodromou *et al.*, 1997; Roe *et al.*, 1999). These studies resulted in a re-interpretation of the

effect of GA on kinases. In the presence of GA, the immature kinase undergoes rapid degradation as a consequence of ubiquitination and subsequent catabolism by the proteasome. As a result, the depletion of the mature kinase can cause cell death.

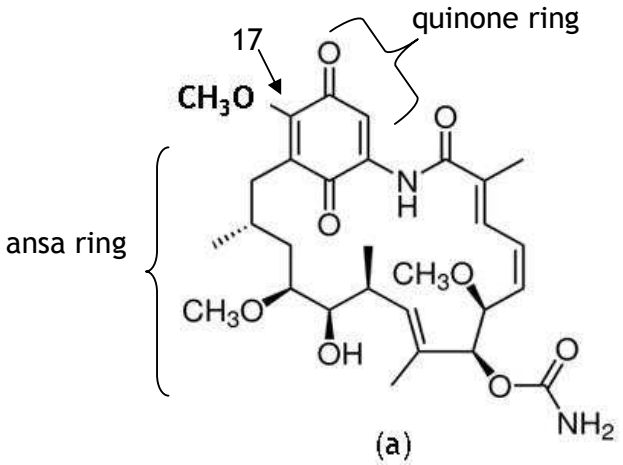
Recently, GA has been used intensively to study the effects of inhibiting Hsp90 in tumour cells. However, GA is poorly soluble in aqueous solution and its hepatotoxicity proved problematic in clinical trials (Supko *et al.*, 1995). Studies by Dikalov and co-workers (2002) showed that incubation of GA with flavin-dependent reductase enzymes transformed GA quinone into a semiquinone. The semiquinone reacts with molecular oxygen to produce superoxide radicals which can cause toxicity (Dikalov *et al.*, 1992) and this causes cell death. By inserting a stronger electron-donating group into the 17-position of GA, it stabilized the quinone and decreased its redox-active potential (Blagg & Kerr, 2006). This new analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG) seems to behave significantly better than GA (Barzilay *et al.*, 2005). 17-AAG has entered Phase I and Phase II clinical trials for cancer chemotherapy. Even though 17-AAG shows promising results in chemotherapy, this analogue still produces hepatotoxicity and its water insolubility makes it difficult to formulate (Jez *et al.*, 2003). In order to improve the solubility of 17-AAG, an extra tertiary amine group was incorporated into 17-AAG which led to the development of 17-demethoxy-17-N,N-dimethylamino-ethylamino-geldanamycin (17-DMAG)(Jez *et al.*, 2003). 17-DMAG is much more water soluble and has entered Phase I clinical trials (reviewed in Bagatell & Whitesell, 2004). Refer to Figure 1.6 for the chemical structure of GA, 17-AAG and 17-DMAG.

Figure 1.6: Geldanamycin and its analogues

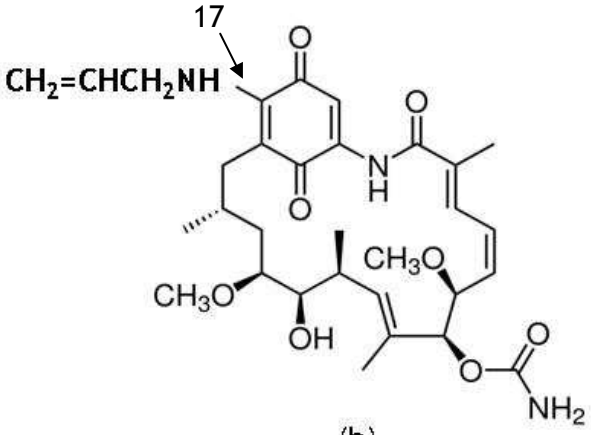
a) Geldanamycin ($C_{29}H_{40}N_2O_9$). As a member of the benzoquinone ansamycin family, GA consists of a quinone ring and hydrophobic ansa bridge.

b) Chemical structure of 17-AAG shows the new substituted group in 17-position of GA (17-AAG, (CH_2=$CHCH_2NH$-).

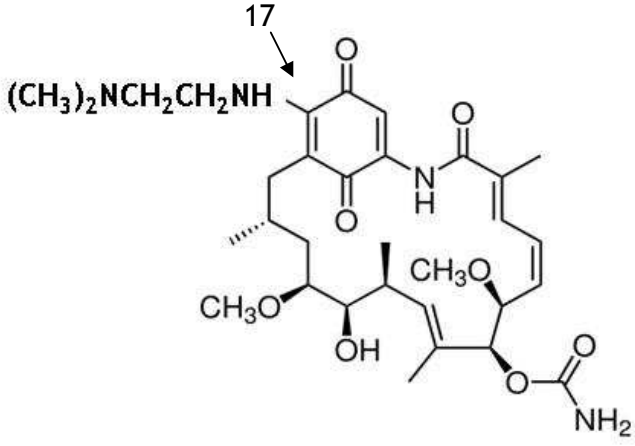
c) 17-DMAG chemical structure shows an extra tertiary amine group inserted in 17-position of GA to produce 17-DMAG ($(CH_3)_2NCH_2CH_2NH$-)


quinone ring
17
CH3O
ansa ring
O
N
H
CH3O
CH3O
OH
O
O
NH2

(a)


17
CH2=CHCH2NH
O
N
H
CH3O
CH3O
OH
O
O
NH2

(b)


17
(CH3)2NCH2CH2NH
O
N
H
CH3O
CH3O
OH
O
O
NH2

(c)

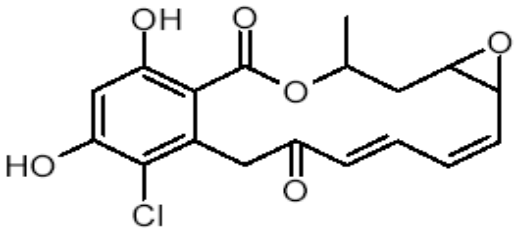
1.3.2 Radicicol

Radicicol is a macrocyclic antibiotic that was first isolated from *Monosporium bonarden* (Delmotte & Delmotte-Plaque, 1953)(see Figure 1.7). Kwon *et al* (1992a) reported that radicicol contributed to the inhibition of the oncogenic kinase Src. Then, it was revealed by Kwon *et al* (1992b) that it is a specific inhibitor of Hsp90. In 1999, the crystal structure of radicicol bound to yeast Hsp90 was solved (Roe *et al*., 1999). Even though radicicol does not have a similar structure to GA, it also inhibits Hsp90 by binding to the N-terminal ATP pocket (Prodromou *et al*., 1997) and it binds with greater affinity compared to GA and 17-AAG (Roe *et al*., 1999). It has been shown to compete with ATP for the binding site of Hsp90 (Roe *et al*., 1999). Radicicol is also known to suppress the activity of various oncogenic proteins such as Ras, Raf and v-src-tyrosine kinase (Kwon *et al*., 1997). Furthermore radicicol has been shown to be active against retinoblastoma cells that are resistant to 17-AAG (Yamamoto *et al*., 2003). However, radicicol lacks anti-tumour activity *in vivo* in experimental models because of its instability (Vogen *et al*., 2002). Besides that there are two analogues of radicicol that show a good affinity for binding to Hsp90 namely, aigialomycin D (Yang *et al*., 2004) and pochonin D (Moulin *et al*., 2005).

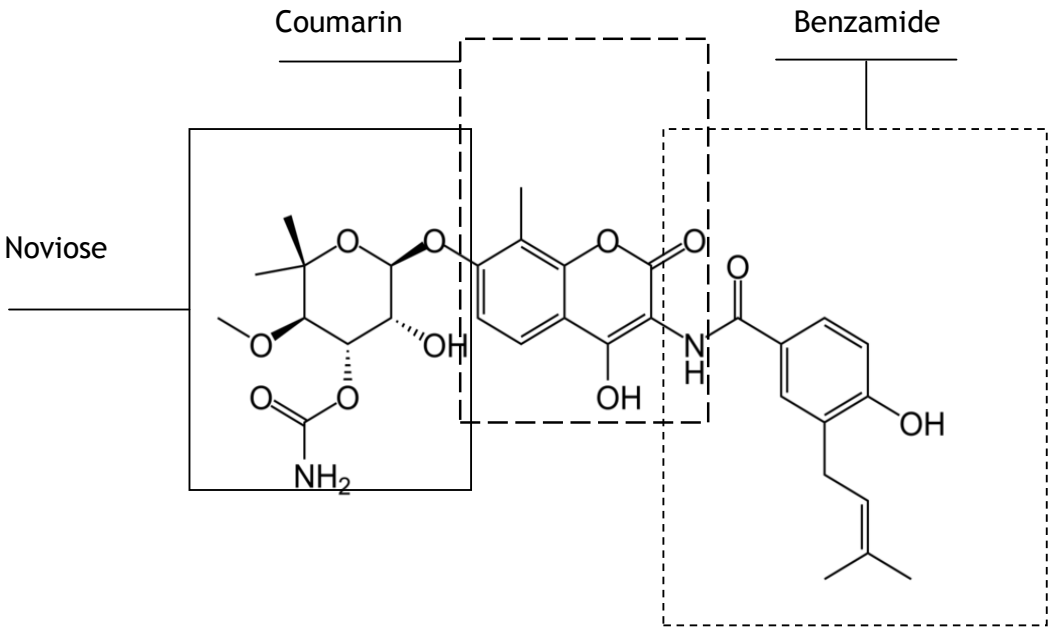
1.3.3 Novobiocin

Novobiocin is a coumarin antibiotic that has been isolated from *Streptomyces* strains and has a potent activity against Gram-positive bacteria (Yu *et al*., 2004)(see Figure 1.7). It has been shown to inhibit the enzyme-catalyzed hydrolysis of ATP and binds to type II topoisomerases including DNA gyrase (Lewis *et al*., 1996; Holdgate *et al*., 1997) and because of that it has

been used for the treatment of bacterial infection. Novobiocin consists of three distinct parts namely the benzamide side chain, the coumarin core and the noviose sugar (Donnelly & Blagg, 2008). The observation that novobiocin inhibits Hsp90 was proposed by Marcu *et al* (2000a). They showed that cell lysates incubated with novobiocin-Sepharose bound to Hsp90, and demonstrated that the novobiocin binding site correlated to residues 538-728 of Hsp90. Removal of amino acids 657-677 from the 538-728 peptide fragment of chicken Hsp90, resulted in a decline in novobiocin binding which demonstrated that residues 657-677 were essential for novobiocin binding. However, subsequent studies suggested that the binding of Hsp90 to novobiocin-Sepharose was non-specific (Whitesell pers. comm. to E. Devaney). Novobiocin was shown to disrupt Hsp90 functions (Marcu *et al.*, 2000b) and cause the destabilization and proteolytic degradation of a number of proteins. Allan and co-workers proposed that novobiocin may lead to substrate release by inducing a conformational change that result in separation of homodimeric C-terminal domains (Allan *et al.*, 2006). *In vitro,* incubation of SkBr3 (human breast cancer cells) with increasing concentrations of novobiocin caused the degradation of Hsp90 client proteins (Marcu *et al.*, 2000b).



(a)



(b)

Figure 1.7: Chemical structure of selected Hsp90 inhibitors

a) Chemical structure of radicicol ($C_{18}H_{17}ClO_6$).

b) Chemical structure of novobiocin ($C_{31}H_{36}N_2O_{11}$) consists of three distinct parts; the benzamide side chain, the coumarin core and the noviose sugar.

1.3.4 Hsp90 inhibitors: Mode of action

Under normal circumstances, Hsp90 acts as a chaperone, aiding the maturation of important client proteins. The binding and hydrolysis of ATP is the key factor for Hsp90 function (Meyer *et al*., 2003; Pearl & Prodromou, 2006). According to Prodromou *et al* (2000), the Hsp90 N-terminal domains transiently binds ATP so that the ATPase cycle is intimately coupled to the opening and closing of a molecular clamp (Figure 1.8). For the N-terminal hydrolysis of ATP, dimerization of Hsp90 is required. According to Pearl & Prodromou (2006), the two halves of the Hsp90 dimer cooperate to achieve a conformation that is able to hydrolyse ATP. ATP binds to Hsp90 and it stabilizes 'a tense conformation' and the functional state of Hsp90, because Hsp90 depends on the presence of the γ-phosphate of ATP. Energy produced by ATP hydrolysis is used to facilitate a conformational change within Hsp90 that clamps Hsp90 around the bound client proteins which leads to proper folding of the client proteins. Hydrolysis of bound ATP releases the polypeptide by opening up the Hsp90 dimer (Young *et al*., 2001). According to Young & Hartl (2000), GA can inhibit the chaperone-mediated folding of Hsp90 client proteins by blocking their ATP-dependent dissociation from Hsp90. It has also been suggested that by binding to inhibitors the formation of closed clamps around the bound client proteins is inhibited (Blagg & Kerr, 2006). As a result, client proteins are released from Hsp90 without achieving their active or mature conformation. These unprocessed chaperone-client protein complexes accumulate within the cell leading to recruitment of E3 ubiquitin ligases that target Hsp90 client proteins for degradation in the proteasome (Sepp-Lorenzino *et al*., 1995; Mimnaugh *et al*., 1996).

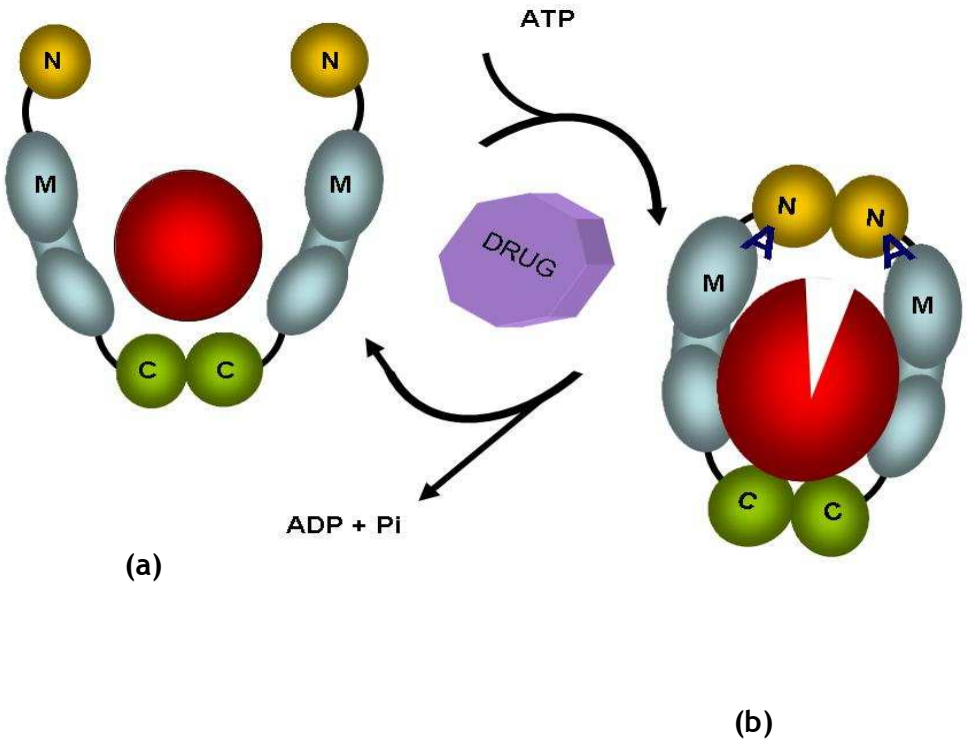


Figure 1.8: Schematic of current model for the conformational changes that accompany binding and hydrolysis of ATP (adapted from Pearl & Prodromou, 2006).

N represents N-terminal, M for M-domain and C for C-terminal domain. Red represents client proteins.

a) Open conformation of Hsp90 clamp. Client proteins can bind to Hsp90 without the presence of ATP. However to achieve the full activation it requires ATP hydrolysis.

b) Closed conformation of Hsp90 dimer in the presence of ATP. In the presence of inhibitor, in the ATP binding pocket, Hsp90 cannot form a closed clamp around bound client proteins leading to degradation of client proteins.

1.4 Hsp90 as a target for anti-tumour drugs

Presently, there is growing evidence that Hsp90 plays a fundamental role in the progress of malignant diseases. Previous studies have shown that Hsp90 was expressed at two- to 10-fold higher levels in tumour cells (lung carcinomas, melanomas and hepatoma) compared to normal cells (peripheral-blood lymphocytes, human umbilical endothelial cells and fibroblasts) and Hsp90 was suggested to play an important role in tumour cell survival (Ferrarini *et al.*, 1992). Hsp90 and its homologue, Grp94, were shown to be over-expressed in human breast cancer (Jameel *et al.*, 1992; Haverty *et al.*, 1997). Furthermore, in tumour cells Hsp90 co-chaperones were observed to be over-expressed, suggesting that a greater amount of Hsp90 in tumour cells might be engaged in active chaperoning (Kamal *et al.*, 2003).

There are several Hsp90 client proteins that are important as possible anti-cancer targets. Most of these require Hsp90 association for their activation. They are mutated p53 (Blagosklonny *et al.*, 1995), Src family kinases (Hatson and Matts, 1994), Wee1 kinase (Alique *et al.*, 1994), transmembrane receptor tyrosine kinases (Chavany *et al.*, 1996), Raf-1 kinase, CK-II (Miyata & Yahara, 1995), Cdk4/Cdk6 (Stepanova *et al.*, 1996), subunits of trimeric GTP-binding proteins (Inanobe *et al.*, 1994), tumour necrosis factor receptor, retinoblastoma protein and focal adhesion kinase (Song *et al.*, 1995; Chen *et al.*, 1996). In addition, certain client proteins that interact with Hsp90 for their stability are oncogenic e.g. ErbB2 transmembrane kinase, Bcr-Abl kinase, Akt kinase, PLK-1, hypoxia-inducible factor 1α and C-RAF (Powers and Workman, 2006). Most of these client proteins represent a group which contribute to the hallmark of cancer (Hanahan & Weinberg, 2000; Powers & Workman, 2006)(Figure 1.9). For

example, Akt kinase is one protein associated with Hsp90. It was shown to be involved in a signal transduction pathway which is responsible for the growth signal, evasion of apoptosis and sustained angionesis (Donnelly & Blagg, 2008). Akt kinase acts as a modulator for phosphatidylinositol 3-kinase (PI-3K) which is responsible for the cell survival signal pathway and the interaction between Akt kinase and PI-3K has been targeted by various anti-cancer drugs (Tsurou *et al.*, 2003).

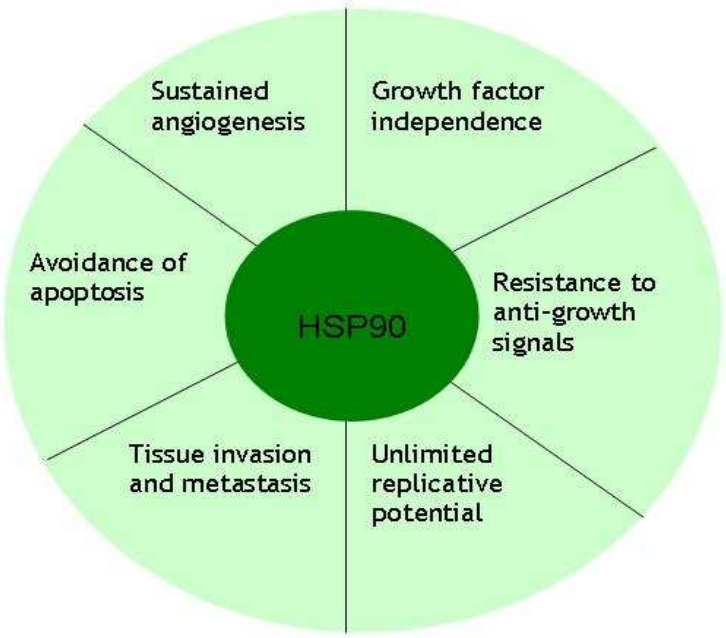


Figure 1.9: Hsp90 and six hallmark traits of cancer.

Hsp90 is essential for stability and function of oncogenic client proteins, which contribute to the hallmark of cancer.

(After Power & Workman, 2006).

In tumour cells, Hsp90 was found to accumulate in multiprotein complexes bound to client and partner proteins while in normal cells Hsp90 was found as a homodimeric protein unbound to partner proteins (Kamal *et al.*, 2003). In addition, Hsp90 in tumour cells has a high ATPase activity and a high affinity for inhibitors such as GA compared to normal cells (Kamal *et al.*, 2003). This high affinity of tumour Hsp90 may be because tumour cells are highly dependent on Hsp90 for growth in the aggressive tumour environment (Blagg & Kerr, 2006). The use of Hsp90 specific inhibitors results in a decline in client protein activity *in vivo* and in the degradation of vital signaling proteins that are involved in cell proliferation, cell cycle regulation and apoptosis (Maloney & Workman, 2002). As a result, unfolded proteins become part of an unproductive heteroprotein complex, which leads to their degradation by the ubiquitin-proteasome pathway (Connell *et al.*, 2001). Targeting a single molecule leads to the degradation of many other proteins, which is why Hsp90 has been suggested as a unique target for cancer therapeutics.

Studies by Hostein *et al* (2001) on human colon cell lines showed that 17-AAG inhibited the RAS-RAF-MEK-ERK1/2 and PI-3K-AKT/PKB pathway by targeting Hsp90 molecules. Subsequently, this was accompanied by cytostasis, cell cycle arrest and cell-line dependent apoptosis. Both signalling pathways require activation by tyrosine kinase ErbB2, which was shown to be an important Hsp90 client protein (Yarden & Sliwkowski, 2001; Citri *et al.*, 2004). Studies by Smith *et al* (2002) showed that ErbB2 over-expression caused a five-fold increase in sensitivity to GA and 17-AAG. These observations suggest the potential use of 17-AAG as an agent for chemotherapy. In phase 1 clinical trials, treatment of two patients with advanced metastatic malignant melanoma showed that B-RAF mutants were very sensitive to 17-AAG-mediated proteosomal degradation (da

Rocha Dias *et al.*, 2005) which may suggest a possible mechanism for the clinical activity of 17-AAG in melanoma. In addition, Pacey *et al* (2006) observed some clinical activity using 17-AAG in breast and prostate cancer. The activity of 17-AAG was shown to be reduced in the presence of P-glycoprotein (P-gp)(Kelland *et al.*, 1999) and it was metabolized by the cytochrome P450, CYP3A4 (Egorin *et al.*, 1998). A second generation of GA derivatives, 17-DMAG has also entered Phase 1 clinical trials. In patients with chemotherapy-refractory acute myelogenous leukaemia, a complete response to treatment in 3 out of 17 patients was observed (Lancet *et al.*, 2010). These results indicate the potential of Hsp90 as a drug target in cancer chemotherapy.

1.5 Hsp90 in nematodes

Nematodes are very diverse organisms and are classified under the phylum Nematoda. According to Blaxter *et al* (1998) nematodes have evolved to be parasitic on multiple occasions and have successfully adapted to non-vertebrate, vertebrate and plant hosts. Despite the tremendous medical and economic importance of nematodes, the great majority of nematodes are free-living. Initially, nematodes were classified based on morphological characteristics derived primarily from light microscopy. Recently, attempts have been made to use molecular methods to classify nematodes. The analysis of small subunit ribosomal RNA genes (SSU rRNA) by Blaxter *et al* (1998) showed that nematodes can be divided into three major clades: Dorylaimia (clade I), Enoplia (clade II), and Chromadorea which also includes the Rhabditida. The Rhabditida can be further divided into Sprurina (clade III), Tylenchina (clade IV) and Rhabditina (clade V). Clade I consists of Dorylaimida, Mermithida, Mononchida, Dioctophymatida and Trichinellida. This clade includes the free-living

microbivores, plant, insect and animal parasitic nematodes. Clade II contains plant parasitic enoplids and triplonchids but no animal parasites. Clade III consists of parasitic species including spirurids, ascaridids, oxyurids and rhigonematids. *B. pahangi* resides in clade III together with *Toxocara cati* and *Parascaris equorum*. Clade IV comprises plant and animal parasites and some free-living species. *Globodera* sp. was placed in this clade together with the vertebrate parasitic Strongyloidoidae. Clade V the Rhabditia, consists of free-living and animal parasitic species. *C. elegans* belongs to clade V with some others important parasitic species such as *Haemonchus contortus* and *Teladorsagia circumcincta*. Figure 1.10 shows a classification of nematodes in different clades based on SSU rRNA (Blaxter *et al.*, 1998; De Ley & Blaxter, 2002).

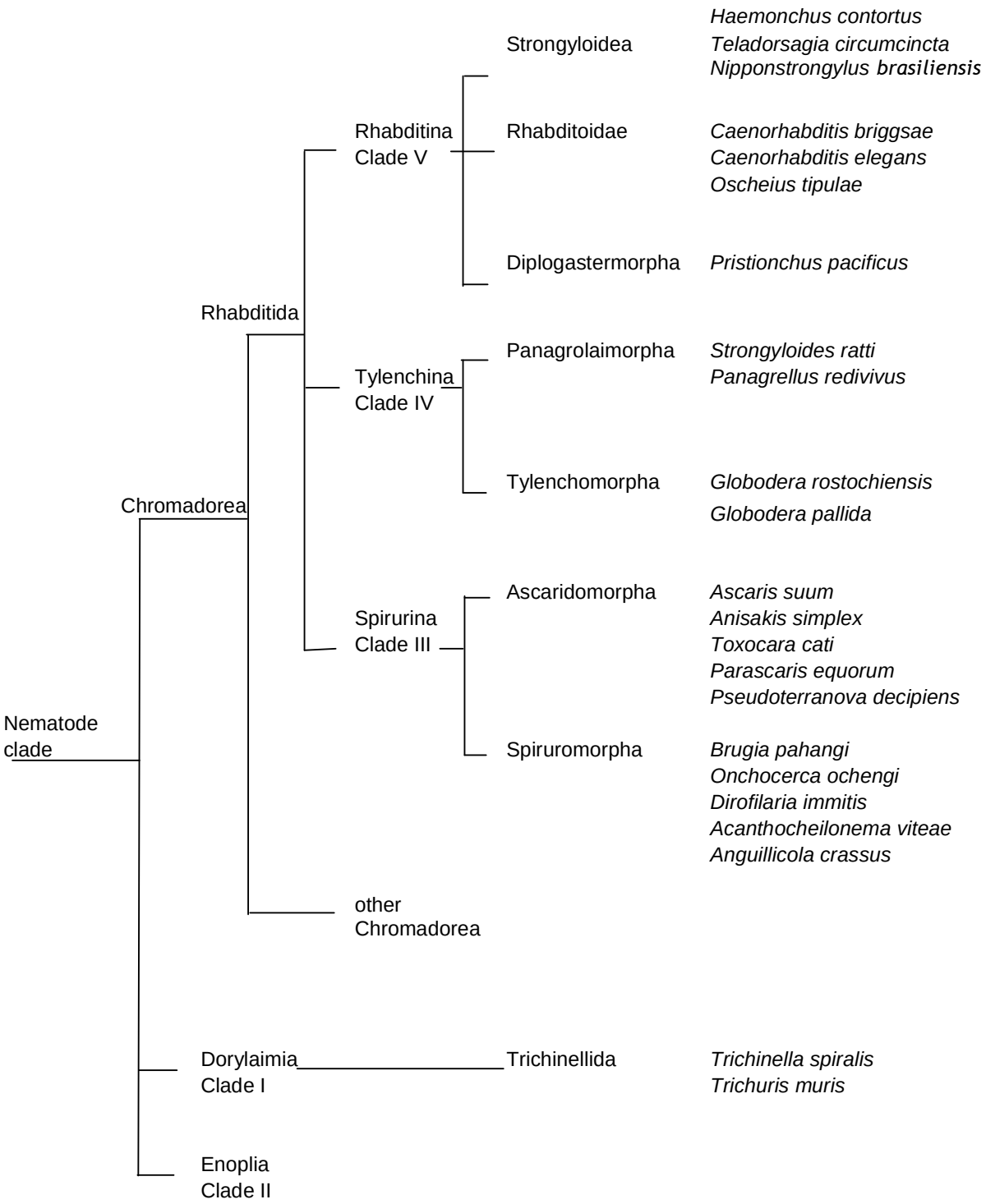


Figure 1.10: Nematode species from different clades of nematode (after Blaxter et al., 1998).

1.5.1 Nematodes life-cycles

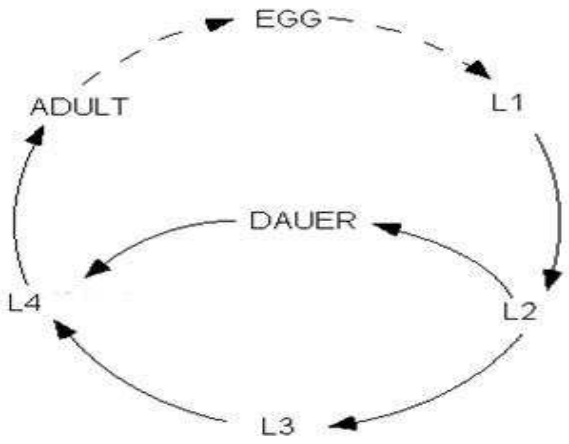
Nematodes show a great deal of diversity in their life-cycles with many free-lving species while others are parasitic in animals and plants. Genarally, nematodes have five developmental stages in their life-cycle; L1, L2, L3, L4 and adult. Free-living species such as *C. elegans* exhibit a direct life-cycle developing from egg to adult (see Figure 1.11a). However, under certain conditions such as an absence of food or over crowding, *C. elegans* can choose an alternative developmental pathway leading to the dauer larva (Cassada & Russell, 1975). In the dauer stage, worms do not feed but can survive under extreme conditions for several months. When conditions become more favourable, development is resumed. The nematodes exit the dauer larval stage and develop into the normal L4 stage before becoming adults. For parasitic species such as the Trichostrongyles, e.g *H. contortus*, part of the life-cycle takes place in the environment. Eggs are passed in the host faeces and L1, L2 and L3 stages develop in the environment. The infective L3 is ingested and L4 and adult stages develop in the definitive host (see Figure 1.11b).

For obligate parasitic nematodes, such as filarial worms, the life-cycle is more complex and requires an intermediate host. Mf to L3 development occurs in the mosquito, while L3 to adult stages takes place in the definitive host. The infection is initiated by the bite of a mosquito which harbours L3 stage parasites. The L3 are released and enter the body of the definitive host. In the definitive host, the L3 undergo two moults before become an adult in the afferent lymphatics. After mating, females produce Mf that circulate in the bloodstream which are then available for a mosquito to ingest with a blood meal (see Figure 1.12).

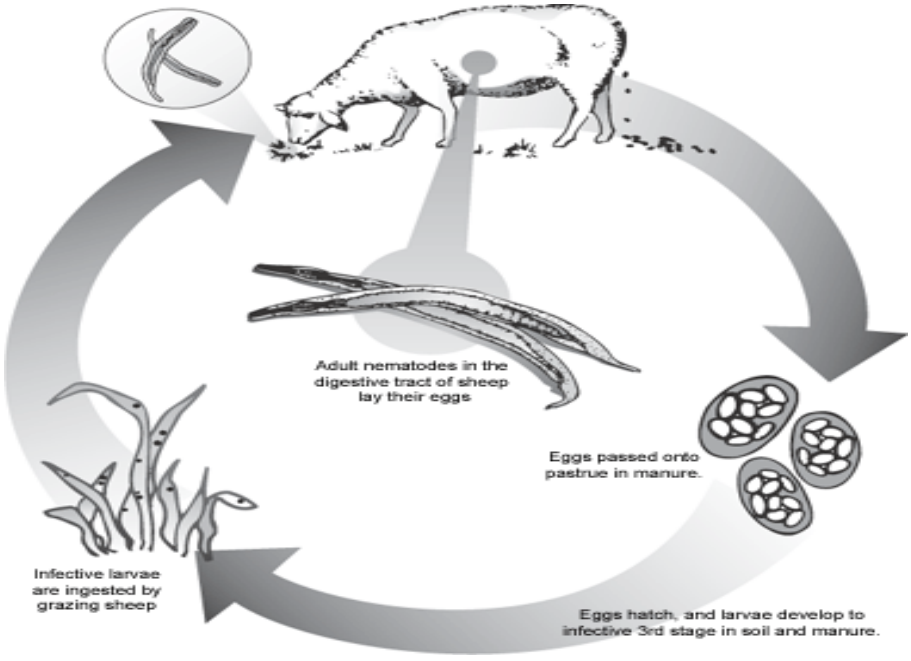
Figure 1.11: Life-cycles of free-living and parasitic nematodes.

a) *C. elegans* life-cycle. As a free-living nematode, the *C. elegans* life-cycle takes place in the environment. Eggs hatch and develop from L1, L2, L3 and L4 before undergo a final moult to adult. Under certain conditions (such as crowded or limitated food source) dauer stages develop. Dauer stages are resistant and non-feeding. When the conditions revert to normal, dauer stages develop into L4 before becoming adult.

b) Parasitic Trichostrongyle life-cycle e.g *H. contortus*, *T. circumcincta.* Eggs are passed in the faeces and hatch. The L1, L2 and L3 develop in the environment. The infection occurs when the definitive host grazes and eat grass containing infective L3. In the definitive host, the L3 develop into L4 and to adult. (Image courtesy of Virginia State University http://pubs.ext.vt.edu/410/410-027/410-027.html)


EGG
L1
L2
L3
L4
ADULT
DAUER

(a)


Adult nematodes in the digestive tract of sheep lay their eggs
Eggs passed onto pastrue in manure.
Eggs hatch, and larvae develop to infective 3rd stage in soil and manure.
Infective larvae are ingested by grazing sheep

(b)

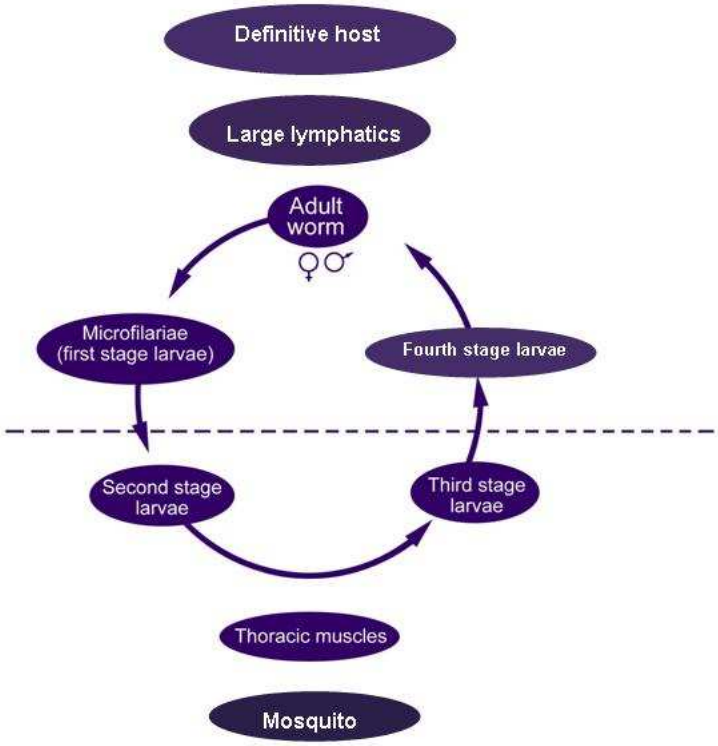


Figure 1.12: Filarial worm life-cycle.

Filarial worms (such as *B. pahangi or B. malayi)* require a mosquito to complete the life-cycle. During a blood meal, an infected mosquito introduces L3 filarial larvae onto the skin of the definitive host, where they penetrate into the bite wound. L3 develop into L4 and adults that commonly reside in the lymphatics. After mating, female worms produce Mf. The Mf migrate into lymph and then circulate in the bloodstream. A mosquito ingests the Mf during a blood meal. The Mf develop into L2 and subsequently into L3. The L3 can infect another human when the mosquito takes a blood meal. (Image courtesy of Medscape http://emedicine.medscape.com/article/998011-overview)

1.5.2 What do we know about Hsp90 in nematodes?

Much of the information on Hsp90 in nematodes comes from studies on *C. elegans*, a free living nematode that is often used as a model for parasitic species. The *C. elegans* genome contains a single *hsp90* gene (*daf-21*) located on chromosome V, which is 74% and 76% identical to human *hsp90α* and *hsp90β* respectively (Birnby *et al.*, 2000). *daf-21* has a molecular weight of approximately 83 kDa as seen by Western blotting. By *in situ* hybridization, *daf-21* mRNA in L1 was shown to be expressed in the germline precursor cells and the head region of *C. elegans* (Inoue *et al.*, 2003). In adult males, *daf-21* was distributed mainly in germline cells similar to hermaphrodites. The over-expression of *daf-21* in the germline suggests that it may be involved in the maintenance and maturation of germline precursors. A strong signal was also observed in spermatocytes but no signal was observed in mature sperm (Inoue *et al.*, 2003). In the same studies, Inoue and co-workers showed that under stress conditions, *daf-21* mRNA was expressed all over the body of *C. elegans*. Interestingly the localization of *daf-21* mRNA and DAF-21 protein are not the same in the L1 and adult stages. *daf-21* mRNA was detected only in germline cells at both the L1 larva and adult stages, whilst the DAF-21 protein was localized not only in germline cells but also in somatic cells at both stages (Inoue *et al.*, 2003).

The transcription of *hsp90* in *C. elegans* is increased 10-15-fold in dauer larvae compared to other life cycle stages (Dalley & Golomb, 1992). The dauer stage is an alternate third stage larva in *C. elegans* that enhances survival of the organism under harsh conditions. In the dauer stage, larvae do not eat because their oral orifice is blocked by an internal plug (Riddle *et al.*, 1981). The reason

why *daf-21* mRNA is highly abundant in dauer stages compared with other life cycle stages remains unknown. It may provide additional chaperone activity under stressful conditions. However, the expression of *daf-21* mRNA decreased sharply within the first 75 minutes of recovery after worms had been stimulated to emerge from the dauer stage (Dalley & Golomb, 1992). It has been suggested that Daf-21/Hsp90 is involved in the formation of dauers in *C. elegans* acting in the same pathway as *daf-11* (a transmembrane guanylyl cyclase) in regulating chemosensory transduction in several types of sensory neurons (Thomas *et al.*, 1993). Furthermore, the dauer is a long-lived stage of *C. elegans* and other studies have also shown that *daf-21* is highly expressed in long-lived mutants of *C. elegans* (Morley & Morimoto, 2004).

Two mutations have been characterised in *C. elegans hsp90* namely, *daf-21(p673)* and *daf-21(nr2081)* (Birnby *et al.*, 2000). *daf-21(p673)* is a point mutation (E292 to K) that causes relatively limited phenotypes. This is not a null mutation and may result in a protein that specifically fails to stabilize chemosensory transduction components because mutants have a range of sensory defects and reduced fertility (Birnby *et al.*, 2000). *daf-21(nr2801)* is a true null mutation and has an 860 bp deletion with a 3-bp insertion. *daf-21(nr2081)* arrests growth at the L2 to L3 stage (Birnby *et al.*, 2000), indicating the importance of Hsp90 in *C. elegans*. RNAi studies on *daf-21* revealed the necessity for Hsp90 in adult worms. Studies by Piano *et al* (2000) showed that injection of dsRNA into hermaphrodite worms results in an embryonic lethal phenotype in the progeny and a defect in egg production, which again suggests, the important roles of Hsp90 in *C. elegans*.

Hsp90 in the filarial parasite *B. pahangi*, is 82% identical to *C. elegans* Hsp90, 67% identical to *D. melanogaster* Hsp90 and 69% identical to human Hsp90 (Thompson *et al.*, 2001). However, the expression of Hsp90 in *B. pahangi* is constitutive and no significant increase in the level of Hsp90 was observed in any life cycle stage of *B. pahangi* exposed to elevated temperatures (Devaney *et al.*, 2005). These results suggest that in *B. pahangi*, Hsp90 is not a major heat-inducible protein. *B. pahangi* Hsp90 contains all five features of Hsp90s that are common to others eukaryotic proteins. However, *B. pahangi* Hsp90 has a long charged region encompassing the amino acids 235–320 (Thompson *et al.*, 2001). *B. pahangi* Hsp90 contains two putative ATP-binding domains (amino acids 93–99 and 135–142) and a large number of potential phosphorylation sites. Analysis by Southern blots showed that *B. pahangi hsp90* is a single copy gene (Thompson *et al.*, 2001), as in *C. elegans*.

Skantar and Carta (2004) showed that the plant parasitic nematodes, *Heterodera glycines* and *Meloidogyne javanica*, contain a single *hsp90* gene similar to *C. elegans* (Birnby *et al.*, 2000) and *B. pahangi* Hsp90 (Thompson *et al.*, 2001). *H. glycines* Hsp90 was shown to be 83% indentical to *B. pahangi* Hsp90, 82% to *C. elegans* and 73% to *D. melanogaster*. Despite the high level of conservation and homology between *C. elegans* Hsp90 and other eukaryotes, *C. elegans* Hsp90 is resistant to GA treatment and failed to bind to GA (David *et al.*, 2003; Devaney *et al.*, 2005). The structure of Hsp90 is well-conserved between *B. pahangi* and *C. elegans*, the only major feature of significance being the shorter CR in *C. elegans* compared to *B. pahangi*.

1.5.3 C. elegans as a model for the study of parasitic nematodes

C. elegans is a small free-living nematode usually found in the soil in temperate climates all around the world. It has a direct life-cycle and is very easy to maintain in the laboratory. It is only 1 mm long. The first paper on the use of *C. elegans* for genetic studies was published in 1974 (Brenner, 1974) and since then, it has become one of the most interesting model organisms to study, especially in developmental biology (reviewed by Gilleard, 2004). A major problem faced by researchers working on parasitic nematode is the lack of *in vitro* culture systems and the requirement to propagate life-cycles *in vivo*. As a result, the technology and genomic resources for parasitic nematode are still lagging behind those available for *C. elegans*.

In addition, there are no reverse genetic methods available to study parasitic nematode gene function (Gilleard, 2004). One of the powerful methods to study gene function is RNA-mediated interference (RNAi)(Fire *et al.*, 1998). This mechanism of gene-silencing was shown to be very reliable and effective in many organisms including insects, planarians, protozoa and mammalian cells (Britton & Murray, 2006). In *C. elegans* RNAi can be achieved by injection of double-stranded RNA (dsRNA) into the adult worm body, by soaking in dsRNA or by feeding the worms with *E. coli* expressing dsRNA (Kamath *et al.*, 2003). Using RNAi to knock-down a gene can result in an observable phenotype. Based on the phenotypes observed it can give a starting point for studying the gene of interest in greater detail. The known phenotypes of RNAi in *C. elegans* can be assessed through the Wormbase website (http://www.wormbase.org/db/searches/basic). In parasitic nematodes, there are several reported successful applications of RNAi e.g. in *Nipponstrongylus brasiliensis* (Hussein *et al.*, 2002), *Heterodera*

glycines and *G. pallida* (Urwin *et al.*, 2002), *Meloloidogyne incognita* (Bakhetia *et al.*, 2005), *B. malayi* (Aboobake & Blaxter, 2003), *Onchocerca volvulus* (Lustigman *et al.*, 2004), *Trichostrongylus colubriformis* (Issa *et al.*, 2005) and *H. contortus* (Kotze & Bagnall, 2006; Samarasinghe *et al.*, 2010). The success of RNAi in these parasitic nematodes suggests that the RNAi pathway does exist in some parasitic species and that some genes can be silenced by dsRNAi (Britton & Murray, 2006; Samarasinghe *et al.*, 2010). Furthermore, the level of target transcript or sites of gene expression influence susceptibility to RNAi by soaking (Samarasinghe *et al.*, 2010). However, the success of RNAi varies enormously with parasite species, life cycle used and genes targeted. RNAi studies by Geldhof *et al* (2005) revealed that the success of RNAi approaches is variable in *H. contortus*. Under certain circumstances, it is possible to suppress gene expression but RNAi only works on a limited number of genes and the effect is often small and hard to reproduce.

C. elegans was the first multi-cellular organism to have its genome completely sequenced in 1998. This provided a very important resource for bioinformatics and functional analysis of *C. elegans* genes. As more genomes for parasitic nematodes become available, it will be possible to identify important genes which can provides a good reference for understanding the molecular basis of the evolutionary adaptations of nematodes. In addition it will provide further understanding of the many adaptations associated with parasitic life-cycles (Wasmuth *et al.*, 2008). The draft sequence of the *B. malayi* genome has been published (Ghedin *et al.*, 2007) and sequencing the *H. contortus* genome is on-going. There also several sequencing projects for other species of veterinary importance which can be accessed through the website (http://sanger.ac.uk/Projects/helminths/).

C. elegans can provide a useful platform to study gene function particularly in parasitic nematodes (Gilleard, 2004). A parasite gene of interest can be expressed in *C. elegans* which then provides a tool to study its function. One of the methods is to introduce the gene of the interest into a *C. elegans* mutant strain to ask whether it can restore the wild type phenotype. The majority of the successful interspecies rescue experiments reported to date come from work using *H. contortus* (Britton & Murray, 2006). For example, *H. contortus* isotype 1 ß-tubulin (*tub-1)* was the first parasitic gene to be functionally expressed in *C. elegans* (Kwa *et al.*, 1995). In *C. elegans*, a mutation in the ß-tubulin gene, *ben-1* conferred resistant to benzimidazole compounds (Driscoll *et al.*, 1989). By expressing the *H. contortus tub-1* susceptible gene in *C. elegans ben-1* mutants, the resistance phenotype was reversed. In contrast expressing the *H. contortus tub-1* resistant gene in the mutant had no effect on the susceptibility of the mutant to benzimidazole (Kwa *et al.*, 1995). This experiment proved that interspecies mutant rescue can be used to study the function of a parasite gene.

1.6 Hsp90 and its role in drug resistance

1.6.1 The ability of Hsp90 to 'buffer' mutations has important implications

Recently, a number of studies have suggested that Hsp90 may be involved in facilitating rapid evolutionary changes in organisms such as *D. melanogaster* (Rutherford & Lindquist, 1998; Sollars *et al.*, 2003), and *Arabidopsis thaliana* (Queitsch *et al.*, 2002). The study of Rutherford & Lindquist (1998) showed that back-crossing a *D. melanogaster hsp90* mutant with a standard laboratory strain resulted in morphological abnormalities in the offspring e.g. deformed eyes or legs, bristle duplications, change in wing shape or venation etc. In addition,

when the wild-type *D. melanogaster* strain was raised on food containing GA, similar abnormalities were produced as observed for the *D. melanogaster hsp90* mutants. It was proposed that these cryptic mutations are not observed when a sufficient amount of Hsp90 is normally expressed. In the plant, *A. thaliana,* Hsp90 was also suggested to act as a buffer for genetic variation (Queitsch *et al.*, 2002). That study extended the early concept of Hsp90 buffering of morphological traits to environmental responses. In *Arabidopsis* they showed that inhibition of Hsp90 function by treatment with GA revealed several phenotypes which were heritable even in the absence of GA. This effect may be conserved in other organisms, potentially influencing the pace and nature of evolution.

1.6.2 Hsp90 and its role in drug resistance in infectious agents

Recent studies on Hsp90 have shown that it is required in *Saccharomyces cerevisiae* to survive the acute stress of drug exposure and also for some types of drug resistance. The original study of Cowen & Lindquist (2005) demonstrated that Hsp90 was important for the acquisition of resistance to azole compounds in *S. cerevisiae*. The azole target Erg11 which is involved in the biosynthesis of ergosterol, a component of fungal membranes (Sanglard, 2002, Odds *et al.*, 2003). Compromising Hsp90 functions with GA or radicicol was sufficient to restore azole sensitivity to otherwise resistant *S. cerevisae*. The emergence of fluconazole resistance under rapid selection was shown to be highly dependent on elevated levels of Hsp90 and this resistance was abrogated when Hsp90 expression was reduced (Cowen & Lindquist, 2005). In that system, inhibition of an Hsp90 client protein, calcineurin, mimicked Hsp90 inhibition suggesting that Hsp90 might act via calcineurin. Importantly, the role of Hsp90 in drug

resistance in *S. cerevisiae* was also conserved in a number of medically important pathogenic fungi such as *C. albicans* and *Aspergillus fumigatus* (Cowen *et al.*, 2009, Singh *et al.*, 2009). Calcineurin is a serine/threonine protein phosphatase (Dobson *et al.*, 1999) and according to Singh *et al* (2009), in *Candida albicans,* Hsp90 inhibition blocks calcineurin activation, which leads to increased sensitivity to the novel anti-fungal compound, echinocandin. These results suggest a role for Hsp90 in acquired drug resistance in fungi.

In *Leishmania donovani* Hsp83, the orthologue of Hsp90, was shown to be expressed at higher levels in isolates from patients unresponsive to pentavalent antimonials, compared to isolates from patients responsive to these drugs (Vergness *et al.*, 2007). The mechanism by which pentavalent antimonials kill the parasite is not well understood, but they may interact with trypanothione, the main reduced cellular thiol of the parasite (Wyllie *et al.*, 2004). *L. donovani* isolates from unresponsive patients were not only resistant to pentavalent antimonials but also exhibited cross-resistance to miltefosine and amphotericin B. Furthermore, transfection of the *hp83* gene from an *L. donovani* resistant strain into an *L. donovani* sensitive strain resulted in an increased resistance to trivalent antimonials. The sensitive isolates overexpressing Hsp83 were shown to be more than two-fold resistant to trivalent antimonials compared to control parasites and also showed cross-resistant to metefosine. The parasites over-expressing Hsp83, were protected from programmed cell death after treatment with trivalent antimonials, suggesting that Hsp83 might increase drug resistance compared to the control group.

1.6.3 Hsp90 and drug resistance in tumour cells

In tumour cells, Hsp90 is an important target for chemotherapy because it is required for the stability and function of many molecules that are important for tumour cell growth. In tumours, Hsp90 interacts with a variety of protein including steroid hormone receptors, Ser/Thr and Tyr kinases and proteins with various other functions (Maloney & Workman, 2002). Several studies in tumour cells suggest that Hsp90 is involved in drug resistance. For example, a breast cancer cell line was shown to be resistant to docetexal chemotherapy due to involvement of the PI-3K pathway which is modulated by Hsp90 (Xing *et al.*, 2008). Another interesting example of Hsp90 involvement in drug resistance in tumour cells comes from studies on malignant melanomas. According to Banerji (2009), malignant melanoma is relatively resistant to most conventional forms of cancer treatment such as chemotherapy and radiotherapy. Studies by Davies *et al* (2002), showed that an Hsp90 client protein, BRAF, is highly mutated in malignant melanoma. Hsp90 inhibition resulted in degradation of the mutant BRAF compared to wild-type BRAF, which suggests that Hsp90 might mediate resistance in malignant melanoma through BRAF. In addition, Hsp90 expression was found to be higher in metastasic melanomas (Faingold *et al.*, 2008; McCarthy *et al.*, 2008). The higher expression level of Hsp90 in resistant tumour cells suggests that Hsp90 may be one mechanism by which tumour cells acquire drug resistance.

A study by Costantino *et al* (2009) on TRAP1, an homologue of Hsp90 which resides in the mitochondria, in drug resistant HT-29 human colorectal carcinoma cells showed that TRAP1 was up-regulated after treatment with three chemotherapeutic agents namely, 5-fluoracil, oxaliplatin and irinotecan

compared to wild-type HT-29 cells. In addition, they also showed that over-expression of TRAP1 leads to 5-fluoracil, oxaliplatin and irinotecan resistant phenotypes in HT-29 cells. In contrast the inhibition of TRAP1 using sperherdin increased the sensitivity of resistant HT-29 to oxaliplatin and irinotecan but not to 5-fluoracil. These results suggest that TRAP1 may be involved in a multi-drug resistant phenotype in *vitro*.

A common mechanism of drug resistance in many tumour cell types is over-expression of drug transporters such as P-gp or detoxification systems. According to Bertram *et al* (1996), Hsp90ß is associated with P-gp in multi-drug resistant colon carcinoma (LoVo) cells and in a murine cell line (S180). These cell lines were shown to be resistant to doxorubicin. In that study, they showed that Hsp90ß was expressed constitutively in the resistant LoVo cell line but not in sensitive LoVo cells. In addition, in the doxorubicin resistant murine cell line, S180, Hsp90ß was also expressed at higher levels compared to a sensitive cell line. In a subsequent study, it was demonstrated that Hsp90ß and P-gp co-precipated in the lysates of resistant LoVo cells, which suggests a direct interaction between P-gp and Hsp90. Furthermore, reducing the Hsp90ß levels by antisense oligonucleotides resulted in a reduced half-life of P-gp which increased the doxorubicin sensitivity of resistant LoVo cells by two-fold (Betram *et al.*, 1996). These results suggest an intracellular co-operation between Hsp90ß and P-gp and the involvement of Hsp90ß in doxorubicin resistance.

1.6.4 Could Hsp90 have a role in anthelmintic resistance in nematodes?

In nematodes anthelmictic resistance is one of the major problems that affects livestock production worldwide. Up to now, the industry has been

dependent on anthelmintic drugs from three different groups (benzimidazoles, imidazothiazoles and macrocylic lactones) for control of infection. Anthelmintic resistance can be defined as a decrease in anthelmintic efficiency against a parasite which is normally susceptible to that drug (Sangster & Gill, 1999). Resistance is a heritable character, therefore many of these nematodes will have inherited their parent's ability to survive exposure to drug (Prichard *et al.*, 1980). The use of anthelmintic drugs to control gastrointestinal nematode put a massive selection pressure on nematode populations which led to emergence of anthelmintic resistance. According to Gilleard (2006), current understanding of the mechanism of benzimidazole resistance is more highly developed, but for imidazothiazole or macrocylic lactones, the underlying mechanisms are not well defined. Studying the mechanisms of anthelmintic resistance in parasitic nematodes is difficult because of the complexity of the life-cycles and because it is not possible to genetically manipulate these organisms. Because of that, *C. elegans* has been used as a model to study the mechanisms of drug resistance in parasitic nematodes.

Benzimidazoles exert their roles by binding to tubulin. Tubulin is a major component of microtubules which play an important role in sustaining the cellular homeostasis in eukaryotic cells. By binding to tubulin, benzimidazoles cause disruption of microtubule structure and function which lead to cell death. Benzimidazole resistance in nematodes appears to be associated with an alteration in ß-tubulin genes, which reduces the high affinity binding of drug to tubulin (Lacey, 1988). In *C. elegans*, sensitivity to benzimidazole is associated with a single gene, *ben-1*, which encodes ß-tubulin isotype-1 (Driscoll *et al.*, 1989). Deletion or mutation of *ben-1* in *C. elegans* does not impair the development of *C. elegans* but it leads to benzimidazole resistance (Driscoll *et*

al., 1989). In the study of Kwa *et al* (1995), the wild-type ß-tubulin isotype-1 gene from *H. contortus* was expressed in benzimidazole-resistant *C. elegans* and restored the sensitivity of *C. elegans* to benzimidazole compunds.

Anthelmintics of the imidazothiazole group were introduced to the market in 1968. The most widely used are levamisole, pyrantel and morantel. Levamisole is a nicotinic acetylcholine (nACh) receptor agonist (Aceves *et al.*, 1970; Aubry *et al.*, 1970) with a selective action on nematode receptors. Levamisole binds to nACh receptors and mimics this neurotransmitter resulting in paralysed worms (Sangster, 1999) and spastic paralysis and egg-laying defects in *C. elegans* (Holden-Dye & Walker, 2007). This muscle paralysis is thought to be caused by activation of the excitatory nACh receptors on the nematode body wall muscle (Martin *et al.*, 1996).). In *C. elegans*, at least four subunits, *unc-38, unc-29, unc-63* and *lev-1* contribute to the levamisole receptor (Culetto *et al.*, 2004). All these genes were suggested to be associated with levamisole resistance in *C. elegans*. Development of levamisole resistance in parasitic nematodes is also likely to be polygenic as reported in *H. contortus* (Sangster *et al.*, 1991) and *Oesophagostomum dentatum* (Varady *et al.*, 1997, Robertson *et al.*, 1999). However the potential genes involved in levamisole resistance have not yet been identified in parasitic nematodes.

The macrocylic lactones that have been used commercially to combat nematode infections are ivermectin, moxidectin and abemectin. Ivermectin is a fermentation product of the micro-organism *Streptomyces avermilitis* (Holden-Dye & Walker, 2007). Ivermectin causes paralysis of worm somatic musculature (Kass *et al.*, 1980; Kass *et al.*, 1982) and inhibits feeding of the nematode by blocking pharyngeal pumping (Geary *et al.*, 1993; Martin *et al.*, 1996; Sangster &

Gill, 1999), causing starvation and worm death. The mechanism of ivermectin resistance is thought to be associated with alterations in the GluCl receptor in *C. elegans* (Cully *et al.*, 1994). As reviewed by Gilleard (2006), the mechanism of ivermectin resistance is complex and multigenic. For example, in *C. elegans* it was shown that resistance to ivermectin requires mutations in at least three genes which are members of the GluCl family, namely in *glc-1, avr-14* and *avr-15* (Dent *et al.*, 2000, Holden-Dye & Walker, 2007). Mutation of any two of these genes did not confer ivermectin resistance on C. elegans (Dent *et al.*, 2000). Furthermore, recent studies in parasitic species suggest the involvement of P-gp in ivermectin resistance in *H. contortus* (Xu *et al.*, 1998). Expression of P-gp mRNA was shown to be higher in ivermectin-selected than unselected strains of *H. contortus*, which suggests that ivermectin resistance in *H. contortus* may be associated with a more efficient drug efflux system.

In tumour cells and fungi, the emergence of drug resistance was suggested to be associated with Hsp90 and its co-chaperones. However, the mechanisms underlying the development of drug resistance in parasitic nematodes are not well understood. Because of that, research addressing the mechanisms of anthelmintic resistance must therefore be a main concern in this field.

1.7 Aims of thesis

The specific aims are detailed below.

- Although the sequence of *daf-21* (Hsp90) in *C. elegans* is highly conserved, it is unique amongst eukaryotes because it fails to bind to GA. In contrast, previous studies have shown that *B. pahangi* Hsp90 can bind GA. The first aim of the project is to determine whether the failure of *C. elegans* Hsp90 to bind to GA is the norm or the exception amongst nematodes and to determine whether GA binding is associated with particular clades of nematodes.

- *B. pahangi* is known to be susceptible to Hsp90 inhibition, but no information exists to explain why GA kills the worm. A second aim of the thesis is to characterise *B. pahangi* Hsp90 in more detail with a view to a better understanding of the function of Hsp90 in nematodes.

- Nematode resistance to anthelmintics threatens the viability of the small ruminant industry in many countries all over the world. In tumours and fungi, Hsp90 is known to be involved in drug resistance. So the third aim of the thesis is to investigate whether Hsp90 plays a role in drug resistance in nematodes.

Chapter 2

Materials and Methods

2 Materials and methods

2.1 GA-binding assay

2.1.1 Nematodes

24 species of nematode from different clades were used for the analysis of GA-binding. These included both free–living and parasitic species. Some nematode strains were received from the *Caenorhabditis elegans* Genetics Centre (CGC, USA) including *C. elegans* Bristol N2, *C. elegans* JT6130, a *daf-21* mutant, *C. briggsae*, *Oscheius tipuleae* and *Pristionchus pacificus*, while others were obtained from relevant laboratories (Appendix 1). Parasitic species analysed included *Trichinella spiralis* and *Trichuris muris* (both clade I). Clade III nematodes included the filarial species (*Brugia pahangi*, *Dirofilaria immitis*, *Onchocerca ochengi*, *Acanthocheilonema viteae*), Ascarid species (*Toxocara cati*, *Ascaris suum*, *Parascaris equorum*), Anisakids (*Anisakis simplex*, *Pseudoterranova decipiens*) and *Anguillicola crassus*. Clade IV species included *Strongyloides ratti*, *Globodera rostochiensis* and *G. pallida* and the free-living species *Panagrellus redivivus*. Clade V species included *Haemonchus contortus*, *Teladorsagia circumcincta*, *Nipponstrongylus brasiliensis* and *Heligmosomoides polygyrus*. Mixed life-cycle stages were used for all free-living nematodes, while for parasitic species, usage was determined by availability of particular life-cycle stages. In most cases adult males and females were analysed with the following exceptions: *H. contortus*, L3; *T. spiralis*, L1 muscle stage larvae; *S. ratti* adults were free-living rather than parasitic females; *Globodera* spp. J2 stages; *O. ochengi* only males were used; *A. simplex* and *P. decipiens* L3 were used.

2.1.2 Maintenance of nematodes

2.1.2.1 Maintenance of free-living species

C. elegans N2, *C. elegans* JT6130 (a *daf-21* mutant which carries a point mutation in *daf-21*, E292 to K)(Birnby *et al.*, 2000), *C. briggsae*, *O. tipuleae* and *P. pacificus* were cultured at 20^{o}C on NGM agar (Appendix 4.3) inoculated with *Escherichia coli* strain OP50 as a food source as described previously (Brenner, 1974). Free-living worms were washed with M9 (Appendix 5.2) several times, concentrated by centrifugation at 2000 rpm for 2 min and stored at -80^{o}C before use.

P. redivivus was received from Prof. Aaron G. Maule (Queen's University Belfast, UK) and was cultured on cooked oats in Petri dishes. 100 g of oats (Quaker Oats) were cooked in some water and poured into Petri dishes (6 x 9 cm). Worms were then poured over the surface of the cold cooked oats and were maintained at room temperature. As the population of the worms bloom, the worms will crawl onto the lid from where they were colleted by washing off the lid with M9. Worms were pelleted by centrifugation at 2000 rpm at 4^{o}C and kept at -80^{o}C before use.

2.1.2.2 Maintenance of parasitic species

For parasitic species, adult stages were obtained from relevant laboratories from their respective hosts and were frozen as soon as possible at -80^{0}C (see Appendix 1). L3 Larval stages of *A. simplex* and *P. decipiens* were obtained from infected fish (kindly provided by Dr. S. Martin and Dr. I. Coombs respectively). *T. spiralis* L1 (kindly provided by Dr. C. Lawrence), muscle stages were obtained by digestion of infected mouse muscle using standard methods

(Wakelin & Wilson, 1977; Knight *et al.*, 2000). *H. contortus* L3 were obtained by harvesting the eggs from infected sheep faeces and culturing to the L3 stage (received from Prof. D. Knox).

The cysts of *G. rostochiensis* (obtained from Dr. J. Jones) and *G. pallida* (Dr. C. Fleming) containing infective stage juveniles (J2) were placed in large Petri dishes half filled with tap water and left for 5 days at room temperature. After 5 days, the water was replaced with tomato root diffusate (TRD) (kindly provided by Dr. J. Jones, Scottish Plant Breeding Institute, UK). The J2 begin to hatch a few days after TRD has been applied. The larval stages of *Globodera* spp. were colleted and cleaned by floating them on a sucrose gradient. The larvae were suspended in 5 ml ddH_2O in a 15 ml conical tube and 5 ml of sucrose solution (Appendix 5.3) was added. 500 µl of ddH_2O was overlaid on top of the surface of sucrose solution which was then centrifuged for 5 min at 2000 rpm. The nematodes will float at the interface between the sucrose and the water overlay. The nematodes were collected and rinsed several times in clean water and kept in -80^{o}C before use.

2.1.3 Protein extraction for GA-binding experiments

Extracts of worms were prepared in ice-cold TNES buffer (Appendix 2.1.1), with protease inhibitors added prior to use (1 tablet per 10 ml TNES) (Complete Mini Protease Inhibitor Cocktail, Roche Diagnostics, UK). Liquid Nitrogen was poured into a mortar, the worm pellet was added and ground to a fine powder using the pestle. The worm extract was then re-suspended in 1.0 ml TNES containing protease inhibitors and the mortar washed out with 0.5 ml TNES. Following incubation on ice for 15 min, the extract was pelleted at 13200 rpm at 4^{o}C for 15 min and the supernatant was retained. In some cases, where

the worm material was very limiting, e.g. *D. immitis* and *O. ochengi*, the lysates were prepared using small glass homogenisers. 100 µl of ice-cold TNES with protease inhibitors was added and the worms were ground. The supernatant was then prepared as described previously.

2.1.4 Protein concentration estimation

Protein concentrations were estimated using the Bio-Rad protein assay in a 96 well microtitre format. Usually samples were diluted 1:50 and then different amount added to the assay e.g. 5, 10, 15, 20 µl. Samples and bovine serum albumin (BSA) standards were incubated in the working reagent (Bio-Rad, UK) for 8 min at room temperature, allowing a colorimetric reaction to occur. The optical density (OD) at 562 nm was measured using OpsysMR (Dynex Technologies) and the protein concentration of the sample (in duplicate) determined through comparison with a standard curve prepared using BSA.

2.1.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Soluble extracts of worms were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-Protean Cell System (Bio-Rad, UK). Protein separation was carried out in 10% polyacrylamide gels (Appendix 2.2.8). Approximately, equal amounts of worms extract (2-4 µg) was mixed with SDS-PAGE sample cocktail (Appendix 2.2.1), boiled for 3 min, centrifuged at 13200 rpm for 3 min and applied to the gel. Following electrophoresis at 200 volts for 60 min in running buffer (Appendix 2.2.4), gels were stained with Coomassie blue (Appendix 2.3.1) for 60 min and destained in the destaining solution (Appendix 2.3.3). In some cases, worms were directly solubilised with appropriate volume of SDS-PAGE sample cocktail and boiled for

3 min followed by centrifugation at 13200 rpm for 3 min. After gel electrophoresis, gels were incubated with gentle agitation in Coomassie blue R-250 (Sigma, UK)(Appendix 2.3.1) for 60 min at room temperature. Gels were then destained in 10% acetic acid solution (Appendix 2.3.3).

2.1.6 Western blotting

Proteins separated by SDS-PAGE were transferred onto nitrocellulose Hybond^{TM}-c extra (NCP) membranes (Amersham, UK) using the Mini Trans-Blot Electrophoretic Transfer Cell System (Bio-Rad, UK) at a constant voltage of 100V for 60 min. The transfer set up consisted of pre-wetting the NCP membrane in transfer buffer (Appendix 2.2.5). The transfer sandwich was created with a fibre pad pre-soaked in transfer buffer. Then one pre-soaked filter paper was laid on top, followed by the equilibrated NCP membrane. The SDS-PAGE gel, having been equilibrated in transfer buffer for 15 min, was then laid on the NCP membrane. Another pre-soaked filter paper was laid on the gel and finally, a pre-soaked fibre pad completing the transfer sandwich. The completed cassette was placed in the module. The same procedure was repeated for a second gel (if required). Following protein transfer, the NCP membrane was stained with Ponceau S (Appendix 2.3.5) for 5 min with agitation to check that the transfer of protein to the NCP membrane was complete. The NCP membrane then was incubated in blocking solution (Appendix 2.2.10) for 60 min at room temperature with gentle agitation.

2.1.7 Antibody detection of transferred proteins

Two antibodies were used in this study to detect Hsp90. The first antibody was raised in rabbits to a recombinant protein equivalent to the C-terminal 238

amino acids of *B. pahangi* (Devaney *et al.*, 2005). The second antibody used was a well-characterised monoclonal antibody AC88, raised to Hsp90 from the water mould, *Achlya ambisexualis*, which recognises Hsp90 from many different species. AC88 was kindly provided by Dr. David Toft (Mayo Clinic College of Medicine, Rochester, USA).

Following the blocking incubation, the NCP membrane was washed 3 x 5 min with washing buffer (Appendix 2.2.11) at room temperature. The primary antibody (*B. pahangi* anti-Hsp90 or AC88 monoclonal anti-Hsp90 antibody) was diluted at appropriate concentration in incubation solution (Appendix 2.2.10) and applied to the membrane overnight at 4^{o}C with gentle agitation. Following overnight incubation, the membrane was washed 3 x 5 min with washing buffer before incubation in either HRP-conjugated anti-rabbit IgG (1:10,000) or HRP-conjugated anti-mouse IgG (1:10,000) (both Sigma, UK) for a further 60 min at room temperature. The NCP membrane was then washed 3 x 5 min in washing buffer. In some experiments, blots were probed with an antibody to actin (Sigma, UK), as a loading control, at a dilution of 1:1000 and followed by anti-mouse IgG as a secondary antibody.

The Enhanced Chemiluminescence (ECL) Plus System was employed (Pierce, UK) to detect any bound antibody by autoradiography. The autoradiograph was subjected to quantitation by scanning and analysing using FluorChem^{TM} IS-5500 software. The Integrated Density Value (IDV) was measured for each sample and subsequently used to calculate the ratio of the signal relative to the loading control (actin).

2.1.8 Preparation of geldanamycin solid support

GA was derivatised as described by Whitesell *et al* (1994). 1.0 mg GA (InvivoGen, UK) was dissolved in 180 µl chloroform. 1,6-Hexamethylenediamine (Sigma, UK) was weighed out and dissolved in chloroform to give a final concentration of 28.7 mg/ml. 71 µl of the 1,6-Hexanediamine in chloroform was added to the GA solution. The mixture was rocked for 4 h at room temperature in the dark to yield 17-hexamethylenediamine-17 demethoxygeldanamycin. A yellow to purple colour change is evidence of this reaction. The mixture was extracted with 1.0 ml of 0.01 M NaOH by centrifugation at 2000 rpm for 2 min. These steps were repeated three times. The mixture was dried under nitrogen and the powder stored at -20^{o}C at this point.

400 µl of DMSO was added to 1.0 mg derivatised GA. Simultaneously, 400 µl of Affi-Gel 10 beads (Bio-Rad, UK) were washed with 1.0 ml cold iso-propanol. The beads were centrifuged at 2000 rpm for 2 min and the supernatant was removed. These steps were repeated four times. Then, the beads were washed with 1.0 ml of DMSO and dried. At this point, the bead volume looks much smaller. 400 µl of ~5 mM 17-hexamethylenediamine-17 demethoxygeldanamycin prepared as described previously was mixed with the beads and agitated for 4 h at room temperature in the dark. At this point the beads were expected to be purple. The supernatant was removed and the beads were washed with 1.0 ml DMSO followed by three washes with 1.0 ml TNES. The beads were rocked overnight in 1.0 ml 1.0 M ethanolamine, pH 8.3 to block non-specific binding. Finally the beads were re-suspended at 50% v/v in TNES. The beads can be stored at 4^{o}C at this point. The same procedures were applied for control beads, reacted with DMSO alone.

2.1.9 GA pull-down assay

50 µl of GA beads were blocked in 1% BSA in TNES (Appendix 2.1.1) for 60 min at room temperature. The beads were then washed 3 x 5 min with 1.0 ml TNES on a rocker and were then centrifuged at 2000 rpm for 2 min. The supernatant was removed and the beads were retained for the binding assay. 300-500 µg of worm protein in a volume 300 µl was mixed with 50 µl packed volume of GA or control beads for 2.5 h at 4^{o}C. The beads were then washed 5 x 10 min with 1.0 ml of TNES buffer by centrifugation. Bound proteins were eluted in 40 µl SDS-PAGE sample cocktail by boiling for 3 min followed by centrifugation at 13200 rpm for 3 min. Proteins eluted from GA beads and control beads were loaded on a 10% SDS-PAGE gel and were analysed by Western blotting as described previously.

2.1.10 The specificity of Hsp90 binding to GA beads

For selected nematode species which showed positive results in pull-down assays, the experiments were repeated in the presence of excess GA to block the binding sites in order to determine the specificity of the binding. Worm extracts were pre-incubated with soluble GA at 2.0 µM or 20.0 µM for 60 min at room temperature then used in a pull-down assay exactly as described above.

2.2 Further characterisation of *Brugia* Hsp90

2.2.1 Analysis of different life-cycle stages of B. pahangi

Three different life-cycle stage of *B. pahangi* were available for the analysis of Hsp90 expression levels. Adults and Mf were obtained from gerbils while L3 were obtained from mosquitoes (*Aedes aegpti*) using standard methods

(Devaney & Jecock, 1991). Gerbils were killed by CO_2 inhalation. Worms were obtained by repeated peritoneal lavage with Hanks Balanced Salt Solution (HBSS, Gibco, UK) at 37°C. Adults were washed in HBSS and were kept at -80°C until they were required for the preparation of parasite extracts. The Mf were collected by washing out the peritoneal cavity with HBSS medium at 37°C. The combined washes were centrifuged 1000 rpm for 5 min. The Mf pellet was then purified to remove red blood cell contamination by adding 1.0 ml sterile ddH_2O, and the Mf were collected by centrifugation at 1000 rpm for 5 min. To separate Mf from contaminating white blood cells, Mf were centrifuged on Histopaque (Sigma, UK). 1.0 ml of Mf suspension was carefully layered onto 5.0 ml of Histopaque and centrifuged at 1200 rpm for 10 min. White blood cells float at the interface, while Mf were colleted from the pellet. The number of Mf in a 20 µl aliquot was counted and the number of Mf in the total volume calculated. The Mf were kept at -80°C until use.

2.2.2 B. pahangi Hsp90 expression level following exposure to heat shock or GA

Heat shock proteins respond to changes in the environment including elevated temperatures and chemical compounds. In order to investigate Hsp90 expression levels after exposure to heat shock or to GA, adult *B. pahangi* were recovered from gerbils as described previously. 10 adult females were cultured in 10 ml RPMI-1640 culture medium (Appendix 4.5) Worms were heat shocked by incubating in a water bath at 41°C for 60 min and were allowed to recover at 37°C for a further 2 h. The control group for the experiment was incubated in a water bath at 37°C for 3 h. Worms were then directly solubilised by boiling in 100 µl SDS-PAGE sample cocktail for 3 min and the supernatant collected by

centrifugation at 13200 rpm for 3 min. 5 µl of supernatant were loaded onto 10% gel and analysed by SDS-PAGE and Western blotting.

In other experiments, 10 *B. pahangi* adult females were cultured in the presence of GA. 1 mg GA was dissolved in 171 µl DMSO to give a stock of 10 mM GA which was then diluted in RPMI-1640 culture medium to give a final concentration of 1.0 µM GA. Adult worms were then incubated in RPMI-1640 containing GA at 37^{o}C in an atmosphere of 5% CO_2 in air for 24 h. The control group for the analysis was incubated in RPMI-1640 culture medium containing DMSO alone for 24 h at 37^{o}C. Worms were directly solubilised in 100 µl of SDS sample cocktail and were centrifuged at 13200 rpm for 3 min. Equal volumes (5 µl) were analysed by SDS-PAGE and Western blotting as described previously using actin as a control.

2.2.3 Estimating the proportion of B. pahangi Hsp90 that binds to GA beads

The aim of this experiment was to investigate the percentage of the cytosolic Hsp90 that could bind to GA beads. Here, the supernatant from one GA pull-down was applied to a second GA pull down, for a total of five times. 50 µl of GA-beads prepared as described in Section 2.1.8 were mixed with 300 µg of *B. pahangi* extract in a volume of 300 µl for 2.5 h at 4^{o}C. The supernatant from the pull-down was retained for the next pull-down. Simultaneously, the beads for the first pull-down were processed as described previously in Section 2.1.9. These steps were repeated five times. Equal volumes of supernatant (20 µl) were loaded onto 10% SDS-PAGE gels and bound protein was analysed by SDS-PAGE and Western blotting. The bands on the autoradiograph were scanned and band densities were obtained using FlourChem^{TM} IS-5500 software.

2.2.4 Detection of Hsp90 in ES products of Mf and adult Brugia

In *B. malayi*, Hsp90 was reported to be secreted as an ES product (Kumari *et al.*, 1994). In order to investigate whether *B. pahangi* secretes Hsp90, two different stages of *B. pahangi* were analysed. Mf and adult *B. pahangi* were recovered from infected gerbils at approximately 120 days post-infection. Adult worms were washed several times in HBSS. After a final wash, approximately 100 *B. pahangi* female and male adult worms were cultured in 40 ml of RPMI-1640 culture medium (Appendix 4.5) at 37^{o}C in an atmosphere of 5% CO_2 in air for 48 h. Medium was changed every 24 h. Spent medium, containing ES products, was passed through a 0.45 µm sterile syringe filter (Iwaki, Japan) to remove Mf and ES was stored at -20^{o}C until analysis. A total of 80 ml spent culture medium was concentrated to approximately 150 µl in a 10,000 Mr cut off Viva Spin 15R Hydrasart (Sartorius Biolab Products, UK) by centrifugation at 3000 g for 45 min. The Mf were collected as described previously in Section 2.2.1. Approximately 1.05 x10^{5} Mf were incubated in RPMI-1640 medium as described previously at 37-^{o}C in an atmosphere of 5% CO_2 in air for 48 h. The medium containing ES products was centrifuged at 1000 rpm for 5 min to remove Mf and the supernatant was processed as described above for adults. To detect the presence of Hsp90 in *B. pahangi* ES products, 10 µl of concentrated ES products were added to 10 µl of SDS-PAGE sample cocktail. The mixture was boiled for 3 min and centrifuged for further 3 min at 13200 rpm. Samples were then analysed by SDS-PAGE and Western blotting.

Gels were initially analysed by Coomassie blue staining but no bands were observed, so a silver staining protocol was used. Following electrophoresis, the gel was incubated in fixative enhancer solution (Appendix 2.3.4.1, Bio-Rad, UK)

for 20 min with gentle agitation. The gel was then rinsed in 400 ml of ddH_2O for 3 x 10 min. ddH_2O was discarded and the gel was stained with staining solution (Appendix 2.3.4.2) for approximately 20 min or until the desired staining intensity was reached. Finally, the gel was placed in stop solution (Appendix 2.3.4.3) containing 5% acetic acid. The gel was then rinsed in ddH_2O for a further 5 min.

2.2.5 Identification of B. pahangi Hsp90-associated proteins

Hsp90 is the central component of a complex chaperone system. In most organisms, Hsp90 associates with a large set of co-chaperones to mediate the activation and maturation of client proteins. Inhibition of Hsp90 function will lead to the disassociation of these co-chaperones and the destabilisation of client proteins. GA was shown to be toxic to *B. pahangi*, but it is not known how inhibition of Hsp90 function kills the worm. In an attempt to address this point, two different approaches were carried out. In the first approach, proteomic analysis of *B. pahangi* adult worms cultured *in vitro* in the presence or absence of GA was carried out. In the second approach, attempts were made to purify Hsp90-associated proteins using a modified pull-down approach (Neef *et al.*, 2010). This modified pull-down relies upon the binding of biotin-labelled GA to Hsp90 protein complexes. The complexes are then isolated using Neutravidin-agarose beads as described below.

2.2.5.1 GA-biotin

B. pahangi protein extracts were prepared in biotin-binding buffer with protease inhibitors added prior to use as described by Neef *et al* (2010). 1.0 mg of worm extract was incubated with 100 µl of GA-biotin (InvivoGen, UK) for 15 h

at 4°C. Hsp90 associated proteins were captured by incubating the mixture with Neutravidin-agarose beads for 90 min at 4°C. The beads were pelleted and the supernatant was discarded and beads were then washed 3 x 1.0 ml in biotin-binding buffer and bound proteins were eluted in SDS-PAGE sample cocktail by boiling for 5 min. Bound proteins were analysed by Coomassie blue staining.

2.2.5.2 Protein lysate preparation for proteomic analysis

B. pahangi adult worms were recovered and processed as described previously in Section 2.2.1. 60 adult worms were cultured in RPMI-1640 culture medium as described previously, containing 1.0 µM GA at 37°C for 24 h. The control group was cultured under identical conditions in the presence of an equivalent volume of DMSO. Both samples were kept at -80°C until required for preparation of the parasite extracts.

Worm extracts were prepared using a mortar and pestle in 2D electrophoresis lysis buffer (Appendix 3.1) with protease inhibitors added prior to use (Roche Diagnostics, UK) exactly as described in 2.1.3. The supernatant was retained for protein precipitation. 4x sample volume of 80% cold acetone was added and incubated on ice for 30 min, following which the extract was pelleted at 13200 rpm at 4°C for 15 min and the supernatant was discarded. The protein pellet was dried for approximately 5 min at room temperature and then re-suspended in 50 µl lysis buffer and the protein concentration estimated using the protein assay as described in Section 2.1.4. Lysis buffer was added to a give final protein concentration of 5 mg/ml.

2.2.5.3 Isoelectric Focusing (IF)

50 µg in 10 µl volume of GA-treated *B. pahangi* lysate was labelled with 1.0 µl Cy3 and 50 µg in 10 µl volume of untreated group was labelled with Cy5 by incubating the mixture for 30 mins on ice in the dark. Cy3 and Cy5 dye were provided by GE Healthcare Bio-Sciences, UK. 1.0 µl of 10 mM lysine was added to each tube and incubated for 10 mins in the dark. The labelled protein lysates were then mixed together and added to 430 µl rehydration buffer (Appendix 3.3) containing Bromophenol blue and DDT. The sample was incubated for 30 min at room temperature in the dark followed by centrifugation at 13200 rpm for 3 min. 450 µl of sample was loaded into an Immobilised pH Gradient (IPG) strip holder using a Gilson pipette. The samples were distributed along the bed of the strip holder.

A 24 cm IPG strip, ph 4-7 (GE Healthcare Bio-Sciences, UK) was removed from the protective package using forceps. The IPG strip was laid into the IPG holder with the sticky surface facing down to make contact with the sample solution. The barcode was placed toward the pointed end of the IPG strip holder. The strip was then overlaid with 1.0 ml mineral oil, sufficient to cover the entire strip to avoid evaporation. The IPG strip was placed on the electrode bed of an IPGphor Isoelectric Focusing System (GE Healthcare Bio-Sciences, UK) with the pointed end of the holder oriented towards the anode. IF was performed at 20^{o}C for approximately 25 h at a limited current of 50 µAmp/IPG strip and accumulated volts from 70000-80000 Vhs. After IF, the IPG strip was removed for analysis or alternatively could be stored at -20^{o}C.

2.2.5.4 Second dimension SDS-PAGE

Prior to SDS-PAGE, the IPG strip was equilibrated in equilibration buffer. The IPG strip was removed from the strip holder and transferred to a cylindrical tube containing the first equilibration buffer (Appendix 3.4) supplemented with 100 mg DTT/10 ml. The IPG strip was incubated for 15 min on a rocker at room temperature. The solution was then poured off and the IPG strip was incubated with a second equilibration buffer (Appendix 3.5) containing 250 mg iodoacetamide/10 ml on a rocker for 15 min at room temperature.

The second dimension 12% gel was prepared using standard recipes (Appendix 3.6). The equilibrated strip was laid onto the top of the SDS-PAGE gel so that there was complete contact between gel and strip, and 1.0 ml of 0.5% agarose in SDS-PAGE running buffer with Bromophenol blue was added to overlay the strip. Electrophoresis was performed using an Ettan DALT*twelve* electrophoresis unit (Amersham Bio-Sciences, UK) in running buffer at constant voltage of approximately 6V for 25 h at 25^{o}C or until the dye front reached the base of the gel. Following electrophoresis, gels were scanned using Typoon 9400 scanner and the images were analysed using the Differential In-Gel Analysis (DIA) software module of the DeCyder batch processor (Amersham Bio-Sciences, UK).

In a further experiment, GA-treated and control worms were analysed separately by 2D-gel electrophoresis. Approximately 50 µg of each worm lysate in 50 µl volume was added to 400 µl rehydration buffer (Appendix 3.3) containing Bromophenol blue and DTT. The sample was incubated for 30 min at room temperature in the dark followed by centrifugation at 13200 rpm for 3 min. 450 µl of each sample was loaded into different IPG strips. The samples were

distributed along the bed of the strip holder, and were processed as described above for IF and second dimension SDS-PAGE. Following electrophoresis, the gels were stained with Coomassie blue G-250 (Appendix 2.3.2) then washed with ddH_2O to remove the blue residue off the staining tray. The gels were then incubated with 10% acetic acid solution (Appendix 2.3.3) with gentle agitation until the desired staining intensity was reached.

The Ettan Spot handling workstation (Amersham Bio-Sciences) was used to spot the differences between the GA-treated and control group. Spots of interest were excised and placed in 1.5 ml eppendorf tubes. The samples were then subjected to in-gel trypsin digest.

2.2.5.5 In-gel trypsin digest

The gel pieces were washed for 60 min in 500 µl 100 mM ammonium bicarbonate followed by a further wash in 50% acetonitrile/100 mM ammonium bicarbonate for 60 min at room temperature. The supernatant was discarded and gel pieces were washed in 500 µl 50% acetonitrile/100 mM ammonium bicarbonate with shaking for 60 min, followed by 50 µl of acetonitrile for 10 min. After 10 min, the solvent was removed and the gel pieces were dried in a vacuum centrifuge. 20 µl of 0.2 µg/µl sequencing grade modified Porcine Trypsin (Promega, UK) in 25 mM ammonium bicarbonate was added to rehydrate each gel piece. 25 mM ammonium bicarbonate was added to cover the gel pieces (~20 µl) and then left overnight at 37^{o}C for digestion. The following day, the tube was centrifuged to pellet the gel pieces. Supernatant in the tube was then transferred to the 96-well plate. 20 µl of 5% formic acid was added to the tube and incubated for 20 min followed by incubation in 20 µl acetonitrile for a further 20 min with gentle shaking. The tube was centrifuged and supernatant

was then transferred into the same well as the first extract. The combination extracts were dried completely in a Speedvac. The dried digested samples were then subjected to analysis by mass spectrometry where all peptides samples were separated on a liquid chromatography-electrospray (LC) system before analysing by ionization/multi-stage mass spectrometry (ESI-MS).

2.2.6 Post-translational modification of Hsp90

Post-translational modifications of Hsp90 are known to be essential in regulating Hsp90 chaperoning functions (Rose *et al.*, 1987; Scroggins *et al.*, 2007). In order to investigate whether post-translational modification could influence the ability of Hsp90 to bind to GA, recombinant human Hsp90α (rHsp90α) and Hsp90 isolated from the breast cancer cell line (SkBr3) were compared. Ideally, purified *B. pahangi* Hsp90 would have been used but was not available. The rHsp90α (StressMarq, Biosciences Inc., UK) used in the study was expressed in *E. coli,* and therefore had no post-translational modifications. SkBr3 cells were obtained from Dr. Tina Rich (Faculty of Veterinary Medicine, University of Glasgow, UK). SkBr3 cells were extracted in extraction buffer (Appendix 2.1.2) by adding 1.0 ml of buffer containing protease inhibitors. The mixture was shaken gently to lyse the cells following which the supernatant was collected by centrifugation at 13200 rpm for 3 min at 4^{o}C. The supernatant was assayed for protein concentration as described previously in Section 2.1.4. Approximately, equal amounts of SkBr3 extract (2-4 µg) was mixed with SDS-PAGE sample cocktail, boiled for 3 min, centrifuged at 13200 rpm for 3 min and loaded onto the 10% gel and analysed by SDS-PAGE and Western blotting. For the pull-down assay, 5 µg of rHsp90α and 500 µg of SkBr3 lysates were used, exactly as described in Section 2.1.9. In order to demonstrate that the binding of

Hsp90α and SkBr3 to GA beads was specific, both samples were subjected to competition assay with free soluble GA. Protein extracts were pre-incubated with soluble GA at 1.0 μM or 2.0 μM for 60 min at room temperature followed by pull-down. Bound proteins were analysed as described previously.

2.2.7 Competition assays with B. pahangi Hsp90

2.2.7.1 Competition assay with GA, ATP and novobiocin

In order to investigate whether the binding of *B. pahangi* Hsp90 to GA beads could be competed with other compounds that bind to Hsp90, a variety of experiments were carried out. Compounds used included Adenosine Tri-Phosphate (ATP) which is known to compete for GA binding, and Novobiocin, a drug which binds at the 3' end of Hsp90, but which can alter the ability of the 5' end of Hsp90 to bind to GA/ATP (Grenert *et al.*, 1997, Garnier *et al.*, 2002). For the competition assay with GA beads, 500 μg of worm extract was pre-incubated with competitors at different concentrations (Table 2.1) for 60 min at room temperature with gentle agitation. The control tube contained only worm extract and control beads. 50 μl of packed volume of GA or control beads, prepared as described previously, was then added to the mixture and incubated for 2.5 h at 4^{o}C. The beads were then washed 5 x 10 min with 1.0 ml of TNES buffer by centrifugation. Bound proteins were eluted in SDS-PAGE sample cocktail by boiling for 3 min and were centrifuged at 13200 rpm for 3 min. Proteins eluted from GA beads and control beads were run on a 10% SDS-PAGE gel and were analysed by Western blotting with antibody as described previously.

2.2.7.2 *B. pahangi* Hsp90 binding to ATP beads

ATP binds to Hsp90 at the N-terminal domain in the same binding site as for GA (Grenert *et al*., 1997, Garnier *et al*., 2002, David *et al*., 2003). These experiments were carried out in order to investigate whether free GA could compete the binding of Hsp90 to ATP beads. For the competition assays with ATP beads, 500 µg of worm extract was pre-incubated with free GA over a range of concentration from 250 nM to 2000 nM for 60 min at room temperature with gentle agitation. 100 µl volume of ATP beads (Sigma, UK) was added to the mixture and incubated for 1.5 h at 4^{o}C. The beads were then washed 5 x 10 min with 1.0 ml of ATP binding buffer (Appendix 2.1.4). Bound proteins were eluted in SDS-PAGE sample cocktail by boiling for 3 min and were centrifuged at 13200 rpm for 3 min. Proteins eluted from GA beads and control beads were run on a 10% SDS-PAGE gel and were analysed by Western blotting with antibody as described previously.

Table 2.1: Concentration of compounds used in competition assays with GA-beads

Competitor	Concentration
Soluble GA	0.0
	25 nM
	50 nM
	100 nM
	200 nM
	1000 nM
ATP	5.0 mM
	10.0 mM
	15.0 mM
	20.0 mM
Novobiocin	2.5 mM
	5.0 mM
	10.0 mM
	20.0 mM

2.2.8 Comparison of B. pahangi and C. elegans Hsp90

2.2.8.1 Hsp90 expression levels in *B. pahangi* and *C. elegans*

Hsp90 is a well conserved protein in most organisms. *C. elegans* Hsp90 was shown to be resistant to GA treatment and failed to bind to GA beads in pull-down assays (David *et al.*, 2003; Devaney *et al.*, 2005). In order to investigate the differences in Hsp90 sequence of *B. pahangi* and *C. elegans*, both sequences were compared using a Clustal W alignment.

In addition, Hsp90 expression levels between *B. pahangi* and *C. elegans* were compared in adult stages. *B. pahangi* adult worms were collected as described in Section 2.2.1, while *C. elegans* adult worms were collected by washing NGM plates with M9. Protein extracts of adult *B. pahangi* and *C. elegans* were prepared as described in Section 2.1.3 and assayed for protein concentration. Equal amounts of protein (2-4 µg) were loaded onto 10% SDS-PAGE gel and analysed by SDS-PAGE and Western blotting using actin as a loading control. The autoradiograph was scanned and analysed using FluorChem™ IS-5500 software. Hsp90 expression levels in *B. pahangi* and *C. elegans* were scored based on the ratio of Hsp90 to actin.

2.2.8.2 *B. pahangi* and *C. elegans* Hsp90 binding to ATP beads

ATP was shown to bind to the N-terminal domain of Hsp90, which is the same binding site for GA. ATP binding affinity can differ from one organism to another. It order to investigate the affinity of Hsp90 binding to ATP beads, *B. pahangi* and *C. elegans* extract were compared. Approximately 300 µg of worm lysates were incubated with 100 µl ATP beads (Sigma, UK) for 60 min at room temperature with gentle agitation. The beads were then washed 5 x 10 min in

ATP binding buffer. The bound proteins were eluted by boiling for 3 min in 30 µl of SDS-PAGE sample cocktail followed by centrifugation at 13200 rpm for 3 min. Samples were analysed by SDS-PAGE and Western blotting. Any band on the autoradiograph was scanned and analysed as described previously.

2.3 Hsp90 and drug resistance

In order to determine whether Hsp90 may play a role in drug resistance in nematodes, a number of experiments were carried out. Hsp90 levels were examined in anthelmintic resistant and susceptible *Teleodorsagia circumcincta*. In addition, Hsp90 expression in various strains of *C. elegans* was assessed. Finally, attempts were made to reduce Hsp90 levels in ivermectin-resistant *C. elegans* to investigate the effect of Hsp90 reduction on susceptibility to ivermectin.

2.3.1 Hsp90 expression levels in drug resistant and susceptible worms

In a previous study on tumour cells, Hsp90 was shown to be expressed at higher level in drug resistant cell lines compared to wild-type cell lines (Bertram *et al.*, 1996, Banerji, 2009; Constantino *et al.*, 2009). However, in nematodes no such information is available. In order to determine the Hsp90 expression levels in resistant and susceptible nematodes, three groups of resistant nematodes were analysed. A *T. circumcincta* resistant isolate (Tci5) and a susceptible isolate (Tci2) were obtained from Prof. David Knox (Moredun Research Institute, UK). Tci5 worms are known to be resistant to anthelmintics from all three broad spectrum families (benzimidazole, ivermectin and levamisole)(Bartley *et al.*, 2005). In contrast the Tci2 isolate is susceptible to all broad spectrum

anthelmintic families. Both of the strains were originally field isolates. *T. circumcincta* resistant and susceptible worms were extracted and assayed as described previously in Section 2.1.3. Approximately equal amounts of protein (2-4 µg) were analysed by SDS-PAGE and Western blotting, using actin as a loading control as described previously.

C. elegans CB3474 and *C. elegans* DA1316 were obtained from the *C. elegans* Genetics Centre (CGC, USA) for analysis. The *C. elegans* CB3474 strain shows resistance to benzimidazoles conferred by a mutation in the *ben-1* gene (ß-tubulin isotype-1) (Driscoll *et al.*, 1989). *C. elegans* DA1316 contains mutations in *avr-14*, *avr-15* and *glc-1* and exhibits high level resistance to ivermectin (Dent *et al.*, 2000). Worms were maintained on standard NGM plates as described in Section 2.1.2.1. For *C. elegans* CB3474 and *C. elegans* DA1316 mixed stages were analysed. Worms were directly solubilised in SDS-PAGE sample cocktail by boiling for 3 min and extracts were collected by centrifugation at 13200 rpm for 3 min. 5 µl samples were loaded onto 10% gels and analysed SDS-PAGE and Western blotting.

Two additional lines of ivermectin-resistant *C. elegans* were also available, IVM-6 and IVM-10. In contrast to *C. elegans* DA1316 and CB3474 which are the product of mutagenesis screen, IVM-6 and IVM-10 worm were selected by growth on increasing levels of ivermectin over a period of time. Therefore, these worms may be more similar to field isolates of drug resistant parasitic nematodes. IVM-6 worms are resistant to ivermectin at 6 ng/ml and IVM-10, to 10 ng/ml (James & Davey, 2009). These strains were obtained from the Institute for Biotechnology of Infectious Diseases, University of Technology Sydney, Australia. IVM-6 and IVM-10 worms were cultured on ivermectin plates containing

drug at 6 ng/ml or 10 ng/ml respectively (Appendix 4.7). For *C. elegans* IVM-6 and IVM-10 worms, adult stages were used for the analysis of Hsp90 levels. 30 worms were solubilised in 30 µl SDS-PAGE sample cocktail by boiling for 3 min and extracts were collected by centrifugation at 13200 rpm for 3 min. 5 µl of samples were loaded onto 10% gels and analysed SDS-PAGE and Western blotting.

2.3.2 C. elegans methods

2.3.2.1 Synchronisation of *C. elegans* cultures

For ivermectin sensitivity assays and RNAi feeding experiments, L4 stage worms were used. In order to get worms at the same stage of development, gravid adult worms were lysed in bleach solution containing 15% sodium hypochlorite solution (Appendix 5.1). Bleaching also cleans the worms of any bacterial contamination from the plates. Worms were cultured on standard NGM plates with *E. coli* OP50 as a food source and were allowed to grow for 3 to 4 days at 20^{o}C so that the plates contained many gravid adults. Gravid adults were collected by washing in M9 buffer in 15 ml conical centrifuge tubes and worms were pelleted at 2000 rpm at 4^{o}C for 2 min. The supernatant was removed without disturbing the worm pellet and 0.5 ml bleach solution was then added to the tube. The mixture was shaken gently for 3-5 min to help to lyse the gravid adults. Eggs were then pelleted by centrifugation at 2000 rpm for 2 min and the supernatant was discarded. Following four washes in M9 buffer, eggs were incubated in M9 and allowed to hatch overnight at 20^{o}C in an incubator. L1 hatched in M9 arrest development. The synchronised L1 stage worms were spotted onto standard NGM plates seeded with OP50 and were allowed to develop to early L4 stage at 20^{o}C before use in ivermectin sensitivity assays or in RNAi experiments.

2.3.2.2 Ivermectin sensitivity assays

Two assays were used for this analysis; a drug cytotoxicity assay and a motility assay. In the drug cytotoxicity assay, the growth of *C. elegans* from egg to gravid adult was scored, while in the motility assays, the motility of worms after exposure to ivermectin at different concentrations for 24 h and 48 h was scored. The drug cytotoxicity assay was applied as described previously by Hart (2006). *C. elegans* N2 and IVM-10 resistant worms were bleached as described previously in Section 2.3.2.1. Eggs were then re-suspended in M9 and 10 µl of the suspension was spotted onto plates containing containing 1 ng/ml, 5 ng/ml, 10 ng/ml ivermectin or DSMO as a control in triplicate and were incubated at 20^{o}C. The worms were allowed to grow for 3-4 days and growth was scored (egg to adult). The motility assay was applied as described previously (Arena *et al.*, 1995). Five L4 stage worms of *C. elegans* N2 or IVM-10 resistant worms were incubated in 24 well plates. Six replicates were made for each dilution of ivermectin. IVM-10 resistant worms were incubated in M9 culture containing 1 ng/ml, 5 ng/ml or 10 ng/ml ivermectin and DMSO as a control in 24 well plates. OP50 was added as a food source. Plates were incubated for 24 h and 48 h at 20^{o}C and worm motility was scored based on the percentage of worms that were still thrashing in the different wells. The activity of the worms was scored (see Table 2.2) and the score was converted to a numerical value.

Table 2.2: Scoring system for motility assay

Activity*	Score
Thrashing	3
Slow	2
Paralysed	1
Dead	0

*** All scores based on the movement or activity of the worm after 24 h and 48 h in liquid culture system containing ivermectin at different concentration (ng/ml). Each worm was observed for 10 sec.**

2.3.2.3 Reducing Hsp90 levels by RNA mediated interference (RNAi)

There are a variety of RNAi approaches that can be applied to *C. elegans* including injection of dsRNA into the hermaphrodite gonad, feeding the worms on lawns of bacteria expressing dsRNA, soaking worms in dsRNA or expressing dsRNA from a transgene (Kamath *et al.*, 2001, 2003). The experiments presented in this thesis involved the feeding of bacteria expressing dsRNA to *C. elegans*. *hsp90(RNAi)* constructs used in this thesis was prepared previously by Dr. Victoria Gillan (University of Glasgow, UK). Two different constructs was used; a 300 bp and a 75 bp construct, as described in Gillan *et al* (2009). Figure 2.2a shows a schematic time frame of a single experiment using RNAi by feeding.

2.3.2.4 Preparation of plates for *hsp90(RNAi),* bacteria preparation and induction

For the RNAi feeding experiment, worms were cultured on RNAi plates containing IPTG and ampicillin. IPTG and ampicillin were added to standard NGM agar (Appendix 4.3) to give a final concentration of 1.0 mg/ml and 25 µg/ml, respectively. Plates were prepared 4-7 days before use and kept at room temperature to allow the plates to dry. RNAi plates must be dried before seeding with bacteria because wet plates will affect the efficiency of RNAi (Kamath *et al.*, 2001).

HT115 (DE3) cells containing the RNAi construct of interest, or containing an empty vector were used in these studies. Bacteria from glycerol stocks were spotted onto L-Broth agar plates (Appendix 4.2) containing 50 µg/ml ampicillin and 15 µg/ml tetracycline. Plates were incubated overnight at 37^{o}C. Next day, one large colony of bacteria was picked and inoculated into L-Broth (Appendix 4.1) containing 50 µg/ml ampicillin and grown overnight with shaking at 37^{o}C in

an orbital incubator. The bacteria were then seeded onto RNAi plates as described above and incubated overnight at room temperature to allow the bacteria to grow and to begin induction by IPTG. The same procedures were applied to the control group, which consisted of bacteria expressing empty vector as a food source.

2.3.2.5 Optimising the conditions for *hsp90(RNAi)*

In order to determine the optimal conditions for *hsp90(RNAi)* and to estimate the reduction of Hsp90 levels in RNAi worms, L4 stages of *C. elegans* N2 were incubated at different temperature or were fed for different time periods. Synchronised L4 were incubated at 16^{o}C, 20^{o}C or 25^{o}C for 24 h feeding time. To determine the reduction of Hsp90 expression levels after 24 h on RNAi or control plates, 30 worms were solubilised in 30 µl of SDS-sample cocktail by boiling for 3 min and collected by centrifugation at 13200 rpm for 3 min. 5 µl of worm extract were loaded on a 10% SDS-PAGE gel and the Hsp90 expression levels were analysed by SDS-PAGE and Western blotting. In other experiments, L4 were incubated at 20^{o}C for 6 h, 12 h or 24 h on RNAi or control plates. Hsp90 expression levels were analysed by SDS-PAGE and Western blotting. Any bands on the autoradiograph were scanned and analysed using FluorChem^{TM} IS-5500 software as described previously in Section 2.1.7. For all experiments, actin was used as a loading control. These experiments were carried out using both the 300 bp and 75 bp *hsp90(RNAi)* constructs.

2.3.2.6 Scoring the efficiency of *hsp90(RNAi)* by counting progeny

To investigate the efficiency of *hsp90(RNAi),* adult worms were fed on the 300 bp or 75 bp *hsp90(RNAi)* construct for a 24 h period and then the number of

progeny were counted. After 24 h on *hsp90(RNAi)* plates, 3 adults were transferred onto each of three fresh NGM plates and were allowed to lay eggs for 24 h at 20^{o}C. Adults were then removed from the plates. The number of eggs laid by adults was counted and plates were incubated for further 24 h at 20^{o}C. Embryonic lethality, early larval phenotypes and post-embryonic phenotypes were scored using a dissecting microscope.

2.3.2.7 Ivermectin sensitivity assays following *hsp90(RNAi)*

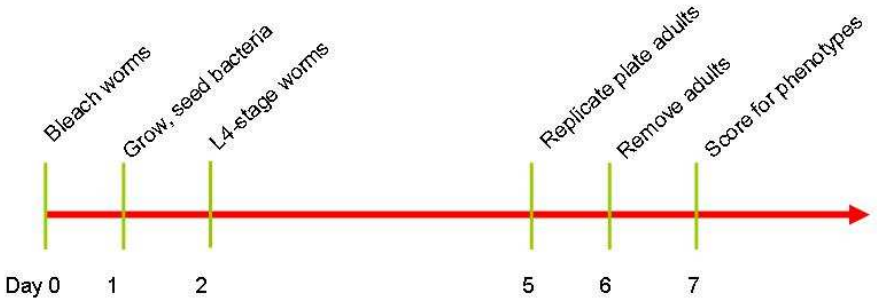
C. elegans N2 and IVM-10 resistant worms were fed on the 75 bp *hsp90(RNAi)* construct or the control empty vector for 24 h at 20^{o}C. After 24 h on RNAi plates, worms were subjected to the drug cytotoxicity assay on plates containing ivermectin at 1, 5 or 10 ng/ml or DSMSO alone as a control. For the motility assay, five worms were transferred into 24 well plates in six replicates. The medium contained 1, 5, or 10 ng/ml of ivermectin or DMSO alone as a control. OP50 was added as food source and the plates were incubated at 20^{o}C for 24 h or 48 h. Figure 2.2b and 2.2c shows a schematic time frame of a single experiment using RNAi by feeding.

Figure 2.1: Schematic time-frame of RNAi feeding experiment.

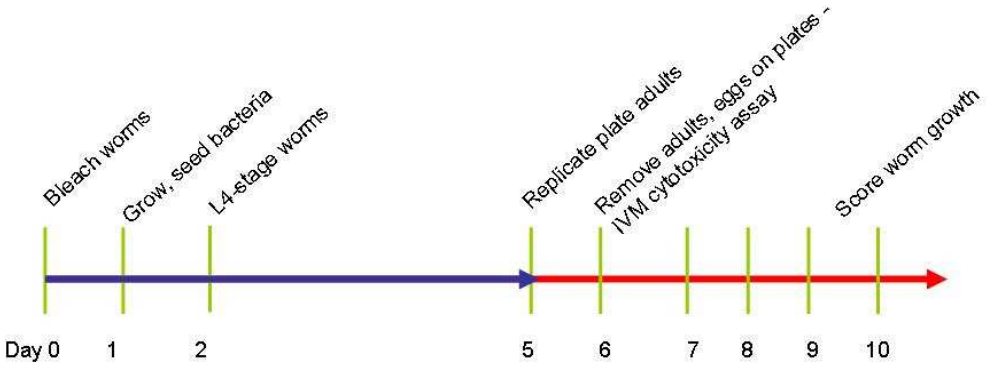
a) Feeding time-line.

b) Schematic of the time-frame of experiment using RNAi followed by ivermectin cytotoxicity assay.

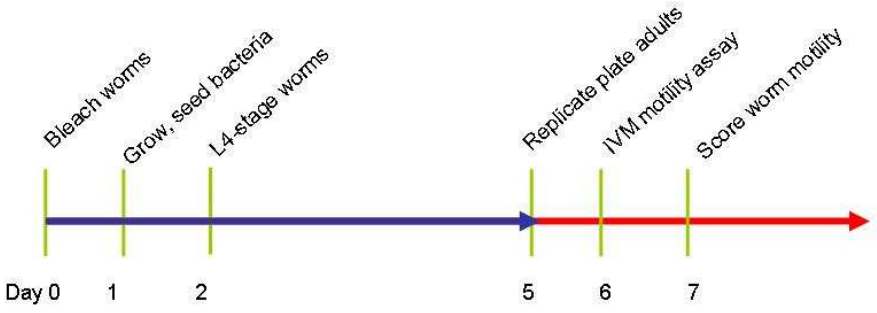
c) Schematic of the time-frame of experiment using RNAi followed by ivermectin motility assay.



(a)



(b)



(c)

Adapted from Kamath *et al* (2003)

2.4 Statistical analysis

The Student t-test and Mann-Whitney *U*-test was used to determine the statistical of significance differences between groups. Differences between groups were considered significant at $p<0.05$.

Chapter 3

Hsp90 And The Biology of Nematodes

3 Hsp90 and the biology of nematodes

3.1 Introduction

Hsp90 is a unique molecular chaperone which is involved in chaperoning important proteins such as kinases, telomerase, helicases and transcription factors. Because it is involved in multiple cellular processes, knock-out of *hsp90* is lethal to most organisms, so the functions of Hsp90 have been studied using inhibitors. One of the best characterised Hsp90 inhibitors is geldanamycin (GA) which is synthesised by *Stremptomyces hygroscopicus*. By binding to Hsp90, GA prevents the binding of ATP, resulting in the destabilisation of client proteins (as reviewed in Chapter 1). The sequence of Hsp90 is highly conserved and in most eukaryotes Hsp90 is inhibited in the presence of GA, the exception to date being *Caenorhabditis elegans*. *C. elegans* Hsp90 failed to bind GA while retaining the ability to bind ATP (David *et al.,* 2003). In that study no discernable effects on larval viability, dauer development, adult fertility and life span were observed despite continuous passage of worms for three generations on plates saturated with GA at 178 µM. Furthermore, *C. elegans* showed no ill-effect when maintained on a lawn of *S. hygroscopicus* secreting GA. In contrast, studies on the filarial nematode *Brugia pahangi*, revealed the importance of Hsp90 in these worms (Devaney *et al.*, 2005). After being exposed to GA at 1.0 µM (the concentration known to inhibit mammalian Hsp90 activity), there was a significant decrease in microfilariae release from adult worms. Similar results were obtained at 500 nM GA but not at 100 nM GA. The female worms appeared flaccid and become progressively more lethargic until, by day 7-8, 100% were dead, while the male worms were more sensitive to GA with 100% dying by day 5-6. This finding suggests that GA has a direct macrofilaricidal effect on *Brugia*.

Moreover, in solid phase pull-down assays, Hsp90 of *B. pahangi* bound to GA as analysed by Western blotting using an antiserum to *B. pahangi* Hsp90. Thus, the effect of GA on filarial worms contrasts with its lack of activity on *C. elegans*. The failure of *C. elegans* Hsp90 to bind GA was suggested to be an example of adaptive evolution, as *C. elegans* shares the same ecological niche of the soil with the *Streptomyces* species that elaborates GA (David *et al.*, 2003). Given the different results with the GA binding assay using extracts of *B. pahangi* and *C. elegans,* some questions arise. Why does Hsp90 of *C. elegans* not bind to GA while Hsp90 of *B. pahangi* does? Is the ability of Hsp90 to bind to GA associated with a particular nematode life-cycle or with a specific clade of nematodes? Is GA resistance or susceptibility the norm amongst nematodes? What are the differences between the structure of non-binding and binding of Hsp90s?

Many nematodes are important parasites of humans, animals and plants and are responsible for significant economic losses worldwide. Nematodes can be grouped into five separate clades based on the phylogenetic analysis of small subunit ribosomal RNAs (as reviewed in Introduction). Nematode life-cycles are very diverse between species. Some species have a free-living larval stage within the environment, e.g. the Trichostrongyles, while some species, e.g. filarial worms, are obligate parasites transmitted by the bite of an infected intermediate host. Furthermore other species are permanently parasitic and transmitted by ingestion of infected tissues, such as *Trichinella spiralis,* a clade I species.

At the start of this study only two species of nematode had been examined for GA binding, *B. pahangi*, a clade III parasitic nematode and the free-living clade V species *C. elegans*. The aim of the studies described in this

chapter was to examine a range of other nematode species for GA binding capacity and to determine whether the ability of Hsp90 to bind GA is associated with a particular clade of nematodes or particular nematode life cycles.

3.2 Results

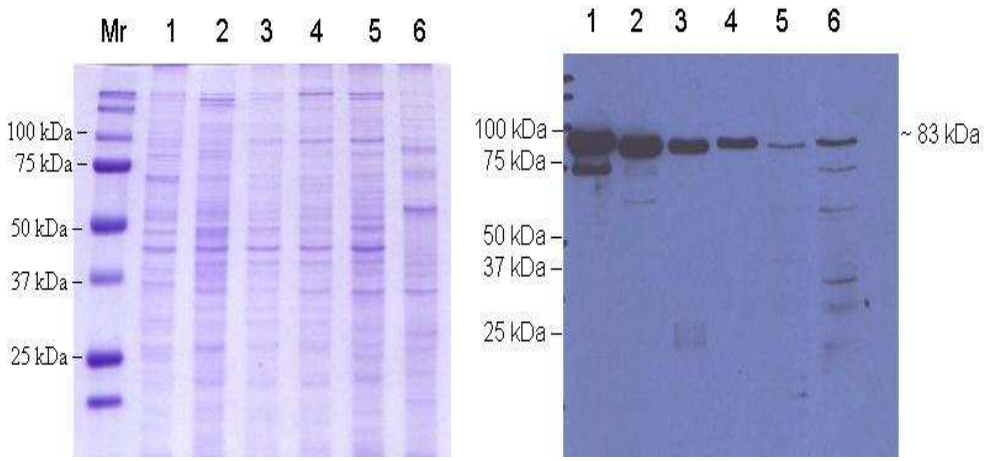
3.2.1 Do other clade V species bind GA?

Initial studies involved analysing extracts of a variety of nematodes to detect the expression of Hsp90 using the *B. pahangi* anti-Hsp90 antibody or the AC88 monoclonal antibody by immuno-blotting. *B. pahangi* lysate was used as a positive control in all experiments because it reacted with both of the antibodies. Any species which showed no reactivity with the *B. pahangi* anti-Hsp90 antibody was tested with the AC88 monoclonal antibody. Nine species of clade V nematode were used for the analysis, of which five were free-living species and four were parasitic. Free-living species were wild-type *C. elegans* N2, *C. elegans* JT6130, a *daf-21* mutant (which carries a point mutation in *daf-21*(E292 to K), *Caenorhabditis briggsae*, *Pristionchus pacificus* and *Oscheius tipulae*. For these worms, mixed life-cycle stages were analysed. All free-living species reacted positively with the *B. pahangi* anti-Hsp90 antibody (see Figure 3.1). The strongest signal was obtained with *B. pahangi* extracts, with *O. ochengi* and *P. pacificus* extracts showing a relatively weak signal. The autoradiograph shown in Figure 3.1b was developed for 10 sec. The signal for *O. tipulae* and *P. pacificus* improved with increased time of exposure (results not shown).

The parasitic species from Clade V analysed were *Haemonchus contortus, Nipponstrongylus brasiliensis, Teladorsagia circumcincta and Heligmosomoides polygyrus*. In all cases, adult males and females were used. All these parasitic species tested reacted with the *B. pahangi* anti-Hsp90 antibody (see Figure 3.2) except for *H. contortus* adults. The anti-Hsp90 monoclonal antibody (AC88) also failed to detect Hsp90 in *H. contortus* adult worms (results not shown). A new

extract of *H. contortus* adult worms was prepared by directly solubilising in SDS-PAGE sample cocktail by boiling for 3 minutes. The soluble supernatant was run on a 10% gel (see Figure 3.3a) and was analyzed by western blot (Figure 3.3b). From the blot it was clear that Hsp90 from *H. contortus* adult worms that were directly solubilised in SDS-PAGE sample cocktail reacted well with the *B. pahangi* anti-Hsp90 antibody, but no signal was obtained from the TNES-soluble extract of *H. contortus* adults. In an attempt to overcome this problem, different protease inhibitors from SIGMA (containing AEBSF, Aprotinin, Bestatin hydrochloride, E-64, Leupeptin hemisulfate salt and pepstatin A) and the Complete Mini Protease Inhibitor from ROCHE (formulation not available) were compared using the standard extraction methods as described before. Then the extracts were run on a 10% gel and analyzed by Western blot. Even using the new protease inhibitor cocktail there was still no signal from *H. contortus* adult TNES-soluble lysates using the *B. pahangi* anti-Hsp90 antibody. This result suggested that the TNES-soluble extract of *H. contortus* adults may be degraded during the preparation of the extract, despite the presence of protease inhibitors. Examination of the Coomassie blue stained gel shown in Figure 3.3a (Lane 3) suggested that this explanation may be correct. Adult worms extracted directly into SDS-sample cocktail show a range of bands of different molecular weight (Lane 2), while the TNES-soluble extract of adult worms shows few bands and an abundance of material at the bottom of the gel (Lane 3). As *H. contortus* adults are known to be rich in proteases (Knox et al., 1993), attempts were made to identify Hsp90 in TNES-soluble extracts of *H. contortus* L3. A TNES-soluble extract of *H. contortus* L3 reacted with *B. pahangi* anti-Hsp90 antibody (Figure 3.3b) and was used for subsequent pull-down assays.

Previous results had shown that *C. elegans* Hsp90 failed to bind GA. Pull-down assays were carried out on a range of free-living species belonging to clade V in order to investigate the GA binding ability of Hsp90 from other free-living nematodes. As shown in Figure 3.4, no binding was observed to GA beads for any free-living species tested. Analysis of clade V parasitic species produced similar results, with no binding observed (Figure 3.5). *B. pahangi* lysate was used as a positive control in all experiments (see Lane 1, Figure 3.4 and Figure 3.5). In all cases, blots were exposed for extended periods of time, but no positive reactions were observed compared to the *B. pahangi* positive control. Each pull-down was repeated at least twice with similar results. These data demonstrated that like *C. elegans*, Hsp90 from the other clade V free-living and parasitic nematodes tested do not bind GA. In all experiments, worm lysate were incubated with control beads in pull-downs and were analysed as for GA beads. Non-specific binding of Hsp90 to control beads was not observed.

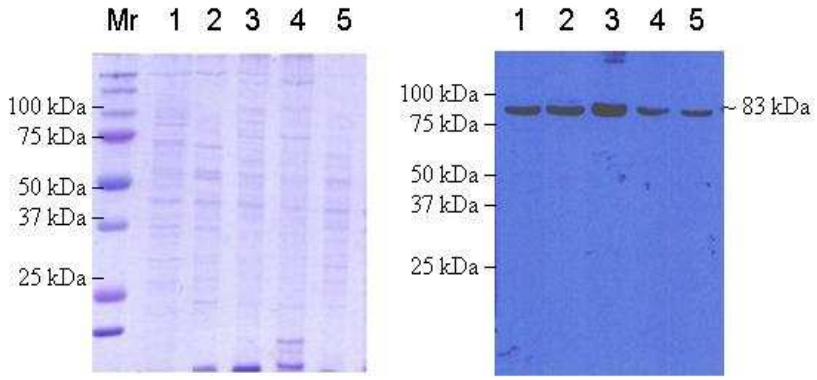


(a) (b)

Figure 3.1: All clade V free-living nematodes were observed to react with the antibody to *B. pahangi* anti-Hsp90.

a) Coomassie blue stained gel of different worm extracts. Approximately equal amounts of proteins were loaded on a 10% SDS-PAGE gel as assessed by Coomassie blue staining for *B. pahangi* (Lane 1), *C. elegans* (Lane 2), *C. elegans* (JT6130) (Lane 3), *C. briggsae* (Lane 4), *O. tipulae* (Lane 5) and *P. pacificus* (Lane 6). Mr represents the molecular weight markers.

b) Free-living clade V nematodes reacted with the *B. pahangi* anti-Hsp90 antibody. A strong signal was observed with *B. pahangi* extract (Lane 1); weaker signals were observed for *O. tipulea* and *P. pacificus* (Lane 5 and Lane 6). Approximately, equal amounts of each extract were analysed on a 10% gel, which was blotted and probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit. The autoradiograph shown was developed for 10 sec.

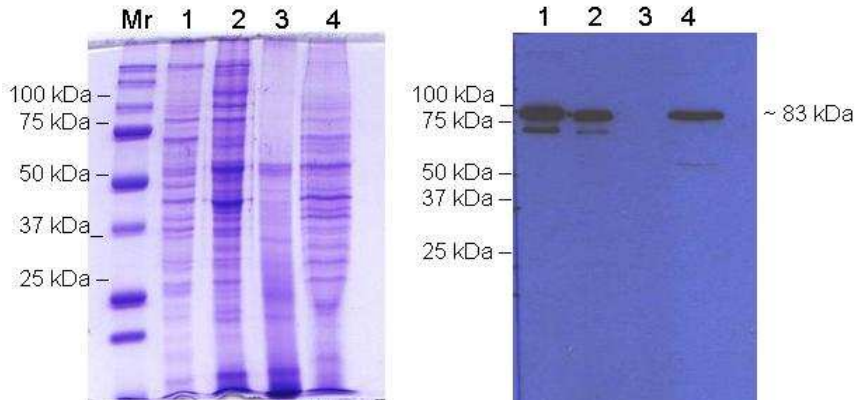


(a) (b)

Figure 3.2: Clade V parasitic nematodes show cross reactivity with *B. pahangi* anti-Hsp90 antibody.

a) Coomassie blue stained gel of clade V parasites. Equal amounts of proteins were loaded on a 10% SDS-PAGE gel. *B. pahangi* (Lane 1), *T. circumcincta* (Lane 2), *N. brasiliensis* (Lane 3), *H. polygyrus* (Lane 4) and *H. contortus* L3 (Lane 5). Mr represents the molecular weight markers.

b) Clade V parasitic nematodes react with the *B. pahangi* anti-Hsp90 antibody. *B. pahangi* (Lane 1), *T. circumcincta* (Lane 2), *N. brasiliensis* (Lane 3), *H. polygyrus* (Lane 4) and *H. contortus* L3 (Lane 5). Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot was probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

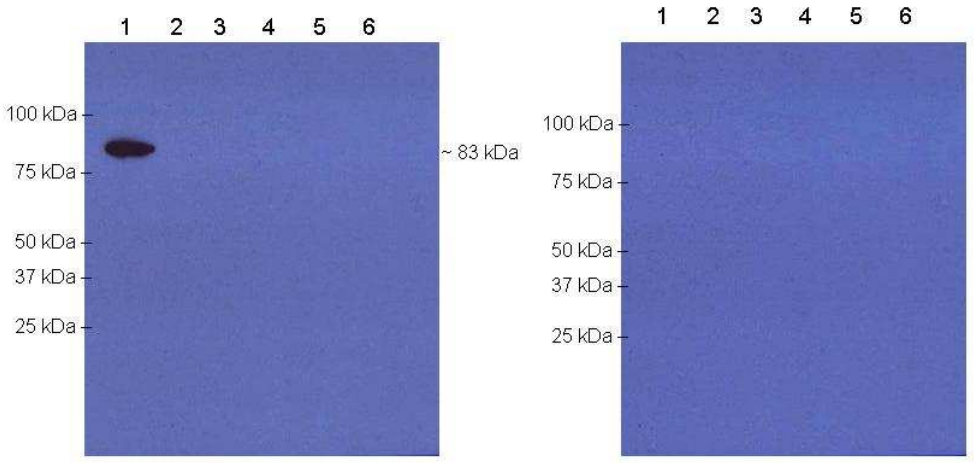


(a) (b)

Figure 3.3: Analysis of *H. contortus* worm extracts with *B. pahangi* anti-Hsp90 antibody.

a) Coomassie blue staining of *H. contortus* extracts. 10% SDS-PAGE gel with *B. pahangi* lysate as a control (Lane 1). An extract of adult *H. contortus* solubilised directly in SDS-PAGE sample cocktail (Lane 2), TNES-soluble extract of *H. contortus* adults (Lane 3) and TNES-soluble extract of *H. contortus* L3 (Lane 4).

b) TNES-soluble extracts of *H. contortus* adults are not recognised by *B. pahangi* anti-Hsp90 antibody. *B. pahangi* extract (Lane 1), adult *H. contortus* solubilised in SDS-PAGE sample cocktail (Lane 2), TNES-soluble extracts of adult *H. contortus* (Lane 3), TNES-soluble extract of *H. contortus* L3 (Lane 4). Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot was probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.



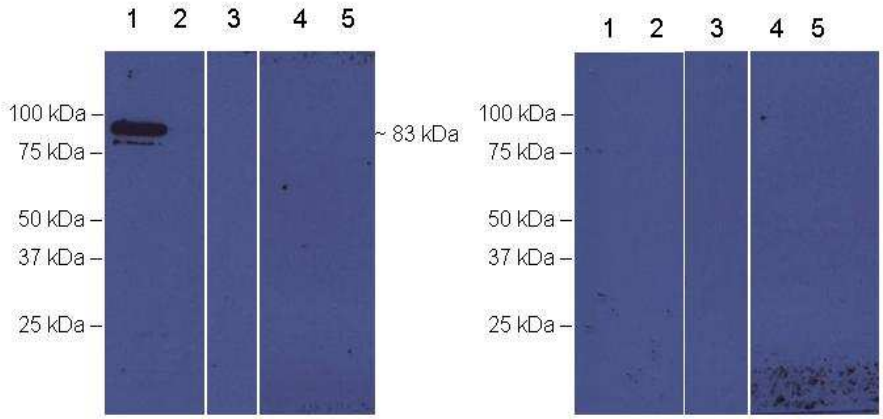
(a) (b)

Figure 3.4: Pull-down assays demonstrate that Hsp90 from free-living clade V nematodes does not bind GA.

a) Pull-down assays with GA beads. *B. pahangi* (Lane 1), *C. elegans* N2 (Lane 2), *C. elegans* (JT6130), *C. briggsae* (Lane 4), *O. tipulae* (Lane 5) and *P. pacificus* (Lane 6).

b) Pull-down assay with control beads. *B. pahangi* (Lane 1), *C. elegans* N2 (Lane 2), *C. elegans* JT6130 (Lane 3), *C. briggsae* (Lane 4), *O. tipulae* (Lane 5) and *P. pacificus* (Lane 6).

GA pull-down assays were carried out with 500 µg of worm lysate incubated with 50 µl of GA beads for 2.5 h at 4^{o} C. The beads were washed extensively in TNES and bound proteins were eluted by boiling in SDS-PAGE sample cocktail. Bound proteins were analysed by SDS-PAGE and immuno-blotting. The blots were probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.



(a) (b)

Figure 3.5: Hsp90 from clade V parasitic nematodes does not bind to GA.

a) Pull-down assay with GA beads. *B. pahangi* (Lane 1), *T. circumcincta* (Lane 2), *N. brasiliensis* (Lane 3), *H. polygyrus* (Lane 4) and *H. contortus* L3 (Lane 5).

b) Pull-down assays with control beads. *B. pahangi* (Lane 1), *T. circumcincta* (Lane 2), *N. brasiliensis* (Lane 3), *H. polygyrus* (Lane 4) and *H. contortus* L3 (Lane 5).

Results shown here from three different pull-down assays. GA pull-down assays were carried out with 500 µg of worm lysate with GA beads. Lysate of worms were incubated with 50 µl of GA beads for 2.5 h at 4^{o} C. The beads were washed extensively in TNES and bound proteins were eluted by boiling in SDS-PAGE sample cocktail. Bound proteins were analysed by SDS-PAGE and immuno-blotting. The blot was probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blots were developed using the Pierce Super Signal West Pico chemiluminescence kit.

3.2.2 Do other clade III species bind GA?

Hsp90 from *B. pahangi*, a clade III nematode, was previously shown to bind GA in pull-down assays and was also sensitive to GA treatment (Devaney *et al.*, 2005). In order to investigate whether the ability to bind GA is shared with other clade III species, several other nematodes were available for analysis including three filarial worms, *Acanthocheilonema viteae, Dirofilaria immitis* and *Ochocerca ochengi,* the Ascarid species, *T. cati, Ascaris suum, Parascaris equorum* and the Anisakids, *Anisakis simplex* and *Pseudoterranova decipiens*. In addition a Dracunculid, species *Anguilicola crassus* was also tested. All filarial worms reacted with *B. pahangi* anti-Hsp90 antibody (Figure 3.6b) as did the ascarid and anisakid species (Figure 3.7b) but no reaction was observed with *A. crassus* (Figure 3.6c). For *A. crassus,* the immuno-blot was repeated using the AC88 monoclonal anti-Hsp90 antibody. However no reaction was observed (see Figure 3.6c).

GA pull-downs assays of clade III parasites showed an interesting variation in results. All filarial nematodes tested were shown to bind to GA (Figure 3.7a), while Hsp90 from the three Ascarid species analysed also bound to GA (Figure 3.7c). However, Hsp90 from the Anisakids (*A. simplex* and *P. decipiens*) did not bind to GA. The autoradiograph was left overnight for further exposure, but still no signal was observed. All experiments were repeated at least twice. As no signal was obtained with the antibody using *A. crassus* extracts, attempts were made to visualise a band in the GA pull-downs with *A. crassus* by Coomassie blue staining of a gel, but no band was observed (results not shown).

For selected species which showed positive results in pull-down assays, the experiments were repeated in the presence of excess GA to block free

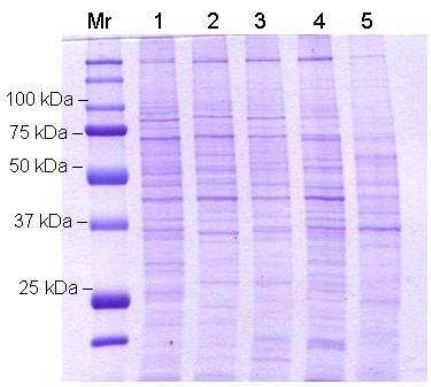
binding sites (see Figure 3.8). Two concentrations of GA were used, 2 μM and 20 μM. The binding of *A. suum* Hsp90 to GA beads was blocked by pre-incubation with GA at 2 μM or 20 μM (Panel a, Figure 3.8). Similar results were observed for *P. equorum* (Panel b, Figure 3.8), *D. immitis* (Panel c, Figure 3.8), and *O. ochengi* (Panel d, Figure 3.8). These results indicate the specificity of the interaction. In separate experiments, male and female worms were available for two species *A. suum* and *T. cati*, so it was possible to compare GA binding of both sexes. The results demonstrate that Hsp90 from males and females of both nematodes bound to GA (Figure 3.9a and 3.9b).

Figure 3.6: Hsp90 from clade III nematode reacted with *B. pahangi* anti-Hsp90 antibody.

a) Coomassie blue stained gel. Gel shows equivalent amounts of proteins from selected clade III nematodes loaded on 10% gel and analysed by Coomassie blue staining. *B. pahangi* (Lane 1), *A. viteae* (Lane 2), *D. immitis* (Lane 3), *O. ochengi* (Lane 4), *A. simplex* (Lane 5).

b) Hsp90 from clade III nematodes is recognised by the *B. pahangi* anti-Hsp90 antibody. The blots were probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. *B. pahangi* (Lane 1), *A. viteae* (Lane 2), *D. immitis* (Lane 3), *O. ochengi* (Lane 4), *T. cati* (Lane 5), *A. suum* (Lane 6), *P. equorum* (Lane 7), *A. simplex* (Lane 8) and *P. decipiens* (Lane 9). Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

c) A TNES-soluble extract of *A. crassus* (Lane 2 and Lane 4) failed to react with the *B. pahangi* anti-Hsp90 or the AC88 anti-Hsp90 antibody. In these experiments a *C. elegans* lysate was used as a positive control (Lane 1 and Lane 3). The blots were probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody (Lane 1 and Lane 2). Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. Lane 3 and Lane 4 were probed with 1:500 dilution of AC88 anti-Hsp90 monoclonal antibody. Bound antibody was detected using a 1:10,000 dilution of anti-mouse IgG. Both blots were developed using the Pierce Super Signal West Pico chemiluminescence kit.



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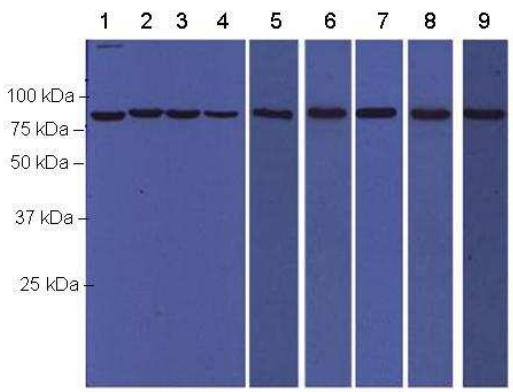
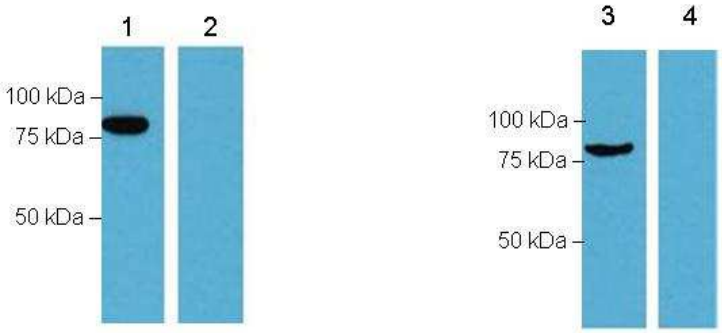


(b)



B. pahangi anti-Hsp90 antibody AC88 anti-Hsp90 antibody

(c)

Figure 3.7: GA pull-down assays using clade III nematodes

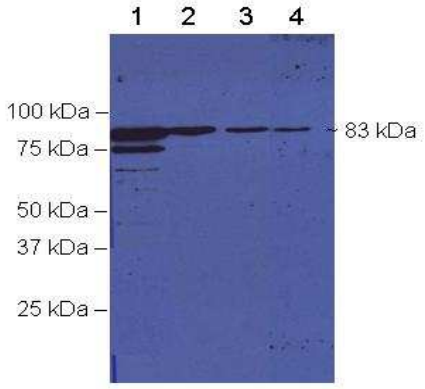
a) Immuno-blot of GA pull-down assays using extracts of filarial worms. *B. pahangi* (Lane 1), *A. viteae* (Lane 2), *D. immitis* (Lane 3) and *O. ochengi* (Lane 4).

b) Immuno-blot of pull-down assays with control beads. Gel loaded as in Panel a.

c) Immuno-blot of GA pull-down assays using extracts of ascarid and anisakid worms. *B. pahangi* (Lane 1), *A. suum* (Lane 2), *P. equorum* (Lane 3) and *T. cati* (Lane 4), *P. decipiens* (Lane 5) and *A. simplex* (Lane 6).

d) Immuno-blot of control beads for the pull-down assays. Gel loaded exactly as in Panel c.

For pull-down assays, 500 µg of proteins extract were incubated with 50 µl of GA beads for 2.5h at 4^{o}C on rocker. The blots were probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.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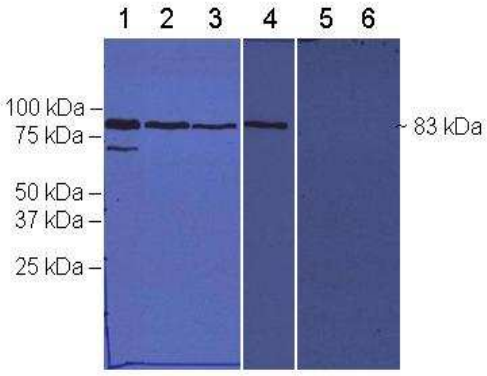
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
~ 83 kDa

(a)


1 2 3 4

(b)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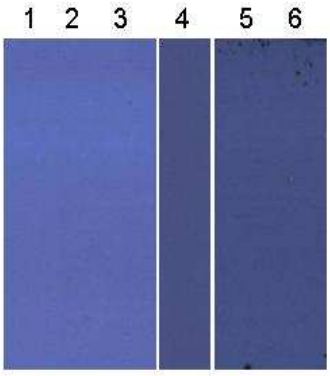
(c)


1 2 3 4 5 6

(d)

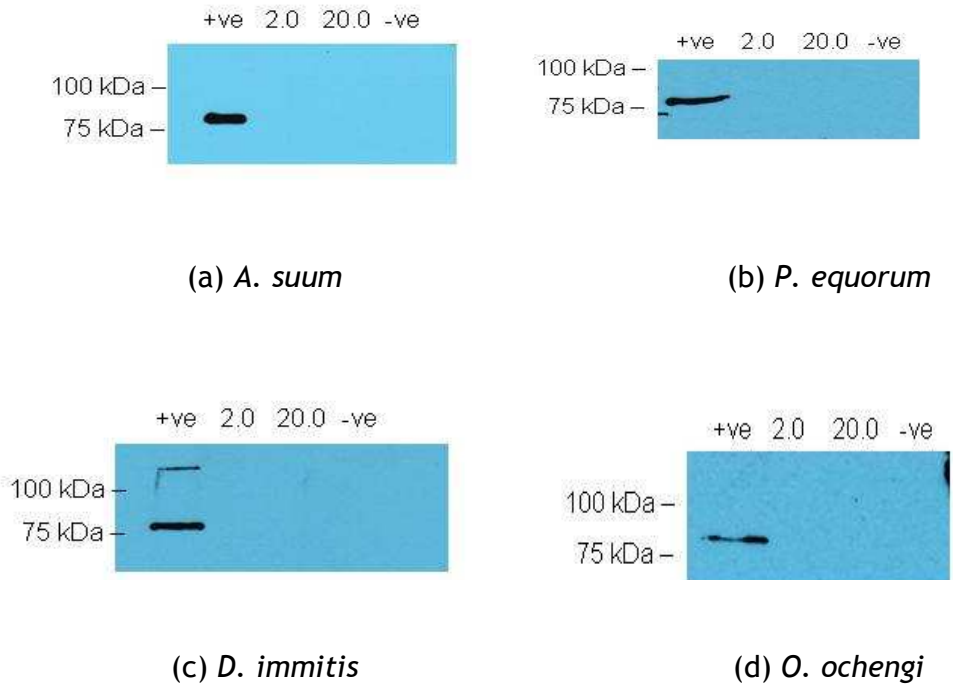


Figure 3.8: The binding of Hsp90 to GA is specific

For selected species which showed a positive result in pull-down assay, the experiments were repeated in the presence of excess GA at 2.0 µM or 20.0 µM to block free binding sites. *A. suum* (Panel a), *P. equorum* (Panel b), *D. immitis* (Panel c) and *O. ochengi* (Panel d).

For the competition assays, 500 µg of worm lysate were first incubated in a total volume 300 µl with GA at 2.0 µM or 20.0 µM for 1 h at room temperature. Then 50 µl of GA beads were added and incubated for further 2.5 h at 4^{o} C. The beads were washed extensively in TNES and bound proteins were eluted by boiling in SDS-PAGE sample cocktail. Bound proteins were analysed by SDS-PAGE and immuno-blotting. The blots were probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

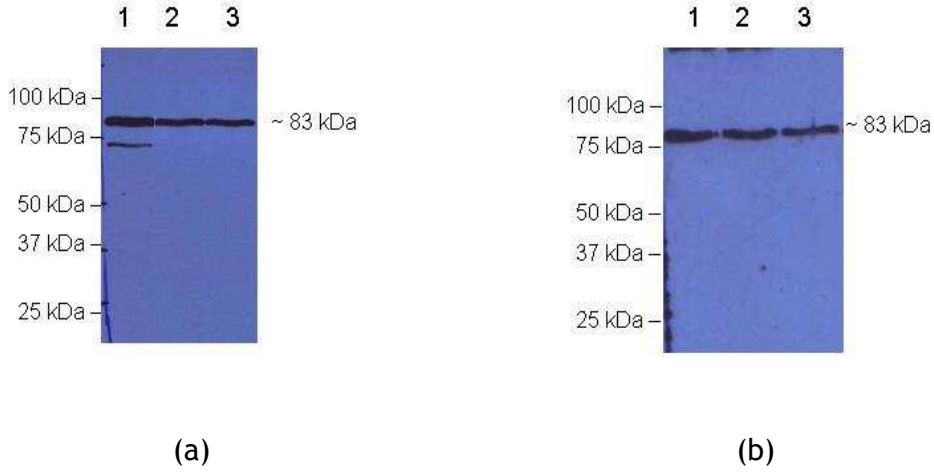


Figure 3.9: The binding of Hsp90 to GA is independent of the sex of the worm.

a) Immuno-blot of GA pull-down assay using *A. suum* male and female worms. *B. pahangi* (Lane 1), *A. suum* female (Lane 2) and *A. suum* male (Lane 3).

b) Immuno-blot of GA pull-down assay using *T. cati* male and female. *B. pahangi* (Lane 1), *T. cati* female (Lane 2) and *T. cati* male (Lane 3).

500 µg of worm lysate of male or female worms were incubated with 50 µl of GA beads for 2.5 h at 4^{o} C. The beads were washed extensively in TNES and bound proteins were eluted by boiling in SDS-PAGE sample cocktail. Bound proteins were analysed by SDS-PAGE and immuno-blotting. The blots were probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

3.2.3 Do nematodes from other clades bind to GA?

Two species of clade I nematode were available for the analysis, *T. spiralis* and *Trichuris muris*. Equal amounts of TNES-soluble protein extracts were loaded onto a 10% SDS-polyacrylamide gel and were analysed to detect Hsp90 on the blots. However, neither species reacted with the *B. pahangi* anti-Hsp90 antibody or with AC88 antibody at a range of antibody concentrations or exposure times (Figures 3.10a and 3.10b). Consequently, it was not possible to analyse GA pull-downs by immuno-blotting. GA and control pull-downs using both *T. spiralis* and *T. muris* were analysed by Coomassie blue staining. For *T. spiralis*, a band of 83 kDa was observed on the gel that was not present using the control beads (Figure 3.10c). However, for *T. muris* no band was observed when GA pull-downs were analysed by Coomassie blue staining (Figure 3.10c). The *T. spiralis* band was excised from the gel and was sent to the Sir Henry Welcome Functional Genomics Unit, University of Glasgow for proteomic analysis. Following trypsin digestion and mass spectrometry, MASCOT searches revealed that the *T. spiralis* band was likely to be Hsp90. Multiple peptide hits showed a high degree of similarity ($p<0.05$) with Hsp90s from many other species. These experiments were repeated in the presence of excess GA to block free binding sites (see Figure 3.10e). Two concentrations of GA were used, 2 μM and 20 μM. At 2 μM GA, the binding of *T. spiralis* Hsp90 to GA beads was completely blocked. This demonstrated the specificity of the interaction of *T. spiralis* Hsp90 with GA.

Figure 3.10: *T. spiralis* Hsp90 binds to GA beads in pull-down assay.

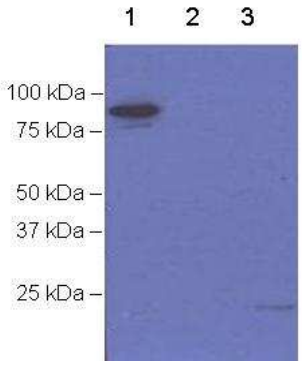
a) Immuno-blot showing that Hsp90 from clade I nematodes is not recognised by the *B. pahangi* anti-Hsp90 antibody. *B.* pahangi (Lane 1), *T.* spiralis (Lane 2) and *T.* muris (Lane 3). The blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

b) Immuno-blot of clade I nematodes failed to reacted with AC88 monoclonal anti-Hsp90 antibody. *C. elegans* (Lane 1), *T. spiralis* (Lane 2) and *T. muris* (Lane 3). The blot probed with 1:500 dilution of the AC88 monoclonal anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-mouse IgG. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit. In this experiment, *C. elegans* lysate was used as a control.

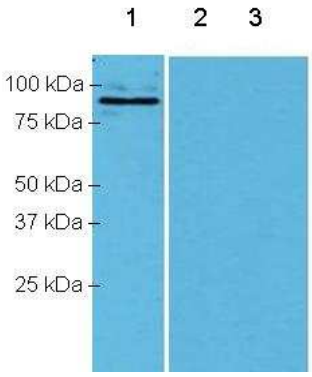
c) Coomassie blue stained gel of GA pull-down assay for clade I nematodes. *B. pahangi* (Lane 1), *T. spiralis* (Lane 2) and *T. muris* (Lane 3). Note the band at ~ 83 kDa in Lane 1 and Lane 2.

d) Coomassie blue stained gel of control beads. Gel loaded as in Panel c. For pull-down assays, 500 µg of proteins extract were incubated with 50 µl of GA beads for 2.5 h at 4^{o}C on rocker. The beads were washed extensively in TNES and bound proteins were eluted by boiling in SDS-PAGE sample cocktail. Bound proteins were analysed by SDS-PAGE and gel was stained using 0.1% Coomassie blue by agitation for 1h at room temperature.

e) Panel e shows a Coomassie blue stained gel of a *T. spiralis* pull-down pre-incubated without GA (Lane 1) and with GA at 2.0 µM (Lane 2) or 20.0 µM (Lane 3) and control beads (Lane 4). 500 µg of worm lysate was incubated in a total volume of 300 µl containing GA at 2.0 µM or 20.0 µM for 1 h at room temperature. Then 50 µl of GA beads were added and incubated for further 2.5 h at 4^{o}C. The beads were washed extensively in TNES and bound proteins were eluted by boiling in SDS-PAGE sample cocktail. Bound proteins were analysed by SDS-PAGE. Gel was stained with 0.1% Coomassie blue.



(a)



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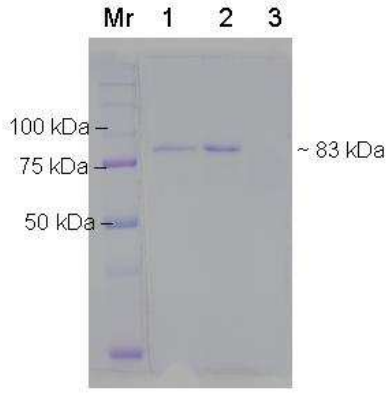
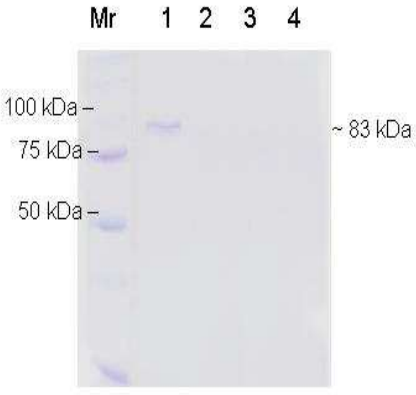


Pull-down with GA beads

(c)

Pull-down with control beads

(d)



(e)

The clade IV nematode species tested were the plant parasitic nematodes, *Globodera pallida* and *Globodera rostochiensis*, a free-living species *Panagrellus redivivus* and the animal parasite *Strongyloides ratti*. The *S. ratti* adults used in the analysis were free-living rather than parasitic. Both plant parasitic nematodes reacted with the antibody, as did *P. redivivus* (see Figure 3.11a). *S. ratti* reacted weakly with the *B. pahangi* anti-Hsp90 antibody although the signal obtained improved with increasing exposure times. Next, pull-down assays were carried out to investigate the ability of Hsp90 from clade IV nematodes to bind GA. From this experiment it could been seen that Hsp90 from the two plant parasitic nematodes failed to bind GA as did Hsp90 from the free-living clade IV species, *P.* redivivus (see Figure 3.11b). Similarly, Hsp90 from *S. ratti* did not bind to GA in pull-down assays (Figure 3.11d). Because the Hsp90 signal in *S. ratti* was relatively weak using the *B. pahangi* anti-Hsp90 antibody, the pull-downs were exposed for prolonged period of time (overnight) but no signal was obtained. These experiments were repeated at least two occasions with similar results Table 3.1 shows the summary of GA pull-down assays with a range of nematodes from different clades.

Figure 3.11: Analysis of clade IV nematodes

(a) Immuno-blot of Hsp90 from clade IV nematodes probed with the *B. pahangi* anti-Hsp90 antibody. *G. pallida* (Lane 2), *G. rostochiensis* (Lane 3), *S. ratti* (Lane 4) and *P. redivivus* (Lane 5). *B. pahangi* lysate was used as a positive control (Lane 1).

b) Immuno-blot of GA pull-down assays. *G. pallida* (Lane 2), *G. rostochiensis* (Lane 3) and *P. redivivus* (Lane 4). For these experiments, *D. immitis* lysate was used as a positive control (Lane 1).

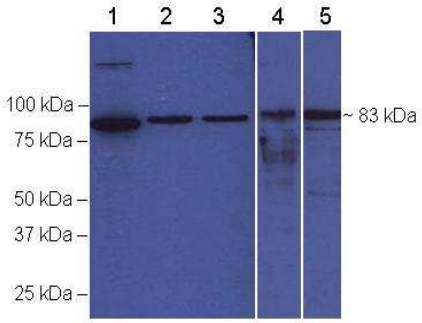
c) Immuno-blot of pull-down assays for control beads. *D. immitis* (Lane 1), *G. pallida* (Lane 2), *G. rostochiensis* (Lane 3) *P. redivivus* (Lane 4).

d) Immuno-blot of GA pull-down assay with *S. ratti* lysate. *S. ratti* (Lane 2). *B. pahangi* lysate was used as a positive control (Lane 1).

e) Immuno-blot of pull-down assays for control beads. *B. pahangi* (Lane 1), *S. ratti* (Lane 2).

Panel a shows a lay-up of GA pull-downs for three separate experiments. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

For pull-down assays (Panel b,c, d and e), 500 µg of proteins extract were incubated with 50 µl of GA beads for 2.5 h at 4^{o}C on rocker. The beads were washed extensively in TNES and bound proteins were eluted by boiling in SDS-PAGE sample cocktail. Bound proteins were analysed by SDS-PAGE and immuno-blotting. The blots were probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.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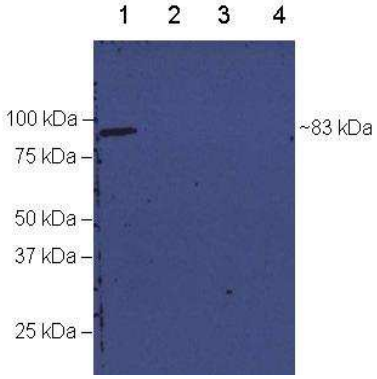
(a)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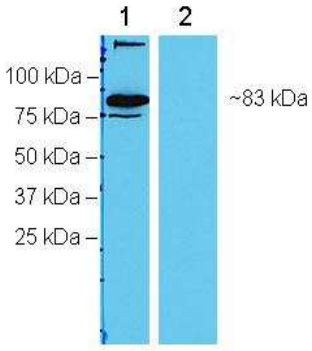
(b)


1 2 3 4

(c)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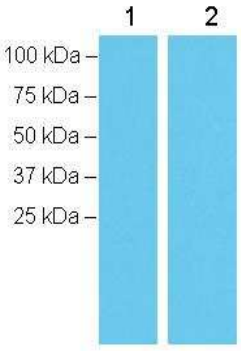
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100 kDa
75 kDa
50 kDa
37 kDa
25 kDa

(e)

Table 3.1: Summary of GA pull-down assays

Species	Clade	Life-cycle stage	Life style	GA binding
T. spiralis	I	L1	Obligate parasite	+
T. muris	I	adult	Parasitic, egg in the environment	?
A. crassus	III	adult	Parasitic, Fl larvae	?
A. simplex	III	L3	Parasitic, Fl larvae	-
P. decipiens	III	L3	Parasitic, Fl larvae	-
A. suum	III	adult	Parasitic	+
P. equorum	III	adult	Parasitic	+
T. cati	III	adult	Parasitic	+
B. pahangi	III	adult	Obligate parasite	+
A. viteae	III	adult	Obligate parasite	+
D. immitis	III	adult	Obligate parasite	+
O. ochengi	III	adult	Obligate parasite	+
S. ratti	IV	adult, FL	Parasitic or FL adult, FL larvae	-
P. redivivus	IV	mixed stages	Free-living	-
G. rostochiensis	IV	J2	Plant parasitic	-
G. pallida	IV	J2	Plant parasitic	-
C. elegans	V	mixed stages	Free-living	-
C. elegans daf-21 mutant	V	mixed stages	Free-living	-
C. briggsae	V	mixed stages	Free-living	-
O. tipulae	V	mixed stages	Free-living	-
P. pacificus	V	mixed stages	Free-living	-
H. contortus	V	L3	Parasitic, FL larvae	-
T. circumcincta	V	adult	Parasitic, FL larvae	-
H. polygyrus	V	adult	Parasitic, FL larvae	-
N. brasiliensis	V	adult	Parasitic, FL larvae	-

FL = free-living - = Negative binding + = Positive binding ? = uncertain GA binding status

3.3 Discussion

As reviewed in Chapter 1, Hsp90 plays a fundamental role in cells by regulating specific client proteins often involved in signalling pathways. These proteins depend on Hsp90 for maturation and stabilisation. Any disruption in this process will lead to degradation of the proteins and to cell death. An important characteristic of Hsp90 is its ability to bind to GA, a specific Hsp90 inhibitor (as reviewed in Chapter 1). According to Whitesell *et al* (1994), most eukaryote Hsp90s are susceptible to GA and will bind to GA in pull-down assays. However, the ability of Hsp90 to bind to GA is not shared by *C. elegans* (David *et al*., 2003) while Hsp90 from the parasitic nematode *B. pahangi* does bind GA (Devaney *et al*., 2005).The results with *C. elegans* were hypothesised to be an example of adaptive evolution as *C. elegans* and *Streptomyces* spp. shared the same ecological niche of the soil (David *et al*., 2003). In this chapter this hypothesis was tested by analysing the GA binding properties of a range of nematode.

Table 3.1 summarises the GA binding assays for the nematodes tested. From these results some interesting conclusions can be drawn. All free-living nematodes tested or those parasitic species which have free-living larval stages in the environment failed to bind GA. Five free-living species from clade V (*C. elegans*, *C. elegans daf-21* mutant, *C. briggsae*, *O. tipulae* and *P. pacificus*) and one species from clade IV (*P. redivivus*) were tested. The parasitic species tested that have free-living larval stages in the environment belong to clade V (*H. contortus*, *N. brasilliensis*, *H. polygyrus* and *T. circumcincta*) and clade IV (*S. ratti*). This group of nematodes are generally gut-dwelling parasites in the mammalian host and are transmitted between hosts when eggs, passed out in the faeces of the infected animal, hatch to release free-living larval stages.

These generally develop to the L3 stage in the environment before infecting a new host by ingestion. The clade IV species, *S. ratti* has a unique life-cycle, which has both a parasitic and a free-living phase. *S. ratti* can undergo two types of development outside of the host: homogonic and heterogonic (Viney, 1996). In homogonic development, the eggs pass out in the faeces, hatch and moult through two larval stages into infective L3. While in heterogonic development, the eggs hatch and moult through four larval stages into dioecious free-living adults (Viney *et al.*, 1993). In the analysis described here, only free-living adult worms were available.

Likewise, none of the clade IV plant parasitic nematodes (*G. pallida* and *G. rostochiensis*) tested could bind to GA. *Globodera* spp. are parasites of plant roots commonly known as potato cyst nematodes, which live in soil. The second stage juvenile worms (J2) are the infective stage in the soil where they penetrate the roots of the susceptible plant and feed on it and develop to adults. The cyst containing eggs remains dormant within the body of the dead female until the proper stimulus to hatch is received. Eggs of potato cyst nematodes can remain dormant and viable within the cyst for 30 years (Winslow & Willis, 1972). In this stage (J2), the nematodes are more resistant to anthelmintic drugs.

For the clade III nematodes, the results were more variable. Some of the species analysed exhibited the ability to bind to GA while some did not. Hsp90 from all filarial worms tested in this study bound to GA. Filarial worms are obligate parasites and their life cycle does not involves any free-living stages in the environment, with arthropods acting as a intermediate hosts for transmission between definitive hosts. In keeping with this result, analysis of Hsp90 from

another obligate parasite from clade I, *T. spiralis,* also showed binding to GA. The life-cycle of *T. spiralis* involves two phases. The first phase is enteral, the worms remain in the intestine, growing from larvae to adults and reproducing. For the second phase known as the parenteral phase, newly hatched larvae migrate to the host's muscle cells and become encysted. Infection occurs when meat contaminated with encysted L1 larvae is eaten.

Analysis of Hsp90 from the Ascarid species (*T. cati, A. suum* and *P. equorum*) shows the ability to bind to GA. For these species, the eggs are passed out in the faeces of the infected animal into the environment, but the larvae are protected within an egg. For example, infection with *Toxocara* occurs after ingestion of eggs, which hatch in the stomach of the definitive host. Alternatively infection can also occur by ingestion of a paratenic host, such as a mouse. All Ascarid parasites have an egg with a thick shell, giving protection against potentially harsh environmental conditions.

The clade III Anasakid parasites, *A. simplex* and *P. decipiens*, did not bind to GA. These parasites have a complex life-cycle in the marine environment. Unembryonated eggs are excreted by mammals such as whales, seals or dolphins and eggs embryonate to form L2 in sea water. When eggs hatch, the larvae are eaten by crustaceans e.g. Eusphausids for *Anisakis* and cyclopod copepods for *Pseudoterranova* (Bristow & Berland, 1992). Similar to the Anasakid, *A. crassus* has a free-swimming larval stage as part of its life-cycle in the environment. *A. crassus* is a parasite of eels where the L2 stage is excreted in the water before being consumed by paratenic or intermediate host e.g. copepods. It develops into the infective stage in the intermediate host before being ingested by the

eel. In the eel, the infective stage moves from the intestine to the swim bladder and develops into an adult.

For some species it was not possible to ascertain with certainty whether Hsp90 bound to GA or not. These species were *A. crassus* and *T. muris*. For both species and for *T. spiralis* from clade I no reactivity was observed with any anti-Hsp90 antibody tested, so the pull-downs could not be analysed by immuno-blotting. For *T. spiralis*, staining a gel of a pull-down with Coommasie blue was sufficient to detect a band running at approximately the correct molecular weight (83 kDa) but not for *A. crassus* and *T. muris*. The *T. spiralis* band was excised from the gel and subjected to mass spectrometry, revealing that the *T. spiralis* band was likely to be Hsp90. Although it was not possible to identify Hsp90 binding to GA in pull down assays using *A. crassus* and *T. muris*, it is not possible to definitively conclude that Hsp90 from these species does not bind to GA. As extracts of *A. crassus* and *T. muris* failed to react with either antibody, the GA-binding status of these species remains unresolved. Further experiment would have to be carried out using a more sensitive detection method e.g. silver staining or perhaps using different life-cycle stages.

The results in this chapter so far suggested that the ability of nematode Hsp90 to bind to GA is correlated with particular nematode life histories. Free-living species and species with a free-living larval stage did not bind to GA. In contrast, species which are permanently parasitic (filarial worms and *T. spiralis*) or species which have free-living stages in the environment enclosed within a protective egg-shell possess a GA-binding Hsp90. However, there are limitations to these results as described above. Overall, these data could be interpreted in the context of adaptive evolution. Adaptive evolution of *C. elegans* Hsp90 would

argue that *C. elegans* Hsp9O is resistant to GA because it shares the same ecological niche as the *Streptomyces* spp. which produces GA. However, whether *C. elegans* and *Streptomyces* spp. do actually share the same spatial and temporal niches within the soil is unknown. Although *C. elegans* can be found in the soil, the predominant in the wild is the dauer larvae, which is usually found in organic-rich environment such as compost. Dauer stages are resistant and non-feeding and the natural food source of *C. elegans* in the wild is unknown (Kiontke & Sudhause, 2006).

A fascinating example of the interaction between a micro-organism that synthesises an Hsp90 inhibitor and its host plant come from the work of McLellan *et al* (2007). They studied a fungus (*Paraphaeospharia quadriseptata*) that synthesises the Hsp90 inhibitor, Monocillin I (MON) and showed that MON could inhibit plant (*Arabidopsis thaliana*) Hsp90. Inhibition of Hsps such as Hsp70 or Hsp90, is known to induce a stress response in many organisms and plants exposed to MON were no exception. Indeed, MON was shown to induce Hsp101, a protein required for thermotolerence. Finally it was demonstrated that co-association of the MON-producing fungus and *A. thaliana* enhanced the tolerance of the plant to heat-stress, suggesting an intricate association between plant and micro-organism.

In order to further investigate the adaptive evolution theory, a collaboration with Dr. Richard Emes (University of Nottingham), analysed *hsp90* sequences from a range of nematodes to determine if there were particular amino acid substitutions associated with GA binding or not. This analysis demonstrated three separate lineages for *hsp90* sequences (Branch A, B and C) which correlated with the ability to bind GA (Figure 3.12). Branch A represents

the group of GA-binding clade III species, Branch B represents the group of non GA-binding clade V species and Branch C represents the group of plant parasitic nematode *hsp90* sequences. While a number of residues showed significant evidence of adaptive evolution, the results did not demonstrate a direct relationship between amino acid substitutions in Hsp90 and GA binding ability (Him *et al.*, 2009). These data suggest that the evolution of *hsp90* may be correlated to key functions other than the ability to bind GA.

While it was not possible to identify specific amino acid sequences associated with GA binding or not, studies in other organisms have demonstrated that point mutations in *hsp90* can alter sensitivity to Hsp90 inhibitors. For example, in yeast an increased sensitivity to GA and radicicol was observed in *hsp90* mutants (Piper *et al.*, 2003a). That study revealed an increased sensitivity to GA of A587T and T101I with T22I, G313S and E381K showing lesser effect. In addition, in chicken Hsp90, mutation of S113A abolished GA binding and K112A reduced the GA binding compared to wild type Hsp90 (Lee *et al.*, 2004). A study on human Hsp90 also showed the effect of mutations in *hsp90* on GA-binding (Onuoha *et al.*, 2007). Mutation of residues L106A and S107A, in the N-terminal of human Hsp90, was shown to be important for the isomeration of GA. As a result of these mutations, the binding of Hsp90 to GA was reduced. S107 is an important element in the catalysis of *trans* to *cis* GA (Lee *et al.*, 2004). S107 acts as a docking site for the binding of *trans* GA and catalyses the conversion to the *cis* isoform. So any mutation at this site, may affect the association and dissociation rates of GA binding, which may explain the differences in GA binding between mutant and wild-type Hsp90.

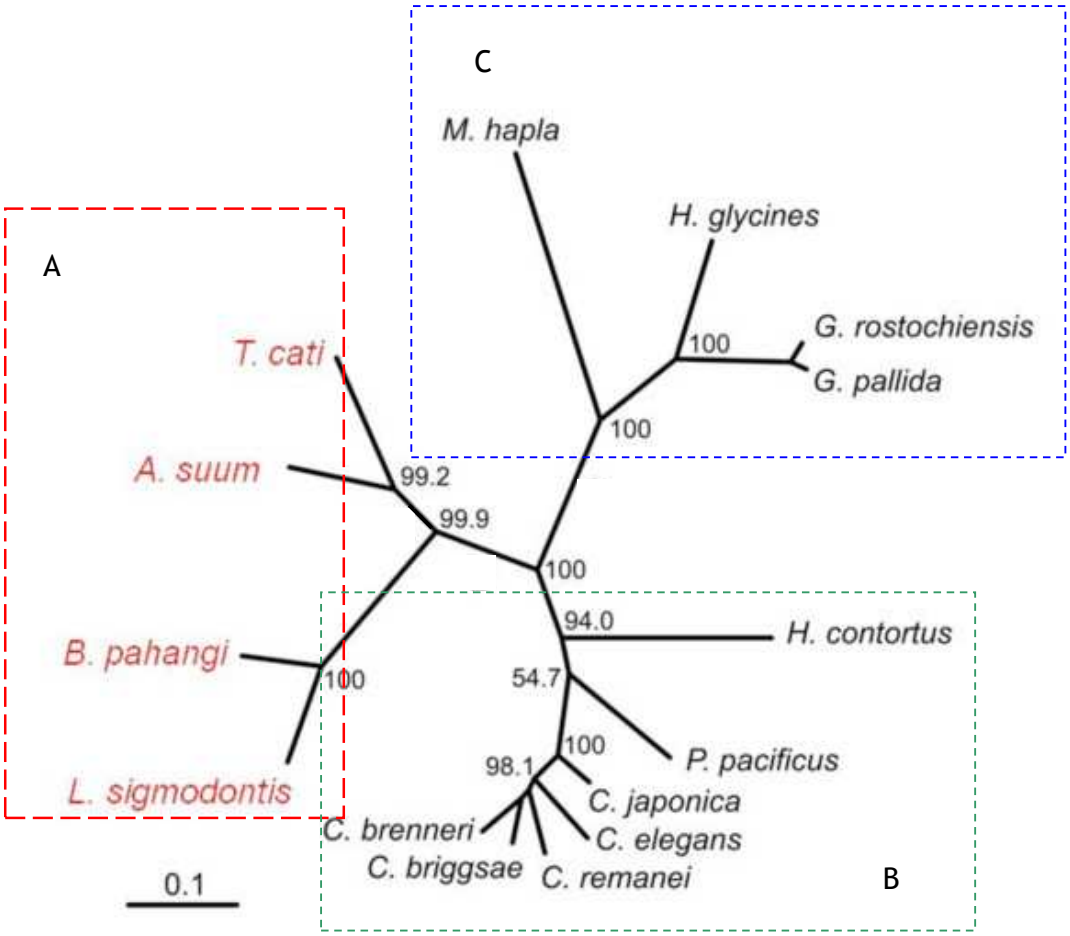

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Figure 3.12: Unrooted phylogenetic tree showing relationship of *hsp90* sequences from fifteen different nematode species.

Three different branches showed significant evidence of adaptive evolution. Branch A represents the group of GA binders (red), B (green) and C (blue) represents the group of non-GA binders. B, non-binding *hsp90* sequences from clade V species and C the plant parasitic nematode *hsp90* sequences.

As GA and related Hsp90 inhibitors, such as radicicol, are produced by micro-organisms, study of the micro-organism Hsp90 might be revealing as presumably inhibition of Hsp90 would be deletenous. In the yeast *Humicola fuscoatra*, a species that produce radicicol, a single conserved amino acid substitution (L33I) in Hsp90 is enough to influence the binding of radicicol without altering the ability to bind ATP or GA (Prodromou *et al.*, 2009). In that study they showed that substitution of leucine to isoleucine alters the size of the hydrophobic drug-binding pocket. As a result of this, there is increased binding of H_2O molecules and hydration of the drug. These findings demonstrate that small alterations in amino acid residues can have major affects on drug binding efficacy.

There are also additional theories on GA binding to Hsp90 that may explain the differences in the ability of nematode Hsp90 to bind to GA. A possible explanation of the differences between *B. pahangi* and *C. elegans* Hsp90 in GA binding is the cellular context of Hsp90; that is the degree of post-translational modifications or the presence of specific co-chaperones in the Hsp90 complex. This is best exemplified by studies in tumour cells where Hsp90 appears to exist in high affinity complexes with a number of co-chaperones (Kamal *et al.*, 2003). In this configuration Hsp90 was shown to be more sensitive to 17-AAG than Hsp90 from normal cells, which exists in a free-uncomplexed state. Hsp90 complexes in tumour cells contain the co-chaperones p23 and Hop, and have a higher affinity for GA than Hsp90 from normal cells. p23 is thought to modulate Hsp90 activity in the last stages of the chaperoning pathway. Hop belongs to the large group of Hsp co-chaperones. It is one of the best studied co-chaperones of the Hsp70/Hsp90-complex and it is essential to link Hsp70 and Hsp90 together. By forming a super-chaperone complex containing many

essential co-chaperones, Hsp90 becomes more active and this helps it carry out its function. In addition, by adding p23, Hop, Hsp70 and Hsp40 to purified Hsp90 the affinity of Hsp90, for 17-AAG was significantly increased (Kamal *et al.*, 2003). These results were substantiated by the studies of Vilenchik *et al* (2004), who reported a 10- to 50-fold difference in binding of PU24FCI, a novel Hsp90 inhibitor, to Hsp90 from tumour cells compared to normal cells.

Piper *et al* (2003a) showed that expressing the human Hsp90ß in *S. cerevisiae* increased the sensitivity of the yeast to GA and radicicol whereas expression of *C. elegans* Hsp90 made the yeast more resistant to GA and radicicol. In yeast, complementation by human Hsp90ß and *C. elegans* Hsp90 requires the presence of the co-chaperones, Sti-1 and p23. Recently Sti-1 has been recognised as an Hsp90-interacting protein in *C. elegans* (Song *et al.*, 2009). This indicates that in the cell, Hsp90 functions in a complex rather than in isolation. In addition, studies from this lab (Gillan *et al.*, 2009), showed that there are fundamental differences between *C. elegans* and *B. pahangi* Hsp90. Injection of *B. pahangi hsp90* into *C. elegans* failed to rescue a *daf-21* mutant, while injection of the wild-type *C. elegans daf-21* rescued the mutant. Interestingly, injection of an *H. contortus hsp90* construct provided a partial rescue. In these studies, wild-type *C. elegans* expressing a translational fusion of *B. pahangi hsp90* gave a positive signal with GA beads in contrast to wild-type *C. elegans hsp90,* showing that even though the *Brugia* protein was translated in *C. elegans*, it was not functional. This suggests that in different nematodes Hsp90 may require different co-chaperones for activity or perhaps reflect difference in the client proteins chaperoned by Hsp90.

While the differences in Hsp90 binding to GA in different species of nematode could be explained by the presence of different co-chaperones or perhaps different client proteins, it might be also relate to differences in post-translational modification of Hsp90. Hsp90 is known to be modified significantly, by phosphorylation, acetylation and glycosylation (Scroggins *et al.*, 2007; Chiosis *et al.*, 2004). These post-translational modifications are known to alter its activity. However to date, very little known of Hsp90 modifications in nematodes.

In conclusion, the results in this chapter demonstrated that *C. elegans* Hsp90 is not unique and that the non-GA binding phenotype is shared by other nematodes from different clades. The ability to bind to GA is associated with the life history of the species. Free-living nematodes and those parasitic species which have a free-living larval stage in the environment do not bind to GA. In contrast, obligate parasite or those parasitic species which have free-living stages enclosed within a protective egg, do bind to GA. However, while these observations may be consistent with the adaptive evolution theory of *hsp90* sequences as proposed by (David *et al.*, 2003), no evidence was produced of specific amino acids that were associated with the binding or non-binding phenotype.

Chapter 4

Further Characterisation of *B. pahangi* Hsp90

4 Further characterisation of *B. pahangi* Hsp90

4.1 Introduction

Lymphatic filariasis is a disabling and disfiguring parasitic disease caused by the adult and developing stages of filarial nematode parasites residing in the lymphatic system of a mammalian host. Nearly 120 million people are infected and, until the advent of the global elimination programme, the number was increasing in affected countries. The disease is caused by the parasitic filarial worms *Wuchereria bancrofti*, *Brugia malayi* or *Brugia timori* that are transmitted by the bite of the mosquitoes acting as their intermediate hosts. Approximately one billion people are faced with the risks of infection and preventing and combating lymphatic filariasis is a long term goal of World Health Organisation (WHO) (http://www.who.int/mediacentre/ factsheets/fs102/en/). However, there are no current drugs which have activity against adult worms. The drugs currently used in control, such as diethylcarbamazine and ivermectin, largely target the microfilariae (Mf). The mechanism by which diethylcarbamazine kills Mf is still poorly understood but it has been suggested that it acts as an inhibitor of arachidonic acid metabolism in Mf (Liu & Weller, 1990) making the Mf more susceptible to host immune attack. As the inhibition of Hsp90 in *Brugia pahangi* by geldanamycin (GA) kills both adult worms and Mf (Devaney *et al.*, 2005) it is relevant to ask whether Hsp90 or its client proteins would be suitable targets for drug development.

In the filarial nematode *B. pahangi*, *hsp90* is a single copy gene which is 69% identical to human *hsp90* (Thompson *et al.*, 2001). *B. pahangi* Hsp90 contains all the features of Hsp90 that are common to other eukaryotic proteins; the N-terminal domain, the C-terminal domain, the M-domain and a charged

region that links the N-terminal domain to the M-domain. In *B. malayi*, Hsp90 was shown to be excreted by adult worms cultured *in vitro* (Kumari *et al.*, 1994). The quantification of excretory/secretory (ES) products released by living worms may be useful for detection of antibody, because these products appear to be more species-specific than crude somatic extracts (de Savigny *et al.*, 1979; Kaushal *et al.*, 1984). In nematodes, most of the information on Hsp90 comes from studies on *C. elegans*. The *C. elegans* genome contains a single *hsp90* gene (*daf-21*) located on chromosome V, which is 74% and 76% identical to human Hsp90α and Hsp90ß, respectively (Birnby *et al.*, 2000). In addition, Hsp90 also has been characterised in *Trichinella spiralis* (Martinez *et al.*, 2001; Martinez *et al.*, 2002) and cloned from *Haemonchus contortus* (Gillan *et al.*, 2009).

As described in Chapter 3, GA inhibits Hsp90 function in most eukaryotes with the exception of *C. elegans* and some other nematodes. However, the mechanism which confers resistance to GA on *C. elegans* Hsp90 is not understood. In most organisms, GA interferes with the folding, maturation, localisation or degradation of certain Hsp90 client proteins and co-chaperones. Hsp90 requires a series of co-chaperones to form a complex required for its function. For example, the Hsp90-dependant activation of kinases requires cell cycle division protein 37 (Cdc37) (Vaughan *et al.*, 2008). In that study, they showed that phosphorylation and dephosporylation of Cdc37 is necessary for the kinase client protein to bind to Hsp90. Most Hsp90 co-chaperones or client proteins have been characterised from human or yeast. However, a recent bioinformatic analysis revealed that the *C. elegans* genome contained most of the well characterised co-chaperones with the exception of cyclophilin-40 (Johnson & Brown, 2009). Cyclophilin-40 is a member the peptidyl-prolyl cis-

trans isomerase family and was shown to interact with steroid receptors such as the glucocorticoid receptor and androgen receptor (Riggs *et al.*, 2004).

The role of post-translational modifications in regulating Hsp90 functions remains unclear. As reviewed previously, post-translational modifications are required for some Hsp90 chaperone functions (Scroggins & Neckers, 2007),. For example, dephosphorylation and re-phosphorylation of Hsp90 regulated the activity of heme-regulated inhibitor kinase, an Hsp90 client protein, (Szyszka *et al.*, 1989) suggesting that Hsp90 phosphorylation is required for its function. In human Hsp90α, acetylation at lysine K294 was shown to reduce Hsp90 activity and chaperone function (Scroggins *et al.*, 2007). K294 resides in the M-terminal domain of human Hsp90α and acetylation of K294 significantly impacts the interaction of Hsp90 with a diverse set of client proteins. These results suggest that acetylation of Hsp90 plays an important role in regulating chaperone function.

The aim of this chapter was to further characterise *B. pahangi* Hsp90. Experiments were carried out to examine the expression profile of Hsp90 in different life-cycle stages, to further characterise the binding of *B. pahangi* Hsp90 to GA, to identify *B. pahangi* Hsp90-associated proteins and to investigate the effect of Hsp90 post-translational modifications on GA binding.

4.2 Results

4.2.1 Hsp90 is expressed in all life-cycles of B. pahangi

Three different stages of *B. pahangi* were available for analysis. Initial experiments focused on the expression of Hsp90 in adult, L3 and Mf by Western blotting using the *B. pahangi* anti-Hsp90 antibody. Anti-actin was used as a loading control for all the experiments. All worm lysates were prepared by grinding in liquid nitrogen as described in Materials and Methods (Section 2.1.3). The protein concentration for each lysate was assayed using the Bio-Rad Protein Assay. Equal amount of proteins were analysed by SDS-PAGE and Western blotting. Hsp90 is expressed in all three life cycle stages (Figure 4.1b) and reacted well with the *B. pahangi* anti-Hsp90 antibody. The autoradiograph was then scanned to quantify the band densities using FlourChem^{TM} IS-5500 software. Figure 4.1c shows the mean ratio of Hsp90 to actin ± SD in each life-cycle stage in three separate experiments. The highest relative level of Hsp90 expression was seen in the Mf, followed by the adult worm with the lowest level in L3. However there was no statistically significant difference between the three groups (p>0.05).

Next the extracts of the three life-cycle stages were used in GA pull-down assays to compare the ability of Hsp90 to bind to GA beads. Surprisingly, only Hsp90 from adult worms bound to the beads (Figure 4.2). Initially, the failure of L3 and Mf extract to bind to GA beads was thought to be due to degradation of the beads. A new batch of GA beads was subsequently derivatised and prepared. Despite the new batch of GA beads, both L3 and Mf stages failed to bind to GA. These experiments were repeated again using fresh worm lysates but the same results were obtained.

Figure 4.1: Hsp90 is expressed in different stages of *B. pahangi*

a) Coomassie Blue stained gel of equal amount of *B. pahangi* lysate from different life-cycle stages. Adult (Lane 1), L3 (Lane 2) and Mf (Lane 3).

b) Immuno-blot of the three life-cycle stages. Hsp90 is expressed in different life-cycle stages and reacted with *B. pahangi* anti-Hsp90 antibody. Adult (Lane 1), L3 (Lane 2) and Mf (Lane 3). Proteins were transferred to NCP using standard methods, then blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot was probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. For actin, the blot was probed with 1:1000 dilution of anti-actin and bound antibody was detected using a 1:10,000 dilution of anti-mouse IgG. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

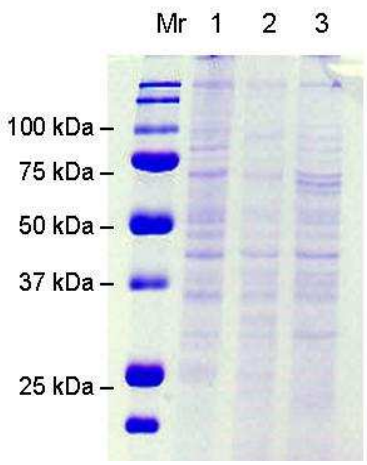
c) The graph shows Hsp90 expression levels in three different life-cycle stages of *B. pahangi* relative to actin as quantified by FlourChem™ IS-5500 software. The results represent the mean ± standard deviation of Hsp90/actin ratio from three different experiments.

Adult 2.10 ± 1.04, L3 0.967 ± 0.33, Mf 2.59 ± 1.32

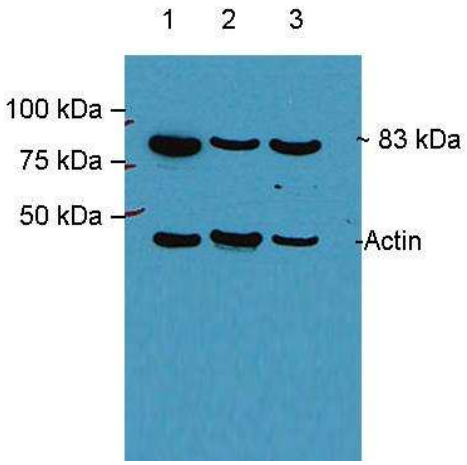
Adult vs L3, p = 0.1641

Adult vs Mf, p = 0.1353

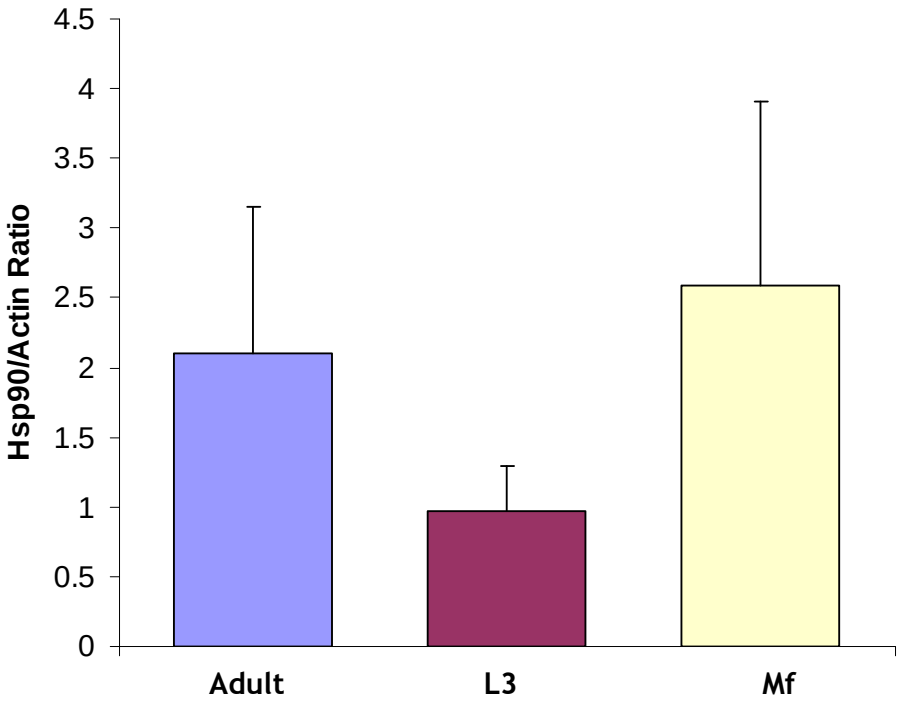
L3 vs Mf, p = 0.1548


Mr 1 2 3
100 kDa –
75 kDa –
50 kDa –
37 kDa –
25 kDa –

(a)


1 2 3
100 kDa –
75 kDa –
50 kDa –
~ 83 kDa
Actin

(b)


Hsp90/Actin Ratio
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Adult
L3
Mf

(c)

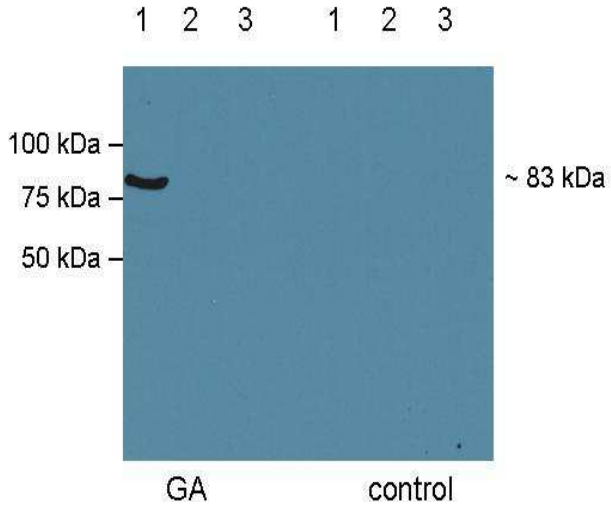


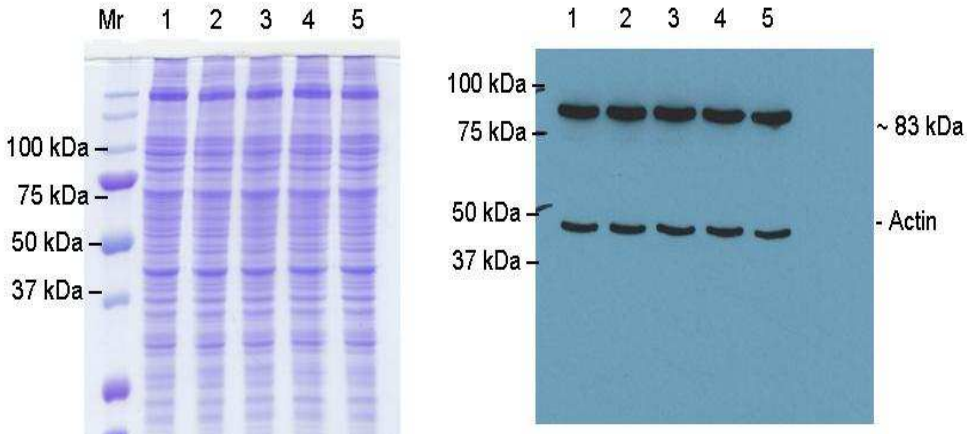
Figure 4.2: Hsp90 from *B. pahangi* L3 and Mf did not bind to GA in pull-down assays.

Western blotting analysis of *B. pahangi* Hsp90 pull-down assay. Adult (Lane 1), L3 (Lane 2) and Mf (Lane 3). For pull-down assays, 300 µg of protein extracts were incubated with 50 µl of GA beads for 2.5h at 4^{o}C on a rocker. The beads were washed extensively in TNES and bound proteins were eluted by boiling in SDS-PAGE sample cocktail. Bound proteins were analysed by SDS-PAGE and immuno-blotting as described previously.

4.2.2 B. pahangi Hsp90 is constitutively expressed and not strongly inducible by heat shock or geldanamycin

As a heat shock protein, Hsp90 responds to stress conditions such as heat and chemicals. In many cellular systems exposure to Hsp90 inhibitors induces a heat shock response. Here I investigated whether Hsp90 levels were up-regulated after exposure to heat shock or to GA. *B. pahangi* adult worms were used in this analysis. For heat shock treatments, 10 adult worms were heat shocked at 41^{o}C for 60 min and allowed to recover for 2 h at 37^{o}C. In addition, one group containing 10 adult worms were incubated at 37^{o}C for 3 h as a control. All worms were solubilised in SDS-PAGE sample cocktail and levels of Hsp90 were then analysed by SDS-PAGE and Western blotting.

In other experiments, 10 adult worms were incubated in 1.0 μM GA for 24 h as described in Materials and Methods (Section 2.2.2), then removed from the drug and directly solubilised in SDS-PAGE sample cocktail. Controls included worms incubated with carrier DMSO or in medium alone. Figure 4.3 shows the autoradiograph of *B. pahangi* Hsp90 expression levels after heat shock or exposure to GA with actin used as a loading control. The autoradiograph shows that no significant increase in the level of Hsp90 was observed in adult *B. pahangi* exposed to elevated temperatures (Lane 5) or treated with GA (Lane 3) compared to control groups (Lane 1, 2 and 4). The bands on the autoradiograph were scanned and band densities were obtained using FlourChem^{TM} IS-5500 software. Statistical significance between groups was calculated based on the density values as quantified by the software. There was no significant difference in the expression level of Hsp90 in any treatment group compared to the relevant control group (p>0.05).



(a) (b)

Figure 4.3: Hsp90 expression levels of *B. pahangi* are not significantly induced by heat shock or GA treatment.

a) Coomassie blue stained gel of worm extracts. Adult *B. pahangi* from each group were analysed on a 10% SDS-PAGE gel. Equal amounts of protein were analysed (as assessed by Coomasie blue staining) of control group without GA (Lane 1), carrier DMSO (Lane 2), treated with GA at 1.0 µM for 24h (Lane 3), control group at 37°C (Lane 4) and heat shocked at 41°C (Lane 5).

b) *B. pahangi* Hsp90 expression is not affected by heat shock or GA treatment. Control group without GA (Lane 1), carrier DMSO (Lane 2), treated with GA at 1.0 µM for 24h (Lane 3), control group at 37°C (Lane 4) and heat shocked at 41°C (Lane 5). All worms were solubilised directly into SDS-PAGE sample cocktail and analysed on 10% SDS-PAGE gels followed by Western blotting. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. For loading control, anti-actin antibody was used at 1:1000 dilution and bound protein was detected using anti-mouse IgG at 1:10,000 dilution. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit and Hsp90 levels were assessed relative to actin, as described previously (data not shown).

GA vs Control, p = 0.6576

DMSO vs Control, p = 0.1618

37°C vs 41°C, p = 0.2123

4.2.3 How much B. pahangi Hsp90 binds to GA beads?

Hsp90 is an abundant protein in cells and it can constitute up to 1-2% of the total cellular protein under normal conditions (Welch & Feramisco, 1982). In these experiments, the proportion of *B. pahangi* Hsp90 that bound to GA was investigated by carrying out a series of pull-down assays. The worm lysate was incubated with GA beads and the non-bound supernatant was retained for the next pull-down. This was repeated five times and the bound proteins were analysed by SDS-PAGE and Western blotting. Figure 4.4a shows a representative autoradiograph of a series of pull-down assays. It can be seen that most of the Hsp90 available to bind to GA beads was present in the first pull-down (Figure 4.4a, Lane 2) with progressively diminishing amounts in subsequent pull-downs. In the fifth pull-down, no band was observed (Lane 6). The autoradiograph was scanned to quantify the band densities using FlourChem™ IS-5500 software. The percentage of Hsp90 binding in each pull-down was calculated relative to the amount of Hsp90 in the starting extract (Figure 4.4a, Lane 1).

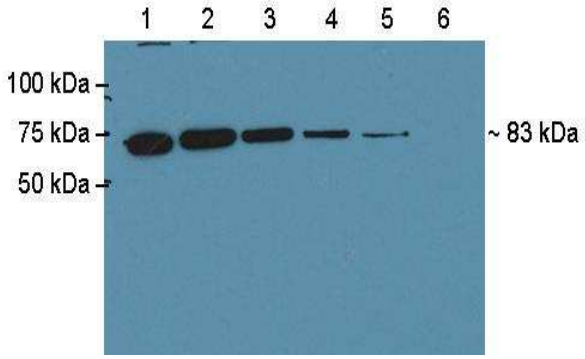
Figure 4.4b shows a graph of the mean percentage of *B. pahangi* Hsp90 bound to GA beads after a series of five pull-down assays calculated from three different experiments. For the first pull-down, approximately 2.88% of the input Hsp90 bound to GA (2.88 ± 0.6025). For the second pull-down approximately 2.10% Hsp90 bound to GA (2.10 ± 0.6401). For the third and fourth pull-downs, approximately 0.93% and 0.39% respectively bound to GA (0.93 ± 0.1305 and 0.39 ± 0.0901), while in the fifth pull-down no signal was observed. Based on these results, approximately 6.31% of the total cellular Hsp90 bound to GA beads in solid pull down assays.

Figure 4.4: Estimating the proportion of *B. pahangi* Hsp90 bound to GA beads

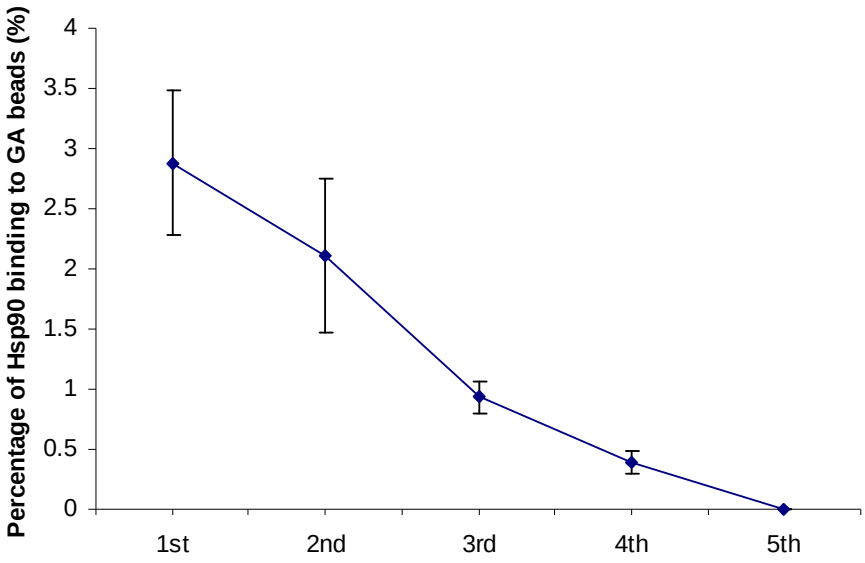
a) Autoradiograph showing the binding of *B. pahangi* Hsp90 to GA beads in a series of pull-down assays. A strong signal is obtained with *B. pahangi* input (Lane 1) and in the first pull-down (Lane 2). Progressively less Hsp90 is present in the second pull-down (Lane 3), the third pull-down (Lane 4), fourth pull-down (Lane 5) with the fifth pull-down (Lane 6) being negative. *B. pahangi* input was analysed at 1:150 dilution.

300 µg of protein extract was incubated with 50 µl of GA beads for 2.5h at 4^{o}C on a rocker. Beads were washed extensively in TNES and bound proteins were eluted by boiling in SDS-PAGE sample cocktail. The supernatant for each pull-down was retained and kept for another round of pull-down. These steps were repeated five times. Then, bound proteins for each pull-down were analysed by SDS-PAGE and immuno-blotting. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

b) Graph shows the proportion of Hsp90 binding to GA beads in pull-down assays. Results presented the mean ± standard deviation of Hsp90 bound to GA beads from three different experiments.


1 2 3 4 5 6
100 kDa
75 kDa
50 kDa
~ 83 kDa

(a)


Percentage of Hsp90 binding to GA beads (%)
4
3.5
3
2.5
2
1.5
1
0.5
0
1st
2nd
3rd
4th
5th

(b)

4.2.4 Hsp90 is secreted by adult B. pahangi

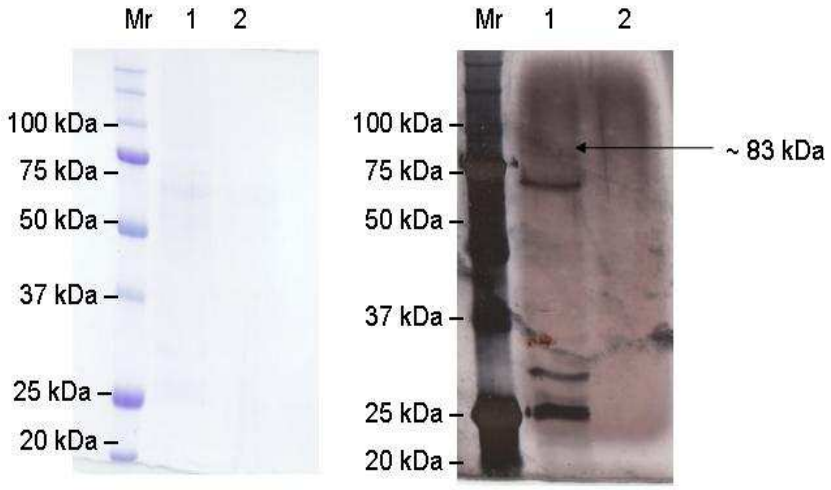
Previous experiments analysing the ES products of adult *B. malayi* showed that Hsp90 is secreted (Kumari *et al.*, 1994). In this section, the ES products of Mf and adult *B. pahangi* were analysed. Mf and adult worms were incubated in RPMI-1640 medium and ES products were collected and concentrated as described in Materials and Methods (Section 2.2.4). Figure 4.5 shows the analysis of ES products from both life-cycle stages. The gel shown in Figure 4.5a was stained with Coomassie blue but this staining method did not detect any bands in the ES products for either stage. The ES products were then analysed using the more sensitive silver staining, as shown in Figure 4.5b. In Lane 1 (adult stage) there were a few bands visible in the ES products but no staining was seen with Mf (Lane 2). In order to validate this observation, concentrated ES from both stages was analysed by SDS-PAGE and Western blotting using the *B. pahangi* anti-Hsp90 antibody. The autoradiograph in Figure 4.5c shows the analysis of ES products detected using *B. pahangi* anti-Hsp90 antibody. Hsp90 was shown to be secreted in ES products of adults (Lane 1) but not Mf (Lane 2).

Figure 4.5: Analysis of ES products of adult and Mf stages of *B. pahangi*

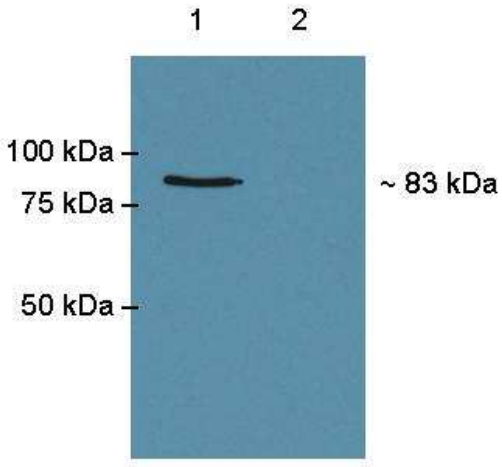
a) Coomassie blue stained gel of ES products from Mf and adult *B. pahangi.* Mr represents the molecular weight marker, adult (Lane 1) and Mf (Lane 2).

b) Silver stained gel of ES products from Mf and adult *B. pahangi*. Mr represents the molecular weight marker, adult (Lane 1) and Mf (Lane 2).

c) Autoradiograph of ES products as detected using *B. pahangi* anti-Hsp90 antibody. Adult stage (Lane 1) and Mf (Lane 2). ES products of both stages were concentrated and solubilised in SDS-PAGE sample cocktail by boiling for 3 min followed by centrifugation at 13200 rpm for 3 min and analysed by SDS-PAGE and Western blotting. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.


Mr 1 2
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
Mr 1 2
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
~ 83 kDa

(a) (b)


1 2
100 kDa
75 kDa
50 kDa
~ 83 kDa

(c)

4.2.5 Identification of B. pahangi Hsp90-associated proteins

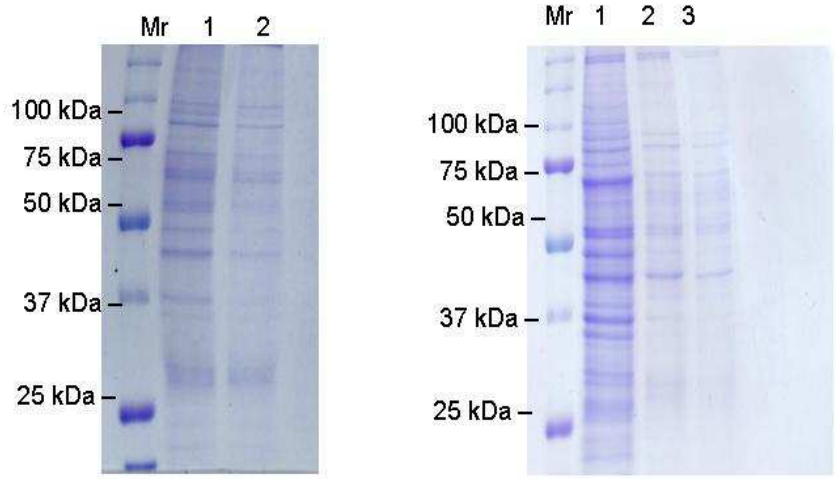
As reviewed by Jackson *et al* (2004) and Wegele *et al* (2004), Hsp90 plays a role as a central component of the assembly and disassembly machine and as such, associates with several groups of proteins. In this section, attempts were made to identify *B. pahangi*-associated proteins by proteomic analysis or using a modified GA-biotin binding assay (Neef *et al.*, 2010).

4.2.5.1 Modified GA-biotin binding assay

This assay relies upon the binding of biotin-labelled GA to Hsp90 protein complexes. The complexes are then isolated using Neutravidin-agarose beads. *B. pahangi* lysate was incubated in the present of GA-biotin, while the control sample was incubated without GA-biotin. The bound proteins were captured by incubating with Neutravidin-agarose beads and analysed as described previously in Materials and Methods (Section 2.2.5.1).

Figure 4.6a shows a representative Coomassie blue gel of *B. pahangi* lysate incubated with GA-biotin or not, followed by Neutravidin capture. It can clearly be seen from the gels, that in the presence of GA-biotin, a number of proteins were present in the eluate of the Neutravidin-agarose beads (Lane 1). However, careful examination of Lane 2 (without GA-biotin) shows that most of the bands were also present, albeit at a lower level, suggesting that binding is mostly non-specific. Attempts were made to overcome this problem by pre-incubating *B. pahangi* lysate with Neutravidin-agarose beads in order to eliminate any proteins that might bind non-specifically to the beads. Following the pre-incubation with Neutravidin-agarose beads, the supernatant was incubated with GA–biotin and processed as described previously. Figure 4.6b

shows the pull-down assay after pre-incubation with Neutravidin-agarose beads. Lane 1 shows the *B. pahangi* proteins bound non-specifically to Neutravidin-agarose beads. The supernatant from this incubation was then mixed with GA-biotin and processed as described previously. Lane 2 shows that a number of proteins were present in the eluate of the Neutravidin-agarose beads. However, many of the bands were also observed in the control lane (Lane 3). Based on these results, this approach was not used further.



(a) (b)

Figure 4.6: GA-biotin binding assay

a) Coomassie blue stained gel showing the proteins bound to GA-biotin as analysed by SDS-PAGE. Bound protein captured by Neutravidin-agarose beads incubated with GA-biotin (Lane 1) or without GA-biotin (Lane 2). 1.0 mg *B. pahangi* lysate was incubated with 100 µl of GA-biotin for 15 h at 4°C. The bound proteins were captured by incubating the mixture with Neutravidin-agarose beads. Bound protein were eluted in 100 µl SDS-PAGE sample cocktail and 20 µl of sample was loaded onto a 10% gel for the analysis. Control (Lane 2) was incubated without GA-biotin and analysed exactly as for Lane 1.

b) Coomassie blue stained gel showing the proteins bound to GA-biotin. *B. pahangi* lysate was pre-incubated with Neutravidin-agarose beads (Lane 1), the remaining supernatant was then incubated with GA-biotin followed by Neutravidin-agarose beads (Lane 2) or without GA-biotin (Lane 3) followed by Neutravidin-agarose beads.

4.2.5.2 Proteomic analysis of *B. pahangi* Hsp90-associated proteins

Hsp90 exists within the cell in complexes with co-chaperones and client proteins. After exposure to GA, Hsp90 undergoes a conformational change with the result that client proteins are released from the complexes and subject to degradation. Thus, it may be possible to identify some Hsp90-associated proteins by their disappearance following GA treatment. *B. pahangi* adult worms were incubated in RPMI-1640 medium containing 1.0 µM GA or DSMSO alone for 24 h and worm lysates were prepared as described in Materials and Methods (Section 2.2.5.1). Equal amounts of protein (50 µg) were labelled with Cy3 for GA-treated group or Cy5 for the control group and the samples mixed and subjected to IF and SDS-PAGE as described in Materials and Methods (Section 2.2.5.3). After SDS-PAGE, the gel was scanned using a Typhoon 9400 scanner. The separate gel images of the treated group (with GA) and the control group (non-treated) were compared using the Differential In-Gel Analysis (DIA) software module of the DeCyder batch processor (Amersham Bio-Sciences, UK). Figure 4.6a shows the 2D-gel image of the treated group with GA at 1.0 µM and Figure 4.7b shows the 2D-gel image of control group. Using the DeCyder batch processor, approximately 2113 spots were detected, of which 96.2% showed similarity in the protein abundance for each sample. Approximately 3.1% of proteins were shown to be up-regulated in the treated group compared to the control group, while approximately 0.7% of the proteins were down-regulated in the treated group compared to the control group. Figure 4.7c shows the spots detected using DIA software. The spots in yellow represent those proteins that differed in abundance as analysed using the software. See Table 4.1 for a summary of the analysis.

Figure 4.7: Proteomic analysis of *B. pahangi* adult extract following GA treatment or control worms.

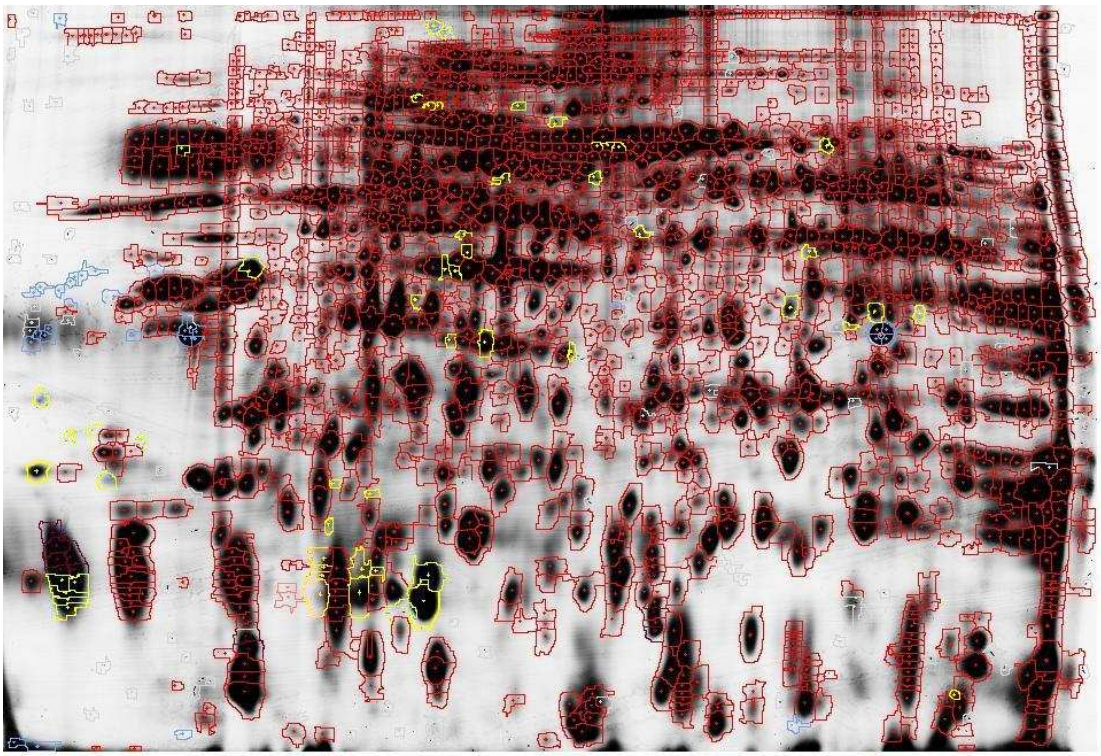
a) Scanned image of a 2D-gel of *B. pahangi* adult worms treated with GA using the Typhoon 9400 Scanner. 60 adult worms were cultured in RPMI-1640 medium containing a final concentration of GA at 1.0 µM at 37^{o}C in an atmosphere of 5% CO_2 in air for 24 h. Equal amounts of protein (50 µg) were labelled with Cy3 for GA-treated and subjected to IF and SDS-PAGE as described in Materials and Methods (Section 2.2.5.3).

b) Scanned image of a 2D-gel of the control group using the Typhoon 9400 Scanner. 60 adult worms were cultured in RPMI-1640 medium containing an equivalent volume of DMSO at 37^{o}C in an atmosphere of 5% CO_2 in air for 24 h. Equal amounts of protein (50 µg) were labelled with Cy5 for control and subjected to IF and SDS-PAGE as described in Materials and Methods (Section 2.2.5.3).

c) Protein spots detected by the DIA software. The red circles represent the spots of equal abundance in both samples, while the yellow circles represent the spot that were differentially expressed in the two samples, as detected by the DIA software.

(a)

(b)



(c)

Table 4.1: Summary of spot statistics as analysed by DIA Software

	Number of spots	Percentage (%)
Increased	61	3.1
Similar	1911	96.2
Decreased	14	0.7
Total	2113	100

Unfortunately it was not possible to identify the up-regulated and down-regulated protein spots because the spot picking equipment required was out of order. In an attempt to analyse the differences, the experiment was repeated using a new fresh worm lysate, prepared exactly as described previously. However, in this experiment, the experimental and the control group were analysed separately. The IPG strip samples of the treated group and the control group were transferred onto different gels. The gels were stained by Coomassie blue, destained and then scanned using the Typhoon Trio Variable Imager. Any differences were spotted manually using the Ettan Spot handling workstation (Amersham Bio-Sciences, UK).

Figure 4.8a and 4.8b show the image of the GA-treated group and the non-treated group as stained by Coomassie blue. Protein spots that are present on the control gel but absent in the GA-treated group are considered to be down-regulated proteins, while protein spots that are absent in the control group but present in GA-treated group are considered to be up-regulated proteins. There were only a few spots in the control group that were absent in the GA-treated group (black circles). In addition, there were two clear protein spots that are present in the GA-treated group compared to the control group (Figure 4.8c, blue circles). Overall, 12 spots were identified of which seven were differently expressed (see Figure 4.8c). Spots of interest were carefully excised and subjected to in-gel trypsin digest following which the dried digested samples were subjected to analysis by mass spectrometry. All peptide samples were separated on liquid chromatography-electrospray (LC) system before analysing by ionization/multi-stage mass spectrometry (ESI-MS) (see Appendix 6). Table 4.2 shows the results of the protein identification of 2D-gel spots in a

comparative screen between GA-treated and control worms. These proteins belong to several groups most of which have structural or chaperone function.

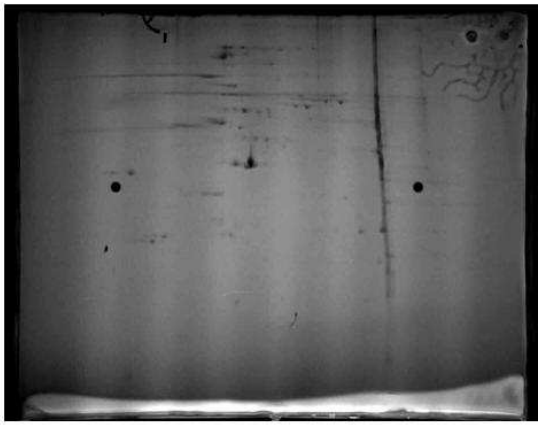
Figure 4.8: 2D-gel image of *B. pahangi* protein lysate as analysed by 2D SDS-PAGE.

a) Image of 2D-gel scanned using Typhoon Scanner represents the GA-treated group. 60 adult worms were cultured in RPMI-1640 medium containing a final concentration of GA at 1.0 µM at 37^{o}C in an atmosphere of 5% CO_2 in air for 24 h. Equal amounts of protein (50 µg) were prepared as described previously and subjected to IF and SDS-PAGE as described in Materials and Methods (Section 2.2.5.3). Gel was stained with Coomassie blue.

b) Image of 2D-gel scanned using Typhoon Trio Variable Imager representing the untreated control group. 60 adult worms were cultured in RPMI-1640 medium containing an equivalent volume of DMSO at 37^{o}C in an atmosphere of 5% CO_2 in air for 24 h. Equal amounts of protein (50 µg) were prepared as described previously and subjected to IF and SDS-PAGE as described in Materials and Methods (Section 2.2.5.3). Gel was stained with Coomassie blue.

c) 2D-image of gel as analysed by Coomassie blue staining showing the spot of interest for the analysis. 12 spots were excised. The black circles represent the spots that decreased in abundance in the GA group. The red circles represent the spots which were similar in abundance in both groups and the blue circles represent the spots which were increased in abundance after exposure to 1.0 µM GA.

(a)



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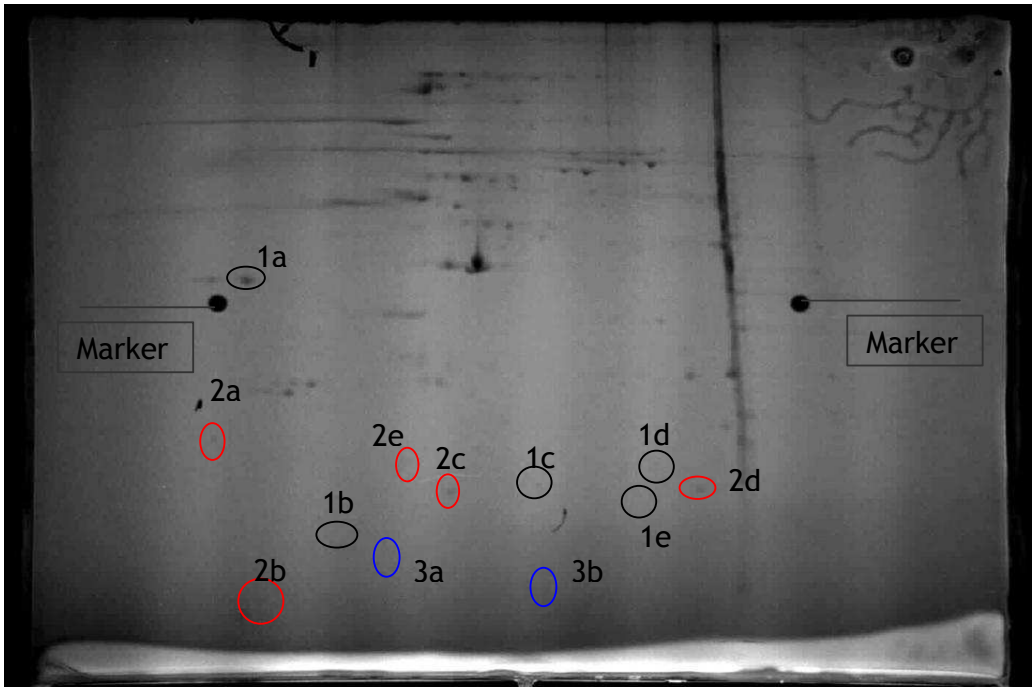


(c)

Table 4.2: Identification of 2D-gel spots differentially expressed in a comparative screen between *B. pahangi* GA-treated group vs control group.

Sample ID*	Protein hit	Hit score	Function
1a	Tropomyosin	1233	Structural
1b	Myosin regulatory light chain 1	469	Structural
1c	Actin-depolymerising factor 1	700	Structural
1d	Actin-depolymerising factor 1	757	Structural
1e	DJ-1 family protein	817	Unknown
2a	Small heat shock protein	194	Chaperone
2b	Alkali myosin light chain	601	Structural
2c	Thioredoxin	219	Antioxidant
2d	Small heat shock protein	814	Chaperone
2e	Tropomyosin	1174	Structural
3a	Myosin regulatory light chain 1	613	Structural
3b	Tropomyosin	701	Structural

*** 1 – down-regulated in GA vs control group, 2 – similar in both groups, 3 – up-regulated in GA vs control group.**

4.2.6 Recombinant human Hsp90α binds to GA beads

Post-translational modifications such as phosphorylation, acetylation, nitrosylation and ubiquitinylation have been shown to regulate the chaperone function of Hsp90. In order to investigate the possible effect of post-translational modifications on Hsp90 binding to GA, the binding of recombinant human Hsp90α (rHsp90α) was compared with native human Hsp90. Human Hsp90 was used in these experiments because recombinant *B. pahangi* Hsp90 was not available for analysis. Native Hsp90 was isolated from SkBr3 cells. The rHsp90α (StressMarq, UK) was expressed in *E. coli* and therefore does not bear any post-translational modification. 8 µg of SkBr3 was analysed by SDS-PAGE. For comparison 2 µg of rHsp90 was analysed (Figure 4.9a). An abundant band at approximately 90 kDa was detected for rHsp90α (Lane 1) but only a faint band for the SkBr3 lysate was detected (Lane 2). Both samples were then tested against *B. pahangi* anti-Hsp90 antibody at 1:5000 dilution or AC88 monoclonal anti-Hsp90 antibody at 1:1000 dilution. Figure 4.9b shows that the *B. pahangi* anti-Hsp90 antibody failed to react with rHsp90α (Lane 1) or SkBr3 lysate (Lane 2). However, the AC88 monoclonal antibody is able to detect Hsp90 in both samples (data not shown). Analysis of pull-down assays showed that Hsp90 from both sources bound to GA beads (Figure 4.9c), which suggests that the presence or absence of post-translational modifications did not affect the ability of human Hsp90 to bind to GA. Blocking assays were then used to show the specificity of the binding by pre-incubation in soluble GA at 1.0 µM or 2.0 µM GA. Figure 4.9d and Figure 4.9e show that the binding of rHsp90α and SkBr3 Hsp90 is blocked by soluble GA, with 1.0 µM GA being sufficient to completely block Hsp90 binding to GA beads for both extracts.

Figure 4.9: Analysis of recombinant human Hsp90α and SkBr3 extract

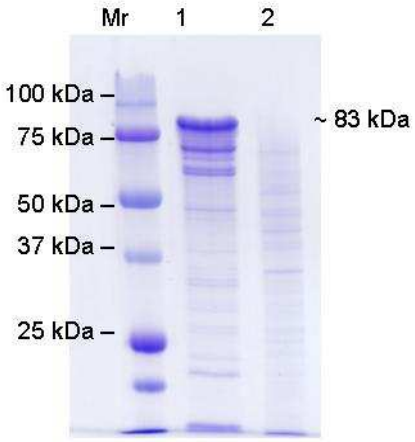
a) Coomassie blue stained gel of rHsp90α and SkBr3 extract. 2 µg of rHsp90α (Lane 1) and 8 µg of SkBr3 lsyate (Lane 2) were analysed. Mr represents the molecular weight marker.

b) Autoradiograph of rHsp90α and SkBr3 extract probed with *B. pahangi* anti-Hsp90 antibody. 2 µg of rHsp90α (Lane 1) and 8 µg of SkBr3 lsyate (Lane 2) were analysed by SDS-PAGE and Western blotting. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

c) Pull-down analysis of rHsp90α and SkBr3 extract with GA beads or control beads. rHsp90α with GA beads (Lane 1), rHsp90α with control beads (Lane 2), SkBr3 laysate with GA beads (Lane 3) and SkBr3 lysate with control beads (Lane 4). 5 µg of rHsp90α or 500 µg of SkBr3 extract were incubated with 50 µl of GA beads for 2.5h at 4^{o}C on rocker. Beads were washed extensively in TNES and bound proteins were eluted by boiling in SDS-PAGE sample cocktail. Then, bound proteins were analysed by SDS-PAGE and immuno-blotting. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:1000 dilution of the AC88 anti-Hsp90 monoclonal antibody. Bound antibody was detected using a 1:10,000 dilution of anti-mouse IgG. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

d) Blocking assays of rHsp90α with soluble GA at 1.0 µM or 2.0 µM GA. rHsp90α without soluble GA (Lane 1), 1.0 µM GA (Lane 2), 2.0 µM GA (Lane 3), DMSO (Lane 4) and control beads (Lane 5). 5 µg of rHsp90α was pre-incubated with soluble GA at different concentration for 1 h at room temperature and subjected to pull-down assay. Bound protein was analysed by SDS-PAGE and Western blotting.

e) Blocking assays of SkBr3 extract with soluble GA at 1.0 µM or 2.0 µM GA. SkBr3 extract without soluble GA (Lane 1), 1.0 µM GA (Lane 2), 2.0 µM GA (Lane 3), DMSO (Lane 4) and control beads (Lane 5). 500 µg of SkBr3 extract was pre-incubated with soluble GA for 1 h at room temperature and processed exactly as rHsp90α.


Mr
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100 kDa
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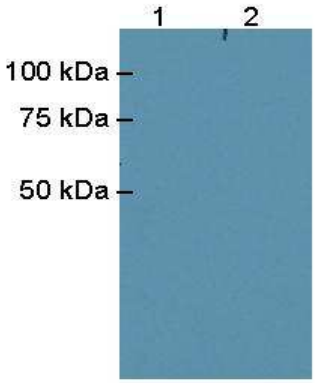
(a)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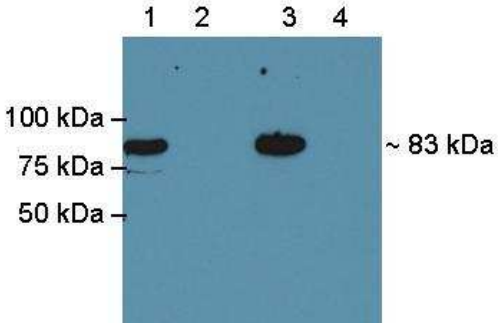
(b)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3
4
100 kDa
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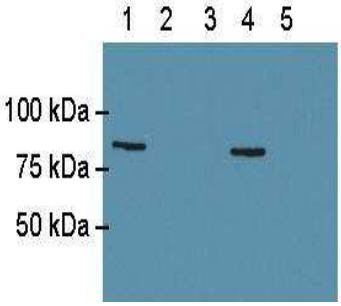
(c)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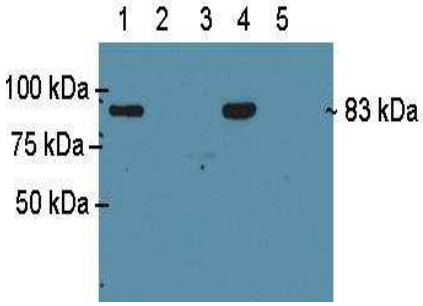
(d)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4
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100 kDa
75 kDa
50 kDa
~ 83 kDa

(e)

4.2.7 Competition assays with B. pahangi Hsp90

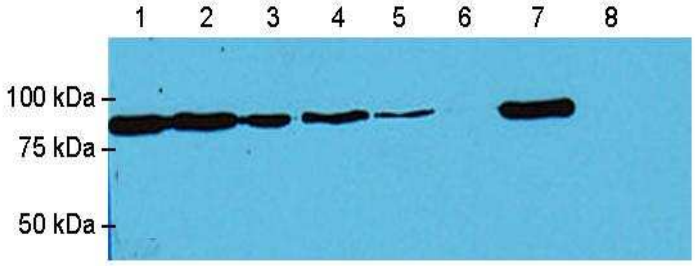
4.2.7.1 Competition assay with soluble GA

To further characterise the binding of *B. pahangi* Hsp90 to GA, I examined the ability of soluble GA to block binding to solid phase GA. The soluble extract of *B. pahangi* worms was pre-incubated with varying concentrations of GA from 25 nM to 1000 nM for 60 min at room temperature. Pull-downs were then carried out as described previously, analysed by immuno-blotting and quantified by scanning of the subsequent autoradiograph. The autoradiograph in Figure 4.10a demonstrates that soluble GA blocks the binding of *B. pahangi* Hsp90 to GA-derivatised beads. At 1000 nM free GA (Lane 6), the binding of *B. pahangi* Hsp90 to GA beads is completely blocked. Overnight exposure of the blot failed to show any band (result not shown). The autoradiograph was then scanned and band densities were obtained using FlourChem^{TM} IS-5500 software. Based on these values, a graph was plotted and analysed. Figure 4.10b shows the competition assay of *B. pahangi* Hsp90 binding to GA pre-incubated with free GA. The IC_{50} value, which represents the concentration of free GA that block the binding of 50% of Hsp90 to GA beads, is 100-200 nM GA.

Figure 4.10: Competition for Hsp90 binding to GA beads with soluble GA.

a) The autoradiograph shows an experiment in which *B. pahangi* extracts were pre-incubated with increasing concentrations of soluble GA. The GA pull-down assays were performed as described previously either using GA beads (Lane 1), pre-incubated with soluble GA at 25 nM (Lane 2), 50 nM (Lane 3), 100 nM (Lane 4), 200 nM (Lane 5), 1000 nM Lane 6), DMSO alone (Lane 7) or control beads (Lane 8). Bound proteins were analysed by SDS-PAGE and Western blotting. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit. The figure shown is representative of three separate experiments.

b) Graph shows the binding of *B. pahangi* Hsp90 to GA beads is competed by soluble GA. At 1000 nM, GA completely blocks the binding of Hsp90 to GA beads. The estimated IC_{50} value is between 100 nM to 200 nM GA. Results presented are the mean ± standard deviation of three different experiments. Binding to GA beads in the absence of GA was set at 100%.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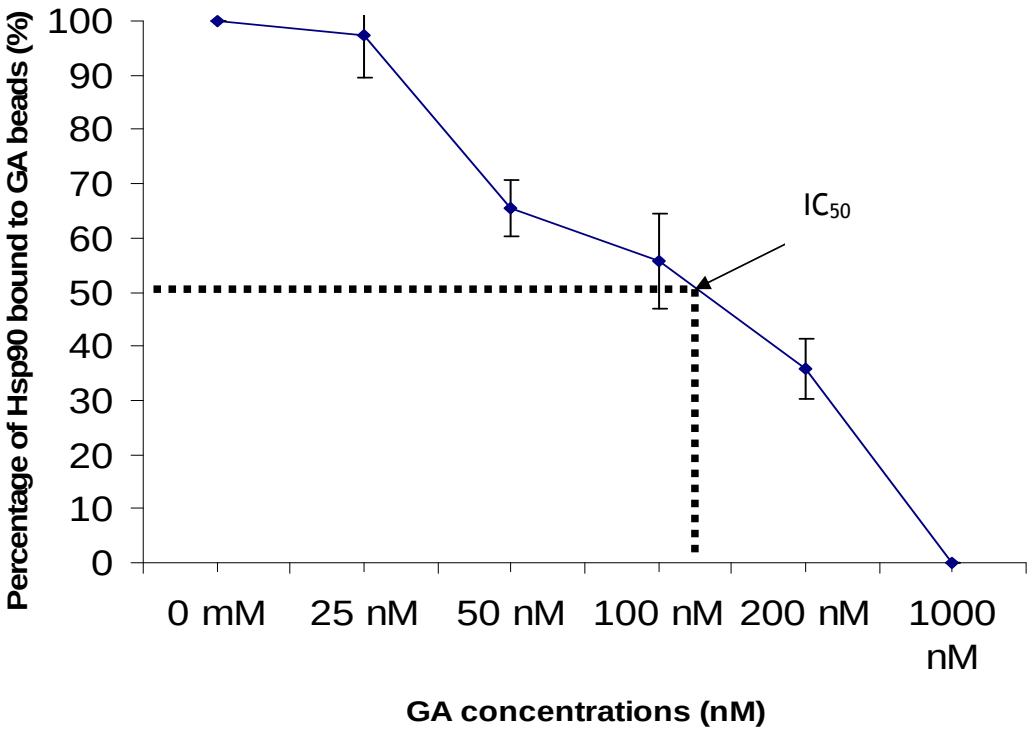
100 kDa
75 kDa
50 kDa

(a)


Percentage of Hsp90 bound to GA beads (%)
100
90
80
70
60
50
40
30
20
10
0
IC50
0 mM
25 nM
50 nM
100 nM
200 nM
1000 nM
GA concentrations (nM)

(b)

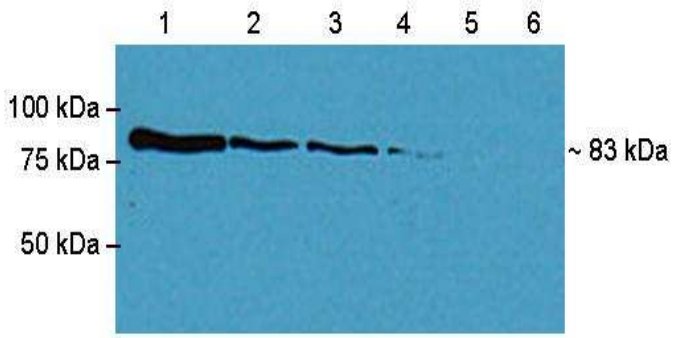
4.2.7.2 The ability of Hsp90 to bind to GA beads is altered by novobiocin

GA and novobiocin do not share the same binding site in Hsp90. However, studies with mammalian Hsp90 have shown that novobiocin can affect the ability of Hsp90 to bind to GA. GA binds to the N-terminal domain of Hsp90 while novobiocin binds to the C-terminal domain. In this experiment, I tested whether novobiocin can affect the binding of *B. pahangi* Hsp90 to GA. *B. pahangi* lysates were pre-incubated with different concentration of novobiocin (from 2.5-20 mM) prior to addition of GA beads (as described in Materials and Methods)(Section 2.2.7.1). The results of a representative experiment are shown in Figure 4.11a. It can be seen that the amount of Hsp90 bound to GA decreased as the concentration of soluble novobiocin increased (Lane 1 to Lane 5). At 20 mM soluble novobiocin (Lane 5), binding was completely inhibited and no signal was obtained even though the blot was exposed overnight (result not shown). Bands on the autoradiograph were scanned and band densities were obtained using FlourChem^{TM} IS-5500 software. Figure 4.11b shows that 10 mM soluble novobiocin was sufficient to inhibit approximately 90% of the binding with an IC_{50} value of approximately 5 mM novobiocin. The IC_{50} value here represents the concentration of soluble novobiocin able to inhibit 50% of Hsp90 binding to GA beads. The ability of free soluble novobiocin to compete with GA beads to bind Hsp90 suggested there is some kind of interaction between the N-terminal domain and the C-terminal domain which affects the binding of Hsp90 to GA.

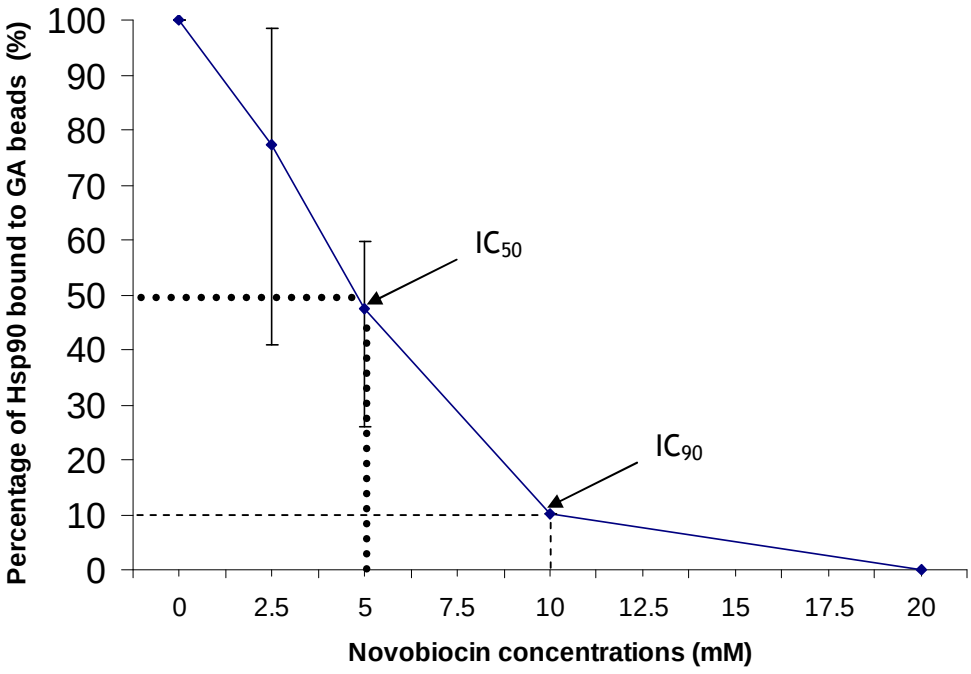
Figure 4.11: Binding of *B. pahangi* Hsp90 to GA beads is altered by excess novobiocin.

a) The autoradiograph shows that soluble novobiocin alters the ability of Hsp90 to bind to GA beads. *B. pahangi* extract incubated with GA beads without soluble novobiocin (Lane 1), with 2.5 mM (Lane 2), 5.0 mM (Lane 3), 10.0 mM (Lane 4), 20.0 mM (Lane 5) novobiocin or with control beads (Lane 6). Lysates were prepared in liquid Nitrogen with a pestle and mortar. 500 µg of lysate in a total volume of 300 µl was pre-incubated with soluble novobiocin (2.5 mM, 5.0 mM, 10.0 mM and 20.0 mM) for 1h at room temperature and then incubated with GA beads or beads alone. Bound proteins were analysed by SDS-PAGE and Western blotting. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit. Exposure 30 sec.

b) Graph shows that the binding of B*. pahangi* Hsp90 is competed by soluble novobiocin. 20 mM novobiocin completely blocks the binding of Hsp90 to GA beads. IC_{50} value is approximately 5.0 mM. Results presented are the mean ± standard deviation from three different experiments. Binding to GA beads in the absence of novobiocin was set at 100%.


1
2
3
4
5
6
100 kDa
75 kDa
50 kDa
~ 83 kDa

(a)


Percentage of Hsp90 bound to GA beads (%)
100
90
80
70
60
50
40
30
20
10
0
IC50
IC90
0
2.5
5
7.5
10
12.5
15
17.5
20
Novobiocin concentrations (mM)

(b)

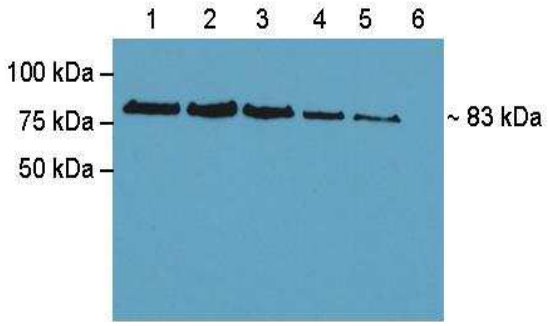
4.2.7.3 ATP affects the binding of *B. pahangi* Hsp90 to GA beads

In mammalian and yeast Hsp90, GA and ATP were shown to bind to the same site in the N-terminal domain of Hsp90. The binding of ATP to Hsp90 followed by ATP hydrolysis is essential for the activity of Hsp90 as reviewed in Chapter 1. In this section the ability of ATP to affect the binding of Hsp90 to GA beads was tested by pre-incubation of *B. pahangi* lysates with ATP at various concentrations (5.0 mM, 10.0 mM, 15.0 mM and 20.0 mM). Pre-incubation of worm lysate with ATP affected the ability of Hsp90 to bind to GA beads. Figure 4.12a shows an autoradiograph of a typical experiment while Figure 4.12b shows that approximately 15.0 mM ATP was sufficient to block 50% of Hsp90 binding to GA beads. However, the highest concentration used in the experiments (20.0 mM ATP) was not able to completely block the binding of Hsp90 to GA beads. The IC_{50} value here represents the concentration of soluble ATP that inhibits 50% of Hsp90 binding to GA beads.

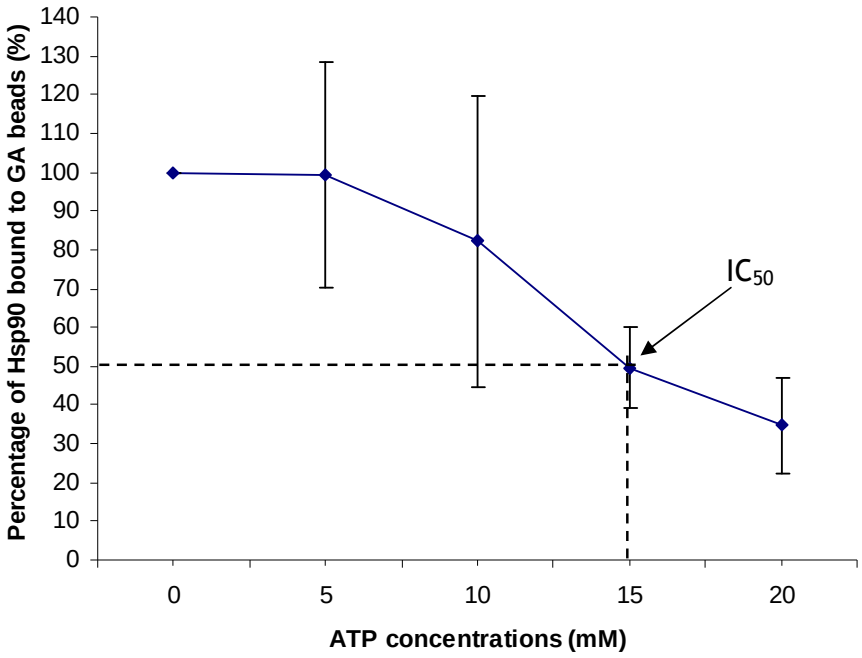
Figure 4.12: ATP alters the ability of *B. pahangi* Hsp90 to bind to GA beads

a) The autoradiograph shows an experiment in which *B. pahangi* lysate was pre-incubated with increasing concentrations of soluble ATP. The solid GA pull-down assays were performed as described previously either using GA beads (Lane 1), pre-incubated with soluble ATP at 5.0 mM (Lane 2), 10.0 mM (Lane 3), 15.0 mM (Lane 4), 20.0 mM (Lane 5) or control beads (Lane 6). Bound proteins were analysed by SDS-PAGE and Western blotting. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit. The figure shown is representative of three separate experiments.

b) Graph shows the binding of *B. pahangi* Hsp90 to GA beads is altered by soluble ATP. The estimated IC_{50} value is 15.0 mM. Results presented are the mean ± standard deviation from three different experiments. Binding to GA beads in the absence of ATP was set at 100%.


1 2 3 4 5 6
100 kDa
75 kDa
50 kDa
~ 83 kDa

(a)


Percentage of Hsp90 bound to GA beads (%)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
ATP concentrations (mM)
IC50

(b)

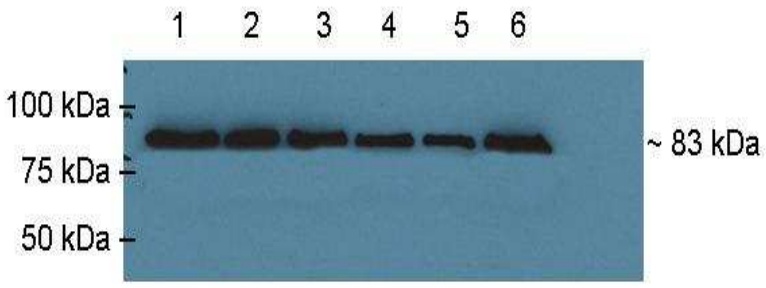
4.2.7.4 The binding of *B. pahangi* Hsp90 to ATP beads is competed by free GA

In further experiments, I investigated whether pre-incubation of *B. pahangi* lysates with soluble GA could inhibit the binding of *B. pahangi* Hsp90 to bind ATP using γ-phosphate immobilised ATP. *B. pahangi* lysates were incubated with soluble GA at different concentrations prior to incubation with ATP beads. Then the bound proteins were analysed by SDS-PAGE and Western blotting (as described previously). Figure 4.13a shows that *B. pahangi* Hsp90 bound to ATP beads and that binding was competed by free GA. The lowest concentration of GA used was 0.25 μM and the highest was 2.0 μM. However, 2.0 μM GA was not sufficient to completely block the binding of Hsp90. Then, the autoradiograph was scanned and band densities were obtained using FlourChem™ IS-5500 software. Figure 4.13b shows that approximately 1.0 μM GA was sufficient to block about 50% of the Hsp90 binding to ATP beads. The IC_{50} represents the value of free GA that inhibits 50% of the Hsp90 binding to ATP beads.

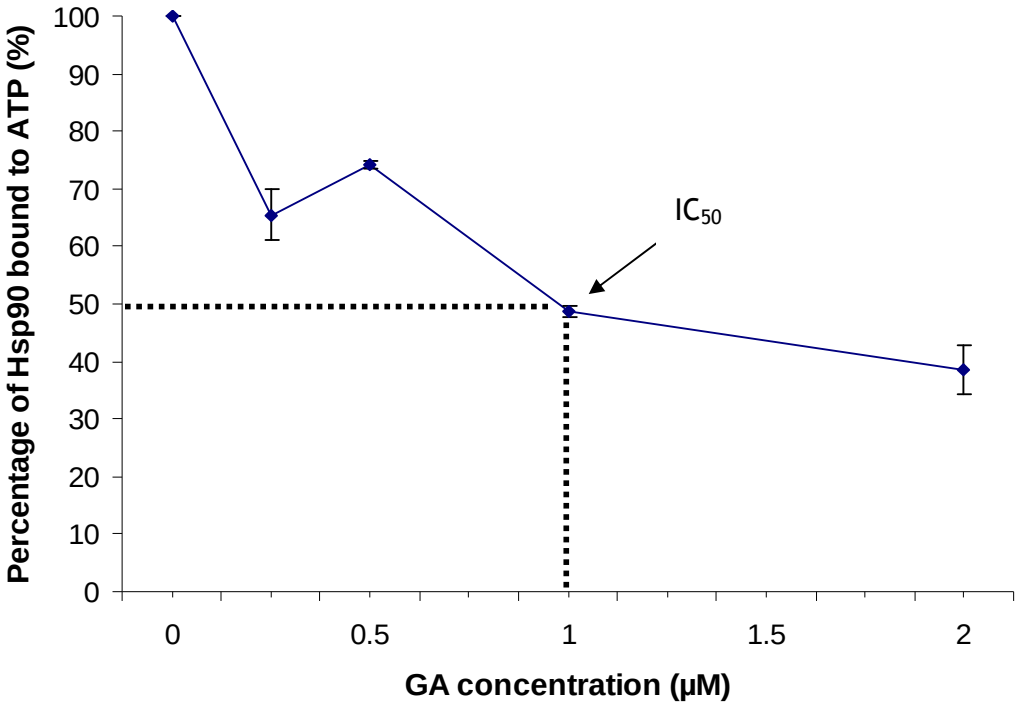
Figure 4.13: Binding of *B. pahangi* Hsp90 to ATP beads is competed by soluble GA.

a) Autoradiograph showing the results of a typical ATP-binding assay. *B. pahangi* lysates were prepared in liquid Nitrogen with a pestle and mortar. 500 µg of lysate in a total volume of 500 µl was pre-incubated with soluble GA at 0.25 µM (Lane 2), 0.5 µM (Lane 3), 1.0 µM (Lane 4), 2.0 µM (Lane 5) or with control beads (Lane 6) for 60 min at room temperature and then incubated with ATP beads or with beads alone. Bound proteins were analysed by SDS-PAGE and Western blotting. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 Ab. Bound Ab was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

b) Graph showing that the binding of *B. pahangi* Hsp90 to ATP is competed by soluble GA. 1.0 µM GA is sufficient to block 50% of Hsp90 binding to ATP beads. Data presented are the mean ± standard deviation from three separate experiments. Binding to ATP beads in the absence of GA was set at 100%.


1 2 3 4 5 6
100 kDa
75 kDa
50 kDa
~ 83 kDa

(a)


Percentage of Hsp90 bound to ATP (%)
100
90
80
70
60
50
40
30
20
10
0
IC50
0
0.5
1
1.5
2
GA concentration (μM)

(b)

4.2.8 Analysis of Hsp90 in B. pahangi and *C. elegans*

In Chapter 3, the ability of *B. pahangi* Hsp90 to bind GA was shown to be shared by some clade III parasitic species and by *Trichinella spiralis* (clade I) but not with parasitic species from other clades or with free-living nematodes. In most eukaryotes, the sequence of Hsp90 proteins is highly conserved. The most obvious explanation for the surprising lack of GA binding by Hsp90 from some worms would be an alteration in the N-terminal domain of the chaperone. In order to investigate the differences between *B. pahangi* and *C. elegans* Hsp90 sequence, an alignment of the predicted amino acid sequence of *B. pahangi* and *C. elegans* was compared (Figure 4.14). This confirmed the high degree of conservation within the whole sequence (Figure 4.14). *C. elegans* Hsp90 is 84% identical (91% similar) to *B. pahangi* Hsp90. The N-terminal domain shows a minimal number of substitutions (see Figure 4.14). The sequence of *C. elegans* Hsp90 is shorter than *B. pahangi* Hsp90, because the charged region contains fewer amino acids.

Despite the high degree of similarity between *C. elegans* Hsp90 and *B. pahangi* Hsp90, *C. elegans* Hsp90 failed to bind GA in solid phase pull-down assays (see Chapter 3). In order to further investigate these contrasting results, Hsp90 expression levels were compared in both species. Worm lysates were prepared as described previously. For this analysis, only adult stages of both nematodes were used. The protein concentration of each lysate was estimated using the Bio-Rad protein assay and equal amounts of protein were loaded onto 10% SDS-PAGE gels and blotted (see Figure 4.15a).

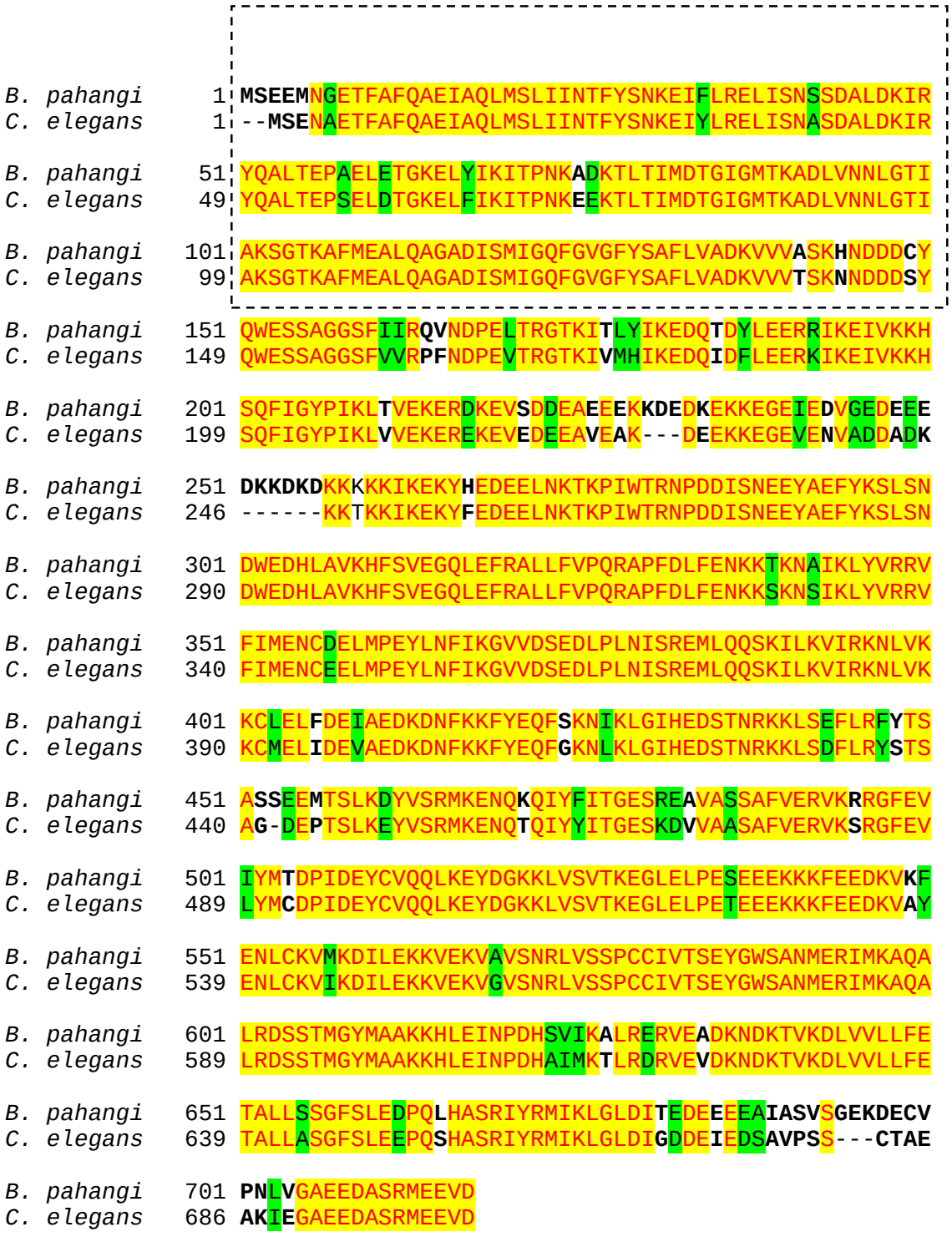


Figure 4.14: Hsp90 is conserved in *B. pahangi* and *C. elegans.*

Figure shows an alignment of the predicted amino acid sequences of *B. pahangi* (Bp, accession number AJ005784) and *C. elegans* (Ce, accession number Z75530) Hsp90s by Clustal-W. Identical amino acids are in red with yellow-shaded box. Green shading represent amino acids that are similar. Bold letters represent amino acids that differ in both sequences. The highly conserved N-terminal domain is indicated by the dashed box.

Figure 4.15b shows the autoradiograph for Hsp90 expression in *B. pahangi* and *C. elegans* with actin as a loading control. A band of ~83 kDa was detected in both species by Western blot using an antibody raised to recombinant *B. pahangi* Hsp90. The autoradiograph was then scanned and band densities were obtained using FlourChem^{TM} IS-5500 software. This analysis showed that Hsp90 is expressed at a relatively higher level in *B. pahangi* compared to *C. elegans* Hsp90 ($p<0.05$).

Previous results showed that soluble GA competes with ATP beads to bind to Hsp90. In *C. elegans,* Hsp90 failed to bind GA but retained its ability to bind ATP (David *et al.*, 2003). To address this issue, I compared the ability of *B. pahangi* and *C. elegans* Hsp90 to bind ATP using γ-phosphate immobilised ATP as previously described (Grenert *et al.*, 1997; Soti *et al.*, 2003). Worm lysates were incubated with ATP beads for 60 min at room temperature. The resin was then pelleted and washed with buffer and analysed by SDS-PAGE and Western blotting. Figure 4.16 shows that both *B. pahangi* and *C. elegans* Hsp90 bind to ATP beads. The results presented are based on three independent experiments.

Figure 4.15: Hsp90 is more highly expressed in *B. pahangi* than in *C. elegans.*

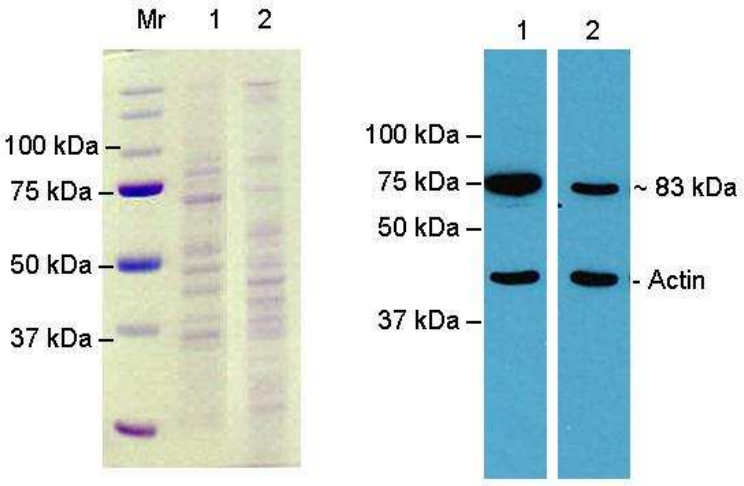
a) Coomassie blue stained gel of *B. pahangi* (Lane 1) and *C. elegans* (Lane 2) adult worm extracts. Mr presents the molecular weight marker.

b) Hsp90 expression levels of *B. pahangi* (Lane 1) and *C. elegans* (Lane 2) with actin as a loading control. Equal amount of proteins were analysed by SDS-PAGE and Western blotting. Proteins were transferred to NCP using standard methods, blocked overnight in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

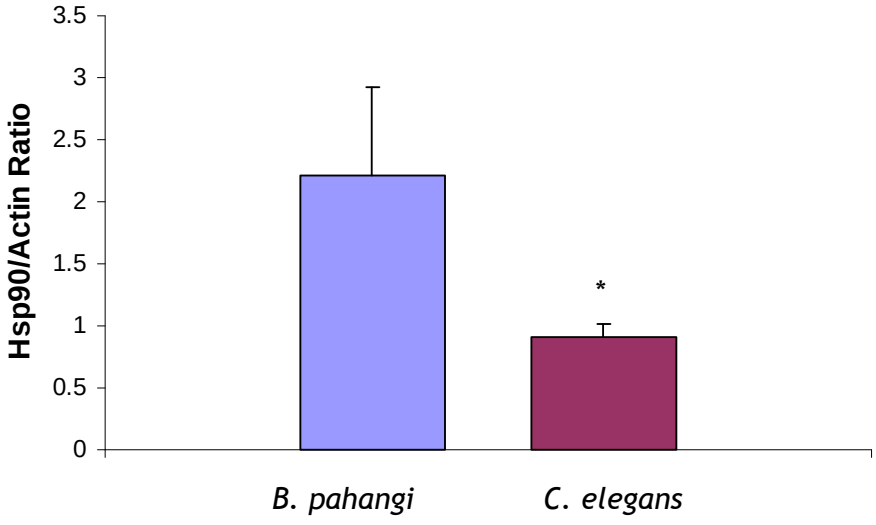
c) The graph shows the expression levels of Hsp90 for *B. pahangi* and *C. elegans* as detected using the *B. pahangi* anti-Hsp90 antibody. The autoradiograph was scanned for densities using FlourChem™ IS-5500 software. Hsp90 expression is significantly higher in *B. pahangi* compared to *C. elegans.* The data represent the mean ratio of Hsp90/actin ± standard deviation from three different experiments.

***B. pahangi* 2.2138 ± 0.9138** ***C. elegans* 0.7155 ± 0.1033**

***B. pahangi* Hsp90 vs *C. elegans* Hsp90 * *p* = 0.0010**


Mr
1
2
100 kDa
75 kDa
50 kDa
37 kDa
1
2
100 kDa
75 kDa
50 kDa
37 kDa
~ 83 kDa
- Actin

(a) (b)


Hsp90/Actin Ratio
3.5
3
2.5
2
1.5
1
0.5
0
*
B. pahangi
C. elegans

(c)

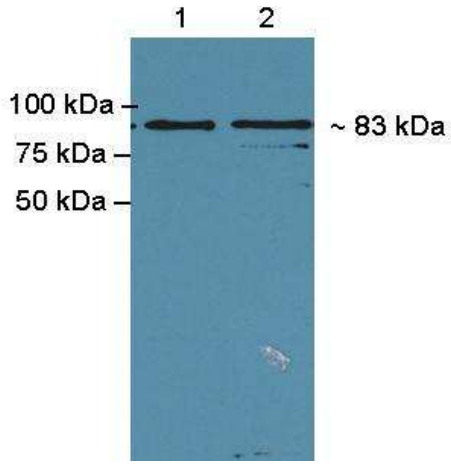


Figure 4.16: *B. pahangi* and *C. elegans* Hsp90 bind to ATP beads.

Autoradiograph shows the comparison of *B. pahangi* (Lane 1) and *C. elegans* (Lane 2) Hsp90 binding to ATP beads. 500 µg of worm lysates were incubated with 100 µl of ATP beads for 60 min at room temperature, pelleted and washed with buffer and bound proteins were analysed by 10% SDS-PAGE and Western blotting which was blotted and probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

4.3 Discussion

The development of a compound with macrofilaricidal activity has long been a goal of the World Health Organisation (WHO). Current treatment of filariasis is largely dependent on treatment with drugs, such as diethylcarbamazine or ivermectin, which kill the Mf but not the adult worm. The only novel chemotherapeutic target currently under development for the filarial nematodes is the *Wolbachia* endosymbiont (Hoerauf, 2008; Supali *et al*., 2008). *Wolbachia* are present in most of the human filariae and were shown to be important for worm fertility and survival (Debrah *et al*., 2007; Hoerauf *et al*., 2008). Previously, Hsp90 was identified as a possible target in *B. pahangi* (Devaney *et al*., 2005). Inhibition of *B. pahangi* Hsp90 by GA not only kills the Mf but also kills the adult worm. Thus Hsp90 might be a useful target for lymphatic filariasis. The aim of this Chapter was to further characterise *B. pahangi* Hsp90.

Results in this Chapter show that Hsp90 is expressed in all *B. pahangi* life cycle stages analysed, at a similar level. However, in the pull-down assay, only adult stage Hsp90 was observed to bind to GA, not Hsp90 from the L3 and Mf. The inability to detect Hsp90 binding to GA beads could perhaps reflect a lack of sensitivity of the assay, or alternatively may suggest that Hsp90 from Mf and L3 is processed in such way that it fails to bind to GA. Nothing is currently known about co-chaperones in *B. pahangi* and whether they may be required for Hsp90 to bind to GA. These data were surprising as GA kills Mf, suggesting that it can bind to Mf Hsp90.

In many eukaryotes, Hsp90 is induced after heat shock or following exposure to stress. However, in this Chapter, Hsp90 expression was not strongly induced by heat shock or GA treatment in *B. pahangi*. These results correlate

well with previous studies on *B. pahangi hsp90* by (Thompson *et al.*, 2001) where it was shown that *hsp90* mRNA levels were minimally induced following 2 h of heat shock (1.2–1.5 fold). In contrast, studies in *C. elegans* using *in situ* hybridisation showed that under stress conditions, *daf-21* mRNA was expressed all over the body of *C. elegans* while under normal conditions, *daf-21* is localised to the germline of adult *C. elegans* (Inoue *et al.*, 2003). This suggested that *daf-21* mRNA was up-regulated by heat shock. The difference between the results of that study using *in situ* hybridisation and the results presented here may relate to the different methods used.

Hsp90 was detected in the ES products of adult *B. pahangi* but not in the ES products of Mf. ES products contain molecules shed from the parasite surface as well as those secreted from the pharyngeal glands, excretory system etc. These results suggest that the secreted Hsp90 might be stage specific but the reason why Hsp90 is secreted in adult ES products of *Brugia* remains unknown. The first discovery of extra-cellular Hsp90 was reported by Ullrich *et al* (1986) in mouse tumour cells. Hsp90 has been identified at the cell surface of other cancer cells where it was shown to be involved in cell motility, invasion and metastasis (Eustace *et al.*, 2004; Sidera *et al.*, 2004). Adult filarial worms secrete many bio-active molecules (Hewitson *et al.*, 2008), and it is possible that Hsp90 may be required for the activity of some of these.

Attempts were made to identify Hsp90 client proteins/co-chaperones using two different methods. The first method, using a modified GA-biotin binding assay, did not identify any potential Hsp90-associated proteins due to the non-specific binding observed in the assay. Attempts were made to overcome this problem but were not successful in increasing the specificity of binding.

However, the original studies by (Neef *et al.*, 2010), showed that this technique was able to detect and identify heat shock factor 1A (HSF1A)-associated proteins from human and yeast using HSF1A-biotin beads.

The second method used a proteomic analysis. Following exposure to GA, Hsp90 complexes undergo a conformational change and as a result the client proteins are released from the complexes and subject to degradation. The rationale of this method was that client proteins would therefore be degraded following exposure of worms to GA. Thus it might be possible to identify proteomic differences between GA-treated and control worms. In the DIGE analysis, a total of 75 proteins (3.8%) were observed to differ in expression level between the groups. Of these, only 14 (0.7%) showed a reduction in expression following GA treatment but unfortunately it was not possible to identify these because of equipment failure. Without knowing the identity of any of these differentially expressed proteins, it is difficult to draw any conclusion from this experiment. It was, however, surprising that so few proteins showed a reduced expression following exposure of adult *B. pahangi* to GA. The second analysis was carried out using a much less sensitive technique and, not surprisingly, few differences were observed. Of the five proteins down-regulated following GA treatment, most were structural proteins. Of these tropomyosin and myosin regulatory light-chain 1, appeared in both the up-regulated and down-regulated list. The reason for this are not clear but it may be that proteolytic fragments of tropomyosin were detected in the GA-treated group. Because of lack of sensitivity of this method, only a single experiment was carried out.

A study by (Tsaytler *et al.*, 2009) using complementary proteomic approaches also identified several Hsp90 protein partners with structural

functions e.g. myosin-9, Filamin A, actin etc. In *C. elegans*, tropomyosin in the muscle wall was suggested to be essential as it is required for the late development of *C. elegans* (Kagawa *et al.*, 2007). Until recently, UNC-45, a muscle-specific protein, was one of the few targets of Hsp90 which had been experimentally validated in *C. elegans* (Barral *et al.*, 2002). According to Barral *et al* (2002), a direct interaction between UNC-45 and Hsp90 occurs as UNC-45 contains a tetratricopeptide repeat domain that binds to the C-terminal of Hsp90. However, further investigations are needed for an improved understanding of the role of Hsp90 in the regulation of the cytoskeleton and contractile activity. Furthermore, different methods need to be adopted to identify Hsp90-client proteins in *B. pahangi*.

A recent study identified Hsp90 interacting proteins in bacteria (*Shewanella oneidensis*, *Shewanella frigidimarina* and *Psychrobacter frigidicola*) that live at different temperatures, using immunoprecipitation followed by a proteomic approach (Garcia-Descalzo *et al.*, 2010). Many of the proteins identified were involved in energy metabolism such as isocitrate lyase, succinyl-CoA synthetase, alcohol dehydrogenase etc. It was hypothesised that Hsp90 and its interacting proteins may play a role in adaptation to cold environments. Echeverria *et al* (2010) also identified Hsp90/p23 client proteins in *Toxoplasma gondii* using a similar co-immunoprecipitation approach. These had multiple functions such as glycolysis, ATP production, protein synthesis, chaperones and foldases, kinases and phosphatases. These studies help explain why Hsp90 is essential in most organisms and suggest that the application of an immunoprecipitation approach to *B. pahangi* might yield more data.

In this Chapter, the binding of *B. pahangi* Hsp90 to GA was further explored. One area of interest was to investigate the amount of Hsp90 that was available for GA binding. Results in this Chapter show that only a small amount of Hsp90 binds to GA, approximately 6.31% of the cellular Hsp90. It is possible that the amount of GA bound to beads is a limiting factor in these experiments. However, this seems unlikely as by the fifth pull-down no binding was observed. Alternatively the low amount of Hsp90 bound to GA beads may reflect steric hindrance between bead-bound GA and soluble Hsp90, or alternatively it may be an accurate reflection of the amount of Hsp90 that can bind GA. If correct, the results suggest that it might be necessary to inhibit only a small percentage of total Hsp90 to kill the worm.

The *B. pahangi* lysate was also subjected to competition assays with GA, ATP and novobiocin. Free soluble GA was shown to inhibit the binding of *B. pahangi* Hsp90 to GA beads in a dose-dependent manner. On the other hand, in the ATP binding assay, soluble GA was also shown to compete for binding of Hsp90 to ATP beads which suggests that ATP and GA share the same binding site in *B. pahangi* Hsp90. These results are consistent with the findings of Grenert *et al* (1997) where they showed that 100-200 nM GA is enough to block 50% of chicken Hsp90 binding to GA beads. Since the amount of GA derivatised to resin is not known and may vary from batch to batch, these values reflect relative IC_{50} values.

Novobiocin, a coumarin type antibiotic inhibits Hsp90 functions in a very different manner to GA and radicicol. Novobiocin was shown to bind to the C-terminal domain of Hsp90, a different binding site from GA. Several point mutations in the N-terminus of chicken Hsp90 did not affect novobiocin binding

and the N-terminus failed to bind novobiocin-beads (Marcu *et al.*, 2000a). Many previous studies have shown that novobiocin affects the interaction of Hsp90 with its client proteins (Marcu *et al.*, 2000a; Katschinski *et al.*, 2002; Haendeler *et al.*, 2003; Yun *et al.*, 2004; Allan *et al.*, 2006). The binding of *B. pahangi* Hsp90 to GA beads was shown to be competed by excess novobiocin. It is noteworthy that the concentration of novobiocin required to inhibit binding of *B. pahangi* Hsp90 to GA beads is very high, in the millimolar range. However, this reflects the concentration of novobiocin required to inhibit Hsp90 binding in mammalian cells (Marcu *et al.*, 2000a) and in *B. pahangi* where novobiocin at concentrations of 200-500 μM kills adult worms (Devaney, unpublished). It has been proposed that the N-terminal and C-terminal domain of Hsp90 interact (Yun *et al.*, 2004). Other studies have demonstrated that novobiocin binding may alter the conformation of the Hsp90 C-terminal domain leading to the dissociation of TPR containing co-chaperones (Marcu *et al.*, 2000a; Marcu *et al.*, 2000b; Soti *et al.*, 2003; Wayne & Bolon, 2007). Taken together these results support the hypothesis that an interaction occurs between domains in Hsp90 which is essential for the chaperoning functions.

One area that remains to be resolved is the lack of binding of *C. elegans* Hsp90 to GA compared to *Brugia*. A possible explanation is that *B. pahangi* and *C. elegans* bear different post-translational modifications that affect GA binding. It was not possible to directly compare recombinant worm Hsp90 with native protein, so a comparison was made between recombinant human Hsp90α with native Hsp90 isolated from SkBr3 cells. Hsp90 is extensively modified and these modifications are known to impact the Hsp90 chaperone machinery. As reviewed previously phosphorylation, acetylation and other modifications of Hsp90 are important elements in regulating the Hsp90 chaperone function (Scroggins &

Neckers, 2007). A recent study of *Plasmodium falcifarum* Hsp90 showed that exposure of infected red blood cells to the histone deacetylase inhibitor, Trichostatin A, had a synergetic effect with GA and inhibited malaria growth (Pallavi *et al*., 2010). These studies suggest that the degree of acetylation of Hsp90 may affect its ability to bind to GA. However, in this Chapter it was shown that both rHsp90α and native Hsp90 bound to GA, suggesting that post-translational modifications of human Hsp90 are not essential for Hsp90 to bind to GA but might be required for other Hsp90 chaperone functions. Thus, while it was not possible to exclude differences in post-translational modifications being important in GA binding, there was no evidence to support it.

The final result in this Chapter showed that *C. elegans* and *B. pahangi* Hsp90 bind ATP similarly even though Hsp90 is expressed at a relatively higher level in *B. pahangi* than in *C. elegans*. However, the analysis of Hsp90 expression level was based on the use of an antibody raised to *B. pahangi* Hsp90, which may have a higher affinity for *B. pahangi* Hsp90 than for *C. elegans* Hsp90. However, the C-terminal domain to which the antibody was raised is very similar between *B. pahangi* and *C. elegans* as shown in Figure 4.14. The ATP binding assay for both nematodes validates previous reports by David *et al* (2003), where they showed that *C. elegans* Hsp90 binds to ATP but not to GA. *C. elegans* Hsp90 and *B. pahangi* Hsp90 are highly conserved (91.4% similar and 84.1% identical) with only minor substitution amino acids in the N-terminal domain. However, these substitutions are unlikely to contribute to conformational differences between *B. pahangi* and *C. elegans* that affect GA binding (Devaney *et al*., 2005). Additionally, the charged region of *B. pahangi* Hsp90 is longer than *C. elegans* Hsp90. A study in yeast showed that the charged region plays an important role in regulating the chaperone function of Hsp90 (Scheibel *et al*., 1999). In future

studies it may be of interest to examine the role of the charged region in *B. pahangi* Hsp90 in more detail.

In conclusion, the mystery of why *C. elegans* Hsp90 fails to bind GA remains unsolved. From other studies in this laboratory, there are clear differences in the function of Hsp90 in the two nematodes e.g. *B. pahangi* Hsp90 was unable to rescue a *C. elegans daf-21* mutant, although the protein was successfully expressed (Gillan *et al.*, 2009). Thus despite a high degree of sequence similarity, Hsp90 appears to be functionally diverse in nematodes. These studies differ from results in yeast, where Hsp90 from human or *Trypanosoma cruzi* can complement a yeast *hsp90* mutant (Palmer *et al.*, 1995) as can *C. elegans hsp90* and human *hsp90*ß (Piper *et al.*, 2003) despite significantly lower levels of identity (60.5% and 60.3%, respectively). According to Gillan *et al* (2009) the overall degree of sequence similarity may not be a good measure of Hsp90 function. Given the interaction between the different domains of Hsp90, it may be important to look outside the N-terminal domain and also to attempt to identify client proteins/co-chaperones which may differ significantly between these two nematodes.

Chapter 5

Hsp90 And Drug Resistance

5 Hsp90 and drug resistance

5.1 Introduction

To date, there are no data on the possible involvement of Hsp90 in drug resistance in nematodes. In this chapter, I investigated the hypothesis that Hsp90 may have a role in drug resistance in nematodes using anthelmintic resistant and susceptible worms. As reviewed in the Introduction, Hsp90 was shown to be involved in drug resistance in tumour cells and fungi. In fungi, the emergence of azole resistance was suggested to be associated with Erg11 (an enzyme in the cytochrome P450 family which is involved in the biosynthesis of ergosterol) and calcineurin. Calcineurin belongs to the protein phosphatase family and was identified as an Hsp90 client protein, involved in regulating numerous responses to membrane stress (Cowen & Lindquist 2005; Cowen *et al.*, 2006; Singh *et al.*, 2009). In contrast, in some tumour cells, P-gp was suggested to be involved in the drug resistance mediated by Hsp90ß (Betram *et al.*, 1996). Thus the question arises: *c*ould Hsp90 have a role in anthelmintic resistance in nematodes?

Gastrointestinal nematode infections can cause great losses in revenue due to decreased livestock production and animal death. Additional losses are suffered due to sub-clinical effects of parasite infection. It is estimated that the cost of controlling gastrointestinal nematode infection is between US$220 to 500 million in Australia (Emery & Wagland, 1991; McLeod, 1995). In the UK itself, the cost of treatment and losses for the sheep industry reach around £84 million annually (Nieuwhof & Bishop, 2005). The most common method to control gastrointestinal nematode infection is by anthelmintic treatment. However,

inconsistent treatment, excessive use and overdose of drugs have caused a new problem to arise, drug resistance.

Anthelmintics are separated into several classes based on their chemical structure and mode of action. To date, three groups of anthelmintic are widely used, namely the benzimidazoles, imidazothiazoles and macrocylic lactones. The most widely used and available anthelmintics belong to the benzimidazole group. Benzimidazoles were introduced to the market in 1961 beginning with thiabendazole followed by albendazole and fenbendazole. Anthelmintics of the imidazothiazole-tetrahydropyridimine group were introduced to the market in 1968. The most widely used are levamisole, pyrantel and morantel. Levamisole is a nicotinic receptor agonist (Aceves *et al.*, 1970; Aubry *et al.*, 1970) with a selective action on nematode receptors. The third group of anthelmintics are the macrocyclic lactones such as ivermectin, moxidectin and abemectin. Macrocyclic lactones were introduced in the early 1980s and since then have become effective anthelmintics for combating gastrointestinal nematode infections.

Anthelmintic resistance is a major problem that threatens the livestock industry throughout the world, especially in countries with hot and humid climates. Such climates provide ideal conditions for the development of free-living stages of nematode parasites on pasture. Several recent investigations have demonstrated a progressive spread of anthelmintic resistance, mainly in nematode populations of sheep (Sargison *et al.*, 2005). This problem became worse when the first multiple drug resistance cases were reported in *H. contortus* from South Africa (Van Wyk *et al.*, 1999), Malaysia (Chandrawathani *et al.*, 2003) and the United States (Mortensen *et al.*, 2003). Anthelmintic

resistance is present in a population when there is a greater frequency of individuals within a population which are able to tolerate an anthelmintic compared to a normal population (Prichard et al., 1980). Several reviews on possible mechanisms of anthelmintic resistance have been published over the last few years (Kaplan, 2004; Jabbar *et al.*, 2006). Since anthelmintics kill the majority of susceptible nematodes, the next generation will consist largely of offspring of the resistant nematodes (Le Jambre, 1985). If the character that provides resistance is controlled by a single major gene, then the resistant population will build up very rapidly (Le Jambre, 1985) and this trait is inheritable.

In the work described in this Chapter, Hsp90 levels were compared in anthelmintics susceptible and resistant worms. The first group studied were field isolates of the Trichostrongylid species, *Teladosargia circumcincta*. Trichostrongylid nematodes are highly prevalent in grazing animals and have developed resistance to anthelmintics. Two UK isolates were compared; Tci5 is resistant to all classes of broad spectrum anthelmintics (Bartley *et al.*, 2005), while Tci2 is susceptible to all of these drugs.

The second group of nematodes compared were *Caenorhabditis elegans* strains that were derived from mutagenesis screens. In these studies, *C. elegans* is exposed to a mutagen and worms are selected by their ability to grow on drug (Driscoll *et al.*, 1989, Dent *et al.*, 2000). The strains used were *C. elegans* CB3474 and DA1316. *C. elegans* CB3474 is resistant to benzimidazole compounds via mutations in the *ben-1* gene which encode ß-tubulin isotype 1 (Driscoll *et al.*, 1989). *C. elegans* DA1316 is an ivermectin-resistant strain which contains mutations in *avr-14*, *avr-15* and *glc-1*, each of which encode glutamate-gated

chloride channel (GluCl) sub-units and exhibits high level resistance to ivermectin (Dent *et al.*, 2000).

Finally, a third group of *C. elegans* were studied; these were wild-type N2 worms which had been continually grown on increasing levels of ivermectin and selected for their ability to survive and reproduce on drug (James & Davey, 2009). These worms were continuously passaged on increasing concentration of ivermectin. IVM-6 worms were resistant to ivermectin at 6 ng/ml, while IVM-10 worms were resistant to 10 ng/ml. Both strains also showed cross-resistance to moxidectin and to other anthelmintics such as levamisole and pyrantel.

The first aim of the studies described in this Chapter was to determine whether Hsp90 expression levels were altered in anthelmintic resistant nematodes. Secondly, experiments were undertaken using the RNAi approach to knock-down *hsp90* levels in *C. elegans* ivermectin-resistant worms, to determine whether Hsp90 is involved in resistance in these worms.

5.2 Results

5.2.1 Hsp90 expression levels in drug resistant nematodes are not significantly different compared to susceptible nematodes

5.2.1.1 *T. circumcincta* isolates – resistance acquired by natural selection in the field

The two *T. circumcincta* isolates used in this study were obtained from the Moredun Research Institute, Edinburgh, UK. *T. circumcincta* extract was prepared as described previously in Materials and Methods (Section 2.1.3) and equal amounts of protein extracts (2-4 µg) were analysed by SDS-PAGE and Western blotting. Figure 5.1a shows a Coomassie blue stained gel, while the corresponding autoradiograph is shown in Figure 5.1b. In this experiment *B. pahangi* extract was used as a control for the antibody and actin was used as a loading control. The autoradiograph shows that there was no difference in Hsp90 expression levels in the *T. circumcincta* Tci5 isolate (Lane 2) compared to the *T. circumcincta* Tci2 isolate (Lane 3). The autoradiograph was scanned and band densities were quantified using using FluorChem™ IS-5500 software. Based on the Hsp90/actin ratio a graph was plotted. The expression level of Hsp90 in the *T. circumcincta* Tci5 isolate was similar to the Tci2 isolate (Tci5 0.9586 ± 0.0461, Tci2 0.7542 ± 0.0318)(p>0.05).

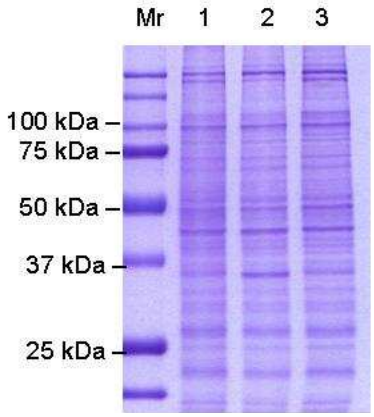
Figure 5.1: Analysis of Hsp90 expression levels in anthelmintic susceptible and resistant *T. circumcincta*

a) Coomassie blue staining of approximately equal amounts of protein. Mr represents molecular marker, *B. pahangi* (Lane 1), *T. circumcincta* Tci5 isolate (Lane 2) and *T. circumcincta* isolate Tci2 (Lane 3).

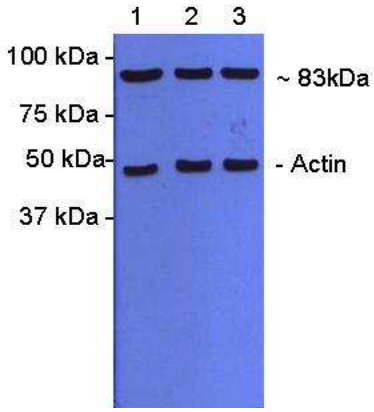
b) Equal amounts of soluble extract of *B. pahangi* (Lane 1), *T. circumcincta* Tci5 (Lane 2) and Tci2 (Lane 3) were analysed by Western blotting using the *B. pahangi* anti-Hsp90 antibody at 1:5000 dilution followed by 1:10,000 dilution of HRP-labelled anti-rabbit IgG (Sigma). Bound antibody was detected by chemiluminescence using Super Signal ® West Pico Kit. Anti-actin was used as the loading control and *B. pahangi* extract was used as a positive control.

c) Graph shows the Hsp90 expression levels in *T. circumcincta* resistant and susceptible isolates. Hsp90 expression was higher in the *T. circumcincta* Tci5 isolate compared to the *T. circumcincta* Tci2 isolate but the difference was not significant between the two isolates ($p>0.05$). Graph presented here shows the mean ± standard deviation of three different experiments.

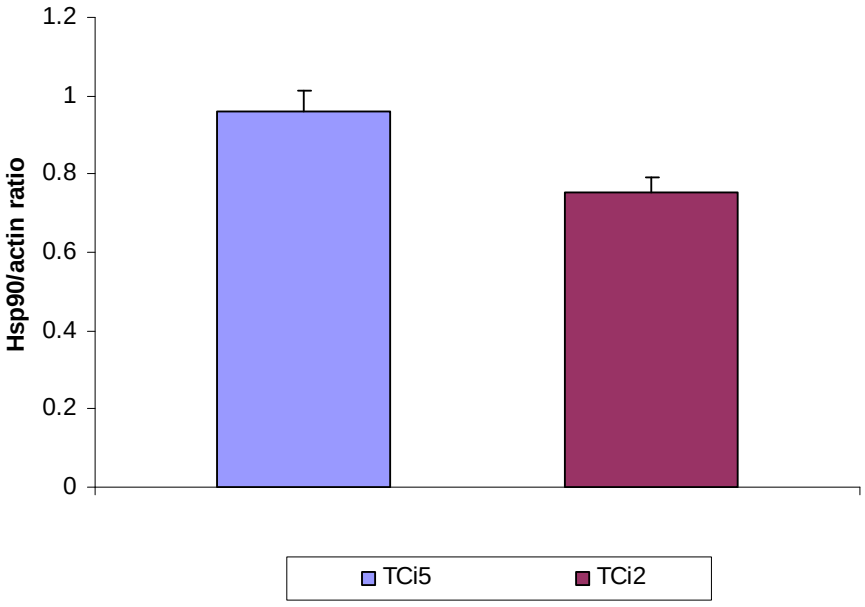
***T. circumcincta* Hsp90 Tci5 vs Tci2, $p = 0.0809$**


Mr
1
2
3
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa

(a)


1
2
3
100 kDa
75 kDa
50 kDa
37 kDa
~ 83kDa
- Actin

(b)


Hsp90/actin ratio
1.2
1
0.8
0.6
0.4
0.2
0
TCi5
TCi2

(c)

5.2.1.2 *C. elegans* resistant strains – resistance acquired by mutagenesis

C. elegans CB3474 is a strain which shows resistance to benzimidazole compounds via a mutation in the *ben-1* gene (Driscoll *et al.*, 1989). *C. elegans* DA1316 is a strain which contains mutations in three separate genes, all of which are required for high level resistance to ivermectin (Dent *et al.*, 2000). Both strains were selected in mutagenesis screens. Levels of Hsp90 were compared in these mutant worms and in wild-type N2 worms by Western blotting. Figure 5.2a shows a Coomassie blue stained gel of the soluble extracts, with the corresponding autoradiograph shown in Figure 5.2b. The autoradiograph was scanned and band densities were quantified using FluorChemTM IS-5500 software. The graph in Figure 5.2c shows Hsp90 expression levels in *C. elegans* N2, *C. elegans* CB3474 and *C. elegans* DA1316.The expression levels of Hsp90 in *C. elegans* N2 was similar compared to the mutant worms (N2 1.3181± 0.0361, CB3474 1.1471± 0.0412, DA1316 1.2853± 0.0685)($p>0.05$).

Figure 5.2: Analysis of Hsp90 levels in *C. elegans* drug resistant strains

a) Coomassie blue stained gel of soluble extracts of *C. elegans* N2 (Lane 1), *C. elegans* CB3474 (Lane 2) and *C. elegans* DA1316 (Lane 3). Mr represents the molecular weight marker.

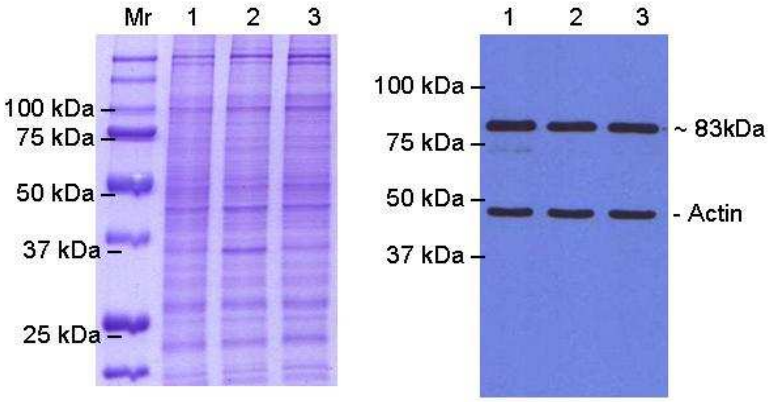
b) Autoradiograph of Hsp90 expression levels in susceptible and resistant *C. elegans*. Extracts were loaded exactly as in Panel a. *C. elegans* N2 (Lane 1), *C. elegans* CB3474 (Lane 2) and *C. elegans* DA1316 (Lane 3). Actin was used as a loading control.

c) The expression level of Hsp90 based on Hsp90/Actin ratio as quantified using FluorChem™ IS-5500 software. Graph presented here shows the mean ± standard deviation of three different experiments.

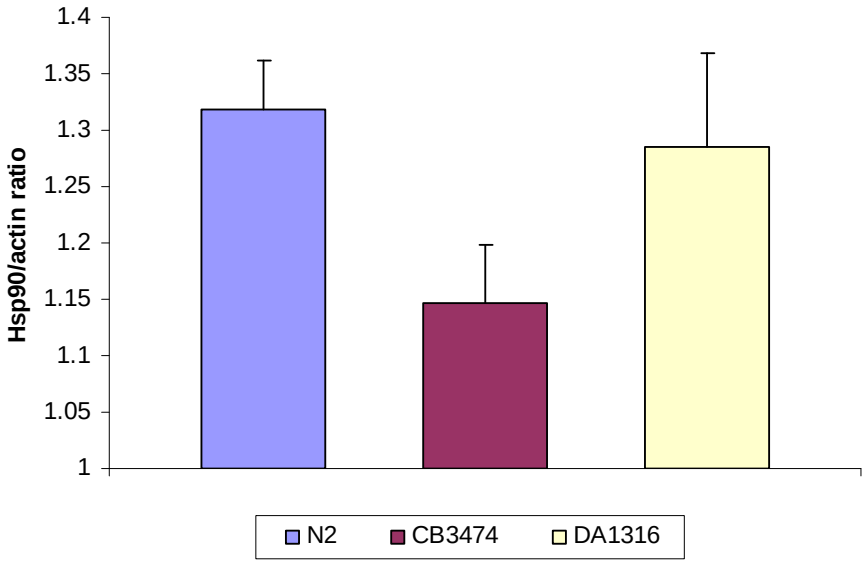
***C. elegans* N2 vs *C. elegans* CB3474, $p = 0.0809$**

***C. elegans* N2 vs *C. elegans* DA1316, $p = 0.6625$**

***C. elegans* CB3474 vs *C. elegans* DA1316, $p = 0.0809$**


Mr
1
2
3
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
1
2
3
100 kDa
75 kDa
50 kDa
37 kDa
~ 83kDa
- Actin

(a) (b)


Hsp90/actin ratio
1.4
1.35
1.3
1.25
1.2
1.15
1.1
1.05
1
N2
CB3474
DA1316

(c)

5.2.1.3 *C. elegans* ivermectin-resistant strains – acquired by selection on drug

In some cases, nematode tolerance to drug can be induced by continuous exposure to drug in the laboratory. In this case, wild-type N2 worms were adapted to culture with ivermectin at 6 ng/ml or 10 ng/ml. Hsp90 expression levels were compared in *C. elegans* N2, *C. elegans* IVM-6 and IVM-10 worms. Figure 5.3a shows a Coomassie blue stained gel of the extracts while Figure 5.3b shows the corresponding autoradiograph of Hsp90 expression levels in ivermectin resistant and susceptible worms. The autoradiograph was scanned and band density was quantified using FluorChem™ IS-5500 software and graph was plotted based on Hsp90/actin ratio. The graph in Figure 5.3a indicates that the expression level of Hsp90 in *C. elegans* N2 was similar compared to the IVM-6 and IVM-10 worms (N2 2.3657±0.1774, IVM-6 1.9683± 0.4080 and IVM-10 1.9104± 0.0668)(p>0.05).

Figure 5.3: Analysis of Hsp90 expression levels in *C. elegans* N2 and ivermectin resistant worms

a) Coomassie blue stained gel of soluble extracts of adult stage of *C. elegans* N2, *C. elegans* IVM-6 and *C. elegans* IVM-10. 30 adult worms were directly solubilised in 30 µl SDS-PAGE sample cocktail by boiling for 3 min. 5 µl of sample extract was loaded onto the gel for the analysis.

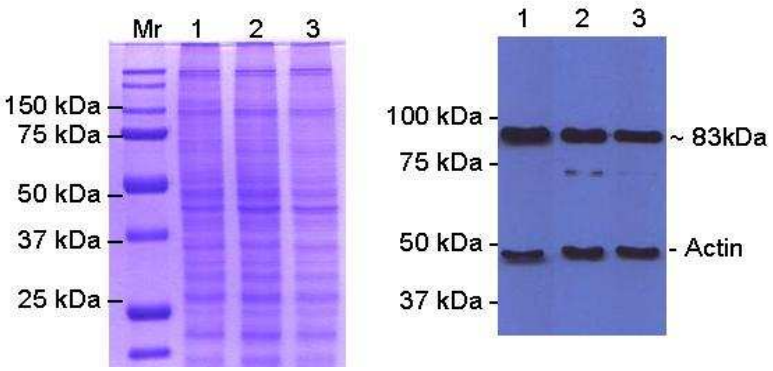
b) Autoradiograph of Hsp90 expression levels as detected using the *B. pahangi* anti-Hsp90 antibody. *C. elegans* N2 (Lane 1), *C. elegans* IVM-6 (Lane 2) and *C. elegans* IVM-10 (Lane 3). *B. pahangi* anti-Hsp90 antibody was used at 1:5000 dilution followed by 1:10,000 dilution of HRP-labelled anti-rabbit IgG (Sigma). Bound antibody was detected by chemiluminescence using Super Signal ® West Pico Kit. Actin was used as a loading control (Mr weight ~42 kDa).

c) The expression level of Hsp90 based on the Hsp90/actin ratio as quantified using FluorChem™ IS-5500 software. Graph presented here shows the mean ± standard deviation of three different experiments.

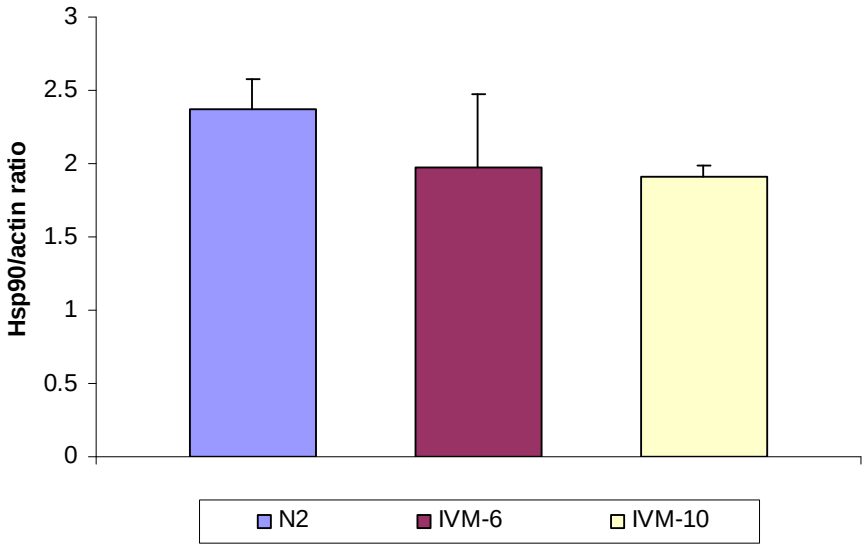
***C. elegans* N2 vs *C. elegans* IVM-6, p = 0.3827**

***C. elegans* N2 vs *C. elegans* IVM-10, p = 0.0809**

***C. elegans* IVM-16 vs *C. elegans* IVM-10, p = 0.6625**


Mr
1
2
3
150 kDa
75 kDa
50 kDa
37 kDa
25 kDa
1
2
3
100 kDa
75 kDa
50 kDa
37 kDa
~ 83kDa
Actin

(a) (b)


Hsp90/actin ratio
3
2.5
2
1.5
1
0.5
0
N2
IVM-6
IVM-10

(c)

5.2.2 Optimising the conditions for hsp90(RNAi)

C. elegans is a useful model for parasitic nematodes, because defining the function of genes of interest in parasitic species can be difficult due to the lack of appropriate knock-out approaches or suitable functional assays (Britton & Murray, 2006). In this section *C. elegans* wild-type worms were used in preliminary *hsp90(RNAi)* experiments to optimise RNAi conditions, prior to using this approach to investigate the possible role of Hsp90 in drug resistance.

5.2.2.1 Investigating the effect of temperature and time of feeding on the efficiency of RNAi

In the initial experiments, wild-type N2 worms were fed on a 300 bp *hsp90(RNAi)* construct described previously (Section 2.3.2.5). RNAi was conducted at different temperatures or for different time periods, as both variables can affect the intake of the construct and the efficiency of the RNAi. The aim of these experiments was to obtain a reduction in Hsp90 levels, while maintaining the viability of the worms. L4 of wild-type N2 were fed on the 300 bp *hsp90(RNAi)* construct for 6 h, 12 h or 24 h at 20^{o}C. 30 worms were then solubilised in 30 µl SDS-PAGE sample cocktail and analysed by Western blotting. Figure 5.4a shows the autoradiograph of Hsp90 expression levels after different feeding duration. At 6 h, there was a small but insignificant increase in Hsp90 expression in RNAi treated worm compared to the control group (Lane 1 and Lane 2)(p>0.05). However, after 12 h of feeding there was a small reduction in Hsp90 expression level in RNAi treated worms compared to the control group (Lane 3 and Lane 4)(p>0.05), with a further reduction after 24 h of feeding (Lane 5 and Lane 6)(p<0.05). In these experiments, Hsp90 levels were reduced by

approximately 42% after 24 h feeding on the *hsp90(RNAi)* construct. Thus subsequent RNAi experiments were carried out at 24 h.

Figure 5.4: Different RNAi feeding periods affect Hsp90 expression levels.

a) Wild-type N2 L4 worms were fed on the *hsp90(RNAi)* construct for 6, 12 and 24 h. Control worms were fed on empty vector under identical conditions. 6 h-Control (Lane 1), *hsp90(RNAi)* (Lane 2), 12 h-Control (Lane 3), *hsp90(RNAi)*(Lane 4), 24 h-Control (Lane 5), *hsp90(RNAi)* (Lane 6). All experiments were carried out at 20^{o}C. Equal volumes of extract (5 µl) were loaded onto a 10% gel and analysed by SDS-PAGE and Western blotting as described previously.

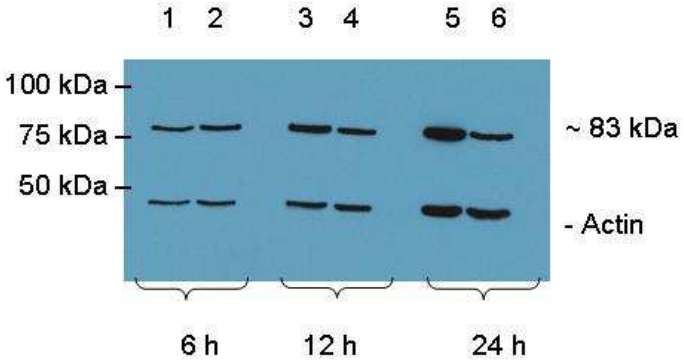
b) Hsp90 expression levels following *hsp90(RNAi)* at 20^{o}C after different feeding times. Results are expressed as ratio of Hsp90/actin in RNAi treated worms compared to controls. All values represent the mean ± standard deviation of three experiments. All percentages are expressed relative to the appropriate control, which was set to 100%.

6 h-106.24% ± 8.87, 12 h-90.70% ± 22.55, 24 h-58.35% ± 2.47,

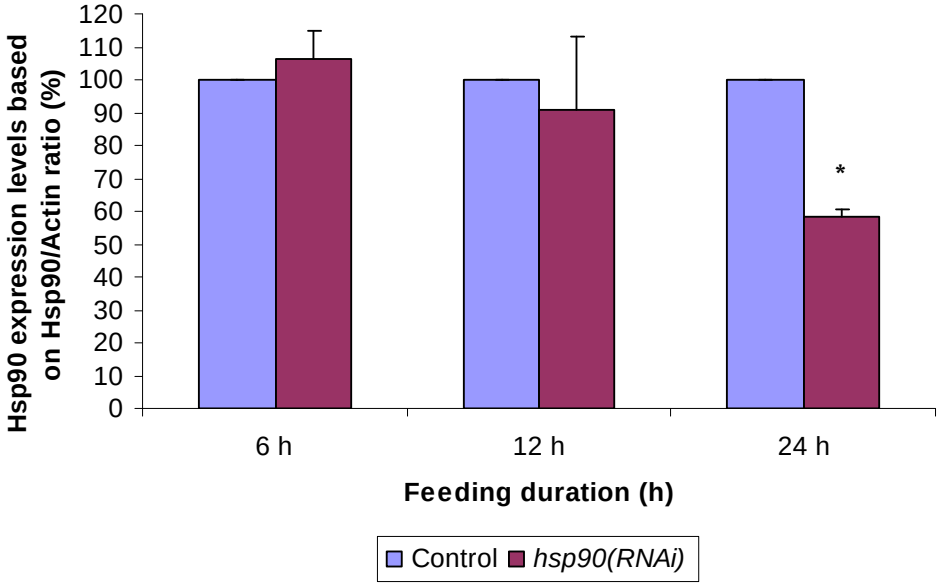
Control vs 6 h feeding on the *hsp90(RNAi)* construct, *p* = 0.311

Control vs 12 h feeding on the *hsp90(RNAi)* construct, *p* = 0.576

***Control vs 24 h feeding on the *hsp90(RNAi)* construct, *p* = 0.001**


1 2 3 4 5 6
100 kDa
75 kDa
50 kDa
~ 83 kDa
- Actin
6 h
12 h
24 h

(a)


Hsp90 expression levels based on Hsp90/Actin ratio (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
*
6 h
12 h
24 h
Feeding duration (h)
Control
hsp90(RNAi)

(b)

In the next experiments, worms were fed on the 300 bp *hsp90(RNAi)* construct for 24 h at different incubation temperatures of 16^{o}C, 20^{o}C or 25^{o}C. Temperature was shown to influence the effectiveness of RNAi in previous studies (Kamath et al., 2003). Figure 5.5a shows the autoradiograph of Hsp90 expression levels of wild-type N2 after 24 h feeding on the 300 bp *hsp90(RNAi)* construct at 16^{o}C, 20^{o}C or 25^{o}C. The autoradiograph was then scanned to quantify Hsp90 expression levels using FlourChem^{TM} IS-5500. Figure 5.5b shows the graph of Hsp90 expression levels after feeding on the 300 bp *hsp90(RNAi)* construct for 24 h at different temperatures. Hsp90 expression levels were reduced at all temperatures tested. However, the greatest reduction was observed at 25^{o}C (49.84% ± 3.82), followed by 20^{o}C (40.48% ± 3.03) and 16^{o}C (9.64% ± 7.30). The reduction in Hsp90 expression was significantly different from the controls at 20^{o}C and 25^{o}C (p<0.05) but not at 16^{o}C.

Figure 5.5: *hsp90(RNAi)* was more efficient at higher temperature.

a) Autoradiograph of wild-type N2 Hsp90 expression levels after 24 h feeding on the 300 bp *hsp90(RNAi)* construct at different incubation temperatures. 16°C-Control (Lane 1), 300 bp *hsp90(RNAi)* (Lane 2), 20°C-Control (Lane 3), *hsp90(RNAi)* (Lane 4), 25°C-Control (Lane 5), *hsp90(RNAi)* (Lane 6). 30 worms were solubilised in 30 µl SDS-PAGE sample cocktail by boiling and equal volumes (5 µl) were loaded onto a 10% gel and analysed by Western blotting.

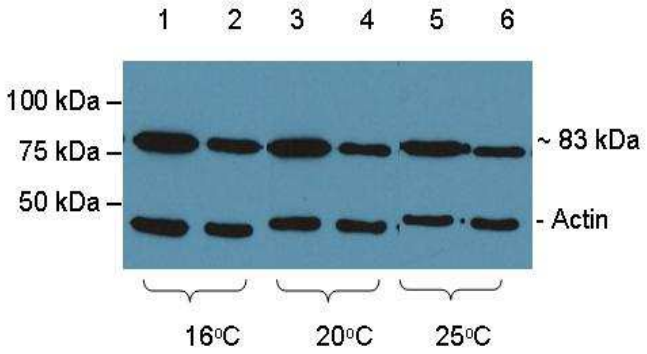
b) Hsp90 levels after 24 h feeding on the 300 bp *hsp90(RNAi)* construct at different temperatures. Results are expressed as the ratio of Hsp90/actin RNAi treated worms compared to controls. All values represent the mean ± standard deviation of three experiments. All percentages are expressed relative to the appropriate control, which was set to 100%.

16° C-90.35% ± 7.30, 20°C- 58.51% ± 3.03, 25°C-50.16% ± 3.82

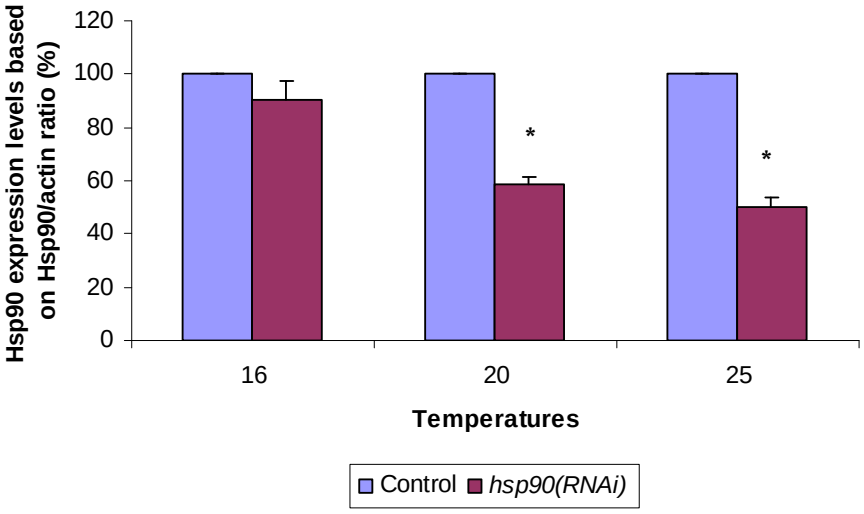
Control vs RNAi treated at 16°C, *p* = 0.168

*** Control vs RNAi treated at 20°C, *p* = 0.02**

*** Control vs RNAi treated at 25°C, *p* = 0.02**


1 2 3 4 5 6
100 kDa –
75 kDa –
50 kDa –
~ 83 kDa
- Actin
16°C
20°C
25°C

(a)


Hsp90 expression levels based on Hsp90/actin ratio (%)
120
100
80
60
40
20
0
*
*
16
20
25
Temperatures
Control
hsp90(RNAi)

(b)

5.2.2.2 Feeding on the 300 bp *hsp90(RNAi)* construct affects the growth of wild-type N2 and produces a phenotype

For the next *hsp90(RNAi)* experiment, L4 were fed at 20^{o}C for 24 h on the 300 bp *hsp90(RNAi)* construct. Three worms were then transferred onto triplicate fresh NGM plates spotted with OP50 and were left for 24 h to let the worm lay eggs. The next day, the numbers of eggs were scored. Figure 5.6 shows the fertility of wild-type N2 after feeding on empty vector or on the 300 bp *hsp90(RNAi)* construct. The P_o wild-type N2 grown on the 300 bp *hsp90(RNAi)* plates had a smaller brood size (58 ± 27) compared to P_o wild-type N2 grown on control plates (control group 223 ± 12)(p<0.05). These experiments confirmed that knock-down of Hsp90 affects the fertility of wild-type N2 worms.

In addition, other phenotypes were observed in P_o RNAi treated worms. Figure 5.7 shows the P_o worms after 24 h feeding on control plates or on the *hsp90(RNAi)* construct. Control worms appeared normal (Figure 5.7a) while some *hsp90(RNAi)* worms developed a protruding vulva (Figure 5.7b) and a 'bagging effect' in which eggs hatched and the larvae were released in the body of the worm (Figure 5.7c). The F_1 generation of control worms were normal (Figure 5.8a), while a protruding vulva was observed in many *hsp90(RNAi)* worms (see Figure 5.8b). The F_1 with protruding vulva were 100% sterile and showed reduced motility. A similar phenotype was previously reported in *hsp90(RNAi)* knock-out worms (Gillan *et al.*, 2009) where they reported the wild-type N2 F_1 generation were infertile.

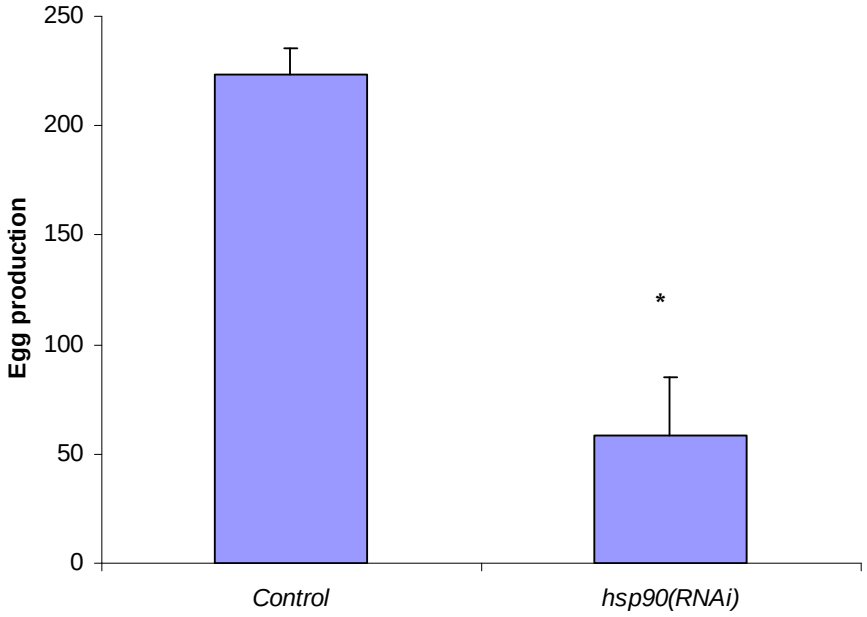


Figure 5.6: Egg counts of wild-type N2 after feeding on the 300 bp *hsp90(RNAi)* construct.

Fewer eggs were produced in wild-type N2 fed on the 300 bp *hsp90(RNAi)* construct compared to the control group fed on empty vector. N2 were fed on the *hsp90(RNAi)* construct for 24 h at 20^{o}C and three worms were transferred onto new NGM plates seeded with OP50. Worms were left for a further 24 h at 20^{o}C and the number of eggs produced was counted.

N2-Control- 223 ± 12

* N2-RNAi- 58 ± 27

***p* = 0.0404**

Figure 5.7: *hsp90(RNAi)* phenotypes observed in wild-type N2 after 24 h feeding on the 300 bp construct.

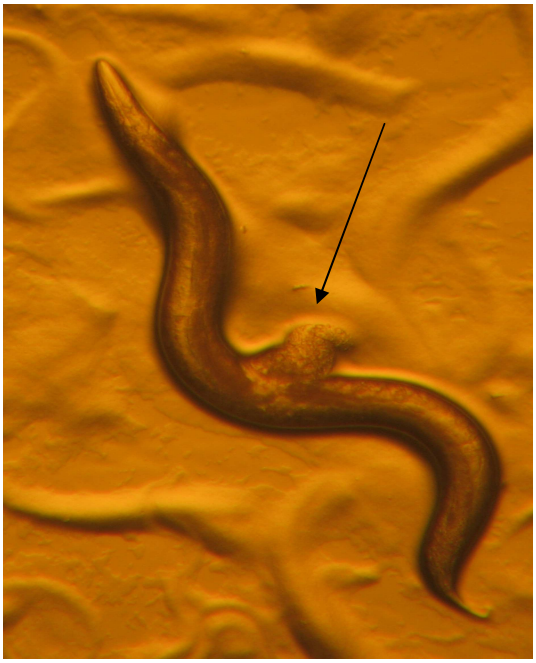
a) N2 P_o after 24 h feeding on the empty vector.

b) N2 P_o after 24 h feeding on the *hsp90(RNAi* construct. Arrow shows the protruding vulva.

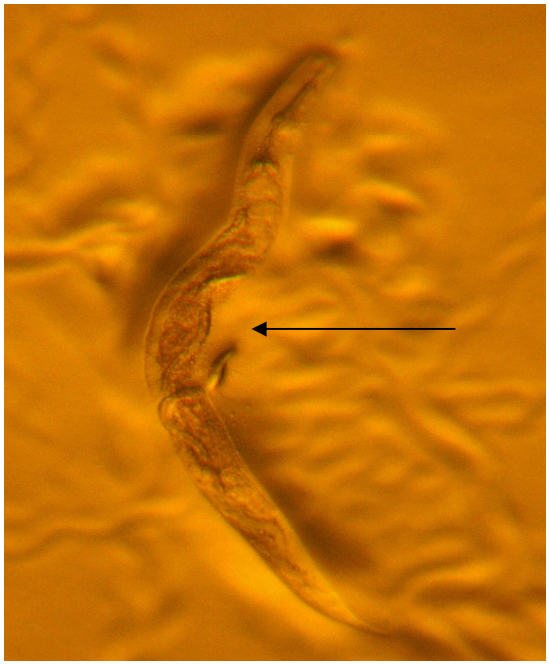
c) P_o of N2 fed on the *hsp90(RNAi)* construct showing a 'bagging effect'. Arrow shows the larvae hatched within the body of parent worm.

Worms were viewed on a Carl Zeiss, Stemin SV 6 microscope and images were captured using Canon Powershot G6 software. Final magnification x75.

(a)



(b)



(c)

Figure 5.8: *hsp90(RNAi)* phenotypes observed in wild-type N2 after 24 h feeding on the 300 bp construct.

a) F_1 of N2 fed on empty vector after 24 h, showing normal development.

b) F_1 of N2 fed on 300 bp *hsp90(RNAi)* construct. Arrow shows protruding vulva.

Worms were viewed on a Carl Zeiss, Stemin SV 6 microscope and images captured using Canon Powershot G6 software. Final magnification x75.

(a)

(b)

5.2.2.3 A shorter *hsp90(RNAi)* construct reduced the Hsp90 expression levels in *C. elegans* N2

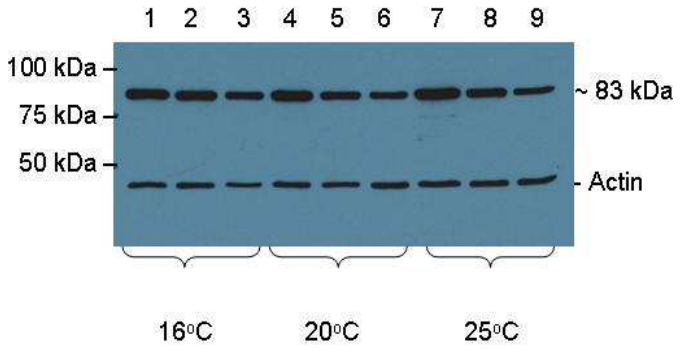
The aim of the *hsp90* knock-down experiments was to reduce the level of Hsp90 in an ivermectin resistant strain of *C. elegans* and to determine whether this reduction in Hsp90 level had any effect on sensitivity to drug. For these experiments it was important that the reduction in Hsp90 level did not affect worm viability. As can be seen in the previous section, the 300 bp *hsp90(RNAi)* construct produced a variety of detrimental phenotypes in N2 worms and therefore was not appropriate for use in these experiments. Based on these observations, the experiments were repeated using a smaller 75 bp *hsp90(RNAi)* construct. This construct was prepared by Dr. Victoria Gillan as described in Materials and Methods (Section 2.3.2.3). In initial experiments, *C. elegans* N2 were fed on the 75 bp *hsp90(RNAi)* construct at different incubation temperatures (16^{o}C, 20^{o}C and 25^{o} C) for 24 h. After 24 h, 30 worms were solubilised in 30 µl SDS-PAGE sample cocktail and analysed by Western blotting. Figure 5.9a shows the autoradiograph of Hsp90 expression in *C. elegans* N2 fed on the 75 bp *hsp90(RNAi)* construct compared to control empty vector or the 300 bp *hsp90(RNAi)* construct at different feeding temperatures. Bands on the autoradiograph were scanned and the reduction in Hsp90 levels was estimated based on the Hsp90 to actin ratio. The reduction of Hsp90 levels was most pronounced in wild-type N2 fed on the longer construct at 20^{o}C or 25^{o}C (Figure 5.9, Lane 6 and Lane 9). However, even using the shorter 75 bp *hsp90(RNAi)* construct a reduction in Hsp90 levels was observed at all temperatures (Figure 5.9b). No phenotypes were observed in wild-type N2 fed on the 75 bp *hsp90(RNAi)* construct but the same phenotypes were observed with the 300 bp *hsp90(RNAi)* construct as described previously. This experiment was repeated

twice under identical conditions with similar results. For the subsequent experiments, the 75 bp *hsp90(RNAi)* construct was used.

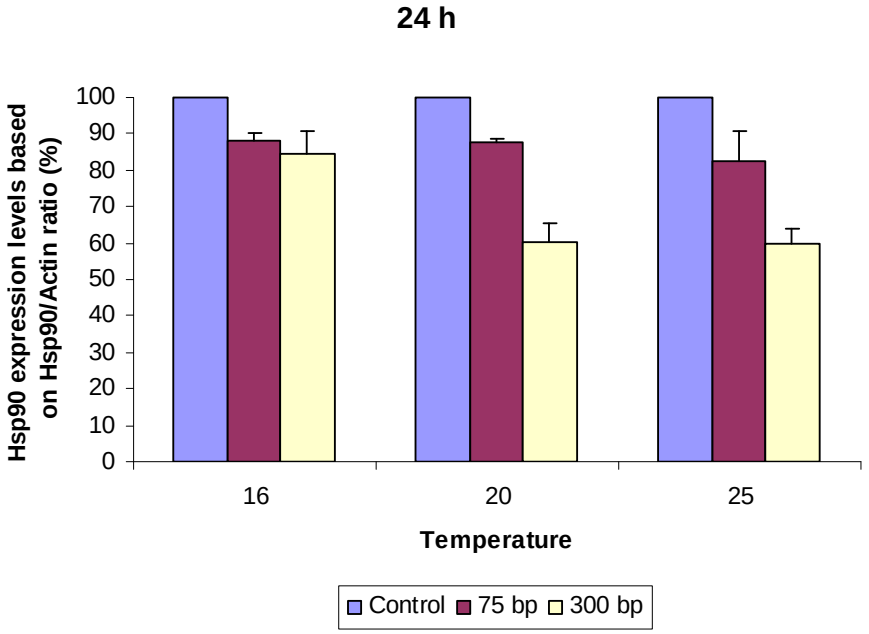
Figure 5.9: A comparison of Hsp90 expression levels in *C. elegans* N2 fed on the 75 bp or 300 bp *hsp90(RNAi)* constructs at different feeding temperatures.

a) Autoradiograph of Hsp90 levels in *C. elegans* N2 fed on control empty vector, 75 bp or 300 bp *hsp90(RNAi)* constructs with actin as a loading control at different feeding temperatures (16^{o}C, 20^{o}C and 25^{o}C) for 24 h. Wild-type N2 fed on control empty vector (Lane 1, 4, 7), wild-type N2 fed on the 75 bp (Lane 2, 5 and 8) or the 300 bp RNAi construct (Lane 3, 6 and 9). An equal volume of extract was analysed on a 10% gel followed by Western blotting. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

b) Graph shows the wild-type N2 Hsp90 expression levels after feeding on the control empty vector, 75 bp or 300 bp construct. The greatest reduction in Hsp90 levels was observed at 20^{o}C or 25^{o}C in worms fed on the 300 bp *hsp90(RNAi)* construct. Results represented the mean ± standard deviation from two different experiments.



(a)



	16°C			20°C			25°C		
Construct	**Empty vector**	**75 bp**	**300 bp**	**Empty vector**	**75 bp**	**300 bp**	**Empty vector**	**75 bp**	**300 bp**
Hsp90 level (%)	**100**	**87.89**	**84.66**	**100**	**87.56**	**60.07**	**100**	**82.39**	**59.95**

(b)

5.2.2.4 Reduction in Hsp90 levels in ivermectin-resistant worms

In fungi and yeast, reducing Hsp90 levels increased the sensitivity of azole-resistant strains to drug. In the experiments presented here, the sensitivity of *C. elegans* IVM-10 worms to ivermectin was investigated following *hsp90(RNAi)* in order to ask whether Hsp90 might have a role in ivermectin-resistance. Based on the *hsp90(RNAi)* results shown previously, the 75 bp *hsp90(RNAi)* construct was used in all experiments at a temperature of 20^{o}C for 24 h feeding time. Hsp90 levels in wild-type N2 and IVM-10 worms were analysed by solubilising 30 worms in 30 µl SDS-PAGE sample cocktail and analysing by Western blotting. Figure 5.10a shows an autoradiograph of Hsp90 expression levels in wild-type N2 and IVM-10 worms after feeding on the 75 bp *hsp90(RNAi)* construct. Hsp90 levels in both strains were reduced following 24 h of RNAi (Lane 2 and Lane 4) compared to control worms (Lane 1 and 3). The Hsp90 reduction levels were estimated based on the density values using FlourChem^{TM} IS-5500 software and a graph was plotted. Figure 5.10b shows that Hsp90 expresson levels were significantly reduced in wild-type N2 worms (21.64% ± 10.75) and in IVM-10 worms (29.72% ± 7.17) following 24 h feeding on the 75 bp *hsp90(RNAi)* construct compared to empty vector control for both groups (p<0.05).

Figure 5.10: Hsp90 levels in wild-type N2 and IVM-10 resistant worms were reduced after feeding on the 75 bp *hsp90(RNAi)* construct

a) Autoradiograph shows Hsp90 levels after 24 h of RNAi with actin as a loading control at 20^{o}C. Wild-type N2 fed on the empty vector construct (Lane 1), 75 bp construct (Lane 2), IVM-10 worms fed on the empty construct (Lane 3) and 75 bp construct (Lane 4). 5 µl of each sample was loaded onto a 10% gel and analysed by SDS-PAGE and Western blotting. Proteins were transferred to NCP using standard methods, blocked o/n in 5% dried milk in PBS/0.5% Tween 20 and then the blot probed with 1:5000 dilution of the *B. pahangi* anti-Hsp90 antibody. Bound antibody was detected using a 1:10,000 dilution of anti-rabbit IgG conjugated to HRP. For the loading control, anti-actin antibody was used at 1:1000 dilution and bound protein was detected using anti-mouse IgG at 1:10,000. The blot was developed using the Pierce Super Signal West Pico chemiluminescence kit.

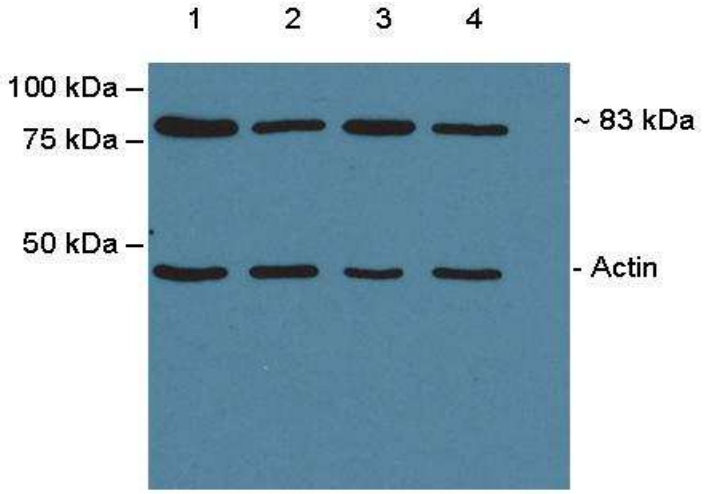
b) Graph shows the Hsp90 expression levels after feeding on the 75 bp *hsp90(RNAi)* construct based on the integrated density value quantified using FlourChem^{TM} Is-5500 software. Results presented the percentage of mean ± standard deviation of Hsp90/actin ratio from three different experiments. The Hsp90 levels of worms fed on the empty vector was set at 100%.

***C. elegans* N2 reduction in Hsp90 level vs N2-Control = 21.64 % ± 10.75**

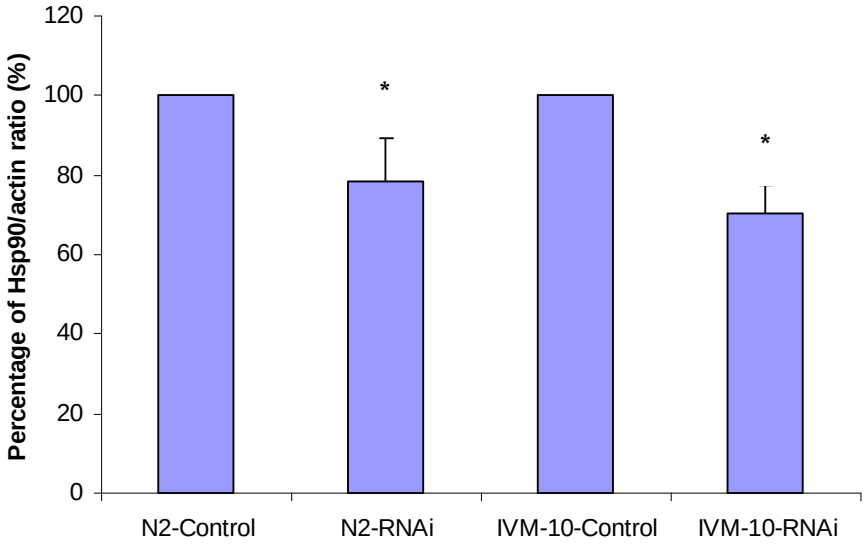
IVM-10 reduction in Hsp90 level vs IVM-10-Control = 29.72 % ± 7.17

*N2-Control vs N2-RNAi, *p* = 0.037

*IVM-10-Control vs IVM-10-RNAi, *p* = 0.009


1
2
3
4
100 kDa –
75 kDa –
50 kDa –
~ 83 kDa
- Actin

(a)


Percentage of Hsp90/actin ratio (%)
120
100
80
60
40
20
0
*
*
N2-Control
N2-RNAi
IVM-10-Control
IVM-10-RNAi

(b)

In addition, egg production by adult worms was also was analysed in these experiments. Three worms were placed onto each of three fresh NGM plates seeded with OP50 after feeding on the 75 bp *hsp90(RNAi)* construct or on empty vector for 24 h. Plates were incubated at 20^{o}C for further 24 h. At this point worms were removed from the plates and the number of eggs counted. Figure 5.11 shows the egg production by wild-type N2 and IVM-10 worms in a typical RNAi experiment. Wild-type N2 fed on the empty vector construct produced 245 ± 75 eggs over the 24 h period, compared to 160 ± 64 eggs by worms fed on the 75 bp *hsp90(RNAi)* construct. IVM-10 worms fed on the empty construct produced 186 ± 68 eggs compared to 126 ± 43 by worms fed on the 75 bp *hsp90(RNAi)* construct. However, the difference is not significant (p>0.05). Further observation of the F_1 generation feeding on the 75 bp *hsp90(RNAi)* construct showed that the F_1 generation were fertile and appeared normal compared to the F_1 generation feeding on the empty construct. This experiment indicated that the reduction in Hsp90 levels by feeding on the 75 bp *hsp90(RNAi)* construct did not significantly affect egg production or worm viability.

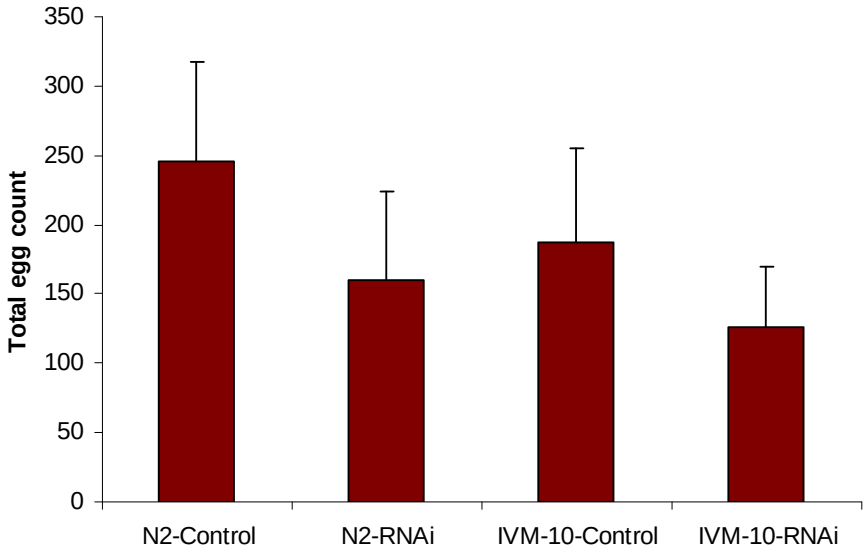


Figure 5.11: Egg production by wild-type N2 and IVM-10 worms fed on the 75 bp *hsp90(RNAi)* construct.

No significant difference was observed in egg production in wild-type N2 and IVM-10 worms fed on the 75 bp *hsp90(RNAi)* construct compared to controls (p>0.05). Wild-type N2 or IVM-10 worms were fed on the 75 bp *hsp90(RNAi)* construct for 24 h at 20^{o}C. Three worms were then transferred onto each of three new NGM plates seeded with OP50. Worms were left for further 24 h at 20^{o}C and the number of eggs counted. Results presented are the mean ± standard deviation of eggs produced by wild-type N2 or IVM-10 worms from three different experiments.

N2-Control vs N2-RNAi, p = 0.1904

IVM-10-Control vs IVM-10-RNAi, p = 0.3827

N2-Control vs IVM-10-Control, p = 0.6625

N2-RNAi vs IVM-10-RNAi, p = 0.6625

5.2.2.5 Reduction in Hsp90 levels alters the ivermectin-resistant phenotype

Previous results showed that Hsp90 levels were reduced in wild-type N2 and IVM-10 resistant worms fed on the 75 bp *hsp90(RNAi)* construct for 24 h at 20^{o}C. However, the RNAi conditions did not affect the growth or the fertility of the worms. The aim of this work was to ask whether a reduction in Hsp90 levels could alter the ivermectin-resistant phenotype of IVM-10 worms. To assess the sensitivity of the RNAi-treated worm to drug, wild-type N2 or IVM-10 worms were fed on the 75 bp *hsp90(RNAi)* construct at 20^{o}C for 24 h. Then 5 worms were transferred to each of 6 wells of a 24-well microtitre plate containing ivermectin at different concentrations (0, 1, 5 and 10 ng/ml). OP50 was added to each well and the plates were incubated at 20^{o}C. Worm motility was scored at 24 h and again at 48 h as described in Materials and Methods (Section 2.3.2.3). Figure 5.12a shows the results of the motility assay at 24 h and in Figure 5.12b, the 48 h results. At 24 h, motility of worms in all groups was similar at all concentrations of ivermectin. However by 48 h, wild-type N2 worms showed a reduced motility which was most apparent at 10 ng/ml ivermectin. In contrast, IVM-10 worms fed on empty vector maintained the tolerance to drug, while worms fed on the 75 bp *hsp90(RNAi)* construct behaved more like the wild-type N2 control i.e. their motility was compromised in the presence of ivermectin. This experiment was repeated three times with similar results.

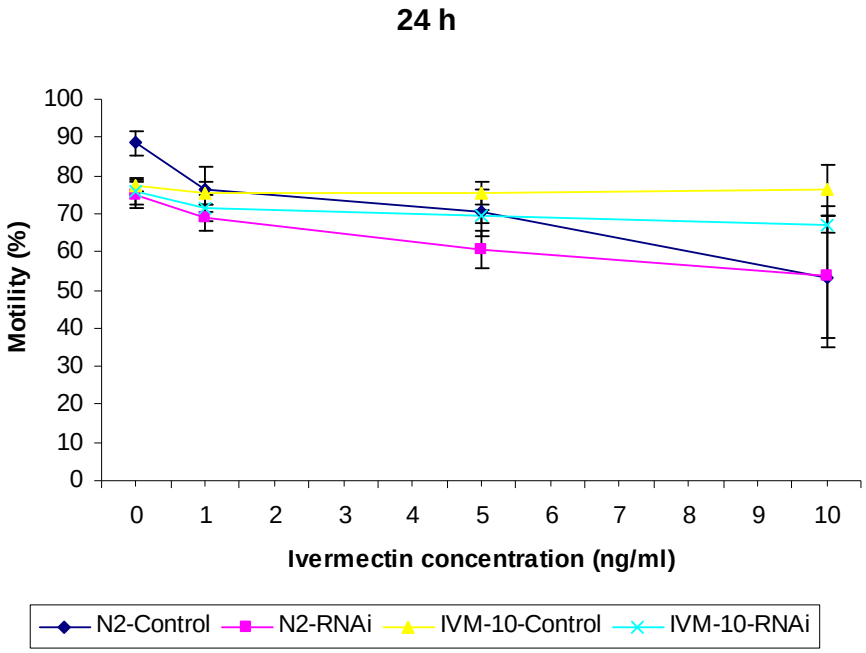
Figure 5.12: A reduction in Hsp90 levels alters the sensitivity of IVM-10 ivermectin-resistant worm to drug.

Wild-type N2 or IVM-10 resistant worms were fed on the 75 bp *hsp90(RNAi)* or on empty vector for 24 h at 20^{o}C, followed by incubation in liquid culture containing ivermectin at different concentrations (0, 1, 5 and 10 ng/ml) at 20^{o}C for 24 h or 48 h . After 24 h (Panel a) or 48 h (Panel b) the motility of the worms was scored. Graph presented here shows the mean ± standard deviation of three different experiments.

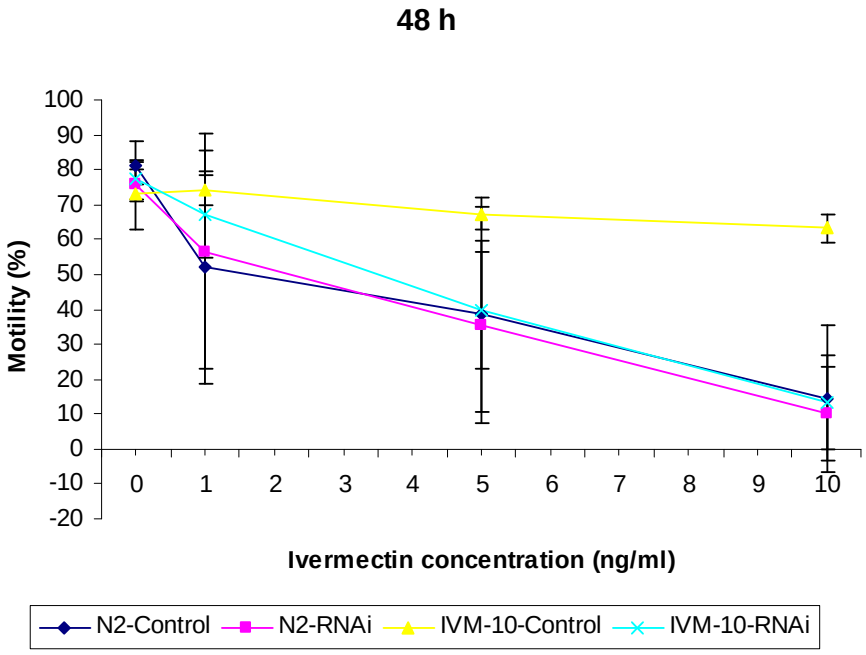
Worm motility was scored based on the activity of the worms in liquid containing different concentrations of ivermectin at 24 h and 48 h. The scoring system for the motility assay is shown below.

Activity	Score
Thrashing	3
Slow	2
Paralysed	1
Dead	0

30 worms for each concentration of drug were scored individually. The score then converted to a numerical value and expressed as a percentage of the maximum score (90 = 30 worms x 3).


24 h
Motility (%)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10
Ivermectin concentration (ng/ml)
N2-Control
N2-RNAi
IVM-10-Control
IVM-10-RNAi

(a)


48 h
Motility (%)
100
90
80
70
60
50
40
30
20
10
0
-10
-20
0 1 2 3 4 5 6 7 8 9 10
Ivermectin concentration (ng/ml)
N2-Control
N2-RNAi
IVM-10-Control
IVM-10-RNAi

(b)

The data shown in Figure 5.12 is presented in more detail in Figure 5.13. At 24 h there was little difference overall in the motility of wild-type N2 and IVM-10 worms fed on the 75 bp *hsp90(RNAi)* construct. However, two groups showed a small but significant difference in motility between RNAi and empty vector controls ($p<0.05$)(Figure 5.13a). The first group was wild-type N2 incubated without ivermectin and the second was IVM-10 worms incubated in 10 ng/ml ivermectin. In both cases, there was a reduction in motility in RNAi-treated worms.

At 48 h, wild-type N2 worms incubated in increasing concentrations of ivermectin showed a progressive reduction in motility, irrespective of RNAi treatment or not, except for those incubated in the highest concentration of ivermectin (10 ng/ml)(Figure 5.13b). In this group there was a significant difference between the motility of RNAi-treated worms compared to controls. No differences were observed in IVM-10 worms incubated without drug or with drug at 1 ng/ml, irrespective of RNAi treatment. However, at higher concentrations of ivermectin (5 and 10 ng/ml), a significant difference was observed between control and RNAi-treated IVM-10 worms. The motility of the RNAi group was significantly reduced compared to the control empty vector. These results indicate that reducing Hsp90 levels in IVM-10 resistant worms alters the sensitivity of the worms to high levels of ivermectin.

Figure 5.13: Motility assays of wild-type N2 and IVM-10 worms following *hsp90(RNAi)*.

Wild-type N2 or IVM-10 ivermectin resistant worms were fed on the 75 bp *hsp90(RNAi)* or on empty vector construct for 24 h at 20^{o}C, followed by incubation in liquid culture containing ivermectin at different concentrations (0, 1, 5 and 10 ng/ml) at 20^{o}C for 24 h or 48 h. The motility of the worms scored after 24 h or 48 h. Graph presented here shows the mean ± standard deviation of three different experiments.

a) 24 h

***N2-Control vs N2- RNAi at 0 ng/ml, *p* = 0.0087**

N2-Control vs N2-RNAi at 1 ng/ml, *p* = 0.0557

N2-Control vs N2-RNAi at 5 ng/ml, *p* = 0.0714

N2-Control vs N2-RNAi at 10 ng/ml, *p* = 0.5582

IVM-10-Control vs IVM-10- RNAi at 0 ng/ml, *p* = 0.3155

IVM-10-Control vs IVM-10-RNAi at 1 ng/ml, *p* = 0.5881

IVM-10-Control vs IVM-10-RNAi at 5 ng/ml, *p* = 0.1387

***IVM-10-Control vs IVM-10-RNAi at 10 ng/ml, *p* = 0.0451**

b) 48 h

N2-Control vs N2-RNAi at 0 ng/ml, *p* = 0.2103

N2-Control vs N2-RNAi at 1 ng/ml, *p* = 0.7475

N2-Control vs N2-RNAi at 5 ng/ml, *p* = 0.3700

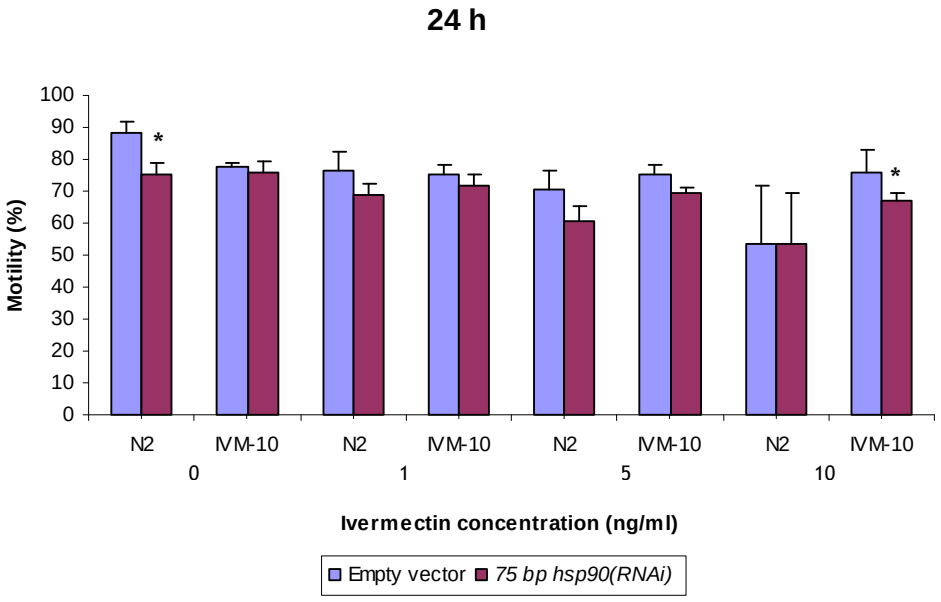
***N2-Control vs N2-RNAi at 10 ng/ml, *p* = 0.0315**

IVM-10-Control vs IVM-10-RNAi at 0 ng/ml, *p* = 0.3674

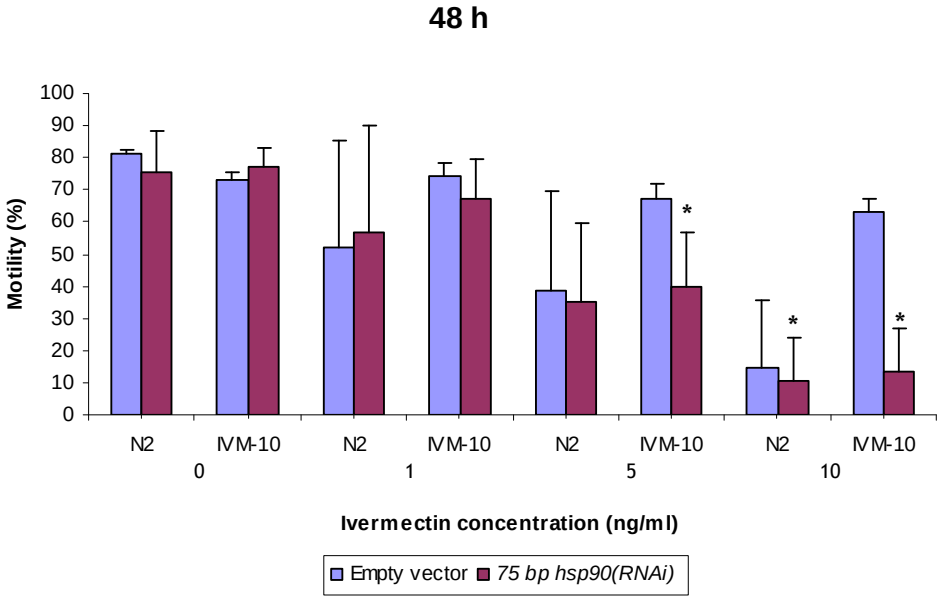
IVM-10-Control vs IVM-10-RNAi at 1 ng/ml, *p* = 0.8037

***IVM-10-Control vs IVM-10-RNAi at 5 ng/ml, *p* = 0.0435**

***IVM-10-Control vs IVM-10-RNAi at 10 ng/ml, *p* = 0.046**


24 h
Motility (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
N2
IVM-10
N2
IVM-10
N2
IVM-10
N2
IVM-10
0
1
5
10
Ivermectin concentration (ng/ml)
Empty vector
75 bp hsp90(RNAi)

(a)


48 h
Motility (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
*
N2
IVM-10
N2
IVM-10
N2
IVM-10
N2
IVM-10
0
1
5
10
Ivermectin concentration (ng/ml)
Empty vector
75 bp hsp90(RNAi)

(b)

5.3 Discussion

Drug resistance is already a serious problem in nematode parasites of livestock and also is also a growing issue in human health e.g. in onchocerciasis (Boussinesq, 2008). While treatment still relies upon the use of anthelmintics, there is a need to understand the mechanisms of drug resistance. Recently, Hsp90 was suggested to mediate drug resistance in fungi (Cowen & Lindquist, 2005; Singh *et al.*, 2009). In this Chapter, the possible role of Hsp90 in mediating anthelmintic resistance was investigated by comparing Hsp90 levels in resistant and susceptible nematodes. Furthermore, in RNAi experiments, levels of Hsp90 were reduced in order to determine whether a reduction in Hsp90 levels could alter the response of drug resistant worms to ivermectin.

The most direct approach to study anthelmintic resistance is by the comparative analysis of resistant and susceptible parasites isolated directly from the field. The *T. circumcincta* isolate, Tci5 was characterised by Bartley *et al* (2005) and shown to be resistant to the three broad spectrum anthelmintic groups. In contrast, the Tci2 isolate is susceptible to all anthelmintic groups. The *T. circumcincta* Tci5 and Tci2 isolates were compared but no significant difference was observed in Hsp90 levels. Next Hsp90 levels were compared in two *C. elegans* strains where resistance was acquired by mutagenesis, namely *C. elegans* CB3464 and DA1316. The CB3464 strain is resistant to benzimidazole compounds, while the DA1316 strain shows a high level of resistant to ivermectin (Dent *et al.*, 2000). Again, no difference in Hsp90 levels was observed between these worms and wild-type N2 controls. These assays were conducted in the absence of drug pressure. In future studies it would be interesting to compare Hsp90 levels in the presence of the relevant drug, to rule out the possibility that

drug exposure induces increased expression of Hsp90. Finally, Hsp90 levels were compared in ivermectin resistant *C. elegans* where resistance was acquired by continuous passage on plates containing drug. These worms were resistant to ivermectin at 6 ng/ml or 10 ng/ml. According to James & Davey (2009), IVM-6 worms show a 4.5-fold difference and IVM-10 worms a 19-fold difference in IC_{50} sensitivity to ivermectin compared to wild-type N2 worms. In that study they also showed that resistance might be associated with increased expression of drug pumps such as *mrp-1* and *pgp-1*. These worms are not only resistant to ivermectin but also showed a cross-resistance to moxidectin, levamisole and pyrantel but not to albendazole (James & Davey, 2009). However, no difference was observed in Hsp90 levels between IVM-6, IVM-10 and wild-type N2 worms.

C. elegans was shown to be a useful model to study parasitic nematodes (Blaxter, 1998; Gilleard, 2004). Using RNAi to knock-down or to reduce the expression of certain genes can result in an observable phenotype. Results presented in this Chapter show that feeding *C. elegans* with *E. coli* expressing dsRNA (300 bp *hsp90(RNAi)* construct) reduced the Hsp90 levels by approximately 40% and produced a phenotype. The cessation of egg production and a protruding vulva was observed in P_0 worms and in the progeny suggesting that Hsp90 is required during oogenesis or embryogenesis. The RNAi study by Piano *et al* (2000) shows that injection of *C. elegans* with *hsp90* dsRNA results in an embryonic lethal phenotype in the progeny and the cessation of egg production in hermaphrodite worms. Gillan *et al* (2009) showed that the progeny of wild-type N2 fed on *hsp90(RNAi)* were infertile and had reduced motility. These results indicate that Hsp90 is important in worm fertility and viability. Because of the deleterious effects observed with the 300 bp *hsp90(RNAi)*

constuct, a shorter construct was used in subsequent experiments. Worms fed on the 75 bp *hsp90(hsp90)* construct did not show any obvious phenotype but Hsp90 levels were reduced.

In the initial RNAi experiments, IVM-6 worms were used but no affect on tolerance to ivermectin was observed when Hsp90 levels were reduced using the 75 bp *hsp90(RNAi)* construct (data not shown). As IVM-10 worms are significantly more resistant to ivermectin, the experiments were repeated using this strain. *hsp90(RNAi)* was carried out and *C. elegans* N2 and IVM-10 worms were subjected to a drug cytotoxicity assay as described in Materials and Methods (Section 2.3.2.2) on NGM plates containing ivermectin at increasing concentrations (1, 5 or 10 ng/ml ivermectin). However, in all cases, the RNAi-treated worms crawled off the drug plates. This experiment was repeated several times with the same results (data not shown). It remains unclear why the RNAi-treated worms should crawl off the plates, but it is possible that a reduction in Hsp90 levels affects the ability of *C. elegans* to sense food, or perhaps more likely induces an aversion response of the worm to ivermectin. In *C. elegans hsp90* was shown to be required for regulation of specific chemosensory behaviours (Birnby *et al.,* 2000; Morley & Morimoto, 2004). Consequently to overcome this problem, worms were subjected to a motility assay in liquid culture containing ivermectin with OP50 as a food source. In the RNAi experiments, reducing Hsp90 levels in IVM-10 ivermectin resistant worms significantly altered the sensitivity to ivermectin but only at 5 and 10 ng/ml ivermectin. It was noteable that only 10-14% of wild-type N2 worms survived after 48 h on 10 ng/ml ivermectin, while more than 63% of control IVM-10 worms survived this concentration of drug. In contrast, the survival of the IVM-10 worms following RNAi was severely compromised. While the results presented in this

Chapter do not indicate how a reduction in Hsp90 levels affects the response to ivermectin, these results do support the hypothesis that Hsp90 may be involved in tolerance of high concentration of ivermection.

The best explanation of how Hsp90 mediates drug resistance comes from studies on yeast (Cowen & Lindquist, 2005; Singh *et al.*, 2009). In azole resistant yeast, resistance was suggested to be associated with calcineurin, an Hsp90 client protein. According to Fox & Heitman (2002), calcineurin acts as a regulator for cell cycle progression, morphorgenesis and virulence. Hsp90 binds to the catalytic subunit of calcineurin keeping it stable and poised for activation (Imai *et al.*, 2000; Kumar *et al.*, 2005). In the presence of azoles, Hsp90 regulates membrane responses that are vital for cells to survive. Importantly Cowen & Lindquist (2005) showed that the response of azole resistant yeast to drug was altered by genetic or chemical inhibition of Hsp90 and that inhibition of calcineurin function by Cyclosporin A or FK506 (inhibitor of calcineurin) phenocopied Hsp90 inhibition. Thus, Hsp90 potentiates drug resistance in yeast via calcineurin.

There are also several reports that suggest that Hsp90 may have a role in drug resistance in tumour cells (Betram *et al.*, 1996; Davies *et al.*, 2002; Xing *et al.*, 2008). For example, Xing *et al* (2008) showed that activation of the phosphatidylinositol 3-kinase (PI-3K/Akt2) pathway has a crucial role in inducing resistance to docetexal in ovarian and breast cancer cells. The PI-3K/Akt2 pathway has been shown to be important in promoting cell proliferation and inhibiting cell death. Most importantly, Akt is a serine-threonine kinase and an Hsp90 client protein, forming a complex with Hsp90 (Sato *et al.*, 2000). Thus, it suggests that in some cancer cells, activation of PI-3K/Akt2 pathway which is

modulated by Hsp90, is important in docetexal resistance (Xing *et al.*, 2008). In another study in cancer chemotherapy, drug resistance was suggested to be associated with P-gp and Hsp90ß (Betram *et al.*, 1996). Hsp90ß was shown to be co-precipitated along with P-gp. P-gp is a member of the ATP-binding cassette transporter family encoded by the gene MDR1 and previously implicated in the multi-drug resistance phenotype (Ueda *et al.*, 1987). The results suggest an intracellular co-operation between Hsp90ß and P-gp and the involvement of Hsp90ß in multi-drug resistant colon carcinoma (Betram *et al.*, 1996). In addition, a study by Liu *et* al (1999) showed that reducing Hsp90 levels using antisense RNA increased the sensitivity of human gastric and hepatic cancer cells to drugs compared to parental cells, which again indicates a possible role of Hsp90 in resistance to chemotherapeutic agents in tumour cells.

In conclusion, the role of Hsp90 in acquired drug resistance in nematodes deserves further study. The mechanisms of anthelmintic resistance in nematodes are not are properly understood but are likely to be complicated. The contribution of Hsp90 to drug resistance might be a general mechanism that relates the cellular stress response with the stress induced by drug exposure. Future investigation of the composition, structure and function of Hsp90 complexes in nematodes may unlock new possibilities for the treatment of anthelmintic resistant nematodes.

Chapter 6

General Discussion

6 General discussion

The exposure of any living cell or organism to environmental stress, including elevated temperature, chemical or oxidative stress, results in the synthesis of proteins known as heat shock proteins (Hsps). Hsps are among the most highly conserved of all proteins and are present in most eukaryotic and prokaryotic organisms. Consequently the study of Hsps has been of much interest in biology. Hsp90, the focus of this thesis, is an essential highly conserved protein, which is present in most organisms tested.

Hsps in host-parasite interactions: What do we know?

The role of Hsps in host-parasite interactions has received considerable attention from both clinical and biological perspectives. From the host point of view, Hsps expressed by invading parasites are potent antigens that induce immune responses (Polla, 1991; Kaufmann, 1992; Maresca *et al.*, 1994). Thus, according to Newport (1991), parasite Hsps are potentially useful in generating vaccines. From the parasite point of view, the synthesis of Hsps may represent a cellular defense mechanism that favours adaptation of the parasite to different environments throughout its life-cycle (Tsuji *et al.*, 1997).

Parasites that are transmitted to mammalian hosts from the environment or from a vector undergo profound changes in temperature during the transition to these hosts (with internal temperatures of 37^{o}C or above). The induction of Hsps commonly accompanies this transition. Numerous studies have demonstrated the developmental regulation of Hsp expression in parasites (Van der Ploeg *et al.*, 1985; Devaney & Lewis, 1993; Ernani *et* al., 1993; Neumann *et al.*, 1993; Hartman *et al.*, 2003); expression differs throughout the life-cycle

both quantitatively and in the types of Hsps induced. For example *Brugia pahangi* requires two hosts to complete its life-cycle and the temperature shift experienced by Mf from 37^{o} C in the mammalian host to 28^{o} C in the mosquito is a requirement for development to proceed (Devaney & Lewis, 1993). According to Jecock & Devaney (1992), different types of Hsps are expressed in the Mf and the L3 of *B. pahangi*. Their observations show that a range of Hsps were expressed in Mf at 37^{o} C and repressed after transfer to 28^{o} C. The most abundant proteins identified were small Hsps and these were shown to be synthesised only in Mf at 37^{o}C (Devaney *et al.*, 1996). In contrast, in the L3, the expression of Hsps is temporary and corresponds with the transition from the mosquito to the mammalian host. These studies show that in the Mf and the L3 of *B. pahangi*, the transmission between hosts is complemented by alterations in gene expression and that temperature induces the expression of certain genes.

For parasitic species that do not involve a vector in their life-cycle, the free-living stage occurs in water or soil. Hsp expression accompanies the transition from the environment into the definitive host. In *Haemonchus contortus,* Hsp20 was identified and shown to be constitutively expressed in the L3, early L4 and adult stages (Hartman *et al.*, 2003). It was proposed that the presence of Hsp20 in the L3 may indicate that this protein plays a role in preparation for the transition from pasture to mammalian host (Hartman *et al.*, 2003). It was also suggested that the presence of Hsp20 in the adult worm may be due to its expression in eggs, as an early adaptation to cold shock when eggs are passed out of the host onto the pasture. Thus, many Hsps appear to be expressed in parasites to co-incide with environmental transitions. In contrast to these findings, Hsp90 is expressed constitutively throughout the *B. pahangi* life-

cycle. This presumably relates to the fact that some Hsps also play important roles under normal conditions of growth.

Why is there a difference amongst nematodes in GA-binding?

Much of the work described in this thesis was concerned with understanding why some nematode Hsp90s bind to GA, while others do not. The variability in GA-binding may relate to differences in nematode life-cycles and the environments in which they live. Nematodes are a remarkably successful group within the Animal Kingdom. While most nematodes are free-living, parasitic species play a significant role in regulating the productivity of wild populations, and impact negatively on human agriculture as well as human health. Nematodes are abundant and have successfully adapted to nearly every habitat from marine to fresh water, from the polar region to the tropics, as well as the highest to the lowest of elevations.

The contradictory affinity of Hsp90 from *Caenorhabditis elegans and B. pahangi* for GA is interesting, given the degree of conservation between the two proteins. The first report on the failure of *C. elegans* Hsp90 to bind to GA came from David *et al* (2003), with similar results reported by Devaney *et al* (2005). C. *elegans* is a free-living nematode that resides in clade V of the nematode phylogeny together with important parasitic nematodes such as *H. contortus*, *Teladosargia circumcincta*, *Heligmosomoides polygyrus* etc. The ecology of these species is very diverse. According to Kiontke & Sudhaus (2006), the actual niche of *C. elegans* in the environment remains unknown. In the wild, *C. elegans* is usually found as a stress-resistant dauer form, which is non-feeding. The study by David *et al* (2003) demonstrated that *C. elegans* is insensitive to GA treatment as exposure to high concentration of GA did not produce any

phenotype. A recent study by Taldone *et al* (2010) validated this finding using a sensitive fluorescence polarisation assay in which GA also failed to bind to *C. elegans* Hsp90. Analysis of *hsp90* sequences from a range of nematode species demonstrated three separate lineages for *hsp90* sequences which correlated with the ability to bind GA (Him *et al.,* 2009). However, in that study, no direct relationship between amino acid substitutions in Hsp90 and GA binding ability was observed. These data suggest that the evolution of *hsp90* may correlate with key functions other than the ability to bind GA.

Darwin argued that natural selection causes traits that aid survival to become more common, while traits that hinder survival become rare. Over many generations, successful random changes are filtered by natural selection and retained. In this way an organism can adapt to its environment. *C. elegans* might be adapted to survive exposure to GA in soil. Interestingly, Hsp90 from parasitic species that have free-living larvae in the environment also failed to bind GA, while Hsp90 from those species that are protected from the environment by an egg shell or are permanently parasitic, did bind to GA. The results presented in this thesis therefore support the 'adaptation theory' as proposed by David *et al* (2003).

Alternatively there are additional explanations that may explain the different sensitivity of *B. pahangi* and *C. elegans* to GA. While *C. elegans* Hsp90 fails to bind to GA, it retains its ability to bind to ATP. Although differences were observed in the level of expression of Hsp90 in *B. pahangi* and *C. elegans,* both proteins bound to ATP beads in binding assay. However, studies using more sophisticated approaches would be necessary to ascertain whether the ATPase activity of *B. pahangi* and *C. elegans* Hsp90 differs. In addition, Hsp90 is known

to be modified significantly, by phosphorylation, acetylation and glycosylation (Chiosis *et al.*, 2004; Scroggins and Neckers, 2007; Scroggins *et al.*, 2007; Yang *et al.*, 2008). These post-translational modifications alter its activity in a range of assays. A recent study on *Plasmodium falciparum* Hsp90 showed that differences in acetylation affected sensitivity to GA (Pallavi *et al.*, 2010). However to date, very little is known of the modifications of Hsp90 in nematodes. In this thesis, no evidence was produced that post-translational modifications are associated with the ability of human Hsp90 to bind to GA, but further studies are required to determine the role of such modifications in GA-binding in nematodes.

Could Hsp90 be a new target for filarial worms?

Previous studies by Devaney *et al* (2005) on *B. pahangi* showed that GA treatment not only kills the Mf but also kills the adult worms *in vitro*. Both male and female worms are susceptible to GA. In addition, the production of Mf by adult was inhibited within 24 h of exposure to the drug. While, the mechanism by which GA kills the worm is unknown, these results indicate that inhibition of Hsp90 activity may represent a novel target for chemotherapy of filariasis. The results in Chapter 4 show that only a small amount of cytosolic Hsp90 bound to GA in the pull-down assays. This may suggest that inhibition of a small proportion of Hsp90 is sufficient to kill the worms. By analogy with its effects on mammalian cells, GA most likely kills filarial parasites by disrupting interactions between Hsp90 and key client proteins that are required for viability. Consequently attempts were made to identify client proteins of Hsp90 in adult *B. pahangi* using a proteomic approach. While key proteins of Hsp90 have been identified in mammalian cells and yeast, fewer interactions

have been identified in nematodes. In *C. elegans*, 68 proteins are predicted to interact with Hsp90 (www.wormbase.org), but mostly of these interactions have not been experimentally validated. In this thesis, several proteins were identified in the proteomic analysis, but these were mostly structural proteins. Although the 2D-gel analysis did not provide a list of possible Hsp90 client protein, further studies should be carried out using the more sensitive DIGE method. Recent studies in other organisms have used a similar approach to successfully identify a range of client proteins or co-chaperones of Hsp90 (Tsayler *et al.*, 2009; Garcia-Descalzo *et al.*, 2010). Hsp90 was shown to be secreted in the ES product of *Brugia malayi* (Kumari *et al.*, 1994) and *B. pahangi* (this thesis). It would be interesting in future studies to compare the ES proteome of adult worms cultured with or without GA to investigate whether any ES products are dependent upon Hsp90 for their activity.

Does Hsp90 mediate drug resistance in nematodes?

The role of Hsp90 in anthelmintic resistance remains unresolved. In fungi, Hsp90 was shown to be involved in drug resistance. Hsp90 was suggested to act as a capacitor of phenotypic variation, buffering genetic variation in *Drosophila melanogaster* (Rutherford & Lindquist, 1998; Sollars *et al.*, 2003) and *Arabidopsis thaliana* (Queitsch *et al.*, 2002). These studies demonstrated that challenging Hsp90 function by mutation, pharmacological means or environmental stress can produce novel heritable phenotypes. The heritable phenotype is passed to the new generation even when Hsp90 levels return to normal. Through studies in *Drosophila* (Rutherford & Lindquist, 1998), a role for Hsp90 in evolutionary adaptation was proposed. Hsp90 functions as a chaperone for mutant proteins, and allows them to act functionally as normal

proteins. These mutations tend to accumulate under the protection of Hsp90. When the accumulated mutations surpass the normal Hsp90 threshold as a result of environmental stress or inhibition of Hsp90 function, various mutations were expressed. Therefore, Hsp90 has the capacity to 'buffer' the expression of mutations and release these in response to environmental stress. The exposure of nematodes to anthelmintic presumably represents a stress and induction of Hsps may help nematodes survive that stress. However, whether Hsp90 may 'buffer' genetic changes in nematodes remains unclear. Results in this thesis suggest that Hsp90 might have a role in drug resistance in *C. elegans*, but the mechanism by which Hsp90 might function in resistance in nematodes remains to be ascertained.

Conclusion

The present study presents a comparative analysis of Hsp90 in nematodes and investigates its possible role in drug resistance. Although a high degree of sequence similarity is observed when comparing Hsp90 amino acid sequences in nematodes, it remains possible that subtle differences in Hsp90 sequence may confer differential sensitivity to GA as recently demonstrated in the yeast, *Humicola fuscoatra*, where a single conservative mutation in Hsp90 altered sensitivity to radicicol (Prodromou *et al.*, 2009). Additionally, defining the Hsp90 complexes in *B. pahangi* and identifying those which are essential for filarial viability may provide additional novel targets for the chemotherapy of filariasis. Future studies should consider the role of client proteins/co-chaperones in Hsp90 activity, the differences in the charged region of Hsp90 and perhaps the internal interactions between domains. Further research addressing the possible roles of Hsp90 in drug resistance should be carried out. This could be achieved

by implementing more sensitive approaches which may be able to identify novel targets that might be responsible for acquired drug resistance in nematodes.

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Appendices

Appendix 1: Worm materials

Nematode	Source
Trichinella spiralis	Dr. C. Lawrence, University of Strathclyde
Trichuris muris	Prof. R. Grencis, University of Manchester
Brugia pahangi	Veterinary Infection and Immunity, University of Glasgow
Dirofilaria immitis	Dr. J. Foster, New England BioLabs
Onchocerca ochengi	Dr. B. Makepeace, Liverpool School of Tropical Medicine
Acanthocheilonema viteae	Prof. W. Harnett, University of Strathclyde
Toxocara cati	Mr. J. McGoldrick, Vet School, University of Glasgow
Ascaris suum	Prof. A. Maule, Queens University Belfast
Parascaris equorum	Prof. J. Matthews, Moredun Research Institute
Anisakis simplex	Dr. S. Martin, University of Aberdeen
Pseudoterranova decipiens	Dr. I. Coombs, University of Glasgow
Anguillicola crassus	Dr. I. Coombs, University of Glasgow
Strongyloides ratti	Prof. M. Viney, University of Bristol
Panagrellus redivivus	Prof. A. Maule, Queens University Belfast
Globodera rostochiensis	Dr. J. Jones, Scottish Crop Research Institute
Globodera pallida	Dr. C. Fleming, Agri-Food and Bioscience Institute, Belfast
Caenorhabditis elegans	*C. elegans* Genetics Center
Caenorhabditis briggsae	*C. elegans* Genetics Center
Oscheius tipulae	*C. elegans* Genetics Center
Pristionchus pacificus	*C. elegans* Genetics Center
Haemonchus contortus	Prof. D. Knox, Moredun Research Institute
Heligmosomoides polygyrus	Prof. R. Maizels, Universitiy of Edinburgh
Teleodorsargia circumcincta	Prof. D. Knox, Moredun Research Institute
Nippostrongylus brasiliensis	Prof. R. Maizels, Universitiy of Edinburgh

Appendix 2: Buffers and stock solutions

2.1 Buffers for use in pull-down assays

2.1.1 TNES buffer

TNES contains 50 mM Tric-HCl pH 7.4, 100 mM NaCl, 2 mM EDTA, 1% Igepal CA-630 (SIGMA, UK) in 100 ml ddH_2O. Store at room temperature. Add protease inhibitor prior to use.

2.1.2 SkBr3 lysis buffer

Buffer consists of 50 mM Tris-HCl, pH 7.5, 1% NP40, 2 mM EDTA, 100 MM NaCl. Store at room temperature. Add protease inhibitor prior to use.

2.1.3 GA-biotin lysis buffer

Buffer consists 20 mM HEPES, 5 nM $MgCl_2$, 1mM EDTA, 100 mM KCl, 0.03% NP-40, 1% Triton X-100. Store at room temperature. Add protease inhibitor prior to use.

2.1.4 ATP-binding buffer

Buffer consists of 20 mm HEPES, 50 mM KCl, 6 mM $MgCl_2$, 0.01% NP40, pH 7.4 in 100 ml total volume. Store at room temperature.

2.2 SDS-PAGE and Western blotting buffers and solutions

2.2.1 1x SDS-PAGE sample cocktail

50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1% Bromophenol blue, 100 mM DTT. Aliquot and store at -20^{o}C before use.

2.2.2 10% (w/v) APS

Dissolve 1000 mg ammonium persulfate (APS) with 10 ml ddH_2O. Aliquot and store at -20^oC.

2.2.3 10% (w/v) SDS

Dissolve 10 g SDS in 90 ml ddH_2O and bring to 100 ml with ddH_2O and store at room temperature.

2.2.4 10x SDS-PAGE running buffer, pH 8.3 (makes 1 L)

Dissolve 30.3 g Tris-base, 144.0 g Glycine, and 10.0 g SDS with ddH_2O up to 1 L of total volume and store at 4^oC before use. Dilute 50 ml of 10x stock with 450 ml ddH_2O for each electrophoresis.

2.2.5 Western blotting buffer/Transfer buffer (makes 1 L)

Dissolve 3.03 g Tris-base, 42.3 g Glycine, add 200 ml Methanol and ddH_2O to 1000 ml. Store buffer at 4^oC before use.

2.2.6 1.5 M Tris-HCl, pH 8.8

Dissolve 27.23 g Tris-base in 80 ml ddH_2O. Adjust to pH 8.8 with HCl. Bring total volume to 150 ml with ddH_2O and store at 4^oC.

2.2.7 0.5 M Tris-HCl, pH 6.8

Dissolve 6 g Tris base in 60 ml ddH_2O. Adjust to pH 6.8 with HCl. Bring total volume to 100 ml with ddH_2O and store at 4^oC.

2.2.8 Gel formulation (10% SDS-PAGE gel)

For 10% resolving gel, add 4.1 ml ddH_2O, 3.3 ml 30% Acrylamide/Bis, 2.5 ml 1.5 M Tris-HCl pH 8.3, 0.1 ml 10% (w/v) SDS, 50 µl 10% (w/v) APS and 5.0 µl TEMED. For 5% stacking gel, add 5.7 ml ddH_2O, 1.7 ml 30% Acrylamide/Bis, 2.5 ml 0.5 M Tris-HCl pH 6.8, 0.1 ml 10% (w/v) SDS, 50 µl 10% (w/v) APS and 10.0 µl TEMED.

2.2.9 5% (w/v) dried milk solution

Dissolve 2.5 g of dried milk to 50.0 ml PBS/Tween20. Prepare fresh as required.

2.2.10 Incubation/blocking buffer for Western blotting

Add 16.0 ml of PBS/Tween20, 4.0 ml of 5% dried milk and 200 µl of FCS to give total volume 20.0 ml. Prepare within 5 min of use.

2.2.11 Washing buffer

Dissolve one pack of PBS (Sigma, UK), 500 µl Tween20 in 1 L ddH_2O. Store at 4^oC.

2.3 Staining solutions

2.3.1 Coomassie blue staining R-250 (makes 2.5 L)

Dissolve 2.5 g of Coomassie blue in 1.125 L methanol, 1.125 L ddH_2O and 250 ml acetic acid and keep at room temperature.

2.3.2 Coomassie blue staining G-250 (makes 500 ml)

Dissolve 2.5 g of Coomassie blue in 250 ml methanol, 50 ml acetic acid and 200 ml ddH_2O and keep at room temperature.

2.3.3 Destaining solution (makes 1.7 L, 10% acetic acid solution)

Add 175 ml of acetic acid and 500 ml methanol to 1.025 L of ddH_2O and store at room temperature.

2.3.4 Silver staining (Bio-Rad, UK. Cat: 161-0449)

2.3.4.1 Fixative enhancer solution (makes 400 ml)

Add 200 ml methanol, 40 ml acetic acid and 40 ml fixation enhancer concentrate, 120 ml ddH_2O to give total volume of 400 ml.

2.3.4.2 Staining solution (makes 100 ml)

Add 35 ml ddH_2O, 5.0 ml silver complex solution, 5.0 ml reduction moderator solution, 5.0 ml image development reagent and 50 ml development accelerator solution. Prepare within 5 min of use.

2.3.4.3 Stop solution (5% (v/v) acetic acid solution)

Add 50 ml acetic acid and ddH_2O up to 1 L and store at room temperature.

2.3.5 Ponceau S

Add 3.0 g of TCA (Trichloroacetic acid) to 100 ml of ddH_2O and add 0.2 g of Ponceau S. Store at room temperature.

Appendix 3: Proteomic analysis buffers and stock solutions

3.1 Lysis buffer

Buffer consists of 8 M urea, 4% CHAPS (w/v) and 100 mM Tris-base in 25 ml. Aliquot and store at -20^{o}C before use. Add protease inhibitor prior to use.

3.2 80% acetone

Add 80 ml acetone to 20 ml ddH_2O (v/v). Store at -20^{o}C before use.

3.3 Rehydration buffer

Buffer consists 6 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT and trace Bromophenol blue. Add 0.5% IPG buffer prior to use.

3.4 First SDS equilibration buffer solution

Buffer consists 6 M urea, 75 mM Tris-base, 29.3% Glycerol (v/v), 2% SDS (w/v), 0.002% Bromophenol blue in 200 ml ddH_2O. Aliquot and store at -20^{o}C. Add 100 mg DDT/10 ml equilibration buffer prior to use.

3.5 Second SDS equilibration buffer solution

Buffer consists 6 M urea, 75 mM Tris-base, 29.3% Glycerol (v/v), 2% SDS (w/v), 0.002% Bromophenol blue in 200 ml ddH_2O. Aliquot and store at -20^{o}C. Add 250 mg iodoacetamine/10 ml equilibration buffer prior to use.

3.6 Gel formulation (12% SDS-PAGE gel)

For 100 ml solution, add 42.25 ml ddH_2O, 31.25 ml 40% Acrylamide/Bis, 25 ml 1.5 M Tris-HCl pH 8.8, 1.0 ml 10% (w/v) SDS, 500 µl 10% (w/v) APS and 33 µl TEMED. Add APS and TEMED just before casting the gel.

3.7 1% Bromophenol blue stock solution

Dissolve 100 mg Bromophenol blue, 60 mg Tris-base with 10 ml ddH_2O. Aliquot and store at -20^oC before use.

Appendix 4: Bacteria and worm culture

4.1 L-Broth, pH 7.0 (makes 1 L)

Dissolve 10 g NaCl, 10 g Tryptone and 5 g yeast to 800 ml ddH_2O and bring the total volume to 1 L. Sterilise by autoclaving and store at room temperature.

4.2 L-Broth agar

Add 7.5 g agar to 500 ml L-Broth and sterilise by autoclaving and store at room temperature.

4.3 NGM agar

Add 3 g NaCl, 17 g agar, 2.5 g Peptone to 975 ml of ddH_2O. Sterilise by autoclaving, cool flask to 55^oC for 15 min. Then add 1.0 ml of 1 M $CaCl_2$, 1.0 ml of 5 mg/ml cholesterol in ethanol, 1.0 ml of 1 M $MgSO_4$, 25 ml of 1 M KH_2PO_4. Store at 4^oC.

4.4 Cholesterol-free NGM

Add 3 g NaCl, 17 g agar, 2.5 g Peptone to 975 ml of ddH_2O. Sterilise by autoclaving, cool flask to 55^oC for 15 min. Then add 1.0 ml of 1 M $CaCl_2$, 1.0 ml of 1 M $MgSO_4$, 25 ml of 1 M KH_2PO_4. Store at 4^oC.

4.5 RPMI-1640 medium for *B. pahangi* culture

Add 1 ml 50% glucose, 100 units/ml streptomycin, 100 µg/ml penicillin to 100 ml RPMI-1640 with containing 5 mM glutamine, 25 mM HEPES (Invitrogen, UK. Cat: 52400-017). Incubate at 37^oC before use.

4.6 Ivermectin stock solution

Dissolve 1 mg ivermectin with 1.0 ml of DMSO to give 1 mg/ml concentration. Pipette 100 µl and add to 10.0 ml DMSO to give a final concentration of 10 µg/ml. Store at -20^oC before use.

4.7 Ivermectin plates

Add 500 µl 25 mg/ml water-soluble cholesterol (Sigma, UK. Cat: C1145-250MG) to 500 ml of cholesterol-free NGM. For 10 ng/ml plates (5 x 10 ml), add 50 µl 10 µg/ml ivermectin to 50 ml NGM to give final concentration of 10 ng/ml. Add 30 µl 10 µg/ml ivermectin to 50 ml NGM to give final concentration of 6 ng/ml. Store plate at room temperature.

2Appendix 5: Other buffers and solutions

5.1 Bleach solution (makes 10 ml)

Add 625 µl 4 M NaOH, 1500 µl sodium hypochlorite to 7875 µl of ddH_2O. Prepare fresh as required.

5.2 M9 buffer (makes 1 L)

Dissolve 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml $MgSO_4$ in ddH_2O and bring up to 1 L and sterilise by autoclaving and store at room temperature.

5.3 50% (w/v) glucose stock

Dissolve 50 g of sucrose to 80 ml ddH_2O. Bring up volume to 100 ml with ddH_2O and store at room temperature.

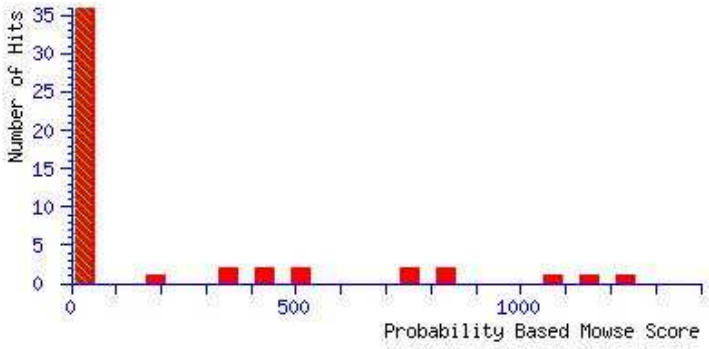
Appendix 6: MASCOT Search Results

6.1 Down-regulated

6.1.1 1a - Tropomyosin

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|42559586 **Mass:** 33215 **Score:** 1233 **Queries matched:** 35 **emPAI:** 5.94
RecName: Full=Tropomyosin; AltName: Full=MOv-14; AltName: Full=Ov-tmy-1

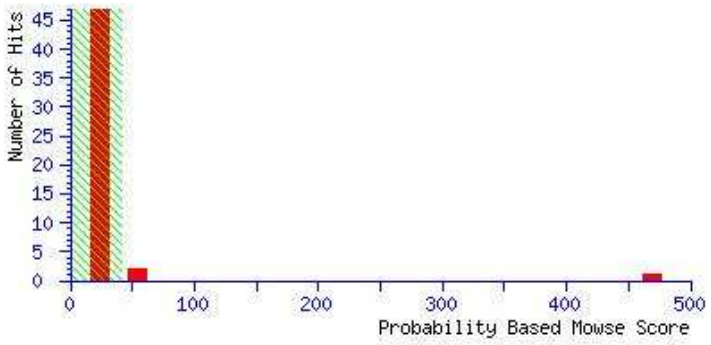
Matched peptides shown in **Bold Red**

```
  1 MDAIKKKMQA MKIEKDNALD RADAAEEKVR QMTEKLERIE EELRDTQKKM
 51 MQTENDLVKA QEDLSVANTN LEDKEKKVQE AEAEVAALNR RMTLLEEELE
101 RAEERLKIAT DKLEEATHTA DESERVRKVM ENRSFQDEER ANTVESQEKE
151 AQLLAEEADR KYDEVARKLA MVEADLERAE ERAEAGENEI VELEEELRVV
201 GNNLKSLEVS EEKALQREDS YQEQIRTVSV RLKEAETRAE FAERSVQKLQ
251 KKVDRLEDEL VHEKERYKNI SEELDQTFQE LSGY
```

6.1.2 1b - Myosin regulatory light chain 1

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|170589459 **Mass:** 18808 **Score:** 469 **Queries matched:** 14 **emPAI:** 5.48
Myosin regulatory light chain 1 [Brugia malayi]

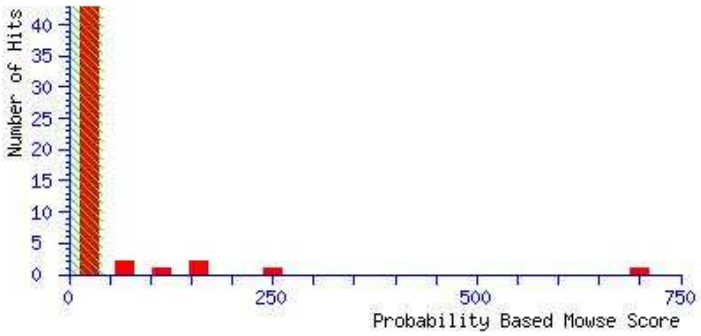
Matched peptides shown in **Bold Red**

```
   1 MSKAAKKKSS KKKSGSEAAQ FDQKTIQEFK EAFGIMDQNK DGIIDKQDLK
  51 DLYAMMGQIA SDAQIDAMIK EAPGPINFTV FLTLFGEKLT GTDPEATIIG
 101 AFQMFDKRDC GKISEDELLK ILQNKRGEPF DDDEIKAMYK GKPPVENGQV
 151 DYKAFAHLIT TGAAEELAKA
```

6.1.3 1c - Actin-depolymerising factor 1

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|170582273 **Mass:** 18826 **Score:** 700 **Queries matched:** 12 **emPAI:** 5.42
actin-depolymerizing factor 1 [Brugia malayi]

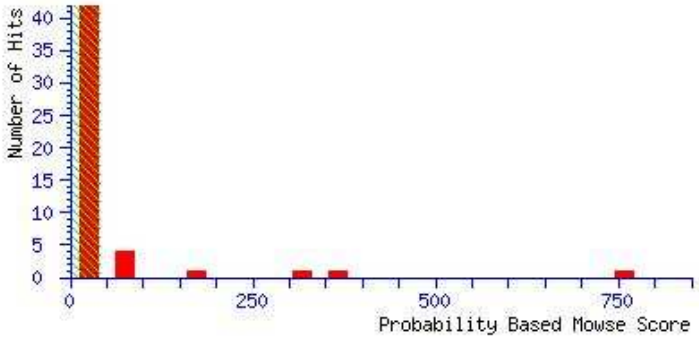
Matched peptides shown in **Bold Red**

```
    1 MSSGVLVNSE CQTVFQQLSE GKHHKLRYII YKIEDKEVVV EAAVSPDELG
   51 VTDDDHDENS KTAYEAFVQD LRERTNGFKD CRYAVFDFKF SCNRPGAGTS
  101 KMDKIVFIQL CPDGAPIKKK MVYASSASAI KSSLGTAKIL QFQVSDDSEI
  151 AHKELLSKLS EKYRDN
```

6.1.4 - Actin-depolymerising factor 1

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|170582273 **Mass:** 18826 **Score:** 757 **Queries matched:** 13 **emPAI:** 5.42
actin-depolymerizing factor 1 [Brugia malayi]

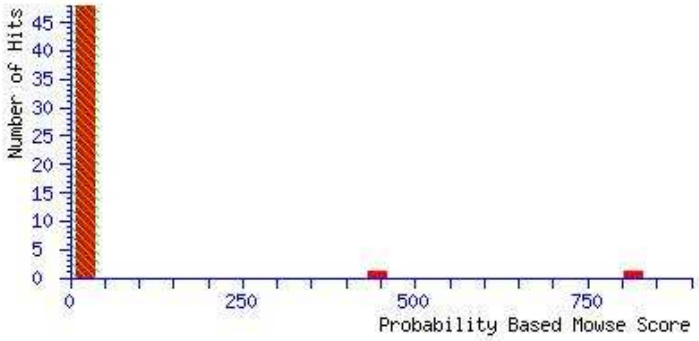
Matched peptides shown in **Bold Red**

```
    1 MSSGVLVNSE CQTVFQQLSE GKHHKLRYII YKIEDKEVVV EAAVSPDELG
   51 VTDDDHDENS KTAYEAFVQD LRERTNGFKD CRYAVFDFKF SCNRPGAGTS
  101 KMDKIVFIQL CPDGAPIKKK MVYASSASAI KSSLGTAKIL QFQVSDDSEI
  151 AHKELLSKLS EKYRDN
```

6.1.5 - DJ-family protein

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|170574881 **Mass:** 19914 **Score:** 817 **Queries matched:** 22 **emPAI:** 13.20
DJ-1 family protein [Brugia malayi]

Matched peptides shown in **Bold Red**

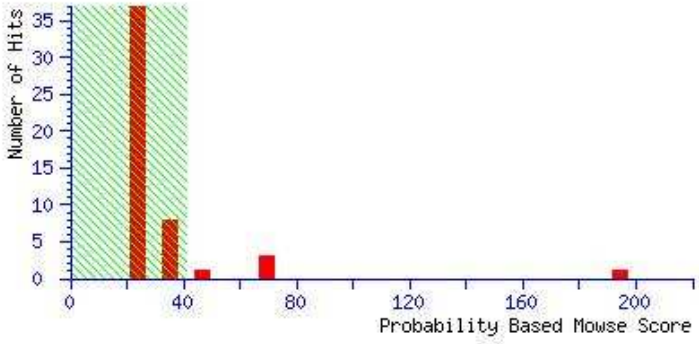
```
   1 MASKTAMVIL AEGAEEMETV IPVDVLRRAG VEVTIAGLLG KNTVKCSRQV
  51 MITPDKALFE VADNKFDVVI LPGGLQGANS LAASDEVGTI LRTQYESGRY
 101 IAAICAAPIA LKSHGIAPGI LLTSHPSVKP KLVEGGYKYS EDRVVTTDHI
 151 VTSRGPGTAL EFALKLVELL VGTEKVKEVS VPMIVKE
```

6.2 Similar in both groups

6.2.1 2a - Small heat shock protein

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|870911 **Mass:** 20239 **Score:** 194 **Queries matched:** 4 **emPAI:** 0.69
small heat shock protein [Brugia pahangi]

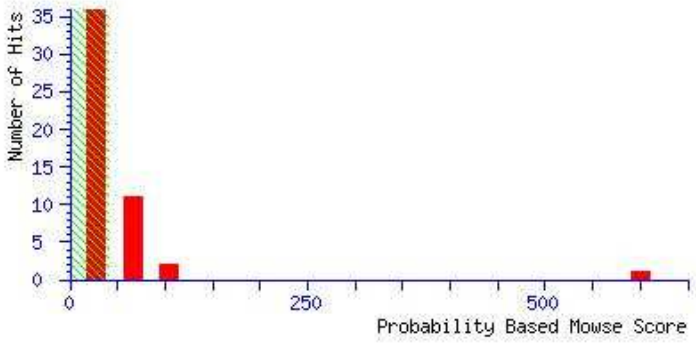
Matched peptides shown in **Bold Red**

```
  1 MSVFRYNPRD YFYTSPMERF IVNLLDNTFD DRYRPLQSVA PYWLNQPVLN
 51 ECNIGNALGE VINEKDKFAI QVDVSHFHPK ELSVSVRDRE LSIEGHHKER
101 NDHSGHGSIE RHFVRKYVMP EEVQTDTIES HLSDKGVLTI CATKTMIGLP
151 AARNIPIRAS PKEPEAGEKS ASNGTGQ
```

6.2.2 2b - Alkali myosin light chain

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|251762815 **Mass:** 16762 **Score:** 601 **Queries matched:** 29 **emPAI:** 21.71
alkali myosin light chain [Setaria digitata]

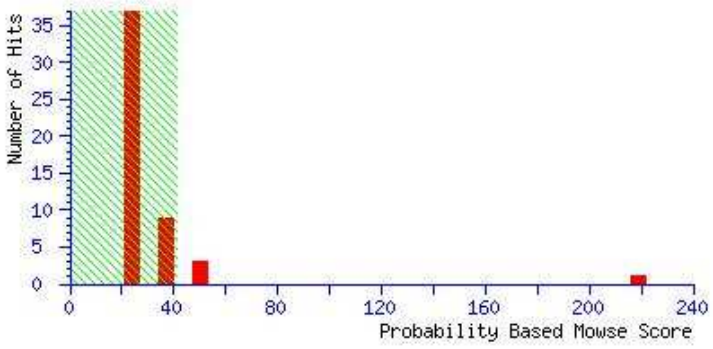
Matched peptides shown in **Bold Red**

```
    1 MLIAELKEIF LLYDEELDGK IDGTQIGDVV RAAGLKPTNA MVVKASGSEY
   51 KRKGEKRLTF EEWMPIYEQL SKEKEQGTFQ DFVEGLKVFD KEESGKIMAA
  101 ELRHVLMALG ERLSAEEADE IMKGCEDAEG MVSYEAFVKK VLAGPFPDD
```

6.2.3 2c - Thioredoxin

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|21436483 **Mass:** 16258 **Score:** 219 **Queries matched:** 6 **emPAI:** 0.91
thioredoxin [Brugia malayi]

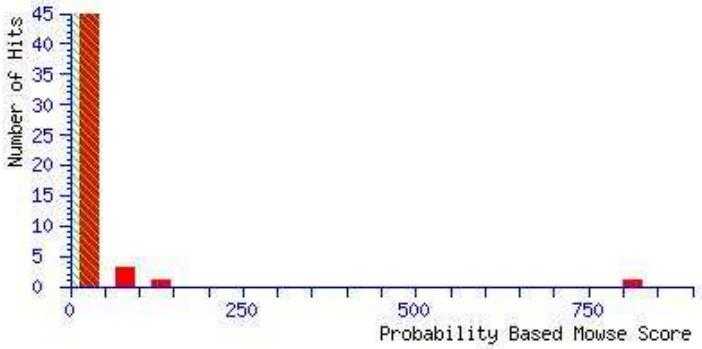
Matched peptides shown in **Bold Red**

```
  1 MADLLANINL KKADGTVKKG SDALANKKVV ALYFSAHWCP PCRQFTPILK
 51 EFYEEVDDDQ FEIVFVSLDH SEEDLNNYVK ESHGNWYYVP FGSSEIEKLK
101 NKYEVAGIPM LIVIKSDGNV ITKNGRADVS GKAPPQTLSS WLAAA
```

6.2.4 2d - Small heat shock protein

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|870911 **Mass:** 20239 **Score:** 814 **Queries matched:** 19 **emPAI:** 12.62
small heat shock protein [Brugia pahangi]

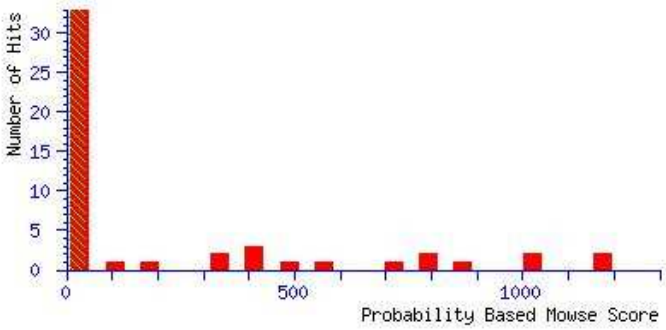
Matched peptides shown in **Bold Red**

```
   1 MSVFRYNPRD YFYTSPMERF IVNLLDNTFD DRYRPLQSVA PYWLNQPVLN
  51 ECNIGNALGE VINEKDKFAI QVDVSHFHPK ELSVSVRDRE LSIEGHHKER
 101 NDHSGHGSIE RHFVRKYVMP EEVQTDTIES HLSDKGVLTI CATKTMIGLP
 151 AARNIPIRAS PKEPEAGEKS ASNGTGQ
```

6.2.5 2e - Tropomyosin

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|224016002 **Mass:** 33537 **Score:** 1174 **Queries matched:** 36 **emPAI:** 9.43
tropomyosin [Ascaris lumbricoides]

Matched peptides shown in **Bold Red**

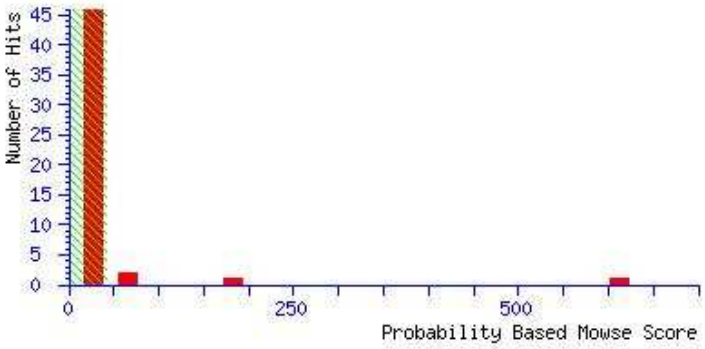
```
   1 MDAIKKKMQA MKIEKDNALD RADAAEEKVR QMTDKLERIE EELRDTQKKM
  51 MQTENDLDKA QEDLSVANSN LEEKEKKVQE AEAEVAALNR RMTLLEEELE
 101 RAEERLKLAT EKLEEATHTA DESERVRKVM ENRSFQDEER ANTVESQLKE
 151 AQMLAEEADR KYDEVARKLA MVEADLERAE ERAEAGENKI VELEEELRVV
 201 GNNLKSLEVS EEKALQREDS YEEQIRTVSA RLKEAETRAE FAERSVQKLQ
 251 KEVDRLEDEL VHEKERYKSI SEELDQTFQE LSGYRSD
```

6.3 Up-regulated

6.3.1 3a - Myosin regulatory light chain 1

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|170589459 **Mass:** 18808 **Score:** 613 **Queries matched:** 11 **emPAI:** 3.46
Myosin regulatory light chain 1 [Brugia malayi]

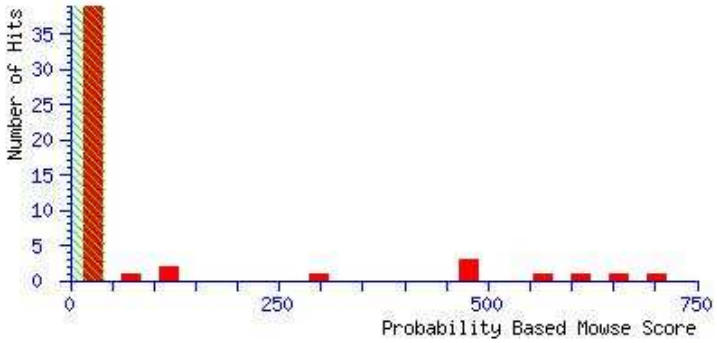
Matched peptides shown in **Bold Red**

```
  1 MSKAAKKKSS KKKSGSEAAQ FDQKTIQEFK EAFGIMDQNK DGIIDKQDLK
 51 DLYAMMGQIA SDAQIDAMIK EAPGPINFTV FLTLFGEKLT GTDPEATIIG
101 AFQMFDKRDC GKISEDELLK ILQNKRGEPF DDDEIKAMYK GKPPVENGQV
151 DYKAFAHLIT TGAAEELAKA
```

6.3.2 3b – Tropomyosin

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 41 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|154466686 **Mass:** 33207 **Score:** 701 **Queries matched:** 42 **emPAI:** 1.63
tropomyosin [Ascaris lumbricoides]

Matched peptides shown in **Bold Red**

```
   1 MDAIKKKMQA MKIEKDNALD RADAAEEKVR QMTDKLERIE EELRDTQKKM
  51 MQTENDLDKA QEDLSVANSN LEEKEKKVQE AEAEVAALNR RMTLLEEELE
 101 RAEERLKLAT EKLEEATHTA DESERVRKVM ENRSFQDEER ANTVESQLKE
 151 VQMLAEEADR KYDEVARKLA MVEADLERAE ERAEAGENKI VELEEELRVV
 201 GNNLKSLEVS EEKALQREDS YEEQIRTVSA RLKEAETRAE FAERSVQKLQ
 251 KEVDRLEDEL VHEKERYKSI SEELDQTFQE LSGY
```