The screening and characterisation of compounds for modulators of Heat shock protein (Hsp90) in a breast cancer cell model

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by

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FEBRUARY 2013

DECLARATION

I declare that this dissertation is my own unaided work, except where acknowledged, submitted for the degree of Doctor of Philosophy in Biochemistry of Rhodes University in the Faculty of Science. It has not been submitted for any degree for examination in any other university.

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Ms Buhle Moyo, February 2013

ABSTRACT

Breast cancer is a leading cause of cancer death in Africa. Hsp90 has been identified as a target for anti-cancer treatments as its inhibition results in the disruption and ubiquitin–proteasome degradation of activated oncoproteins. Currently, there are no US Food and Drug Administration approved Hsp90 inhibitor drugs and existing Hsp90 inhibitors such as geldanamycin and novobiocin are hepatotoxic and display a low affinity for Hsp90, respectively. Therefore, there is a need for the development of Hsp90 inhibitors with improved inhibitory properties. In this study twelve natural compounds bearing a quinone nucleus were screened and characterised for the modulation of Hsp90. The compounds analysed formed three series; the sargaquinoic acid (SQA), naphthoquinone, and pyrroloiminoquinone alkaloid series. Certain compounds exhibited half maximal inhibitory concentrations of between 3.32 μM and 12.4 μM, while others showed no antiproliferative activity at concentrations of up to 500 μM in the MDA-MB-231 breast adenocarcinoma cell line. Immunofluorescence and Western analyses indicated that the modulation of Hsp90 and partner proteins by SQA was more similar to that of novobiocin. Isothermal titration calorimetry analyses suggested that SQA interacted with Hsp90β with a low affinity, and saturation-transfer difference nuclear magnetic resonance confirmed that this interaction with Hsp90β occurred through the methyl moiety bound to 1, 4 benzoquinone of SQA. Pulldown assays indicated SQA disrupted the association between Hsp90 and Hop dose-dependently, more similarly to novobiocin. Immunofluorescence and Western analyses performed on naphthoquinone and pyrroloiminoquinone alkaloid compounds indicated modulation of Hsp90 and Hsp90 partner proteins by the compounds. Naphthoquinone compounds were prioritised for analysis for binding to Hsp90β over the pyrroloiminoquinone alkaloid compounds. Lapachol interacted with Hsp90β with a low affinity however; this interaction was thought to be too weak to disrupt the association of Hsp90 and Hop. The remaining naphthoquinone compounds showed no interaction with Hsp90β, thus allowing the determination of a preliminary structure-activity relationship for these compounds. To the best of our knowledge, this is the first study to describe a systematic subcellular analysis of the effects of geldanamycin and novobiocin in comparison to sargaquinoic acid and compounds of the naphthoquinone and pyrroloquinoline scaffold on Hsp90 and its partner proteins.

LIST OF ABBREVIATIONS

17-AAG	17-N-allylamino-17-demethoxygeldanamycin
17-DMAG	17-dimethylaminoethylamino-17-demethoxygeldanamycin
αLAP	α-lapachona
ADMET	Absorption, distribution, metabolism, excretion and toxicity
ADP	Adenosine diphosphate
Aha	Activator of Hsp90 ATPase 1
ATPase	Adenosine triphosphatase
BBL	Bromo-β-lapachona
Bis-Tris	2-[Bis (2-hydroxyethyl) amino]-2-(hydroxymethyl)-1, 3-propanediol
BSA	Bovine serum albumin
C-	Carboxyl-terminal
CAL	C-alil-lausona
Cdc37	Cell cycle division 37 homologue
CHIP	Carboxyl terminus of the Hsc70 interacting protein
DMEM	Dulbecco's Modified Eagle Medium
DMN	Damirone C
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DP	Aspartate-proline
DSN	Discorhabdin A
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGCG	(-)-Epigallocatechin-3-gallate
EnR	Endoplasmic reticulum
ER	Oestrogen receptor
FCS	Foetal calf serum
G1	First gap phase
G2	Second gap phase
GA	Geldanamycin
GR	Glucocorticoid receptor
Grp94	Glucose-regulated protein 94
HBL	Hydroxy-β-lapachona
HDACi	Histone deacetylase inhibitor
HER	Human epidermal growth factor receptor
Hip	Hsp70-interacting protein
Hop	Hsp70/Hsp90 organising protein
HRP	Horse radish peroxidase
HS	Heat shock
H.sapiens	Homo sapiens
Hsc70	Heat shock cognate protein 70 kDa
HSF	Heat shock factor
Hsp	Heat shock protein
Hsp40	Heat shock protein 40 kDa
Hsp70	Heat shock protein 70 kDa
Hsp90	Heat shock protein 90 kDa
HtpG	High temperature protein G

IC_{50}	Half maximal inhibitory concentration
Ig	Immunoglobulin
IP	Immunophilin
IPI-504	17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITC	Isothermal titration calorimetry
LAP	Lapachol
LogP	Octanol-water partition coefficient
M-	Middle
MKM	Makaluvamine M
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MWCO	Molecular weight cut off
NBL	Nor-β-lapachona
N-	Amino-terminal
N.A.A	No antiproliferative activity
N.D	Not determined
NOV	Novobiocin
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PR	Progesterone receptor
pSTAT3	Phosphorylated signal transducer and activator of transcription 3
PTX	Paclitaxel
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
RIBS-1	*N*-1-β-D ribofuranosyl makaluvamine
RIBS-3	*N*-1-β-D-ribofuranosyl makaluvic acid
RNA	Ribonucleic acid
RNPs	Ribonucleoproteins
S.cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
siRNA	Short interfering Ribonucleic Acid
SQA	Sargaquinoic acid
STAT3	Signal transducers and activators of transcription 3
STD NMR	Saturation-transfer difference nuclear magnetic resonance
TBS	Tris-Buffered Saline
TBS-T	Tris Buffer Saline with Tween 20
TEMED	N, N, N', N'-tetramethylethylenediamine
TNBC	Triple-negative breast cancer
TPR	Tetratricopeptide repeat
TRAP-1	Tumour necrosis factor receptor-associated protein 1
Tris	Tris-2-amino-2-hydroxymethyl-1, 3-propanol
WGA	Wheat germ agglutinin
YT	Yeast extract-Tryptone media

LIST OF SYMBOLS

α	Alpha
Å	Angstroms
β	Beta
CO_2	Carbon dioxide
°C	Degrees Celsius
γ	Gamma
g	Gram(s)
κ	Kappa
kcal	Kilocalories
kDa	Kilodaltons
L	Litre(s)
µ	Micro
µcal	Microcalorie (s)
µg	Microgram(s)
µl	Microlitre(s)
µm	Micrometre
µM	Micromolar
mg	Milligram(s)
ml	Millilitre(s)
mM	Millimolar
min	Minutes
M	Molar
mol	Mole(s)
nm	Nanometre
nM	Nanomolar
%	Percentage or g/100 ml
pH	Hydrogen ion concentration
sec	Seconds
V	Volts
v/v	Volume to volume ratio
w/v	Weight to volume ratio
x g	Relative centrifugal gravitational force

LIST OF AMINO ACIDS

Name	**One Letter Code**	**Three Letter Code**
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Methionine	M	Met
Proline	P	Pro
Threonine	T	Thr
Tyrosine	Y	Tyr
Serine	S	Ser
Valine	V	Val

LIST OF RESEARCH OUTPUTS

Buhle Moyo, Soumya S. Daturpalli, Tatenda C. Munezdimwe, Kevin Lobb, Denzil R. Beukes, Michael Davies-Coleman, Sophie E. Jackson, Gregory L. Blatch, and Adrienne L. Edkins. Quinone-bearing compounds sargaquinoic acid and lapachol modulate Hsp90 localisation and chaperone association. In preparation for submission to *Biological Chemistry*.

Jo-Anne de la Mare, Lara Contu, Morgan Hunter, **Buhle Moyo,** Jason Sterrenberg, Lorraine Mutsvunguma, Karim Dhanani and Adrienne L. Edkins. Breast Cancer: Development in molecular approaches to diagnosis and treatment. Submitted to *Patents in Anti-Cancer Drug Discovery.*

Earl Prinsloo, Leanne C. Cooper, **Buhle Moyo**, Jo-Anne de la Mare, Jessica C. Lawson, Adrienne L. Edkins and Gregory L. Blatch .(2010). Heat shock proteins in normal and cancer stem cell biology: implications for regenerative and chemotherapeutic medicine in Stem Cell, Regenerative Medicine and Cancer; pages 693-713

Buhle Moyo, Adrienne L. Edkins and Gregory L. Blatch. (2011). The characterisation and screening of novel compounds for modulators of heat shock protein 90 (Hsp90) function in breast cancer cells. Oral presentation at CARISA Workshop Cape Town, South Africa.

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CHAPTER 1

LITERATURE REVIEW
HYPOTHESIS
PROBLEM STATEMENT
AIMS AND OBJECTIVES

1.1. Breast cancer: an increasing burden in Africa

The term cancer refers to a broad category of diseases in which a population of abnormal cells shows excessive proliferation, invades surrounding tissues at the primary site and may metastasize to other sites (Clark, 1991). The defining characteristics of cancer cells, the hallmarks of cancer, were originally categorised as having six features and have subsequently been revised to ten features (Hanahan and Weinberg, 2011). These hallmarks comprise sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism, genome instability and mutation, inflammation and evading immune destruction (Hanahan and Weinberg, 2011).

Breast cancer is a collective term that describes a heterogeneous disease which can be differentiated at the clinical and molecular level (Sørlie *et al*., 2001, Perou *et al*., 2000). Breast cancers are currently categorised into five subtypes, each with distinct characteristics, clinical outcomes and responses to therapy (Gatza *et al*., 2010; Stephens *et al*., 2009; Chin *et al*., 2006). A new biological categorisation system was introduced by the St Gallen International Expert Consensus based on the expression of the oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER2) and Ki-67 antigen (Goldhirsch *et al.,* 2011). These subtypes are the luminal A (ER and/or PR positive, HER2 negative, low Ki-67 index), luminal B HER2 negative (ER and/or PR positive, HER2 negative, high Ki-67 index), luminal B HER2 positive (ER and/or PR positive, HER2 overexpressed, any Ki-67 index), HER2 positive (ER and PR negative, HER2 overexpressed) and basal-like or triple-negative (ER and PR negative, HER2 negative) (Goldhirsch *et al.,* 2011).

Recently, Curtis *et al*. performed an integrated genome and transcriptomic analysis of 1992 breast cancer tumours supplied from tumour banks in Canada and the United Kingdom (Curtis *et al*., 2012). Copy number variations, acquired somatic copy number aberrations and single nucleotide polymorphisms of these tumours were associated with expression in approximately 40 % of genes. The analysis of paired DNA–RNA profiles revealed novel breast cancer subgroups each with distinct clinical outcomes (Curtis *et al*., 2012). It is thought that these findings will benefit future patients as they may be able to receive a more tailored therapy. Although the incidence of breast cancer cases remain lower in third world countries compared to that of developed countries, it is on the increase and the lower numbers may be because of a lack of data availability and/or under reporting (Fregene and Newman, 2005). In Africa, an estimated 715 000 new cases of cancer and 542 000 cancer deaths occurred in 2008, with breast cancer being the most commonly diagnosed form and

second leading cause of cancer death (Jemal *et al*., 2012). It is estimated that over 50 % of breast cancer deaths occur within women who live in developing countries and that by 2020, 70 % of all new cancer cases, of which a substantial amount will be breast malignancies, will occur in people in developing countries (Fregene and Newman, 2005).

Despite the rates of the incidence of breast cancer in countries such as Uganda and Algeria almost doubling over the last twenty years, women from Southern African countries have the highest incidence of breast cancer of all African women (Jemal *et al*., 2012; Vorobiof *et al*., 2001). Breast cancer is the most common cancer in South African women, with one in twenty nine women at risk of developing the disease in their lifetime (South African National Cancer Registry, 2001). Furthermore, women of African descent have been shown to have a predisposition to triple-negative breast cancer (TNBC), a biologically aggressive form of the disease (Livasy, 2009; Fregene and Newman, 2005). TNBC makes up 10 - 20 % of all breast cancers and generally affects younger women (Morris *et al*., 2007). These tumours which are HER2, ER and PR negative (Dawson *et al*., 2009; Nofech-Mozes *et al*., 2009), are associated with poor clinical outcomes and have been shown to metastasize to distant sites (Dent *et al*., 2009, Heitz *et al*., 2009; Nofech-Mozes *et al*., 2009). Only 30 % of women with TNBC live past 5 years and most die from the disease despite adjuvant chemotherapy treatment (Dent *et al*., 2007).

As the understanding behind the molecular mechanisms of breast cancer has increased, so has the identification of novel targets and the development of targeted therapies (Di Cosimo, and Baselga, 2008). An early example of the use of targeted therapy would be the utilisation of the anti-HER2 monoclonal antibody trastuzumab (Herceptin^{TM}) in treating patients with HER2 positive breast cancer (Vogel *et al.*, 2002; Slamon *et al*., 2001). To date, a significant improvement in the overall prognosis in patients with this breast cancer subtype has been documented (Di Cosimo and Baselga, 2008). The success of trastuzumab cannot be disputed; however, as with several other targeted therapies, treatment is confined to one breast cancer subtype. The development of agents that target multiple signal transduction pathways identified as important in the progression of breast cancer would be advantageous, as they could be used across subtypes. An example such a target is the family of proteins known as molecular chaperones (Mosser and Morimoto, 2004).

1.2. Molecular chaperones: novel targets for anti-cancer therapies

The term molecular chaperone was first used by Laskey *et al*. (1978) to describe the ability of the protein nucleoplasmin to prevent histone aggregation and allow their assembly into nucleosomes. The term was refined by Ellis *et al*. (1987) to define a ubiquitous class of highly conserved proteins that assist in the assembly and disassembly of incorrectly folded proteins without becoming a permanent part of that protein complex. Chaperones play important roles in maintaining the equilibrium between the synthesis and degradation of many proteins (Söti *et al*., 2005; Young *et al*., 2001). Different chaperones function to prevent protein aggregation and misfolding and thus maintain the normal protein folding environment within cells (Donmez *et al*., 2012; Yamagishi *et al.*, 2012; Jana *et al.*, 2009; McLean *et al*., 2002; Young *et al*., 2001). These chaperone proteins play significant roles in the stress-response (Young *et al*.,2001) and regulate proteins involved in several important functions which include, but are not limited to, cell proliferation and apoptosis (Luo *et al*., 2006; Mosser and Morimoto, 2004).

Molecular chaperones have emerged as a promising new target for novel anti-cancer therapies as they have been found to be involved in several key processes associated with carcinogenesis. Long-term exposure to various stressors in cells such as stress hormones, hypoxia, heat, acidosis and heavy metals lead to the aggregation and/or misfolding of proteins, which has a deleterious effect on cell function (Yang *et al*., 2013; Garcia *et al*., 2012; Flint *et al*., 2012; Wu *et al*., 2012). The upregulation of molecular chaperones within stressed cells increases their survival in tissues when exposed to such stressors (Csermely *et al*., 1998). Several chaperones known as heat shock proteins (Hsps) accumulate within the cells under such conditions (Ritossa *et al*., 1962). The Hsp response was first discovered in 1962 by Ritossa and colleagues, when it was observed that a brief increase in temperature resulted in the expression of a small group of *Drosophila melanogaster* genes that were later shown to encode Hsps.

1.3. Heat shock proteins

Hsps are a family of highly-conserved proteins within both prokaryotic and eukaryotic cells (Kregel, 2002). A large number of Hsps with varying molecular masses from 1- 100 kDa have been identified and categorised based on their size and function, including Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsps (15–30 kDa) (Powers and Workman, 2007; Young *et al*, 2004). The larger Hsps are adenosine triphosphate (ATP)-dependent, while the smaller ones act independently of ATP (Kregel,

2002). These proteins are localised within numerous subcellular locations, including the cytosol, mitochondria, endoplasmic reticulum (EnR) and nucleus of cells (Kregel, 2002). The increase in Hsp expression within stressed cells has been identified in different solid tumours and may reflect the capability of cancerous cells to sustain homeostasis under the conditions of the tumour microenvironment (Bagatell and Whitesell, 2004) as well as to increase the chances of survival of cells exposed to stressors (Kregel, 2002). The induction of the expression of genes encoding Hsps in response to various stresses is mediated by a family of transcription heat shock factors (HSFs). In humans six types of HSFs have been identified namely HSF1, HSF2, HSF4, HSF5, HSFX and HSFY, with the best described being HSF1 (Xu *et al.*, 2012). HSF3 has been described for mice but not humans (Fujimoto *et al.*, 2010). The activity of HSF1 is regulated by heat shock protein 90 (Hsp90), with which it forms a complex in the cytoplasm (Zou *et al*., 1998). Upon exposure to elevated temperatures, stress, or in the presence of certain Hsp90 inhibitors, HSF1 dissociates from Hsp90 and is rapidly activated from a monomeric to a trimeric form, which constitutively localises to the nucleus (Shamovsky *et al*., 2008, Zou *et al*., 1998) and HSF1 accumulates in stress granules (Holmberg *et al*., 2000; Jolly *et al*., 1999; Cotto *et al*., 1997; Jolly *et al*., 1997). HSF1 has subsequently been shown to dissociate from these granules and re-localise in a diffuse pattern within the cells during recovery from stress (Jolly *et al*., 1999). In addition to regulating Hsp expression, HSF1 also modulates the expression of numerous other genes essential for cell survival under stress conditions (Guertin and Lis, 2010; Page *et al*., 2006), and as such, influences many cellular processes including cell development and differentiation (Abane and Mezger, 2010; Fujimoto *et al*., 2008; Trinklein *et al*., 2004a; Trinklein *et al*., 2004b), protein translation, ribosome biogenesis, cell cycle control and glucose metabolism (Dai *et al*., 2007; Page *et al*., 2006). It is known that despite being exposed to the stressors of the tumour microenvironment, the survival of cancer cells is partially made possible by HSF1 (Dai *et al*., 2007). HSF1 expression has been shown to be increased in certain cancer cells, lines and tumour tissues including breast cancer (Khaleque *et al*., 2008; Khaleque *et al*., 2005 and Hoang *et al*., 2000). Breast cancer cells also show an increased incidence of HSF1 localised to the nuclei of cells (Santagata *et al*., 2011). This increase in nuclear HSF1 was demonstrated to be associated with poor outcomes in breast cancer and as such HSF1 may be a potential therapeutic target and prognostic marker (Santagata *et al*., 2011).

1.4. Heat shock protein 90 kDa (Hsp90) family of proteins

Hsp90 is a ubiquitous, highly abundant ATP-binding molecular chaperone which accounts for 1 - 2% of the total protein content within an unstressed cell (Welch and Feramisco, 1984). Two distinct

genes encode the cytosolic isoforms of this highly conserved protein, namely Hsp90α (84 kDa) and Hsp90β (83 kDa) (Krone and Sass, 1994; Moore *et al*., 1989). These two proteins are highly conserved and closely related (Figure 1.1). Hsp90β is constitutively expressed (Meng *et al*., 1993) whereas Hsp90α is inducible in response to cellular stress (Hickey *et al*., 1989). Owing to the difference in their amino acid sequences, these two isoforms may vary in their chaperone activity (Pepin, 2001) and may exhibit differential binding to client proteins (Millson *et al*., 2007).

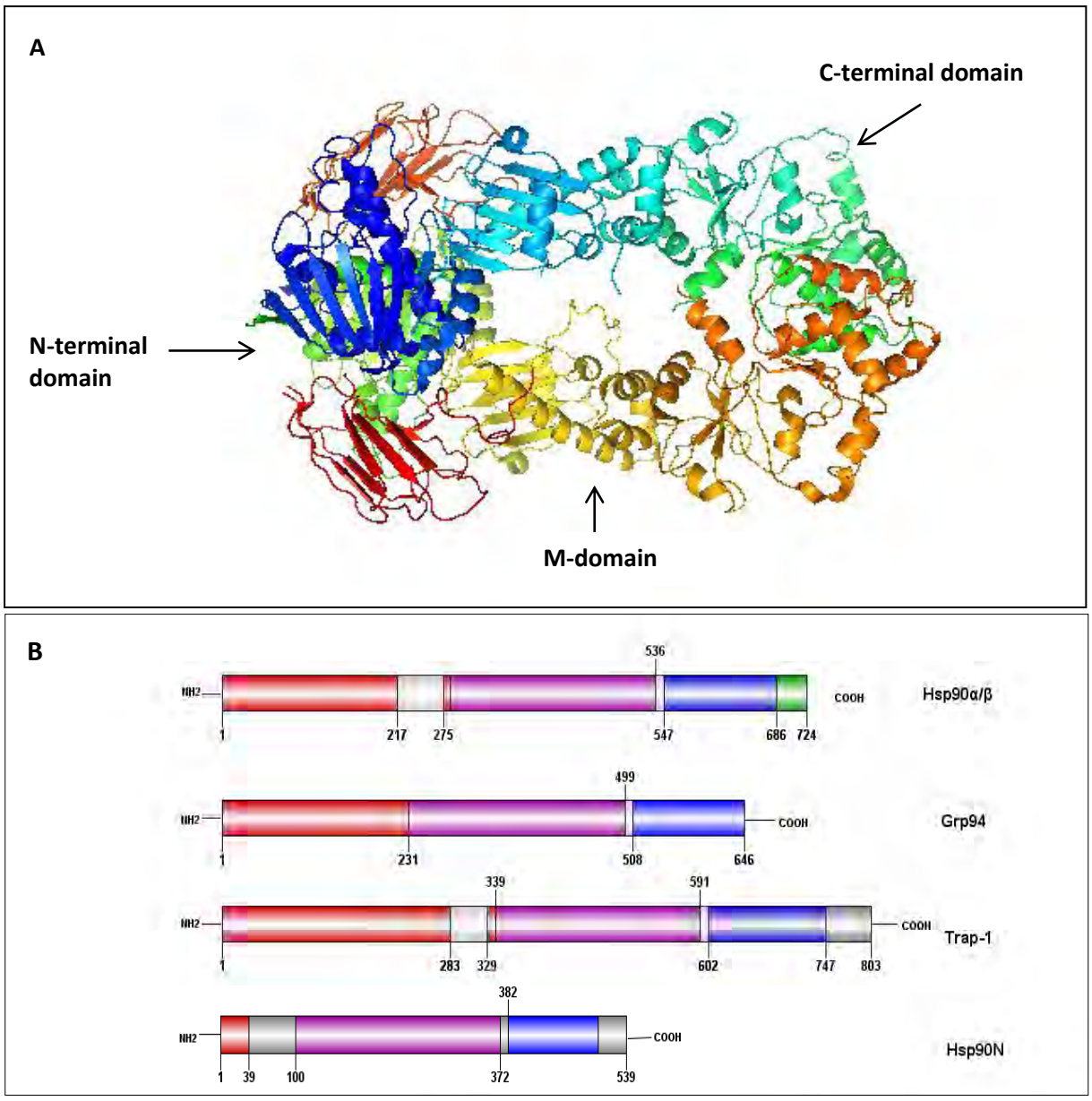


Figure1.1: Overview of Hsp90α/β homodimer domains and structure. (A) Structure was obtained from the protein data bank (PDB) (Berman *et al*., 2003) (2CG9) with Sba1 removed and visualised with PyMol (version 1.3)[1]. The 25 kDa amino (N)-domain is linked to the 35 kDa middle (M)-domain through a charged linker region joined to a 12 kDa carboxyl (C)-terminal domain (Pearl and Prodromou, 2006). N-, M- and C-terminal domains are indicated with arrows. (B) Schematic diagram of the domain structure of the human isoforms of Hsp90 (adapted from Buchner, 1999). N-terminal adenosine triphosphatase (ATPase) domains are shown in red, middle domains in purple and C-terminal domains are shown in blue, methionine-glutamic acid-glutamic acid-valine-aspartic acid (MEEVD) motif is shown in green. Hsp90 domains were drawn using DOG 2.0: Illustrator of Protein Domain Structures[2].

The Hsp90 family also consists of glucose-regulated protein 94 (Grp94) in the EnR of eukaryotes, which is required for the maturation and secretion of insulin-like growth factors (Wanderling *et al*., 2007) and tumour necrosis factor receptor-associated protein 1 (TRAP-1) in the mitochondrial matrix

[1] http://www.pymol.org/
[2] http://dog.biocuckoo.org/down.php

which protects cells against oxidative stress (Hua *et al*., 2007; Felts *et al*., 2000). Both Grp94 and TRAP-1 have adenosine triphosphatase (ATPase) activity but no known co-chaperones have been identified for either protein (Leskovar *et al.,* 2008; Dollins *et al*., 2007; Frey *et al*., 2007). A membrane associated variant of Hsp90, Hsp90N, discovered in 1998, shares high sequence homology with the α and β isoforms but the 25 kDa ATP binding N-terminus of this protein is replaced with a unique sequence of 30 amino acids (Zurawska *et al*., 2008; Grammatikakis *et al*., 2002; Schweinfest *et al*., 1998). It remains to be seen whether Hsp90N is a true Hsp90 isoform or if it is an artefact of cDNA synthesis or a chromosomal rearrangement that occurred within the human T-lymphoblastoid cell line in which it was discovered (Zurawska *et al*., 2008). Furthermore, the existence of an Hsp90N gene in the human genome is yet to be shown and no Hsp90N homologues in genomes of other eukaryotic organisms have been identified (Zurawska *et al*., 2008). Members of the Hsp90 family identified outside of the *Homo sapiens (H.sapiens)* species include murine Hsp84 and Hsp86 (corresponding to Hsp90α and Hsp90β in *H.sapiens* respectively), Hsp83 in *Drosophila,* Hsp82 in *Saccharomyces cerevisiae (S.cerevisiae)* and high temperature protein G (HtpG) in the bacterial cytosol.

Hsp90 primarily functions to assist in the conformational regulation of client proteins, many of which are involved in signal transduction (Zhao, *et al.,* 2005; Pratt and Toft 1997). The list of Hsp90 clients continues to grow with the BioGrid[3] database currently reporting that 1382 and 278 proteins interact physically or genetically with *H.sapiens* Hsp90α and *S.cerevisiae* Hsp82 respectively. It has been established in *S.cerevisiae* strains expressing *H.sapiens* Hsp90, that certain Hsp90 client proteins, namely HSF and v-Src tyrosine kinase, are activated more efficiently by Hsp90α than Hsp90β (Millson *et al*., 2007). Similarly, the effects of certain inhibitors such as PU-H71 (Chan *et al*., 2008) and radicicol (RAD) (Millson *et al*., 2007) are dependent on the Hsp90 isoform which they are acting upon.

1.4.1. *Structure and Characterisation of Hsp90*

The structure of Hsp90 (Prodromou *et al*., 1997) is homologous to other ATPases in the GHKL (gyrase, histidine kinase, MutL) superfamily (Bilwes *et al*., 2001; Ban *et al*., 1999; Wigley *et al*., 1991). This protein exists as a homodimer (Iannotti *et al*., 1988; Koyasu *et al*., 1986; Ullrich *et al*., 1986) with two or three phosphate molecules bound per monomer (Iannotti *et al.*, 1988) (Figure 1.1). The formation of Hsp90α-Hsp90β heterodimers has been documented (Minami *et al*., 1991) and at

[3] http://www.thebiogrid.org

higher temperatures (Nemoto and Sato, 1998; Lanks, 1989), or in the presence of divalent cations (Jakob *et al.*, 1995) Hsp90 oligomers can occur. Hsp90 functions as part of a multi-chaperone complex in the cytosol in association with chaperones such as Hsp70 and co-chaperones including Hsp40, Hsp70/Hsp90-organizing protein *(*Hop), cell cycle division 37 homologue (Cdc37) and p23 (Pratt and Toft, 2003; Smith, 1993).

1.4.1.1. The N-terminal domain

The N-terminal domain of Hsp90 consists of an anti-parallel, eight-strand β sheet with nine helix bundles that fold to form an α and β sandwich motif (Stebbins *et al.*, 1997). A 15 Å-deep opening exists within the helical side of the sandwich, which forms an ATP/adenosine diphosphate (ADP) binding site (Grenert *et al.*, 1997; Prodromou *et al.*, 1997). This site is unique as it contains a Bergerat fold (Bergerat *et al*., 1997) that is characteristic of the GHKL class of ATPases (Dutta and Inouye, 2000). The exchange of nucleotides and ATP hydrolysis at the N-terminal ends (Obermann *et al*., 1998; Prodromou *et al.*, 1997) is required for Hsp90 function and Hsp90-mediated protein folding (Panaretou *et al.*, 1998). Hsp90 has low basal ATPase activity with the maximum number of enzymatic reactions catalysed per second of Hsp90β being 1.7×10^{-3} s^{-1} (McLaughlin *et al*., 2002). Therefore, when acting alone Hsp90 has very little ATPase activity (Obermann *et al*., 1998). The Hsp90 inhibitors geldanamycin (GA) and RAD are known to interact with Hsp90 through this N-terminal site (Roe *et al*., 1999; Grenert *et al.*, 1997). The N-terminal and M-domains of Hsp90 are linked through a flexible, charged linker in eukaryotic cells which is missing in bacterial systems (Hainzl *et al*., 2009).

1.4.1.2. Charged Linker Region

In eukaryotes the N- and M- domains of Hsp90 are separated by a region known as the charged linker which is an unstructured, charged region of amino acids (Pearl and Prodromou, 2006). The same region that separates the N- and M-domains in bacterial HtpG is much shorter (Shiau *et al*., 2006), while that of *S.cerevisiae* Hsp82 was thought to be dispensable as a minimal length has been shown to be required for chaperone activity and as the sequence of this region did not appear important (Hainzl *et al*., 2009). Recently it was found, that beyond a necessary minimum amino acid length, the length of the charged linker did not significantly influence chaperone function for mammalian Hsp90 (Tsutsumi *et al*., 2012). Furthermore it was demonstrated that in addition to providing the flexibility necessary for domain rearrangement in the ATP cycle, this region in both mammalian Hsp90 and *S.cerevisiae* Hsp82 was required for binding co-chaperones, such as the

activator of Hsp90 ATPase 1 (Aha1), and plays a role in modulating Hsp90 chaperone activity in a co-chaperone, client protein and possibly environment-specific form (Tsutsumi *et al*., 2012).

1.4.1.3. The M-domain

The M-domain comprises two α-β-α motifs joined together by several α-helices (Meyer *et al*., 2003). The major role of this domain is to differentiate between assortments of client proteins and to adjust for proper substrate activation (Hawle *et al*., 2006). Results obtained from mutagenesis experiments indicate that the M-domain has an important function in the binding of client proteins to Hsp90 (Meyer *et al*., 2003) and may be involved in the binding of substrates (Park *et al*., 2011; Street *et al*., 2011; Vaughan *et al*., 2006; Meyer *et al*., 2003). This domain is also thought to have a role in modifying ATP hydrolysis by interacting with the γ-phosphate of ATP molecules bound to the N-terminal ATP binding site of Hsp90 (Meyer *et al*., 2003; Söti *et al*., 2003). Furthermore, the M-domain interacts with the co-chaperone Aha1, enhancing the association between itself and the N-terminal domain of Hsp90 and resulting in an increase in the rate of ATP hydrolysis (Söti *et al*., 2003). The presence of a substrate binding region within the M-domain on the interior cleft of the Hsp90 homodimer has recently been shown (Street *et al*., 2012) and it has been suggested that a secondary cluster of substrate contacts drives an N-terminal domain orientation change on the opposite Hsp90 monomer (Street *et al.,* 2012).

1.4.1.4. The C-terminal domain

The C-terminal domain of Hsp90 is the dimerisation region of the protein (Harris *et al*., 2004; Yamada *et al*., 2003; Minami *et al*., 1994). In eukaryotes this domain contains the conserved pentapeptide MEEVD that is involved in binding to the 34 amino acid tetratricopeptide repeat (TPR) domains of co-chaperones, such as Hop and C-terminus of the Hsp70 interacting protein (CHIP) (Pearl *et al*., 2008; Zhang *et al*., 2005; Odunuga *et al*., 2003; Scheufler *et al*., 2000). The determined crystal structure of the C-terminal dimerization domain of Escherichia coli (*E.coli)* HtpG has shown that a potential client binding site exists between residues 548–557 (Harris *et al*., 2004). Mutagenesis of the same residues was shown to eliminate binding of the glucocorticoid receptor (GR) to Hsp90 (Fang *et al*., 2006). Despite these findings it is still largely unknown whether the C-terminal domain is involved in interacting with other Hsp90 client proteins.

The Hsp90 C-terminal domain has been proposed to contain a second possible nucleotide binding site, although this putative site is less well characterised than its N-terminal counterpart (Garnier *et al*., 2002; Söti *et al*., 2002, Marcu *et al*., 2000a; Marcu *et al*., 2000b). Unlike the N-terminal ATP binding site, a crystal structure for the C-terminal region of Hsp90 bound to any inhibitors is yet to be published. The evidence of a second ATP binding site within the C-terminus of Hsp90 has been demonstrated through fluorescence microscopy, isothermal titration calorimetry (ITC) and differential calorimetry experiments (Garnier *et al*., 2002) however the presence of this C-terminal ATP binding site was not distinctly shown in the elucidated full-length structures of *E. coli* HtpG (Shiau *et al*., 2006), *S.cerevisiae* Hsp82 (Ali *et al*., 2006) and canine Grp94 (Dollins *et al*., 2007). Novobiocin (NOV) was the first C-terminal inhibitor (Marcu *et al*., 2000a) and remains the most common Hsp90 inhibitor used to study the C-terminal domain. The NOV binding site within the C-terminal region of Hsp90 has been shown to be close to the dimerization domain at amino acids 538–728 (Marcu *et al*., 2000a). Furthermore, NOV was shown to efficiently compete with ATP for binding and to disrupt co-chaperone binding (Marcu *et al*., 2000a). It was thought that the ATP binding site of NOV only became available once the N-terminal site is occupied (Söti *et al*., 2003) and that this may be because of cooperativity between the N- and C-terminal domains of Hsp90 (Ratzke *et al*., 2010). It has also been shown that in addition to adenine nucleotides, that this site was able to bind other purines and pyrimidines (Söti *et al*., 2003). Furthermore, as well as disrupting the ability of Hsp90 to undergo N-terminal dimerization, the binding of a small molecule to this C-terminal site may also prevent the dissociation of the C-terminal dimerization domains (Ratzke *et al*., 2010). The C-terminal binding site bound to NOV has been modelled and the proposed model indicates that NOV predominantly interacts with one monomer of the Hsp90 homodimer (Matts *et al.*, 2011). The data presented by Matts *et al*. (2011) and Ratzke et al. (2010) suggests that the binding of a small molecule to the Hsp90 C-terminus occurs while this domain is open, preventing dimerization and continuation through the catalytic cycle.

1.4.2. *Hsp90 Multi-chaperone Cycle*

The Hsp90 homodimer can exist in two conformations; either an open or a closed conformation. The interaction of Hsp90 and its client proteins has been well studied with steroid hormone receptors (Pratt and Toft, 1997). The open, V-shaped conformation exists with the two N-termini of the homodimer physically apart (Bron *et al.*, 2008; Shiau *et al*., 2006) (Figure 1.2). Binding of ATP results in a rotation of approximately 120° of the lid of the pocket thus bringing the N-termini close to each other, with further conformational changes resulting in the creation of a twisted Hsp90 homodimer (Ali *et al*., 2006; Meyer *et al*., 2003). In this closed state, the Hsp90 client proteins are held inside

(Terasawa *et al*., 2005) and the M-domain interacts with the closed N-terminal lid allowing the active site to interact with the ϒ phosphate of the bound ATP, resulting in the hydrolysis of the β-ϒ phosphodiester bond (Ali *et al.*, 2006). ATP hydrolysis causes the dissociation of the homodimer and the return of Hsp90 to the open conformation with the N-termini apart and open lid segment (Hahn*,* 2005).

In eukaryotes, the regulation of Hsp90 function is dependent on a dynamic association between Hsp90 and more than twenty co-chaperones and chaperones such as Hsp70 and Hsp40 (Pearl and Prodromou, 2006). For example, Aha1, Cdc37, p23 modulate the rate of Hsp90 hydrolysis while, the conformational flexibility of Hsp90 is regulated by p23, Sgt1, and the binding of specific substrates to Hsp90 regulated through Hop, Cdc37, Sgt1 (Forafonov *et al*., 2008; Zhang *et al*., 2008a; McLaughlin *et al*., 2006; Roe *et al*., 2004; Meyer *et al*., 2004; Panaretou *et al*., 2002). A partial list of these co-chaperones and Hsp90-assocated chaperones is given in Table 1.2.

The Hsp90 chaperone cycle of most client proteins has been known to occur in a similar manner to that proposed for the PR (Smith, 1993). Figure 1.2 depicts a simplified, schematic representation of the Hsp90 chaperone cycle, detailing the minimal systems required. Following this model, a newly synthesised or misfolded client protein interacts with Hsp70, Hsp40 and the adaptor Hsp70-interacting protein (Hip) to form an early complex. An intermediate complex has been suggested in which Hop interacts with Hsp90 stabilising a client loading conformation that favours an interaction with Hsp70 (Southworth and Agard, 2011), thus delivering the early complex to Hsp90. This has been confirmed by findings that Hsp90-Hop complexes promote the binding of Hsp70 resulting in complexes with 2:1:1 ratios of Hsp90, Hop and Hsp70, respectively (Ebong *et al*., 2011). It has also been proposed that Hsp70 binds to the TPR1 domain of Hop through its C-terminal EEVD motif and also makes additional contacts with N-terminal and M-domains of Hsp90, in order to deliver the client protein to the Hop stabilised V-shaped inter-dimer cleft of Hsp90 (Southworth and Agard, 2011). This asymmetric interaction with Hsp90 has also been described for the co-chaperones Cdc37 (Vaughan *et al*., 2006), Aha1 (Retzlaff *et al*., 2010) and PPIase (Li *et al*., 2011). Hsp90 is then stabilised as Hop dissociates from the complex and is replaced with co-chaperone p23 (Johnson and Toft, 1995). The mature complex is eventually formed upon the binding of cyclophilin 40 to Hsp90 (Ratajczak *et al*., 1996). Finally, the co-chaperone Aha1 binds to the M-domain of Hsp90, resulting in conformational changes in the complex, which stimulates the Hsp90 ATPase activity (Meyer *et al*., 2004). Upon ATP hydrolysis, the correctly folded client protein is released from Hsp90 (Terasawa *et*

al., 2005). The multi-chaperone complex dissociates and the client protein can enter the cycle again by interacting with Hsp40 and Hsp70.

It has been suggested that in isolation, Hsp90 does not follow a sequential order of states but that the conformational transitions and nucleotide binding may be random as they are driven by thermal variation (Ratzke *et al*., 2010). It has been shown that nucleotides can bind to the N-termini in both the open and closed state without the requirement of the protein to be in a particular conformation (Ratzke *et al*., 2010).

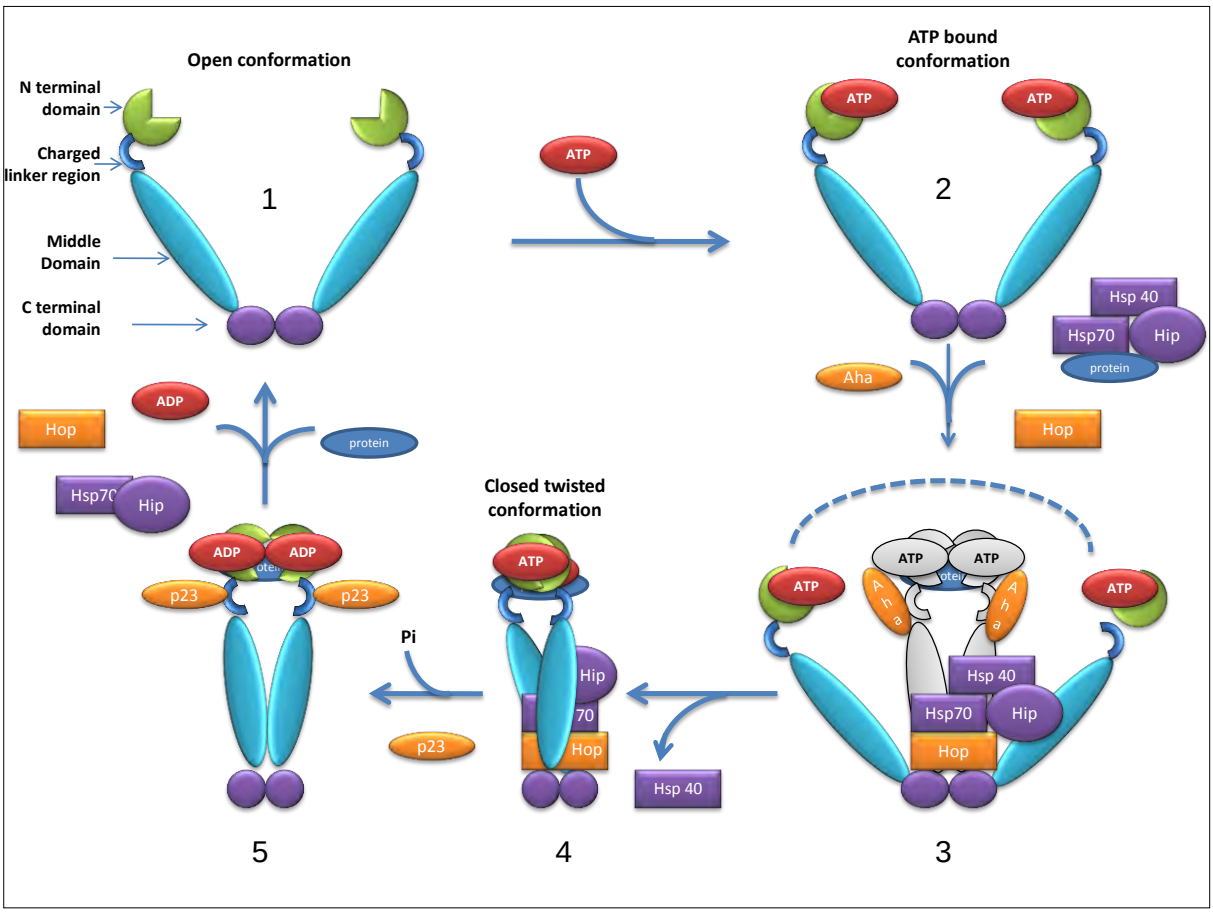


Figure 1.2: Schematic representation of the ATP dependent Hsp90 chaperone cycle (adapted from Trepel *et al*.**, 2010).** (1) Hsp90 consists of an N-terminal domain, charged linker region, M and C-terminal domain and exists in an open conformation with the two N-termini apart (2). The client protein associates with Hsp70, Hsp40 and Hip (Hsp70-interacting protein), binds to Hsp90 via the adaptor protein Hop and displaces Hsp40 to form an intermediate complex (3). ATP binding to Hsp90 brings the N-termini close to each other, resulting in the formation of a compacted, twisted Hsp90 homodimer (4). Hop is replaced by immunophilins (IPs) and p23 (5) and, upon ATP hydrolysis, the correctly folded client protein is released from Hsp90.

1.4.3. *Regulation of Hsp90 activity by Hsp90 partner proteins*

Hsp90 co-chaperones participate in modulating Hsp90-chaperone activity and may or may not exhibit chaperone activity independent of Hsp90 (Caplan *et al*., 2003). Several Hsp90 co-chaperones were initially isolated and thus identified from mammalian or yeast cell extracts through the co-purification with Hsp90 or through physical or genetic interactions with Hsp90 in *S. cerevisiae* (Zuehlke and Johnson, 2010). Hsp90 co-chaperones can generally be divided into two groups; TPR containing and non-TPR containing proteins. Several chaperones and Hsp90 co-chaperones are shown in Table 1 with Hsp70, p23/Sba1, Aha1 and Hop being discussed further.

1.4.3.1 Hsp70

The human Hsp70 family consists of at least 13 isoforms that vary from 66 kDa to 78 kDa in size and include the constitutively expressed heat shock cognate 70 (Hsc70), EnR-localised binding immunoglobulin protein, mitochondrial mtHsp70 and stress-inducible Hsp70 (Hsp72) (Brocchieri *et al.*, 2008). These proteins facilitate the folding of a wide spectrum of proteins in an ATP-dependent manner (Szabo *et al.*, 1994) and are controlled by nucleotide exchange factors (Briknarová *et al.*, 2001; Sondermann *et al*., 2001; Harrison *et al*., 1997) and Hsp40s (Lu and Cyr, 1998). Each Hsp70 protein is made up of three domains; an N-terminal 44 kDa ATPase (Flaherty *et al*., 1990), 18 kDa peptide binding (Zhu *et al*., 1996; Wang *et al.,* 1993) and lastly 10 kDa C-terminal variable region, that constitutes part of the substrate-binding domain and facilitates interactions with other chaperones such as Hsp90, Hsp40 and CHIP (Li *et al*., 2006; Brinker *et al*., 2002; Demand *et al*., 1998; Freeman *et al*., 1995). Hsp70 functions in various processes, including the folding and refolding of both newly synthesised and aggregated proteins respectively, membrane translocation and maintenance of secretory proteins, and maintenance of secretory and regulatory proteins respectively (Young *et al*., 2003; Hartl and Hayer- Hartl, 2002; Ryan and Pfanner, 2002). Hsp70 functions in the early stages of protein folding by stabilising nascent polypeptide chains until sufficient protein has been synthesised to allow correct protein conformation to be achieved before delivering some proteins to Hsp90 (Calderwood *et al*., 2010). Hsp70 is overexpressed in many different cancer cells (Cornford *et al*., 2000; Kaur and Ralhan, 1995; Ciocca *et al*., 1993), can be induced via activation of heregulin β1 in breast cancer cells (Khaleque *et al*., 2005) and inhibits programmed cell death and senescence when expressed at high levels (Garrido *et al*., 2006; Gabai *et al*., 1998). Apart from being vital for cancer cell survival and growth, Hsp70 overexpression is associated with poor clinical outcomes (Cornford *et al*., 2000; Kaur *et al*., 1995; Ciocca *et al*., 1993). Furthermore, Hsp70 expression has been reported to be induced following treatment with the

Hsp90 inhibitor 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) for several cancers (McCollum *et al*., 2006; Clarke *et al*., 2000).

Table 1.1: **Partial list of Hsp90 co-chaperones and co-activators** (adapted from Zuehlke and Johnson, 2010; Buchner, 1999).

Chaperone/Co-chaperone	Hsp90 binding site	Defining characteristics	References
Aha1	M-domain	Activator of Hsp90 ATPase activity	Meyer *et al*., 2004
CHIP	None	TPR domain, ubiquitin ligase, binds Hsp70	Connell *et al*., 2001
Cyclophilin 40	C-terminus	Chaperone TPR domain, peptidyl-prolyl isomerase	Mayr *et al*., 2000
FK506 binding protein 51, FK506 binding protein 52	C-terminus	Chaperone, TPR domain, Prolylisomerase,	Cox *et al*., 2007
Hip	-	TPR domain, Hsp70 cofactor	Höhfeld *et al*., 1995
Hop	C-terminus	TPR domains, binds Hsp70 and Hsp90	Prodromou *et al*., 1999; Chen and Smith, 1998
Hsp70	-	Chaperone, delivers client protein	Bukau et *al*., 2006 Tavaria *et al*., 1996
p23	N-terminus	Chaperone, stabilises closed conformation of Hsp90	Ali *et al*., 2006; Johnson *et al*., 1995
Cdc37	N-terminus	Kinase-specific cofactor, binds kinase clients	Caplan *et al*., 2007
Protein phosphatase 5	C-terminus	TPR domain protein-serine phosphatase,	Hinds and Sanchez, 2007
Suppressor of second gap phase (G2) allele of skp1	N-terminus	TPR domain and domain homologous to p23	Martins *et al*., 2009

1.4.3.2 Non-TPR containing Co-chaperones

1.4.3.2.1 Aha1

Aha1 binds to Hsp90 directly (Retzlaff *et al*. 2010) through its N-terminal domain to the M-domain of Hsp90 (Lotz, 2003) and activates Hsp90 ATPase activity by modulating the catalytic loop of the M-domain of Hsp90 (Meyer *et al*., 2004; Lotz *et al*. 2003; Panaretou *et al*., 2002). Aha1 is the most

powerful known activator of Hsp90 ATPase activity (Meyer *et al*., 2004; Panaretou, *et al*., 2002). Aha1 has also been found to bind to the C-terminal domain of Hsp90 and it is thought that it may modulate ATPase activity through this C-terminal interaction; although this remains to be demonstrated experimentally (Retzlaff *et al*., 2010).

1.4.3.2.2 p23/Sba1

Mammalian p23 and its *S. cerevisiae* homologue Sba1 act at a later stage in the Hsp90 ATPase cycle and reduce the rate of ATPase activity (McLaughlin *et al*., 2006; Richter *el al*., 2004; McLaughlin *et al*., 2002; Panaretou *et al.*, 2002). Although Sba1 and p23 possess the ability to bind to Hsp90 in a nucleotide-free state, they are known to bind to the nucleotide bound form with a higher affinity (McLaughlin *et al*., 2006; Siligardi *et al*., 2004). Two molecules of p23/Sba1 bind to the N-terminal domains of Hsp90 in the dimerised state (Ali *et al*., 2006) and thus trap Hsp90 in the ATP hydrolysis state (McLaughlin *et al*., 2006; Ritcher 2004). Furthermore p23 and Sba1 also bind to the M-domain of a monomer (Martinez-Yamout *et al*., 2006), promoting and stabilising the closed conformation of Hsp90 (Richter *et al*., 2004; Siligardi *et al*., 2004). The promotion of the diminished state of Hsp90 by p23 and inhibition of the ATPase activity results in the stabilisation of client proteins complexes to Hsp90 as demonstrated for the PR and GR (Kosano *et al.*, 1998; Dittmar and Pratt 1997).

1.4.3.3 TPR Containing Co-chaperones

TPR domains are degenerate sequences of 34 amino acids on proteins (Hirano *et al.*, 1990) of which there are several hundred in mammalian genomes (Caplan *et al.,* 2003). As TPR motifs are involved in a variety of interactions unrelated to chaperones, not all will interact with Hsp90 or Hsp70 (Blatch and Lässle, 1999).

1.4.3.3.1 Hop

Hop is a highly conserved, 60 kDa eukaryotic protein that exhibits no independent chaperone activity (Flom *et al*., 2007; Odunuga *et al*., 2004). There are discrepancies in the literature regarding the oligomeric state of this co-chaperone with some studies indicating that it exists as monomeric protein (Li *et al,* 2011; Yi *et al*, 2010; Young *et al.*, 1998), while others state that is a dimeric protein (Onuoha *et al*., 2008; Flom *et al*., 2007; Prodromou *et al*., 1999) and one study provides evidence for the existence of both monomeric and dimeric forms (van der Spuy *et al*., 2001). The dimeric form of Hop has been shown to have butterfly-like quaternary structure (Onuoha *et al*., 2008). Despite the

inconsistency in the reports of its oligomeric state, Hop has been shown to be composed of three TPR domains namely TPR1, TPR2A and TPR2B and two smaller aspartic acid proline (DP) domains composed of highly homologous α-helical folds (DP1 and DP2) (Schmid *et al*., 2012), with the structure arranged as TPR1-DP1-TPR2A-TPR2B-DP2 (Nelson *et al*., 2003; Prapapanich *et al*., 1998). The TPR2A domain of Hop has recently been shown to be the high affinity Hsp90 binding site while the TPR1 and TPR2B bind to Hsp70 with moderate affinity (Lee *et al*., 2012a; Schmid *et al*., 2012). Hop is thus able to interact with both Hsp90 and Hsp70 through the C-terminal MEEVD (Scheufler *et al*., 2000) and GPTIEEVD motifs, respectively (Odunuga *et al.*, 2003). This interaction results in the stabilisation of the intermediate Hsp70–Hsp90–client protein complex and allows Hop to transfer client proteins from Hsp70 to Hsp90 during the chaperone cycle (Wegele *et al*., 2006). X-ray crystallography recently revealed that the two Hop TPR2A and TPR2B domains are associated though a linker that positions their peptide-binding sites in opposite directions, and allows the simultaneous binding of TPR2A to the Hsp90 C-terminal domain and of TPR2B to Hsp70 (Schmid *et al*., 2012). Furthermore, it has been shown that TPR2A and TPR2B also interact with the Hsp90 M-domain (Schmid *et al*., 2012). The function of the DP domains was previously unknown but have recently been shown to have roles in client activation as the TPR1–DP1 module is thought to act as an Hsp70–client delivery system for the TPR2A–TPR2B–DP2 segment, which is essential for client activation *in vivo* (Schimd *et al*., 2012).

Hop also functions to inhibit the ATPase activity of Hsp90 and stabilises Hsp90 in its open conformation, thus preventing nucleotide-induced closure (Li *et al*., 2011, Onuoha *et al*., 2008). Furthermore it has been shown that one Hop per Hsp90 homodimer is sufficient to inhibit the ATPase activity of Hsp90 (Li *et al*., 2011) and more recently, that the main component of Hop required for Hsp90 inhibition is the TPR2A–TPR2B segment (Lee *et al*., 2012a; Schmid *et al*., 2012).

1.4.4 Regulation of Hsp90 by post translational modification

Hsp90 is regulated by various post-translational modifications, including phosphorylation, S-nitrosylation, acetylation, oxidation, ubiquitination and methylation, which affect co-chaperone association and/or ATP binding (Li *et al*., 2012).

Hsp90 is a phosphoprotein containing several serine, threonine, and tyrosine phosphorylation sites (Mollapour *et al*., 2011a; Mollapour *et al*., 2011b; Mollapour *et al*., 2010; Old *et al*., 2009; Garnier *et al*., 2001; Mimnaugh *et al*., 1995; Miyata and Yahar, 1992; Rose *et al*., 1987). Phosphorylation affects Hsp90 chaperone activity in different ways (Mollapour and Neckers, 2012) with some studies

showing an increase in chaperone activity (Duval *et al*., 2007), whilst others show an inhibition of Hsp90 activity (Wandinger *et al*., 2006).

S-nitrosylation is a reversible post-translational modification of Hsp90 through nitric oxide in which a nitrogen monoxide group covalently binds to a thiol side chain of a cysteine residue (Mollapour and Neckers, 2012). It has been reported that the S-nitrosylation of cysteine 597 of Hsp90 results in the inhibition of chaperone and ATPase activity (Retzlaff *et al*., 2009; Martínez-Ruiz *et al*., 2005).

Acetylation involves the addition of acetyl groups, usually to the lysine residues of proteins (Mollapour and Neckers, 2012) with lysine 294 in the M-domain being recognised as an important acetylation site of Hsp90 (Kovacs *et al*., 2005). Hsp90 acetylation was first shown in response to treatment with the histone deacetylase inhibitor (HDACi), Romidepsin (Yu *et al*., 2005). Romidepsin increased the steady state acetylation of Hsp90 and destabilised its interaction with several other client proteins such as HER2 and Raf-1 and decreased binding to ATP (Yu *et al*., 2005). Hyper-acetylation has been shown to promote the extracellular localisation of Hsp90α, increasing the association of Hsp90α with matrix metalloproteinase 2 and promoting invasion of breast cancer cells (Yang *et al*., 2008).

Oxidative stress may result in Hsp90 post-translational modification (Mollapour and Neckers, 2012) as it may cause lipid peroxidation which leads to accumulation of thiol-reactive α, β-unsaturated aldehydes, including 4-hydroxy-2-nonenal and 4-oxo-2-nonenal (Carbone *et al*., 2005). 4-hydroxy-2-nonenal targets Cys572 of Hsp90 and inhibits its ability to chaperone clients (Carbone *et al*., 2005). Lysines are also post-translationally modified by ubiquitination (Carbone *et al*., 2005). Treatment with the signal transduction inhibitor Hypericin resulted in increased Hsp90 ubiquitination, inhibition of chaperone activity and dissociation of mutant p53, Cdk4, and Plk from Hsp90 complexes (Blank *et al*., 2003).

The methylation of Hsp90 by lysine methyltransferase Smyd2 in various cell lines has recently been described (Donlin *et al*., 2012). This form of post translational modification of Hsp90 was shown to be necessary for the maintenance and function of skeletal muscle and contributed to the formation of a protein complex containing Smyd2, Hsp90, and titin (Donlin *et al*., 2012).

1.5 Hsp90 client proteins

Hsp90 client proteins are highly diverse and include members of the steroid receptor family and other transcription factors, receptor tyrosine kinases, serine-threonine kinases, cell cycle regulators, telomerase and many other proteins which participate in multiple signal transduction pathways contributing to cancer progression (Zhang and Burrows, 2004; Pratt and Toft, 1997). It is for this reason that Hsp90 has been referred to as the "hub of protein homeostasis" (Taipale *et al*., 2010) and as the "master regulator of master regulators" (Lu *et al*., 2011). The clients of Hsp90 include proteins that contribute to all ten hallmarks of cancer (Hanahan and Weinberg, 2011) (Table 2). Current research is directed at targeting complexes between Hsp90 and client proteins in cancer cells. The ability of small molecules to inhibit Hsp90 function, resulting in the disruption of multiple oncogenic clients simultaneously, has become a key focus in cancer research as it represents a novel target for anti-cancer therapies (Maloney and Workman, 2002).

Table 1.2: Selected examples of Hsp90 client proteins involved in the ten hallmarks of cancer.

Cancer cell hallmarks	**Examples of Hsp90 client proteins**	**References**
Evasion of apoptosis	Insulin-like growth factor receptor, AKT	Nielsen *et al*., 2004; Sebti and Der, 2003
Self-sufficiency in growth signalling,	HER2	Kiguchi *et al.,* 2001
Insensitivity to anti-growth signals	Cyclin-dependent kinase 4	Chin *et al.,* 1998
Tissue evasion and metastasis	Met receptor tyrosine kinase, matrix metalloproteinase 2	Curran and Murray, 1999; Polette and Birembaut, 1998; Schmidt *et al.,* 1997
Limitless replicative potential	Telomerase	Bryan and Cech, 1999; Shay and Bacchetti, 1999
Sustained angiogenesis	Hypoxia-inducible factor 1, AKT	Liao and Johnson, 2007; Phung *et al*., 2006
Deregulating cellular energetics	Hypoxia-inducible factor 1	Kondo *et al.,* 2002
Genome instability and mutation	p53	Lengauer *et al.,* 1998
Avoiding immune destruction	Ephrin type-A receptor 2	Annamalai *et al.,* 2009; Kawabe *et al.*, 2009
Tumour promoting inflammation	Signal transducers and activators of transcription 3 and 5	Yu *et al*., 2009

1.5.1 HER2

HER2, also known as ErbB2 and neu2, is a tyrosine kinase whose stability depends on Hsp90 function (Xu *et al*., 2002). This 185 kDa transmembrane glycoprotein belongs to the ErbB family of epidermal growth factor receptors, which also includes HER1, HER3 and HER4 (Yarden and Sliwkowski, 2001). HER2 is able to form homodimers and heterodimers with the other members of the ErbB family (Coussens *et al.*, 1985). ErbB family receptors are made up of specific structural regions: an extracellular domain containing cysteine rich regions, a membrane-spanning region, an intracellular protein-tyrosine kinase domain and a C-terminal tail (Ullrich *et al*., 1984). ErbB receptors, with the exception of HER2, which is a ligand-less receptor, are activated through interaction with growth factors belonging to the epidermal growth factor/neuroregulin family, resulting in the formation of homo- and heterodimers with different signalling capacities (Yarden and Sliwkowski, 2001). HER mediated signal transduction is required for the development and maintenance of epithelial tissues (Carraway *et al*., 1997) and overexpression of HER2 results in irregular signalling which may result in the formation of aggressive tumour cells (Hung *et al.* 1986). Approximately 25 % of breast cancers are HER2 positive (Slamon *et al.*, 1989; Slamon *et al*., 1987). The overexpression of HER2 has been shown to be associated with poor clinical outcomes and an aggressive clinical phenotype including high-grade tumours (Slamon *et al.*, 1989; Slamon *et al*., 1987) which exhibit increased resistance to chemotherapeutic agents (Yu and Hung, 2000). This resistance is thought to occur as a result of the stimulation of the phosphatidylinositol 3-kinase (PI3K) - serine/threonine kinase AKT pathways (O'Brien *et al*., 2010).

1.5.2 Serine/threonine kinase AKT

Activation of the PI3K/AKT pathway causes the proliferation, growth and migration of cells, as well as angiogenesis, and causes the inactivation of a number of apoptotic pathways (Vivanco and Sawyers, 2004). AKT is the downstream target of PI3K, which is stabilised by Hsp90 and activated through phosphorylation of Thr208 and Ser473 (Alessi *et al*., 1997). Three forms of AKT have been identified, namely AKT1, AKT2 and AKT3 (Masure *et al.,* 1999*;* Nakatani *et al.,* 1999*;* Coffer *et al.,* 1998). AKT is responsible for many proliferative and anti-apoptotic effects induced by the binding of peptide growth factors to its transmembrane receptors (Andjelković *et al*., 1996; Franke *et al*., 1995; Kohn *et al*., 1995). Once activated, AKT translocates from the cytoplasm to the membrane (Santi and Lee, 2010), nuclei (Andjelković *et al*., 1997; Meier *et al*., 1997) and mitochondria (Bijur and Jope, 2003) of cells. AKT activation results in the stimulation of first gap phase (G1) progression, resulting in cell growth (Rossig *et al*., 2001), and is also responsible for desensitizing cells to the apoptotic

stimuli caused by growth factors such as insulin-like growth factor I (Dudek *et al*., 1997). These processes occur as a consequence of the regulation by AKT, of several transcription factors required for the expression of genes involved in apoptosis, through the phosphorylation and inactivation of members of the apoptotic machinery such as Bax (Yamaguchi and Wang, 2001) and Bad (Datta *et al*., 1997). AKT is negatively regulated by the dephosphorylation of Thr308 and Ser473 by protein phosphatase 2 and PH domain leucine rich repeat phosphatase, respectively (Brognard *et al*., 2007; Andjelković *et al*., 1996). Regulation may also occur through the dephosphorylation of PI3K by phosphatase and tensin homologue deleted on chromosome ten (PTEN) (Sun and Steinberg, 2002). AKT was first shown to be deregulated in ovarian cancer two decades ago (Staal, 1987). Later studies showed that AKT2 was overexpressed in ovarian cell lines and tumours (Cheng *et al*., 1996). The subsequent siRNA silencing of AKT was found to prevent the transformation of the same cell lines (Cheng *et al*., 1996). Since then, AKT overexpression has been shown to be associated with resistance to chemotherapeutic drugs and poor prognosis in various cancers including breast (Perez-Tenorio and Stal, 2002), prostate (Kreisberg *et al*., 2004), pancreatic (Schlieman, *et al*., 2003) and brain cancers (Ermoian *et al*., 2002).

1.5.3 Signal transducers and activators of transcription 3

The transcription factor, signal transducers and activators of transcription 3 (STAT3), has been identified as an Hsp90 client protein, shown to bind to Hsp90 directly (Prinsloo *et al*., 2012; Sato *et al*., 2003) and is constitutively activated in various human cancers (Mora *et al*., 2002; Garcia *et al*., 1997; Zhang *et al.*, 1996). STAT3 belongs to family of STATs of which there are seven different proteins in mammals, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6, which carry out various functions and have roles in differentiation (Yanagisawa *et al*., 1999; Nakajima *et al*., 1996; Yamanaka *et al*., 1996), proliferation (Thierfelder *et al*., 1996), development (Planas *et al*., 1997; Takeda *et al*., 1997), apoptosis (Fukada *et al*., 1996), and inflammation (Catlett-Falcone *et al*., 1999). STAT3 is activated by Tyr70 phosphorylation following stimulation and activation of the Janus kinases by extracellular signals such as cytokines or growth factors (Hirano *et al*., 2000). In addition to tyrosine phosphorylation, STAT1, STAT3, STAT4, STAT5a, and STAT5b are occasionally phosphorylated on Ser727 residues, (Decker and Kovarik, 2000). Activated STAT3 dime rises through Src-homology 2 domains, and translocates from the cytoplasm to the nucleus, binds to STAT-specific DNA-response elements of target genes and induces the transcription of numerous genes involved in cell cycle progression, proliferation, migration and invasion, and survival (Buettner *et al*., 2002). In healthy cells, STAT3 phosphorylation is tightly regulated by protein tyrosine phosphatases (Kim *et al*., 2010). STAT3 is found in an activated phosphorylated form in more than 50 % of primary breast

tumours (Diaz *et al*., 2006; Gritsko *et al*., 2006; Dolled-Filhart *et al*., 2003; Watson and Miller, 1995) and is linked to poor clinical outcomes (Diaz *et al*., 2006). The inhibition of STAT3 results in increased apoptosis, chemosensitivity, and decreased angiogenesis (Gritsko *et al*., 2006; Kotha *et al*., 2006; Leslie *et al*., 2006), making it target for breast cancer treatment.

1.6 Hsp90 as a target for anti-cancer therapies

Hsp90 has not always been seen as a cancer drug target, but has now been referred to as the "cancer chaperone" (Neckers, 2007; Whitesell and Lindquist, 2005). Hsp90 is between 2 - 10 fold overexpressed in cancer cells, indicating that it is required for cancer cell growth and/or survival (Ferrarini *et al.*, 1992). Hsp90 is thought to buffer unstable mutant proteins within cancer cells and it is known that a number of mutated oncogenic client proteins are more dependent upon Hsp90 than their wild-type counterparts (Grbovic *et al*., 2006; da Rocha *et al*., 2005; Shimamura *et al*., 2005). Hsp90 within cancer cells exists in an activated, highly complexes form that has a higher affinity for and is between 20 and 200-fold more sensitive to Hsp90 inhibitors than uncomplexed Hsp90 found within normal cells (Kamal *et al*., 2003).

Hsp90 is an attractive target for anti-cancer treatments as it regulates multiple oncogenic signalling pathways, therefore, Hsp90 inhibition would potentially result in the simultaneous disruption and ubiquitin–proteasome degradation of client oncoproteins (Zhang and Burrows, 2004). This would greatly reduce the possibility of drug resistance developing from molecular feedback loops and the activation of alternative pathways.

Hsp90 inhibition should be explored for the treatment of breast cancer as it has the potential to target all five subtypes of the disease. Current targeted therapies in breast cancer target HER2, ERs and PRs. All three of these proteins are clients of Hsp90 and, as such, Hsp90 inhibition would result in the targeted degradation of these proteins in breast cancer cells, as has already been shown for HER2 in HER2 positive xenograft models (Bagatell *et al*., 2001; Münster *et al*., 2001). Chemotherapeutic resistance in breast cancer cells has been shown to involve the PI3K/AKT/PTEN pathway (Xing *et al*., 2008). As AKT is an identified Hsp90 client, Hsp90 inhibition could effectively result in the disruption of this pathway and the re-sensitization of these cells to chemotherapeutic agents. Results from a Phase 1 clinical trial demonstrated that the combined treatment of 17-AAG (Tanespimycin^{TM}) with the breast cancer drug trastuzumab results in clinical activity in patients with trastuzumab resistant tumours (Modi *et al*., 2007).

1.7 Hsp90 Inhibitors

Despite the fact that many Hsp90 inhibitors also target other cellular proteins, several Hsp90 inhibitors are being clinically evaluated[4] some of which are shown in Figure 1.3. Most of the Hsp90 inhibitors available are from natural sources or synthetic derivatives of natural compounds. However, there is yet to be an approved Hsp90 inhibitor drug on the market. Four different mechanisms for inhibiting the function of Hsp90 are known: (1) competitive inhibition of ATP binding, (2) targeting and disruption of Hsp90-co-chaperone interactions, (3) targeting and disruption of Hsp90-client protein interactions, and (4) interference with post-translational modification of Hsp90 (Li *et al*., 2009a).

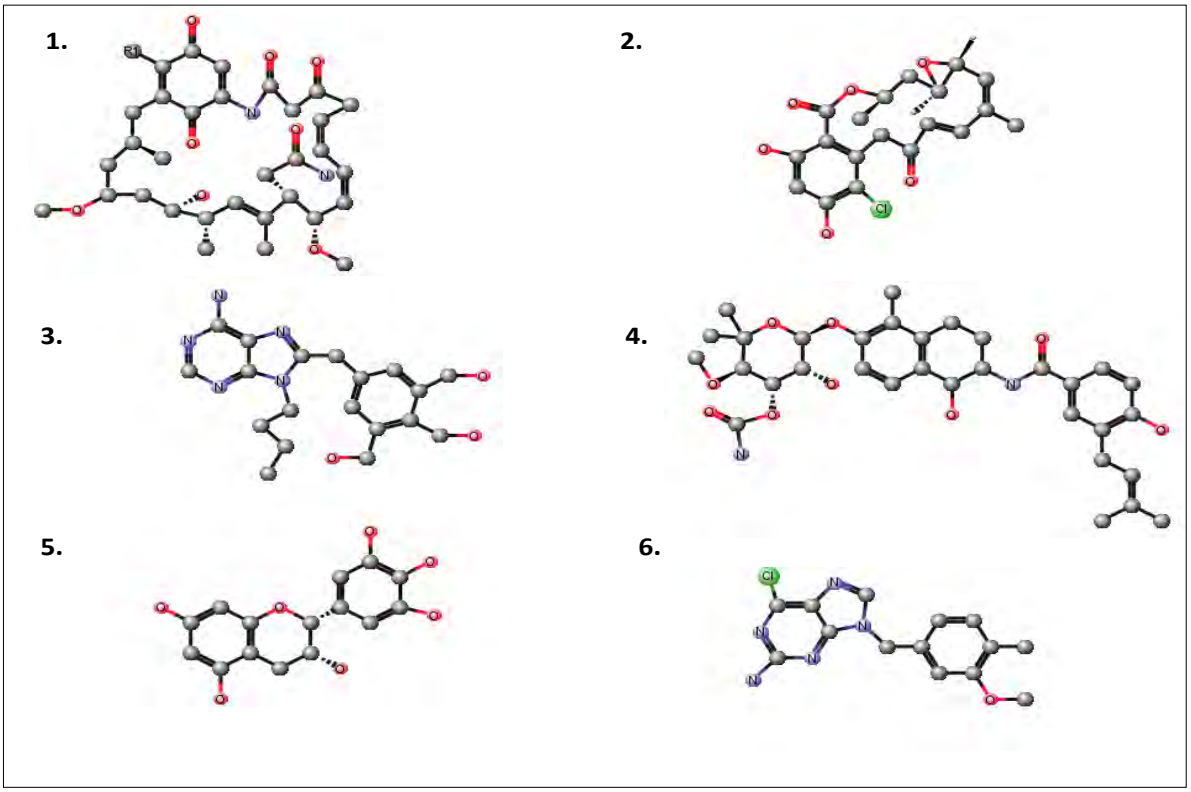


Figure 1.3: Chemical structures of selected inhibitors of Hsp90, some of which have reached clinical trial stage (adapted from Li *et al*., 2009a and Chiosis *et al*., 2004). 1. Benzoquinone ansamycin family. For geldanamycin R =, CH_3O- 17-AAG R1 = CH_2=$CHCH_2NH$ and 17-dimethylaminoethylamino-17-demethoxygeldanamycin R = $(CH_3)_2NCH_2CH_2NH$-), 2. Radicicol, 3. PU3, 4. Novobiocin, 5. (-)Epigallocatechin-3-gallate and 6. BIIB021. Structures were drawn and displayed using MarvinSketch (version 5.10.1)[5].

1.7.1 N-terminal Hsp90 inhibitors

[4] http://chemicaltrials.org/
[5] http://www.chemaxon.com/products/marvin/

Most of the Hsp90 inhibitors discovered and developed to date are shown to bind to the N-terminal domain of Hsp90. The publication by Whitesell and Neckers detailing the inhibition of the Hsp90-Src complex by GA (Whitesell *et al*., 1994) arguably began the interest in the use of Hsp90 inhibitors in the treatment of cancers.

1.7.1.1 Benzoquinone ansamycins

Several members of the benzoquinone ansamycin family are known to be competitive inhibitors of ATP in Hsp90 (Sydor *et al*., 2006; Hollingshead *et al*., 2005; Roe *et al*., 1999). These compounds are represented by GA, a natural antibiotic isolated from *Streptomyces hygroscopicus* (DeBoer *et al*., 1970) that was initially thought to be a kinase inhibitor but was later shown to bind to Hsp90 (Whitesell *et al*., 1994). Structure-activity analysis has shown the 2, 3 double bond of GA to be crucial for forming a stable conformation with Hsp90 (Roe *et al*., 1999). The 11-hydroxy and 17-methoxy moieties of GA form hydrogen bonds with Hsp90, thus improving the anti-cancer activities of this compound (Roe *et al*., 1999). GA binds to Hsp90α, Hsp90β, Grp94 and TRAP-1 comparably (Felts, 2004), but despite being a powerful anti-cancer agent, was found to be highly hepatotoxic (Supko, *et al*., 1995). This finding necessitated the development of derivatives with improved toxicological characteristics, such as 17-AAG, the water-soluble 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (Hollingshead *et al*., 2005) and 17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (IPI-504) (Sydor *et al*., 2006).

1.7.1.2 Resorcinol class of inhibitors

RAD is a natural antibiotic initially isolated from *Monocillium nordinii* and *Monosporium bonorden* and falls into the resorcinol class of Hsp90 inhibitors (Delmotte and Delmotte-Plaque, 1953). RAD binds to the ATP binding site on the N-terminal region of Hsp90 and thus disrupts ATPase activity in a similar manner to GA (Roe *et al*., 1999). Unlike GA, however, RAD binds to Hsp90α and Hsp90β with a 5- and 10-fold greater affinity, respectively compared to Grp94 or TRAP-1 (Schulte *et al*., 1999). Some of the anti-tumour activity of RAD can be attributed to its ability to disrupt Hsp90-mediated activation of protein kinases, similar to that of GA (Roe *et al*., 1999). RAD binds to the N-terminal ATP binding site of Hsp90 and has a 50-fold greater affinity for this region than GA (Roe *et al*., 1999). Although RAD has potent *in vitro* activity, the compound is chemically unstable within *in vivo* systems and lacks significant activity, possibly owing to the multiple electrophilic sites that it carries, which lead to metabolic deactivation and decomposition (Winssinger *et al*., 2009; Yang *et al*., 2004; Agatsuma *et al*., 2002). Synthetic RAD derivatives have been developed in which the epoxide

groups were replaced with cyclopropyl derivatives (Yang *et al*., 2004) and in which an oxime chemotype was inserted into the conjugated dienophile thus reducing the reactivity of the compound (Ikuina *et al*., 2003; Agatsuma *et al*., 2002; Soga *et al*., 1999). These compounds show improved *in vivo* efficacy, with some compounds showing an even greater affinity for Hsp90 than RAD (Barluenga *et al*., 2008).

The compound CCT018159 with a 3, 4-diarylpyrazole resorcinol was shown to inhibit the N-terminal ATPase of *H.sapiens* Hsp90 and *S. cerevisiae* Hsp84 (Cheung *et al*., 2005). Treatment of cancer cells with the compound resulted in client protein degradation, induction of Hsp70 and pro-apoptotic activity (Sharp *et al*., 2007; Cheung *et al*., 2005).

1.7.1.3 Purine, imidazopyridine, thieno pyrmidine

A novel class of Hsp90 inhibitors with the purine scaffold was developed by Rosen and colleagues and shows a similar phenotype to GA in breast cancer cells (Chiosis *et al*., 2001). The first compound from this series, PU3, bound moderately to Hsp90 and was shown to be more soluble but less effective than 17-AAG (Chiosis *et al*., 2001). The further development of these purine scaffold compounds resulted in the development of the BIIB021 (Kasibhatla *et al*., 2007), the first fully synthetic Hsp90 inhibitor to enter into clinical trials. BIIB021 has completed several Phase 1 and Phase 2 clinical trials for different tumours including hormone receptor positive metastatic breast cancer and gastrointestinal stromal tumours[6]. A novel imidazopyridine Hsp90 inhibitor CUDC-305 developed by Curis[7] has shown favourable tumour retention, thought to be because of its high lipophilicity as it has a calculated octanol-water partition coefficient of 4.0 and high oral bioavailability (Bao *et al*., 2009). This compound has been shown to increase survival in intracranial glioblastoma models, a first for any reported Hsp90 inhibitor (Bao *et al*., 2009). The compound 2-aminothieno [2,3-d]pyrimidine analogue 14 (NVP-BEP800), produced by Novartis[8] and selected for further development, binds to Hsp90 with a high affinity and has shown efficacy in a subcutaneous BT474 human breast tumour xenograft model, and has good oral bioavailability (Brough *et al*., 2009).

[6] http://clinical trials.gov
[7] http://www.curis.com/index.php
[8] http://www.norvartis.com

1.7.1.4 Peptide Mimetics

The peptidomimetic Shepherdin was developed to mimic the region of survivin, 174-L87, that interacts with Hsp90 on its N-terminal ATP binding site (Plescia *et al*., 2005). Similar to other inhibitors targeting the N-terminal region of Hsp90, treatment with survivin results in the inhibition of chaperone activity, degradation of client proteins and antiproliferative and pro-apoptotic activity (Plescia *et al*., 2005). Survivin has been shown to be cytotoxic to acute myeloid leukaemia cells and in tumour xenografts models, as it causes Hsp90 inhibition, degradation of client proteins and interference of mitochondrial function (Gyurkocza *et al*., 2006). The molecule 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside is a non-peptidic compound that was developed to mimic the Hsp90 inhibitory properties of Shepherdin (Meli *et al*., 2006). Like Shepherdin, treatment with 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside results in Hsp90 client protein degradation, antiproliferative and pro-apoptotic activity in several of tumour cell lines (Meli *et al*., 2006).

1.7.2 C-terminal Hsp90 inhibitors

Although less well characterised than the N-terminal domain, owing to the lack of structural information, several Hsp90 inhibitors targeting the C-terminal domain have been identified and it is evident that these compounds interfere with Hsp90 chaperone activity, resulting in anti-cancer effects (Li *et al*., 2009a).

1.7.2.1 Novobiocin and its analogues

NOV, a coumarin antibiotic isolated from the *Streptomyces* species that binds and inhibits bacterial DNA gyrase to block bacterial DNA synthesis (Gellert *et al*., 1976) represents a different strategy for inhibiting Hsp90 as it targets and binds weakly to Hsp90 through its putative C-terminal ATP binding site (Marcu *et al*., 2000a; Marcu *et al*., 2000b). NOV binding to Hsp90 results in decreased chaperone dimerization, diminished ATPase activity and reduced association of Hsp70 and p23 with Hsp90 (Yun *et al*., 2004). This results in improperly folded proteins being targeted for the ubiquitin proteasomal degradation pathway (Whitesell *et al*., 1992). Although NOV and its analogues have more acceptable toxicity and bioavailability than the benzoquinone ansamycin family of Hsp90 inhibitors, they exhibit a low affinity for Hsp90 and generally require high concentration for any observable effect (Matthews *et al*., 2010; Burlison *et al.,* 2008). Therefore, there is a need for the development of NOV analogues that have a higher affinity for Hsp90.

1.7.2.2 (-)-Epigallocatechin-3-gallate

(EGCG) is a polyphenolcatechin Hsp90 inhibitor found in green tea that is thought to function by binding to the C-terminal domain of Hsp90, thus disrupting interactions between Hsp90, p23 and Hsc70 and leading to client protein degradation (Li *et al*., 2009b). It has been proposed that EGCG binds to, or at least positions itself adjacent to, the putative C-terminal domain resulting in a conformational change in Hsp90 structure and inhibition Hsp90 dimerization (Yin *et al*., 2009). Participants are currently being recruited for Phase 1 clinical trials with this compound for breast cancer patients[9].

1.7.2.3 1, 4 Naphthoquinone scaffold family

The central coumarin ring of NOV is structurally similar to the 1, 4 naphthoquinones core scaffold which has since been identified as a new class of Hsp90 inhibitor and is hypothesized to bind to Hsp90 in a similar region to that of NOV (Hadden *et al*., 2009). Several 1, 4 naphthoquinone analogues have been identified as 'hit compounds' and were shown to have antiproliferative and HER2 degradation activity in breast cancer cell lines (Hadden *et al*., 2009). Blagg and colleagues performed a high–throughput screen to identify several 1, 4 "hit" naphthoquinone compounds from which analogues were synthetically prepared and tested for their efficacy against Hsp90 through *in vitro* assays (Hadden *et al*., 2009). Benzamide and acetamide derivatives were synthesised and the smaller acetamides were found to exhibit better inhibition of Hsp90 than their benzamide counterparts, while compounds that contained a 3-position p-methoxy aryl ring system were found to be even less active (Hadden *et al*., 2009). This was thought to be as a result of the increased steric bulk preventing these analogues from binding to the proposed C-terminal Hsp90 binding site (Hadden *et al*., 2009).

1.7.2.4 Other types of Hsp90 inhibition

As Hsp90 activity may be modulated through post-translational modification, several Hsp90 inhibitors have been developed to take advantage of this detail. HDACis such as LAQ824 (dacinostat) (Fuino *et al*., 2003; Nimmanapalli *et al*., 2003) and FK228 (depsipeptide) (Yu *et al*., 2002) inhibit Hsp90 activity by acetylation, resulting in client protein degradation *in vitro* and *in vivo*. However, HDACis display broad specificity and, as such, their use as Hsp90 inhibitors remains a concern as they may alter the acetylation of chromatin and other non-histone proteins (Ma, 2009).

[9] http://clinicaltrials.gov

1.7.3 Novel chemotypes

Several novel chemotypes of Hsp90 inhibitors (Figure 1.4) have been reported and are currently undergoing clinical trials (Kim *et al.,* 2009). SNX-5542 is a compound developed by Serenex[10] shown to be non-toxic in xenograft models (Huang *et al*., 2009) while showing partial tumour regressions (Chandarlapaty *et al*., 2008) and is both water soluble and orally bioavailable (Huang *et al*., 2009). SNX-5542 has also been shown to have greater efficacy than 17-AAG in mouse xenograft models (Chandarlapaty *et al*., 2008) and several Phase 1 clinical trials[1] are being performed with an oral formulation of this compound. STA-9090 (ganetespib), KW-2478, and BIIB028 (structure not reported) are compounds under development by Synta Pharmaceuticals[11], and Biogen[12] respectively. STA-9090 underwent a Phase 1 clinical trial in combination with doxataxel (Kauh, 2012) and a Phase 3 clinical trial via intravenous (IV) formulation with docetaxel[10]. KW-2478 is in Phase 1/2 clinical trials in combination with bortezomib for multiple myeloma[13] and BIIB028 has completed Phase 1 clinical trials[14] in subjects with solid tumours. XL888 by Exelixis[15] and Hsp990 (a resorcinol derivative; structure not reported) by Novartis[16] are Hsp90 inhibitors that have reached Phase 1 clinical trial via oral formulation while the Hsp90 inhibitor MPC-3100 by Myriad Pharmaceuticals[17], completed Phase 1 clinical trials in 2011.

[10] http://www.serenex.com/
[11] http://www.syntapharma.com/prdHsp90.aspx
[12] http://www.biogenidec.com/
[13] http://www.syntapharma.com/prdHsp90.aspx
[14] http://clinicaltrials.gov
[15] http://www.exelixis.com/pipeline/xl888
[16] http://www.novartisoncology.us/research/pipeline/hsp990.jsp
[17] http://www.myriadpharma.com/

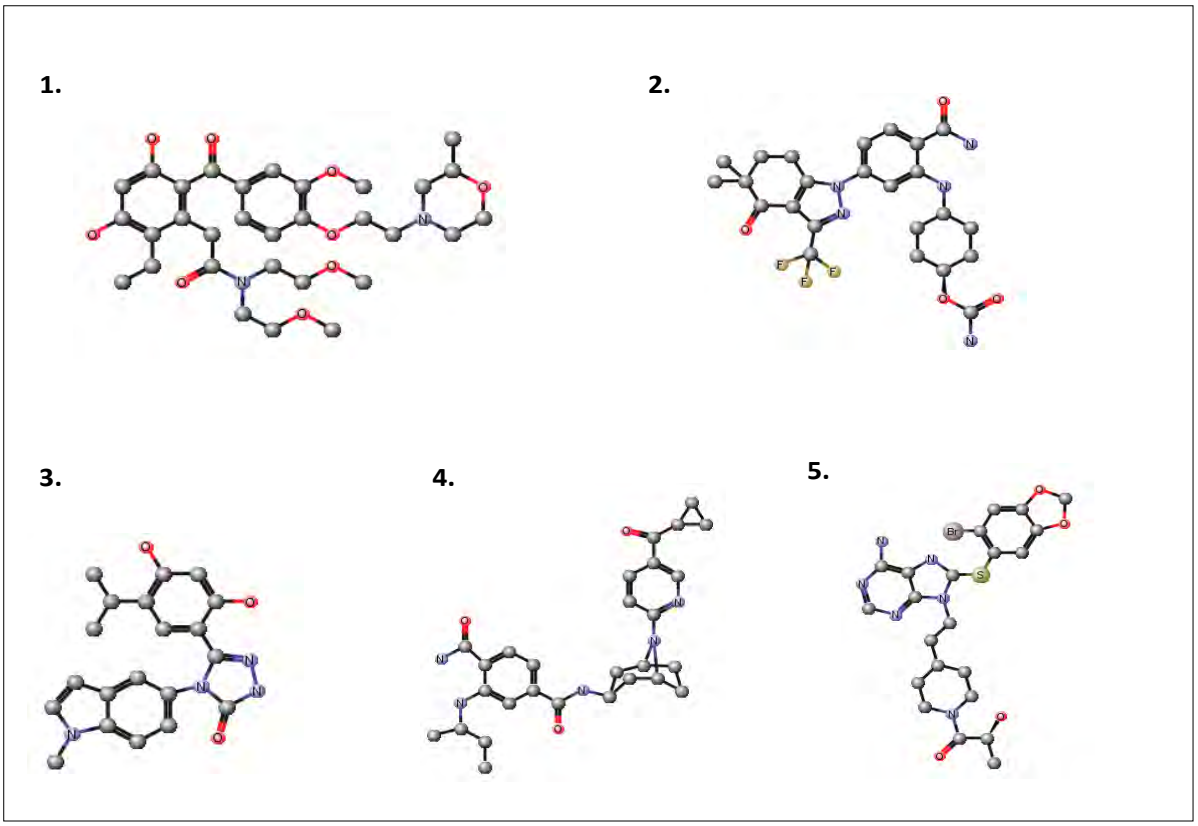


Figure 1.4: Chemical structures of Hsp90 inhibitors with a novel chemotype. 1. KW2478, 2. SNX-5542, 3. STA-9090, 4. XL888 and 5. MPC-3100. Structures were drawn and displayed using MarvinSketch. (version 5.10.1)[18].

1.7.4 Inhibition of specific isoforms of Hsp90

It is interesting to note that the effects of certain inhibitors are dependent upon the Hsp90 isoform on which they are acting (Chan *et al*., 2008). GA and RAD have been reported to interact with Hsp90α and Hsp90β with comparable efficacy; whereas certain purine-scaffold compounds, such as PU-H71, have been found to be more effective against Hsp90α than Hsp90β (Chan *et al*., 2008). The compound 50-N-ethylcarboxamidoadenosine only binds to Grp94 and no other Hsp90 isoform (Rosser *et al*., 2000). It is unclear and remains to be seen whether the isoform specificity of an Hsp90 inhibitor could be a therapeutic advantage (Li *et al*., 2009a).

1.8 Role of natural products in anti-cancer therapies

Natural products have been used medically since ancient times (Lee *et al*., 2012b) and, owing to the vast number of plant, animal, marine and microbial species found in nature, their chemical diversity is expected to be substantial (da Rocha *et al*., 2001). This chemical diversity is thought to have

[18] http://www.chemaxon.com/products/marvin/

evolved in order to provide species with self-defence mechanisms against predators (da Rocha *et al*., 2001). More than 60 % of the available anticancer drugs, of which some are Hsp90 inhibitors, are derived from plant and natural sources (Cragg and Newman, 2005) and numerous marine organism derived compounds are in pre-clinical and early clinical evaluation as anticancer targets as shown in Table 1.3.

Table.1.3: Natural product-derived anti-cancer agents, their sources, and their mechanism (adapted from da Rocha *et al.,* 2001). [19]

Compound	Mechanism of action	Type of cancer targeted	Original source	Clinical trial status reached	References
Vincristine	Inhibits microtubule assembly	Leukaemia, lymphoma, breast, lung	Plant	Phase 3/4	Noble , 1999; DeVita *et al.*, 1970
Paclitaxel	Stabilises microtubules, leading to mitotic arrest	Ovary, breast, lung, bladder, head and neck	Plant	Phase 3/4	Bertino 1997; Wani, 1971
Discodermolide	Stabilises microtubules, leading to mitotic arrest	Lung	Marine	Phase 1	Mita *et al*., 2004; Honore, *et al*., 2003; Kowalski, *et al*., 1997
Dolastatin 10	Inhibits microtubules and pro-apoptotic effects	Small-cell lung, prostate	Marine	Phase 2	Vaishampayan *et al.,* 2000 ; Poncet *et al*., 1999
Etoposide	Inhibits topoisomerase II activity	Small cell lung	Plant	Phase 3/4	Harvey, 1999; Liu, 1989
Topotecan	Inhibits topoisomerase I activity	Ovarian, lung and paediatric	Plant	Phase 2/3	Creemers *et al*., 1996
Daunomycin	Inhibits topoisomerase II activity	Leukaemia	Microbe	Phase 3/4	Gieseler *et al.*, 1997
Doxorubicin	Inhibits topoisomerase II activity	Lymphoma, breast, ovary, lung, sarcomas	Microbe	Phase 3/4	Gieseler *et al.*, 1997
Idarubicin	Inhibits topoisomerase II activity	Breast, leukaemia	Microbe	Phase 3/4	Gieseler *et al.*, 1997
Bryostatin 1	Activation of PKC	Experimental	Marine	Phase 2	Varterasian *et al*., 2001; Pettit *et al*., 1996,
Actinomycin	Targets DNA conformation present within the transcriptional complex	Sarcoma, germ-cell tumours	Microbe	Phase 3/4	Sobell, 1985
Ecteinascidin 743	Alkylation of DNA	Small cell lung, ovarian, soft tissue carcinoma	Marine	Phase 2	Laverdiere *et al*., 2003 ; Li *et al*., 2001; Hendriks, 1999
Bleomycin	Prevents cells from entering visible mitosis	Germ-cell, cervix, head and neck	Microbe	Phase 3/4	Nagatsu *et al*.,, 1979
Flavopiridol	Blocks cell-cycle progression at G1 or G2	Experimental	Plant	Phase 1/2	Keeland, 2000; Worland, 1993
Aplidine	Inhibition of cell-cycle progression	Experimental	Marine	Phase 1	Maroun *et al*., 2006; Geldof *et al*., 1999
Novobiocin	Targets Hsp90 function	Experimental	Microbe	Phase 1	Eder *et al*., 1999
Radicicol	Targets Hsp90 function	Experimental	Microbe	Pre-clinical development	Soga *et al*., 1999; Schulte and Neckers, 1998

1.8.1 Marine organism derived agents

It is not known how many species of organism reside within the ocean but the number of prokaryotic cells has been estimated to be approximately 10^{29} (Whitman *et al*., 1998) and the

[19] Microbe refers to either a bacterial or fungal species

number of eukaryotic species estimated at approximately 2.2 million (Mora *et al*., 2011). An excess of 3000 new compounds have been isolated from marine organisms, many of which have entered clinical trials (Cragg *et al*., 1997b). Additionally, many of these novel compounds have been found to be cytotoxic against multiple tumour types (Cragg *et al*., 1997b). Selected examples of marine-derived compounds with anti-cancer properties are shown in Table 1.3. Didemnin B, a cyclic depsipeptide isolated from *Tridemnum solidum,* was the first anti-tumour compound isolated from a marine organism to enter clinical trials (Mittelman *et al*., 1999; Shin *et al*., 1994). Didemnin B functions by inhibiting protein synthesis and induces G1 cell cycle arrest (Geldof *et al*., 1999). Several ecteinascidins have been isolated from *Ecteinascidia turbinate* (Sakai *et al.*, 1992). The ecteinascidin, ET-743, has been shown to exhibit anti-tumour activities at nanomolar and sub-nanomolar concentrations (Izbicka *et al.*, 1998). Anti-tumour effects have been observed in Phase I clinical trials with ET-743 at concentrations of less than 2 $mg.m^{-2}$ body surface (Le Cesne *et al*., 2005). The dolastatins are peptides that were originally isolated from a sea mollusc, *Dolabella auricularia* (Pettit *et al.,* 1982). These peptides have cytotoxic activity and the compound Dolastatin 10 which functions by inhibiting microtubule assembly, resulting in the cell-cycle arrest in metaphase, entered Phase 1 and Phase 2 clinical trials (Pathak *et al*., 1998; Bai *et al.,* 1990).

1.8.2 Plant derived agents

Many compounds of plant origin are used in anti-cancer treatments with a large number remaining at the experimental phase (da Rocha *et al*., 2001) (Table 1.3). The compounds paclitaxel (PTX) and docetaxel of the taxane family are examples of synthetic drugs in clinical use that were originally isolated from plant material. PTX was originally obtained from the bark of the pacific Yew tree, *Taxus brevifolia* (Wani *et al*., 1971) and docetaxel, a semi-synthetic analogue of PTX, synthesised from 10-deacetyl baccatin isolated from the needles of the European Yew tree, *Taxus baccata* (Appendino, 1995). Both have been shown to have anti-tumour properties that are effective against numerous malignancies including breast (Jones *et al.*, 2005) and ovarian (Vasey *et al*., 2004) cancers. PTX functions by stabilising microtubules, eventually leading to the mitotic arrest of treated cells (Wani *et al*., 1971). Irinotecan and topotecan, derivatives of camptothecin, exhibit anti-tumour properties against colorectal and ovarian cancer, respectively (Bertino, 1997; Creemers *et al*., 1996). These compounds were originally extracted from the bark and wood of Nyssacea *Camptotheca accuminata* and function by inhibiting topoisomerase I (Liu, 2000). Flavopiridol is a synthetic compound originally isolated from the leaves and stems of *Amoora rohituka* and *Dysoxylum binectariferum* (Kelland, 2000; Worland, 1993; Harmon *et al*., 1979). This compound functions by interfering with the

phosphorylation of cyclin-dependent kinases, resulting in the inhibition of their activation which blocks cell-cycle progression at G1 or G2 (Kelland, 2000, Worland, 1993).

1.8.3 Microbe derived agents

Antibiotics with anti-tumour properties form an important part of anti-cancer agents and include the ansamycin, actinomycin, bleomycin, mitomycin and aureolic acid families (Cragg *et al*., 1997a). Rapamycin, which was discovered in a screen for anti-fungal agents (Sehgal *et al.*, 1975), was later shown to have antiproliferative properties and as a result found use as a novel chemotherapeutic agent (Dilling *et al*., 1994). Rapamycin was isolated from the bacterium *Streptomyces hygroscopicus* and has been shown to inhibit signalling pathways required for T-cell activation and proliferation by blocking progression of the cell cycle at the mitotic phase-to-late G1 in several cell lines (Alberts *et al*., 1993). Although the original compounds isolated from natural sources are often not used as drugs themselves, they do serve an important role as lead compounds that could result in the production of novel drugs (Cragg and Newman, 2005).

1.9 Problem Statement and Aim

The molecular chaperone Hsp90 is a promising target for anti-cancer treatments and its inhibition has the potential to result in the simultaneous disruption and ubiquitin–proteasome degradation of activated client oncoproteins. Many of these oncoproteins control numerous signalling and self-renewal pathways required for the maintenance and progression of various cancers. Four different mechanisms for inhibiting the function of Hsp90 are known: (1) competitive inhibition of ATP binding, (2) targeting and disruption of Hsp90-co-chaperone interactions, (3) targeting and disruption of Hsp90-client protein interactions and (4) interference with post-translational modification of Hsp90 (Li *et al*., 2009a). Although several inhibitors are being clinically evaluated there are no approved Hsp90 inhibitor drugs available to the public. Several promising Hsp90 inhibitors including GA face problems such as high toxicity and poor solubility in aqueous media. There remains therefore, a need for the identification and development of compounds that have a high affinity for Hsp90 and that display an acceptable clinical toxicity. Compounds isolated from natural and biological sources are structurally diverse and an important source of novel hit compounds with the potential of being developed into anti-cancer drugs. Currently, more than 60 % of the available anti-cancer drugs are derived from natural sources including plants, microbes and marine organisms (Cragg and Newman, 2005), revealing that natural sources bear a vast quantity of compounds of great structural diversity. The aim of this study was to screen, identify and

characterise novel and natural compounds of marine and terrestrial origin for potential modulators of Hsp90 function.

1.10 Hypothesis

Small novel, organic molecules with a quinone ring and putative anti-cancer activity will interact directly or indirectly with Hsp90.

1.11 Broad Objectives

The objectives of this study were to screen and characterise potential anti-cancer compounds in terms of their ability to interact directly or indirectly with Hsp90 and/or to inhibit Hsp90 chaperone activity. The compounds selected for analysis were classified into three groups: (1) Sargaquinoic acid (SQA), (2) naphthoquinones and (3) marine pyrroloiminoquinone alkaloid compounds. The objectives of the project were:

1. *In silico* prediction of the drug-like properties and *in vitro* determination of the antiproliferative activity of natural and terrestrial compounds (CHAPTER 2).

2. Determination of the effects of SQA on the subcellular localisation and expression of Hsp90, Hsp90 associated chaperones, co-chaperones and client proteins in a breast cancer cell model and the determination of the biophysical interaction of Hsp90 and SQA (CHAPTER 3).

3. Determination of the effects of naphthoquinone and marine pyrroloiminoquinone alkaloid compounds on the subcellular localisation and expression of Hsp90, Hsp90 associated chaperones, co-chaperones and client proteins in a breast cancer cell model and the determination of the biophysical interaction between Hsp90 and naphthoquinone compounds (CHAPTER 4).

CHAPTER 2

STRUCTURAL SIMILARITY-BASED SCREENING AND EVALUATION OF THE DRUG-LIKENESS OF QUINONE-BEARING COMPOUNDS IN BREAST CANCER CELLS

2.1. Introduction

As Hsp90 is a promising cancer drug target, a structural similarity-based screening method was deemed as an appropriate approach in the identification of 'hit' compounds with Hsp90-modulatory activity from a small library of chemically similar compounds. A 'hit' compound may be defined as a non-reactive compound (Rishton, 2003) of known structure and purity, with a confirmed minimal *in vitro* potency (Wunberg *et al*., 2006). Several methods and technologies are used in the discovery of novel hit compounds, which include *de novo* design, and the use of *in silico*, *in vitro* and *in vivo* screening tools. 'Lead' compounds may be defined as compounds that not only exhibit *in vitro* potency but also show selectivity towards a specific target. Furthermore, for a compound to be considered as a 'lead' it should exhibit specific binding towards the target and the biophysical and absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of the compound should be known (Wunberg *et al*., 2006).

In silico screening is advantageous in the drug discovery field as it allows for the identification of drug-like compounds or fragment-like molecules through the use of screening tools such as the rule-of-five (Lipinski *et al*., 1997) or the rule-of-three (Congreve *et al*., 2003) respectively. The rule-of-five defines the desired physicochemical properties of a compound that allow for oral absorption (Lipinski *et al*., 1997). The rule was developed after a pattern in the physicochemical properties was observed following the screening of 2245 compounds from the World Drug Index (Lipinski *et al*., 1997). The majority of compounds screened displayed a molecular mass of less than 500 $g.mol^{-1}$, were composed of less than 10 hydrogen bond acceptors and 5 hydrogen bonds and possessed an octanol-water partition coefficient (logP) value of less than 5 (Lipinski *et al*., 1997). Following the publication of the rule-of-five, numerous other publications appeared defining additional rules and refinements to compliment the original rules. These properties include the polar surface area, a computational molecular property indicative of lipophilicity, and the number of rotatable bonds; an indication of the conformational flexibility (Veber *et al*., 2002; Kelder *et al*., 1999).

In vitro and *in vivo* screening allows for the generation and determination of the ADMET properties of compounds thus allowing for the assessment of the druggability of a compound. Toxicity or cytotoxicity determination forms an important part of the lead optimisation process and is required in the early stages of compound screening to allow for modifications to be made to compounds to improve the toxicity profile of the compounds belong screened. There are several methods for determining the potency of compounds including the 3-(4, 5-dimethylthiazzolyl-2)-2, 5-diphenylterazolium bromide (MTT) assay which is a well characterised cell proliferation assay that

has been described as the gold standard to which new cell proliferation assay methods are compared (Abate *et al*., 2004). This assay is a rapid and quantitative colorimetric assay that allows for the determination of cell viability by measuring the change of the yellow tertazolium to dark purple formazan crystals catalysed by mitochondrial dehydrogenase in metabolically active cells thus allowing for the antiproliferative effects of a compound to be determined in a cell-based system (Wu *et al*., 2008).

As reviewed in Chapter 1, 1, 4 naphthoquinones represent a new class of Hsp90 inhibitor (Hadden *et al*., 2009) and have been shown to possess antiproliferative activities in several cell lines (Chung *et al*., 2004; Kongkathip *et al*., 2003). The 1, 4 naphthoquinone, lapachol (LAP), has known anti-tumour activity in various cancer types (Santana *et al*., 1980). It is believed that this anti-tumour activity of LAP and naphthoquinones in general may be because of their interaction with nucleic acids resulting in the inhibition of DNA replication and RNA synthesis (Murray and Pizzorno, 1998). Similarly, as quinoid compounds, the makaluvic acids, makaluvamines and damirones are thought to exhibit their antiproliferative effects through a similar inhibition mechanism (Antunes *et al*., 2005a). The discorhabdins represent a novel class of anti-tumour compound owing to their high toxicity, although at present, it is thought that the antiproliferative activities of these compounds may be non-specific (Antunes *et al*., 2005a).

The aim of this chapter was to employ a structural similarity-based screen to identify potential modulators of Hsp90 function from naphthoquinone compounds of terrestrial origin and marine pyrroloiminoquinone alkaloid compounds. The antiproliferative activity of each compound in the MDA-MB-231 cell line and the three-dimensional similarity of the compounds were determined. A preliminary pharmacological screening of the compounds to determine their drug-likeness by utilising Lipinski's rule-of-five (Lipinski *et al*., 1997) was performed.

2.2. Materials

The triple negative, breast epithelial adenocarcinoma cell line, MDA-MB-231 (ATCC HTB-26) was provided by Dr Sharon Prince (Department of Human Biology, Faculty of Health Sciences, University of Cape Town). General reagents were purchased from Sigma-Aldrich (Germany) or Saarchem (Merck, South Africa). Tissue culture reagents (Dulbecco's Modified Eagle Medium [DMEM] with GlutaMAX™-I, foetal calf serum [FCS], 10 x Trypsin-Ethylenediaminetetraacetic acid [EDTA] and Penicillin/Streptomycin/Amphotericin) were from Gibco, Invitrogen (Paisley, United Kingdom) and Biowhittaker (United Kingdom). Tissue culture plasticware was from Corning Incorporated (United

States of America). Cell Proliferation Kit [MTT] (cat no.: 11465007001) was purchased from Roche Diagnostics GmbH (Germany). Manufacturers or suppliers of any other specialised reagents are referenced in-text.

2.3. Methods

2.3.1. *Isolation of compounds and origin of compounds*

SQA was isolated from S*argassum heteropyllum* as described by Afolayan *et al.* (2008) and provided by Dr Denzil Beukes from the Department of Pharmaceutical Chemistry, Rhodes University. The naphthoquinone LAP was isolated from *Tabebuia* sp. (*Tecoma*) and the derivatives, α-lapachona (αLAP), c-alil-lausona (CAL), bromo-β-lapachona (BBL), nor-β-lapachona (NBL) and hydroxy-β-lapachona (HBL) were synthesised as described by Sunassee *et al*., 2013. The marine pyrroloiminoquinone alkaloid compounds damirone C (DMN), discorhabdin A (DSN), makaluvamine M (MKM), *N*-1-β-D ribofuranosyl makaluvamine (RIBS-1), *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3) were isolated from the marine sponge *Strongylodesma aliwasliensis* as described by Antunes *et al*., 2005b. Both the naphthoquinone and marine pyrroloiminoquinone alkaloid compounds were provided by Professor Davies-Coleman from the Department of Chemistry, Rhodes University. The structures of all the compounds were confirmed by nuclear magnetic resonance and compounds were dissolved in DMSO.

2.3.2. *Cell culture and maintenance of cell lines*

The MDA-MB-231 cell line was cultured at 37 °C with 10 % carbon dioxide (CO_2) in a humidified incubator in DMEM with GlutaMAX^{TM} supplemented with 5 % (v/v) heat inactivated FCS and 100 U.ml^{-1} penicillin, 100 µg.ml^{-1} streptomycin and 12.5 µg.m^{-1} amphotericin. The cell line was verified as being free from mycoplasma infection by immunofluorescence microscopy analysis following staining with Hoechst 33342 and by polymerase chain reaction analysis.

2.3.3. *Determination of the drug-likeness of the compounds through Lipinski's rule-of-five*

The drug-likeness of SQA, the naphthoquinone and pyrroloiminoquinone alkaloid series of compounds were analysed according to the rule-of-five (Lipinski *et al*., 1997). The characterised Hsp90 inhibitors GA and NOV were also assessed and screened against the same parameters.

2.3.4 Generation of the three-dimensional structures of compounds

The three dimensional structures of SQA, the naphthoquinone and pyrroloiminoquinone alkaloid series of compounds were generated and compared to those of GA and NOV. The structures were drawn in MarvinSketch (version 5.10.1)[20] in which, the conformational isomers of each compound was generated and the lowest energy conformational isomer displayed.

2.3.5 Determination of the antiproliferative activity of marine and terrestrial compounds in triple negative breast cancer cell line

The antiproliferative effects of the anti-cancer compound PTX, the naphthoquinone and pyrroloiminoquinone alkaloid series were determined in the MDA-MB-231 breast cancer cell line using the Cell Proliferation Kit (MTT) according to the manufacturer's instructions. The experiment was performed at 37 °C, 10 % CO_2. A total of 6000 cells per well were seeded into a 96-well plate and were allowed to adhere overnight. The different concentrations of each compound were prepared by five-fold serial dilutions. Compounds were added to each well at a concentration range of between 0 – 20 µM and 0 to 500 µM for compounds which, showed no antiproliferative activities within the lower concentration range. Each compound was tested in triplicate on each plate and each plate repeated in triplicate. Cells were treated with a dimethyl sulphoxide (DMSO) vehicle control at the same concentration of each compound at its highest concentration. The DMSO concentration did not exceed 1 % of the final concentration in media at any time. Four different concentrations of each compound were tested. Samples were incubated for 96 hours after which, MTT reagent was added to each well and incubated for a further 4 hours followed by the addition of the sodium dodecyl sulphate (SDS)-containing solubilisation reagent to each well. The plates were incubated overnight. The colour change of the yellow tertazolium to dark purple formazan crystal catalysed by mitochondrial dehydrogenase by metabolically active cells was quantified by measuring the absorbance of the samples at 550 nm using a Powerwave spectrophotometer (Biotek). The

[20] http://www.chemaxon.com/products/marvin/

percentage survival of the cells was determined for each compound at each concentration by comparing the values following compound treatment to those of the DMSO control. The percentage survival of each compound was plotted graphically and the half-maximal inhibitory concentration (IC_{50}) value of each compound was calculated by plotting the % survival of MDA-MB-231 breast cancer cells against the log of the concentration of each compound used. The IC_{50} was calculated to compare the relative cytotoxicity of each compound using a linear regression model in Microsoft Excel 2010. Three replicates per experiment were conducted, and three independent experiments were performed. The IC_{50} value was calculated for each compound and used as a parameter to compare the relative cytotoxicity of each test compound. A Statistical analysis of the percentage of viable cells along with concentration of compounds used to treat the cells was calculated using employing the one-way analysis of variance (ANOVA) in Microsoft Excel 2010. A probability of 0.05 or less was considered to be statistically significant.

2.4 Results and Discussion

2.4.1 Validation of compounds through the preliminary pharmacological profiling of the compounds through Lipinski's rule-of-five

Lipinski's rule-of-five (Lipinski *et al*., 1997) was used in this study as a screening tool to determine the drug- or lead-likeness of all the compounds being investigated in this study. The characterised Hsp90 inhibitors GA and NOV were included as controls in the screening process. Compounds that conform to the rule-of-five and are considered as ideal should violate no more than one of the rules detailed by the rule-of-five. Although Lipinski's rule-of-five addresses the potential use of compounds in whole animals and the work in this dissertation examines the use of the compounds in a cell culture model, it was important to determine the usefulness of the compounds for future applications as compounds which fail the rule-of-five could be limited in their use for further studies.

Table 2.1: **Drug-likeness analysis of compounds using the rule-of-five for the naphthoquinone series of compounds; lapachol (LAP), α-lapachona (αLAP), c-alil-lausona (CAL), nor-β-lapachona (NBL), bromo-β-lapachona (BBL) and hydroxy-β-lapachona (HBL) and sargaquinoic acid (SQA).**

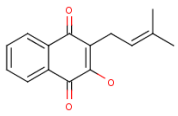
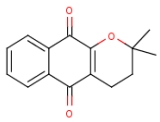
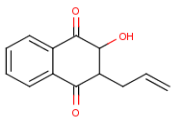
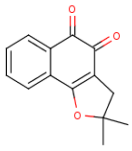
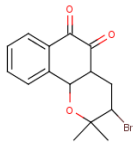
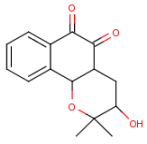
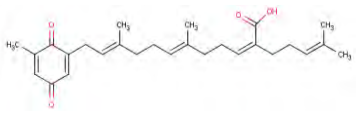
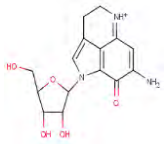
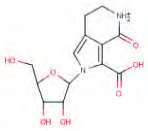
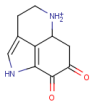
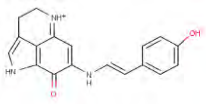
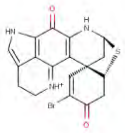
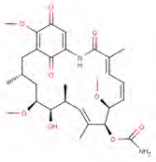
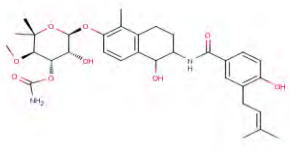
Name of compound	2D Chemical structure	Molecular Weight: [g.mol^{-1}] (Ideal <500)	H-bond Donors (Ideal <5)	H-bond acceptors (Ideal <10)	LogP (Ideal <5)	Violations
LAP $C_{15}H_{14}O_3$		242.26	4	3	2.59	0
αLAP $C_{15}H_{14}O3$		242.26	3	3	2.15	0
CAL $C_{13}H_{12}O_3$		216.23	4	3	2.44	0
NBL $C_{14}H_{12}O_3$		228.24	3	3	2.18	0
BBL $C_{15}H_{15}BrO_3$		323.18	4	3	3.37	0
HBL $C_{15}H_{16}O_4$		260.28	5	4	1.83	0
SQA $C_{27}H_{36}O_4$		424.57	1	4	7.20	1

Table 2.2: **The Drug-likeness analysis of compounds using the rule-of-five for the marine pyrolloimoquinone alkaloid series of compounds; *N*-1-β-D ribofuranosyl makaluvamine (RIBS-1), *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3), damirone C (DMN), makaluvamine M (MKM), discorhabdin A (DSN) and of geldanamycin (GA) and novobiocin (NOV**).

Name of compound	2D Chemical structure	Molecular Weight: [g.mol^{-1}] (Ideal < 500)	H-bond Donors (Ideal < 5)	H-bond acceptors (Ideal <10)	LogP Ideal <5)	Violations
RIBS-1 $C_{15}H_{18}N_3O_5$		320.32	5	6	-1.95	1
RIBS-3 $C_{13}H_{17}N_2O_7$		313.28	5	7	-1.82	1
DMN $C_{10}H_{11}N_2O_2$		191.20	2	2	0.42	0
MKM $C_{18}H_{16}N_3O_2$		306.33	3	5	1.85	0
DSN $C_{18}H_{15}BrN_3O2_S$		417.30	2	5	1.24	0
GA $C_{29}H_{40}N_2O_9$		560.63	4	11	2.62	2
NOV $C_{31}H_{36}N_2O_{11}$		612.62	6	13	2.86	4

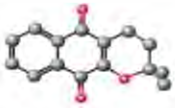
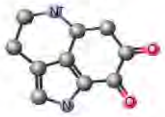
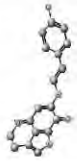
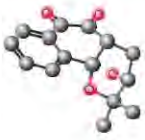
To pass Lipinski's rule-of-five, a compound should violate no more than one of the four rules. It is interesting to note that all the compounds under investigation in this study passed the rule-of-five as shown in Table 2.2 and Table 2.3 thus suggesting that these compounds would remain active if administered orally (Lipinski *et al*, 1997). Although the known Hsp90 inhibitors GA and NOV, analysed in this study failed to satisfy the requirements of the rule it must be remembered that most of the drugs and compounds that fail the rule-of-five include antibiotics such as GA and NOV and that most of these compounds are still bioavailable as they possess groups that act as substrates for transporters (Walters and Numchuk, 2003; Lipinski *et al*., 1997).

2.4.1 Determination of the three-dimensional structures of the compounds investigated in this study

The two-dimensional representation of compounds allowed for structural comparisons to be made between compounds but did not allow for the prediction of the true conformation of the compounds to be made. Atoms may appear spatially separated when represented two-dimensionally, but may be shown to exist closer to each other when represented three-dimensionally owing to the preference of a compound for a particular conformational state. The structures of the naphthoquinone series and marine pyrolloimoquinone alkaloid series of compounds were drawn, converted and cleaned to generate their three-dimensional structures in MarvinSketch (version 5.10.1)[21] as shown in Table 2.3.

[21] http://www.chemaxon.com/products/marvin/

Table 2.3: The three-dimensional representations of lapachol (LAP), α-lapachona (αLAP), c-alil-lausona (CAL), nor-β-lapachona (NBL), bromo-β-lapachona (BBL) and hydroxy-β-lapachona (HBL), *N*-1-β-D ribofuranosyl makaluvamine (RIBS-1), *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3), damirone C (DMN), makaluvamine M (MKM), discorhabdin A (DSN), geldanamycin (GA) and novobiocin (NOV).

Compound Name	three-dimensional Structure	Compound Name	three-dimensional Structure
LAP		SQA	
αLAP		RIBS- 1	
CAL		RIBS-3	
NBL		DMN	
BBL		MKM	
HBL		DSN	
GA		NOV	

The differences in the details of the structures of the compounds within the naphthoquinone series were evident from the two-dimensional depiction but these differences became more apparent when the compounds were depicted three-dimensionally. The predicted three-dimensional structures depicted LAP, αLAP and CAL as having conformations in which all the rings were arranged linearly. These findings are in agreement with the published crystal structure of LAP which, indicated that the aromatic ring system was planar with the allylic side chain positioned perpendicularly to the ring system (Larsen *et al*., 1992). Conversely, the published crystal structure of BBL indicated that the atoms comprising the core naphthoquinone ring and the adjacent furan ring were co-planar to each other, with the furan ring assuming a distorted half-chair conformation (Cremer and Pople 1975).The three-dimensional predictions generated in this study for NBL, BBL and HBL showed that the naphthoquinone rings were arranged in a similar planar arrangement and a half-chair conformation adopted by the pyran ring of NBL, and furan rings of HBL and BBL. The correlation between the published structural information and the three-dimensional predictions for the naphthoquinone compounds validate the findings made in this study. It was therefore, predicted that the cell biological and biophysical properties of these compounds would be dependent on the variation in the position of the conjugated carbonyl groups on the naphthalene ring and the differences in the conformations of the compounds. It was thus predicted that LAP, αLAP and CAL would exhibit similar cell biological and biophysical activities to each other and that BBL, HBL and NBL would exhibit similar activities to each other.

Table 2.1 also showed DMN as existing within a single plane, while the sugar moieties present in RIBS-1 and RIBS-3 adopted a perpendicular conformation to the pyrroloquinoline ring in the compounds. It was therefore, predicted that DMN, RIBS-1 and RIBS-3 would have similar cell biological and biophysical activities to each other. The three-dimensional structures of DSN and GA appeared to be unique within the compounds analysed as both contained large ring structures. DSN was comprised of a fused ring structure of azacarbocyclic spirocyclohexanone and pyrroloiminoquinone rings with a central sulphur atom. GA comprised a planar benzoquinone within a fifteen-carbon ansa ring and an amide group at the opposite end of the compound. Excluding LAP, CAL and αLAP, the compounds shown to exhibit linear conformations were SQA, NOV and MKM with the three-dimensional conformations of SQA and NOV appearing more similar to each. It was therefore, predicted that the cell biological and biophysical activities of SQA and MKM would be similar to those of NOV owing to the similarity in their three dimensional conformations, while GA and DSN would have distinct activities.

2.4.2 Determination of antiproliferative activity of marine and terrestrial compounds in a triple negative breast cancer cell line

Many methods have been developed to measure the antiproliferative effects of compounds in cells including traditional methods such as the direct counting of viable cells, or the measurement of metabolic activity and cellular DNA content. The use of the MTT assay to indirectly quantitate viable cells and thus assess cell proliferation has been widely exploited (Farabegoli *et al*., 2007; Thangapazham *et al*., 2007; Yu *et al*., 2007). The MTT assay may be considered superior to traditional cell counting methods, which, although may be simple and inexpensive tend to be very time consuming and are prone to inaccuracies owing to human error (Kanemura *et al*., 2002). The naphthoquinone series and marine pyrolloimoquinone alkaloid series of compounds were tested in order to determine their potential antiproliferative activity in the triple-negative, metastatic MDA-MB-231 breast cancer cell line and to determine their IC_{50} values (Figure 2.1). The MDA-MB-231 cell line was selected as a TNBC model as this form of the disease is biologically aggressive and women of African descent have been shown to have a predisposition to this form of breast cancer. Therefore, the identification and development of any compounds with antiproliferative activity against the TNBC model could potentially benefit African women in the long-term. The taxol, PTX, which exhibits anti-cancer activity against a wide range of solid tumours (Sackett and Fojo, 1997; Rowinsky and Donehower, 1995; Horwitz, 1992), was used as a control compound to validate the assay. Published values for the IC_{50} value for PXT in the MCF-7 breast cancer cell line of 100 nM (Dwight *et al*., 1997) are similar to the values obtained in this study of 106 ± 2.08 nM thus verifying the validity of the results obtained from this assay. IC_{50} values for SQA, NOV and 17-AAG were obtained from literature values for the compounds. The IC_{50} values to be used in this study were 90.2 µM for SQA (de la Mare *et al.,* 2012), 260 µM for NOV (Radanyi *et al*., 2008) and 200 nM for 17-AAG (Münster *et al*., 2002).

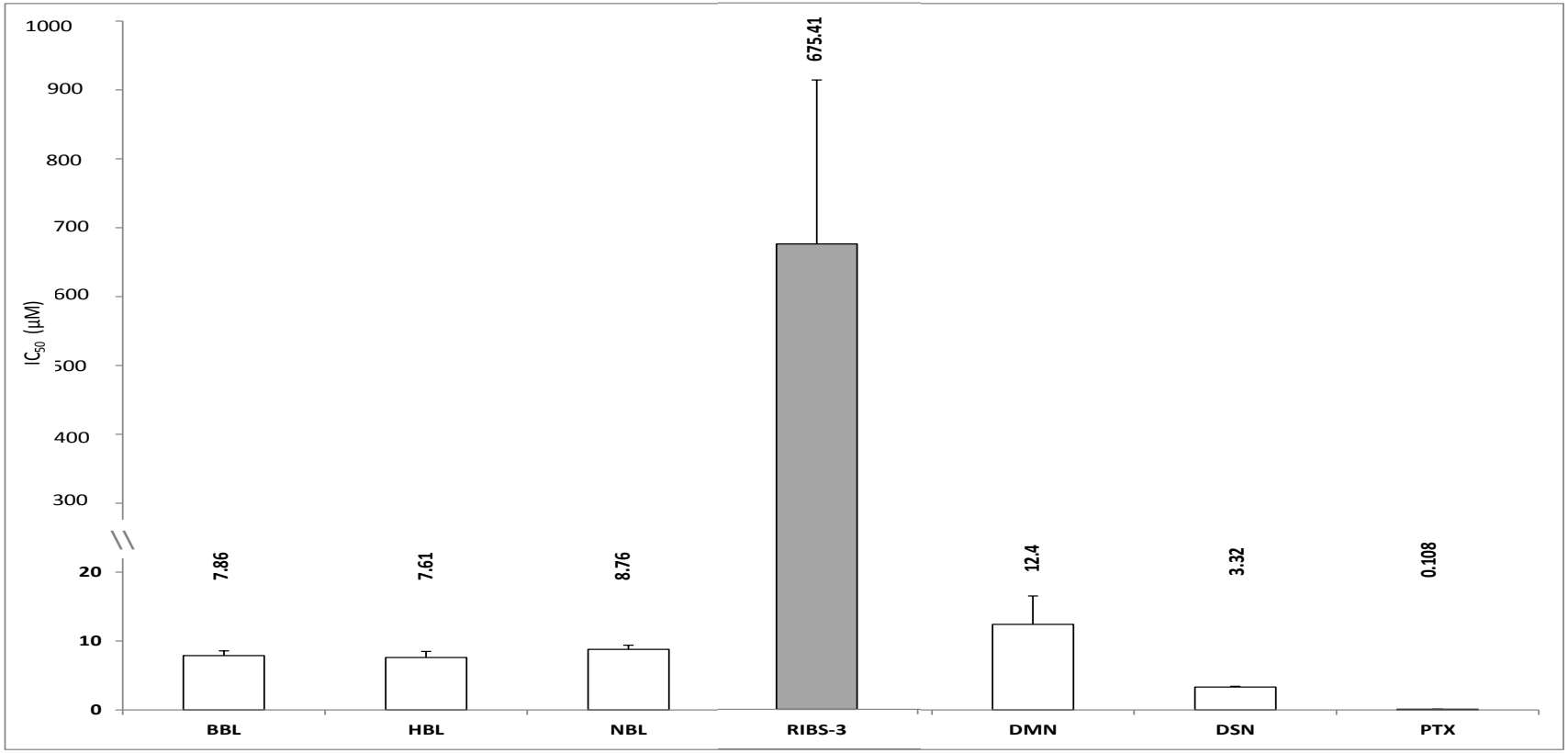


Figure 2.1: Graphical representation of the antiproliferative activities of naphthoquinone and pyrroloiminoquinone alkaloid series of compounds. The IC_{50} concentration of each compound is given above each bar representing each compound. Paclitaxel (PTX) was used a control and was shown to have an average IC_{50} of 0.108 nM in MDA-MB-231 cells. The naphthoquinone series of compounds bromo-β-lapachona (BBL), hydroxy-β-lapachona (HBL) and nor-β-lapachona (NBL) are shaded grey, marine pyrroloiminoquinone series of compounds *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3), damirone C (DMN) and discorhabdin A (DSN) are shaded white. The error bars represent the standard deviation where n = 9. The statistical significance of the differences in percentage survival relative to the DMSO control was calculated using ANOVA. *** denotes treatments where $P \leq 0.05$.

Compounds from the isomeric naphthoquinones, 1, 4 naphthoquinone and 1, 2 naphthoquinone series, showed differential antiproliferative activities in the MDA-MB-231 cell line. The 1, 4 naphthoquinones LAP, αLAP and CAL did not exhibit any antiproliferative activities following treatment at concentrations as high as 500 µM and as a result the IC_{50} values could not be calculated. Treatment with the 1, 2 naphthoquinones BBL, HBL and NBL resulted in IC_{50} values of 7.86 ± 0.854, 7.61 ± 1.068 and 8.78 ± 0.733 µM respectively in the same breast cancer cell line. These antiproliferative activities were found be significant at the $P < 0.05$ level for NBL [$F(1, 4) = 17.1$, $P = 0.01$], BBL [$F(1, 4) = 17.1$, $P = 0.01$] and HBL [$F(1, 4) = 17.1$, $P = 0.01$]. Although the naphthoquinone compounds were only tested in MDA-MB-231 cell line in this study, the same compounds were tested in the MCF-12A cell line in which they were shown to have similar antiproliferative effects to those found in the MDA-MB-231 cell line (Cockburn, Ph.D. thesis, 2013). Similarly, compounds of the naphthoquinone series were shown to have similar antiproliferative effects in the WHCO1 cell line (Sunassee *et al*., 2013). These two findings suggested that the antiproliferative effects of the naphthoquinone series of compounds on the MDA-MB-231 cell line were not necessarily cell-type specific. Furthermore, our findings were similar to those of the study performed in the WHCO1 oesophageal cell line, which showed that the antiproliferative effects of LAP and αLAP were approximately fifteen and eighteen times less than that of BBL, respectively (Sunassee *et al*., 2013). The same study also found similar IC_{50} values for BBL and NBL as those determined in this study (Sunassee *et al*., 2013). Although LAP has been demonstrated to exhibit anti-cancer properties for cancer types including, prostate, cervical and liver cancers (Lee, 2006; Lee *et al*., 2005) it exhibits low plasma concentrations even at high doses and is thus unable to be therapeutically effective when administered (Block *et al*., 1974). It is possible that when taken orally LAP may be metabolized before adequate plasma concentrations are reached as low oral bioavailability is common with drugs that are poorly water-soluble (Matsumoto and Zografi, 1999; Chiou and Riegelman, 1971). The demonstration that LAP displays low oral bioavailability despite satisfying the rule-of-five shows that although it is a good indicator of oral bioavailability, the rule-of-five remains a prediction tool that should be used to support *in vitro* and *in vivo* data as it provides a limited amount of information. It would be of interest to determine whether compounds such as NBL, BBL and HBL, which showed greater antiproliferative activities to LAP in this and a previous study (Sunassee *et al*., 2013) would have improved therapeutic properties compared to LAP and whether the naphthoquinone series of compounds generally display poor oral bioavailability.

The difference in the antiproliferative activities confirmed the prediction that a structure-activity relationship (SAR) may exist for the naphthoquinone series of compounds. A SAR analysis indicated

that the antiproliferative activity of the naphthoquinone series of compounds against the MDA-MB-231 cell line may have been predominantly influenced by the structural variation in the position of the conjugated carbonyl groups to the naphthalene ring as the 1, 2 naphthoquinones elicited antiproliferative activities in the low micromolar range but the 1, 4 naphthoquinones did not. Further to this, the presence of hydroxyl and bromo-substituents in HBL and BBL, respectively, increased the antiproliferative activity of the 1, 2 naphthoquinones compared to NBL, indicating that the addition of electronegative substituents to the pyran ring, forming a furan ring increased the antiproliferative activity of these compounds even further. However, antiproliferative assays would have to be conducted on compound libraries of naphthalene compounds with single chemical changes between chemotypes to validate these proposals.

The anti-cancer activities of the pyrroloiminoquinone alkaloid family have been described, and their anti-cancer properties thought to be a consequence of their unique highly-fused structures (Delfourne, 2008). The marine pyrroloiminoquinone compounds MKM and RIBS-1 did not display antiproliferative activities in breast cancer cells treated up to concentrations of 500 µM and appeared to increase cell growth. Cells treated with RIBS-3, DMN and DSN of the same series of compounds displayed IC_{50} values of 678.4 ± 238.2, 12.4 ± 4.13 and 3.32 ± 0.111 µM respectively. Statistical analysis using ANOVA indicated that these IC_{50} values were significant for treatments with RIBS-3 [$F(1, 4) = 9.18$, $P = 0.04$], DMN [$F(1, 4) = 17.0$, $P = 0.01$] and DSN [$F(1, 4) = 17.1$, $P = 0.01$] The discorhabdin family, which contain azacarbocyclic spirodienones in addition to pyrroloquinoline rings, has been shown to have high antiproliferative activities in several cell lines (Antunes, 2005a). In this study DSN was the found to be the most toxic compound within the pyrroloiminoquinone alkaloid series and indeed of all the compounds investigated. DMN displayed an IC_{50} value of 12.4 µM, and interestingly the incorporation of additional functional groups to the pyrolloimoquinone group resulting in the compounds MKM and RIBS-1 resulted in the loss of antiproliferative activity for these compounds. This SAR analysis suggested that the antiproliferative activity of these compounds is derived from the pyrroloquinoline ring and that any additional functional group to this moiety may have resulted in the diminished antiproliferative activity with the exception of the addition of an azacarbocyclic spirodienones for the compound DSN.

The IC_{50} values determined in this study indicated that the concentrations necessary to produce antiproliferative activities for the compounds under investigation fall within the low micromolar range. As a nanomolar range is preferable for the development of lead candidates to drug candidates there may be a need for the synthetic modification of these compounds in order to

improve their antiproliferative activity profile. It would be important to determine and compare the IC_{50} values of all the compounds analysed in this study in several cancer cell lines and non-cancerous cell lines to allow for the identification of compounds that are bio-selective; that is compounds with the ability to elicit at least a five-fold higher inhibitory effect in cancer cell lines than in normal cell lines (Dumont *et al*., 2007). Although a structural similarity-based screening was performed in this study, an *in silico* ligand docking approach would have complemented the findings of this study. This approach would have been particularly advantageous in terms of compounds of the marine pyrroloiminoquinone alkaloid series of compounds as there are no reports within the literature regarding their interaction with Hsp90. This approach would have allowed for the preliminary identification of functional groups important for Hsp90 binding and thus would allow for the development of analogues of compounds with higher Hsp90 binding affinities. The *in silico* ligand docking approach would have been advantageous as it would provide a more reliable predication of compound binding to Hsp90. Furthermore, this approach would allow for the sites of interaction to be determined and predict the domains with which the compounds could potentially interact. Nonetheless, the screening approach detailed in this dissertation has allowed for a structure-activity relationship to be determined for the compounds described in this study.

2.5 Conclusions

In conclusion, all potential modulators of Hsp90 screened in this study passed Lipinski's rule-of-five, and it was interesting to note that the commercially available Hsp90 inhibitors GA and NOV both failed the same test reiterating the need to support *in silico* data with *in vitro* and *in vivo* data. A SAR was developed from the structural similarity-screen utilised in this study to characterise the compounds under investigation. This screening method was used to predict the cell biological and biophysical effects of the compounds analysed in this study on Hsp90. It was predicted that in addition to the variation in the position of the conjugated carbonyl groups, the biological and biophysical activities of the naphthoquinone compounds would be based on the conformational similarity that existed within the compounds. It was thus predicted that the 1, 2 naphthoquinones BBL, HBL and NBL would exhibit similar biological activities to each other and that the 1, 4 naphthoquinone compounds LAP, CAL and αLAP, would exhibit biological activities similar to each other. As the 1, 4 naphthoquinone scaffold has been suggested to be a new class of Hsp90 inhibitor, it was predicted that the compounds LAP, CAL and αLAP would bind to Hsp90 and modulate the cell biological activities of this protein. A SAR was identified for the marine pyrroloiminoquinone series of compounds and it was thought that the additional of certain functional groups to the

pyrroloquinoline ring may have resulted in diminished antiproliferative activities. It was predicted that the effects of the compounds DMN, RIBS-1 and RIBS-3 on Hsp90 would be similar, and that DSN and GA would exhibit distinct effects on Hsp90. The conformational similarity of SQA, MKM, and NOV resulted in the prediction that these three compounds may exert similar Hsp90 modulatory properties.

CHAPTER 3

SCREENING OF THE MARINE ALGAL COMPOUND SARGAQUINOIC ACID FOR MODULATION OF HSP90

3.1. Introduction

Various secondary metabolites have been described from more than 60 species of algae belonging to the *Sargassum* genus (*Sargassaceae, Fucale*s) (Blunt *et al*., 2008). Several pre-clinical studies on small molecules isolated from these species have revealed different physiological and biological activities including anti-tumour (Dias *et al*., 2005), anti-angiogenic (Dias *et al*., 2005), anti-coagulant (Athukorala *et al*., 2007) and anti-viral (Zhu *et al*., 2006) activities. *S. siliquosum* J. G. Agardh has recently been found to possess significant free radical scavenging activity against hydroxyl, hydrogen peroxide and nitric oxide free radicals thus suggesting a therapeutic use for compounds isolated from this species in the prevention of diseases such as cardiovascular and neurodegenerative diseases, diabetes and cancer (Corpuz *et al*., 2013). Plastoquinone and plastohydroquinone compounds isolated from brown algae include sargaquinone, sargahydroquinoic acid and SQA (Reddy and Urban, 2009; Afolayan *et al*., 2008). These two families of compounds differ primarily in the linear terpene chain moiety which has hydroxyl and carbonyl groups in various positions. SQA comprises a 2-methyl,-1, 4, benzoquinone with an isoprenoid side chain in position 5. Both SQA and SQHA are thought to be potential lead compounds against diabetes as they are the active compounds of *S.yezoense* which has been demonstrated to have beneficial effects on glucose and lipid metabolism by activating both peroxisome proliferator-activated receptors A and peroxisome proliferator-activated receptors C (Kim *et al*., 2012). SQA has also been reported to be a neuroactive substance that promotes neurite outgrowth and the survival of rat pheochromocytoma cells (Tsang *et al*., 2005; Tsang and Kamei, 2004) and displays moderate acetylcholinesterase inhibitory activity thus identifying this compound as a potential drug candidate in the treatment of Alzheimer's disease (Choi *et al*., 2007). SQA has also been shown to induce apoptotic effects within the HaCaT human keratinocyte line, (Hur *et al*., 2008) and MDA-MB-231 breast cancer cell line, in which it causes G1 arrest (de la Mare *et al*., 2012). This compound displays weak antimicrobial activity against *Bacillus subtilis* and moderate anti-tumour activity (Reddy and Urban, 2009). More recently, SQA was suggested to have therapeutic applications in inflammatory diseases as it prevented nitric oxide production in macrophages via the blockade of nuclear factor kappa-light-chain-enhancer of activated B cells activation (Kang *et al*., 2013). As a result of the varied attractive therapeutic properties and quinone structure of this compound, the efficacy of SQA as an Hsp90 modulator was investigated.

The specific objectives to this study were to investigate the ability of SQA, NOV and 17-AAG to modulate the subcellular localisation and expression levels of Hsp90, Hsp70, Hop, and selected Hsp90 client proteins in the MDA-MB-231 breast cancer cell line. Hsp90 is known to reside predominantly in the cytoplasm (Gething and Sambroock, 1992) but has also been shown to localise to the nuclei of both unstressed and stressed cells (Gasc *et al*., 1990; Sanchez *et al*., 1990; Collier and Schlesinger, 1986) as well as breast cancer cells (Yano *et al*., 1996). The Hsp90 cochaperone Hop has previously been reported as a protein that resides mainly in the cytoplasm (Longshaw *et al*., 2004). However, it has also been observed within the nuclei of cells (Lässle *et al.*, 1997) and shows increased nuclear localisation upon cell cycle arrest at G1/initiation of DNA synthesis phase (Longshaw *et al.*, 2004). More recently, Hop was shown to colocalise with actin in lamellipodia in the Hs578T breast cancer cell line (Willmer *et al*., 2013). Hsp70 is known to be predominantly localised to the cytoplasm of cells but has been shown to accumulate with the nuclei of cells under stress conditions (Diehl *et al.*, 2003; Chu *et al.*, 2001; Nollen *et al.*, 2001).

The ability of SQA, NOV and 17-AAG to induce a stress-response was investigated through analysing changes in HSF1 localisation following compound treatment. HSF1 is known to be expressed in the cytoplasm and is shuttled into nucleus upon HS and cellular stress conditions and localises in stress granules within the nucleus (Jolly *et al*., 2002; Cotto *et al*., 1997). Several Hsp90 inhibitors have been shown to stimulate a stress-response. For example, GA stimulates a stress-response by inducing the dissociation of complexes HSF1 from Hsp90 (Whitesell *et al*., 2003) and increasing the stability of active HSF1 trimers during HS and delaying their disassembly during recovery (Conde *et al*., 2009). The effects of NOV on HSF1 are thought to be mediated through changes in Hsp90 autophosphorylation rather than the dissociation of HSF1 from Hsp90 (Conde *et al*., 2009). The ability of SQA to bind to and interact with Hsp90β was investigated through ITC and saturation-transfer difference nuclear magnetic resonance (STD NMR) and the ability of SQA treatment to disrupt the association of Hsp90 and Hop *in vitro* was evaluated and compared to that of GA and NOV treatment.

3.2. Materials

The MDA-MB-231 (ATCC HTB-26) breast cancer cell line was provided by Dr Sharon Prince (University of Cape Town, South Africa). General reagents were purchased from Sigma-Aldrich (Germany) or Saarchem (Merck, South Africa). Tissue culture reagents (Dulbecco's Modified Eagle Medium [DMEM] with GlutaMAX™-I, FCS, 10 x Trypsin-EDTA and

Penicillin/Streptomycin/Amphotericin) were from Gibco, Invitrogen (Paisley, United Kingdom) and Biowhittaker (United Kingdom). Tissue culture plasticware was from Corning Incorporated (United States of America). Hybond nitrocellulose membrane was from Bio-Rad (United Kingdom). Mouse anti-Hsp90α/β [F-8] (cat no: sc-13119), mouse anti-Hsp70/Hsc70 [W27] (cat no: sc-24), rat anti-HSF1 [10H8] (cat no: sc-13516), rabbit anti-AKT1/2/3 [H-136] (cat no: sc-9312), rabbit anti-STAT3 [H-190] (cat no: sc-7179), mouse anti-pSTAT3 [B7] (cat no.: 8059) antibodies were from Santa Cruz Biotechnology (United States of America). Mouse anti-Hop [STIP, p60] (cat no.: DS14F5) was from Stressgen, United States of America. Rabbit anti-histone H3 (cat no.: 9715) was from Cell Signaling Technology (United States of America). Rabbit anti-actin (cat no.: A2103), Alexa® Fluor 488 donkey anti-mouse IgG [H+L] (cat no.: A21202), Alexa® Fluor 633 donkey anti-goat IgG [H+L] (cat no.: A21082), Alexa Fluor® 555 IgG [H+L] donkey anti-rabbit (cat no.: A31572) antibodies were from Invitrogen (Paisley, United Kingdom). Mouse anti-fibrillarin [38F3] (cat no.: ab4566) antibodies were from Abcam (United Kingdom). Zeba™ Spin Desalting Columns 7K MWCO 0.5 ml, Hoechst 33342 solution (cat no.: 62249) was from ThermoScientific (United States of America). Goat anti-mouse IgG F (ab') 2 Polyclonal Antibody, HRP (cat no.: 474 1806) was purchased from Kirkegaard and Perry Laboratories (United States of America). Donkey anti-rabbit IgG polyclonal antibody, HRP (cat no: NA 934V) and the Enhanced Chemiluminescence™ kit (cat no.: RPN2106) were purchased from GE Healthcare (United Kingdom). Amicon Ultra Centrifugal filters-15 Ultracel® 30K were from Millipore (Ireland). Recombinant human Hsp90β protein (cat no.: SPR-102) and recombinant human Hop protein (cat no.: SPR-302) were purchased from Stressmarq (Canada). GA (cat no.: 30562-34-6) was purchased from BioMol International (United States of America). 17-AAG (cat no.: A8476-500UG), 17-DMAG (cat no.: D5193-1MG) and NOV (cat no.: N1228-25G) were purchased from Sigma-Aldrich (Germany). Manufacturers or suppliers of any other specialised reagents are referenced in-text.

3.3. Methods

3.3.1. *Cell culture and maintenance of cell lines*

The MDA-MB-231 cell line was cultured as described in Section 2.3.1.

3.3.2. *Immunofluorescence microscopy analyses of the Hsp90 complex*

Cells were seeded at a density of 0.5 x 10^5 cells per well onto ethanol-sterilised coverslips in a 24-well plate. Cells were allowed to adhere overnight and 17-AAG, NOV or SQA was added to the wells

of each plate at the IC_{50} concentrations of 200 nM, 260 µM and 90 µM respectively. 17-AAG was selected as a positive control of an N-terminal Hsp90 inhibitor and NOV as a positive control of a C-terminal Hsp90 inhibitor. Cells were treated for 5 hours with each compound, as this was found to be an optimal time for treatment with the compounds prior to analysis, and incubated at 37 °C, 10 % CO_2. Control cells were treated with DMSO at an amount equivalent to that of the highest concentration of compound used. In each instance, the DMSO concentration did not exceed 0.1 % of the final volume in media. An HS treatment was performed in which cells were incubated with media pre-warmed to 42 °C for 1 hour followed by a recovery phase for 1 hour at 37 °C. Following each treatment, cells were rinsed in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and fixed in ice-cold methanol. Cells were blocked in filter sterilised 1 % (w/v) bovine serum albumin (BSA)/PBS or BSA in PBS containing 0.1 % (v/v) Nonidet-P40 for 30 minutes and incubated with primary antibody at either a 1:100 or 1:50 dilution in 0.1 % BSA/PBS overnight at 4 °C. Cells were washed twice in 0.1 % (w/v) BSA/PBS followed by an incubation step with species specific secondary antibodies conjugated to the appropriate fluorescent dye at 1:1000 dilutions in 0.1 % (w/v) BSA in PBS for 1 hour in the dark with gentle agitation. Cells were washed twice in 0.1 % (w/v) BSA /PBS and a final wash performed with 1 $ug.ml^{-1}$ Hoechst 33342 in distilled water. Negative controls were included to determine the background fluorescence and non-specific staining of the secondary antibodies. Cells were incubated with secondary antibodies conjugated to fluorescent dyes at 1:1000 dilutions in 0.1 % (w/v) BSA in PBS under the same conditions as the test experiments. Coverslips were air-dried and mounted onto microscope slides with Dako fluorescent mounting media (cat no.: S3023) (Dako, United States of America). Microscope slides were evaluated using a Zeiss LSM Meta 510 confocal laser scanning microscope (Carl Zeiss, GmbH, Germany). Images were captured using either the 40x oil immersion or 63 x oil-immersion Plan-Apochromat objectives using excitation laser wavelengths 405 nm (Hoechst 33342) , 488 nm (Alexa Fluor 488), 543 nm (Alexa Fluor 555) and 633 nm (Alexa Fluor 633) as required. Data was analysed using AxioVision 4.8.2 LE software (Carl Zeiss Microscopy GmbH, Germany). Images are representative of at least two independent experiments and three different randomly selected image fields. The number of cells in each sample was recorded, quantified and cells classified as exhibiting either (i) cytoplasmic only, (ii) nuclear only or (iii) cytoplasmic and nuclear localisation patterns.

3.3.3. *Harvesting of cells for expression level analysis*

Cells were seeded out at a density of 1 x 10^6 cells per well directly into 6-well plates. Cells were allowed to adhere overnight and either 17-AAG, NOV or SQA added to each well of each plate at

concentrations of 200 nM, 260 µM and 90 µM respectively. 17-AAG was selected as a positive control of an N-terminal Hsp90 inhibitor and NOV as a positive control of a C-terminal Hsp90 inhibitor. Negative control cells were treated with DMSO at an amount equivalent to that of the highest concentration of compound used. In each instance, the DMSO concentration did not exceed 0.1 % of the final volume in media. Cells were incubated at 37 °C, 10 % CO_2. One set of plates was removed following a 2 hour incubation period and cells were harvested, while the second set of plates was removed after a 24 hour incubation period. Control DMSO treated cells were treated for a 24 hour incubation period. Cells were washed once in PBS, and harvested in 100 µl sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample denaturing and reducing buffer (62.5 mM Tris-HCl, pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 5 % (v/v) β-mercaptoethanol, 0.05 % (w/v) bromophenol blue). Lysates were collected, boiled for 5 minutes and analysed by SDS-PAGE (Laemmli, 1970) and Western analysis (Towbin, 1979).

3.3.4. *SDS-PAGE analysis of proteins*

Proteins were separated by discontinuous SDS-PAGE as described by Laemmli (1970) using a 4 % SDS-PAGE stacking gel (0.5 M Tris-HCl, pH 6.8) pre-cast on top of a 12 % SDS-PAGE resolving gel (1.5 M Tris-HCl, pH 8.8). Equal numbers of cells were loaded into each lane and proteins were separated by electrophoresis at 120 V for approximately 1.5 hours in SDS-PAGE running buffer (0.25 mM Tris, 192 mM glycine, 1% (w/v) SDS) using a Bio-Rad Protean III electrophoresis system (Bio-Rad, United Kingdom). The approximate molecular weight of the sample proteins was estimated by using either peqGOLD Protein Marker II or Protein Marker IV (PEQLAB, Germany).

3.3.5. *Chemiluminescence-based Western analysis of proteins*

Western analysis was performed according to Towbin *et al*. (1979). Proteins were separated by electrophoresis on a discontinuous SDS-PAGE gel. The gel, nitrocellulose membrane and 3 MM Whatman filter paper were pre-equilibrated in ice-cold transfer buffer (25 mM Tris, 192 mM glycine, 20% (w/v) methanol). SDS-PAGE gel and nitrocellulose membrane were placed between sheets of 3 mm Whatman filter paper, placed in a cassette and subjected to electrophoresis using a Bio-Rad Western transfer set (Bio-Rad, United Kingdom) at 100 V for 2 hours. Successful transfer of protein onto the nitrocellulose membrane was confirmed by staining with Ponceau S stain (0.5 % (w/v) Ponceau S, 1 % (v/v) glacial acetic acid) for 1 minute. The nitrocellulose membrane was subsequently destained with distilled water. The nitrocellulose membrane was blocked for 1 hour at room

temperature in blocking solution (5 % (w/v) fat-free milk powder in Tris buffered saline (TBS)-T (50 mM Tris-HCl, 150 mM NaCl, 0.2 % (v/v) Tween-20, pH 7.5 or 1 % BSA in TBS-T) and incubated with specified primary antibodies according to the manufacturer's recommendations in blocking solution overnight at 4 °C. Nitrocellulose membrane was washed twice in TBS-T for 20 minutes each and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody according to the manufacturer's recommendations in blocking solution for 1 hour at room temperature with gentle rocking. Nitrocellulose membrane was washed four times for 15 minutes each and the proteins on the membrane detected with a combination of prepared and commercial chemiluminescence reagents on a Chemidoc™ EQ system (Bio-Rad, United Kingdom). Actin or histone H3 was used as a loading control in each case with the exception of pSTAT3 in which case protein levels were measured against total STAT3. Three independent experiments were performed for each compound. Densitometry analysis for each instance was performed using ImageJ software (MacBiophotonics, Canada) to allow for the comparison of trends between independent experiments.

3.3.6. *Hsp90 expression and purification*

Recombinant hexahistidine-tagged Hsp90 was expressed and purified from C41 *E.coli* cells transformed with a pET28a-based Hsp90β plasmid. Cells were grown in 2 x YT media (1.6 % (w/v) tryptone, 1 % (w/v) yeast extract and 0.5 % (w/v) sodium chloride) supplemented with 100 $\mu g.ml^{-1}$ ampicillin until the cells reached the exponential growth phase when the absorbance at 600 nm was between 0.6 and 0.8. Protein expression was induced at 37 ˚C with 0.5 mM IPTG and the temperature immediately reduced to 28 ˚C. Cell cultures were grown at 28 ˚C overnight with agitation. Cells were harvested by centrifugation at 12227 x *g* for 15 minutes, 4 °C using JLA.8100 rotor in an Avanti™ J-20 Xp1 Centrifuge (Beckman Coulter, United States of America) and pellets re-suspended in cell lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM imidazole, 0.5 % Triton X-100, complete mini EDTA-free protease inhibitor cocktail tablet (cat no: 05 056 489 001, Roche, Germany) (1 tablet per 50 ml buffer, pH 8.0). Cells were sonicated using an UltraSonic Processor (MiSonic Incorporated, United States of America) at power setting 8 for a pulsing time of 15 seconds on and 45 seconds off, for a total pulsing time of 5 minutes at 4 °C. Cell extracts were clarified by centrifugation at 23666 x *g* for 30 minutes, 4 °C using JA 25.50 rotor in an Avanti™ J-20 Xp1 Centrifuge (Beckman Coulter, United States of America). The resulting supernatant was passed through 0.22 µm pore size filters (GVS Filter Technology, United States of America) and loaded onto 3 x 5 ml HisTrap™ HP columns (cat no: 17-5248-01 GE Healthcare, United Kingdom) pre-equilibrated in 50mM Tris pH 8.0 on an ÄKTA Basic™ FPLC™. (GE Healthcare, Sweden). Proteins with a lower

affinity for the column than the hexahistidine-tagged Hsp90 were washed off in 50 mM Tris, 20 mM imidazole, pH 8.0 until the absorbance at 280 nm was less than 0.1. Hexahistidine-tagged Hsp90 was eluted with 50 mM Tris, 300 mM imidazole, pH 8.0 at 2.00 ml.min^{-1}. Protein fractions containing Hsp90 were collected and dialysed in 20 mM Bis-Tris pH 6.0 overnight. The dialysed protein was filtered through 0.22 µm pore size filtered and loaded onto a MonoQ 10/10 ion exchange column (GE Healthcare) pre-equilibrated with 20 mM Bis-Tris pH 6.0 on an ÄKTA Basic ™ FPLC™. Proteins were eluted with a linear gradient of 0 -100 % 20 mM Bis-Tris, 1 M sodium chloride) pH 6.0 for 90 minutes with a flow rate of 1.00 ml.min^{-1}and fractions containing Hsp90 collected and pooled together. Hsp90 was dialysed overnight in 50 mM Tris, 1 mM dithiothreitol (DTT), pH 7.4, concentrated by centrifugation, snap-frozen in liquid nitrogen and stored at -80 °C until required.

3.3.7. *Isothermal titration calorimetry analyses*

ITC experiments were performed with assistance from Ms Soumya Daturpalli (University of Cambridge, United Kingdom) in a VP-ITC MicroCalorimeter (MicroCal Inc., Northampton, Massachusetts, United States of America) at 30 °C. Purified Hsp90β protein was dialysed in ITC buffer (50 mM Tris, 6 mM $MgCl_2$ 20 mM KCl, 1 mM β-mercaptoethanol, pH 7.4) overnight prior to use. The dialysate was kept for the preparation of the ligand to ensure samples were in identical buffer solutions. The concentration of dialysed Hsp90β was determined by subtracting the absorbance value at 280 nm from that at 320 nm and using the extinction coefficient 53360 M^{-1} cm^{-1}. Ligand and protein solutions were degassed for 10 minutes immediately prior to use. 17-DMAG was selected as a positive control for a compound binding to Hsp90 as it is water soluble. Purified Hsp90 was loaded into the sample cell and the concentration of the ligand to be injected was at least twenty times in excess of the protein for compounds with unknown Hsp90 binding capacities and fifteen times in excess for 17-DMAG. The reference cell contained the same buffer used for protein and ligand preparation and sample and reference cells contained equivalent amount of DMSO as the ligand. An initial injection of 2 µl was followed by further titrations of 10 µl volumes of ligand solution from the syringe into the sample cell. Intervals of 180 seconds were used between injections. The change in binding enthalpy (ΔHb), binding constant (Kb) and binding stoichiometry (n) were determined using Origin 7.0 software. Following the optimisation of the system, the experiment was performed once for each compound.

3.3.8. *STD NMR of Hsp90β with SQA*

STD NMR experiments were performed in collaboration with Dr Kevin Lobb (Department of Chemistry, Rhodes University). 1 mg SQA was prepared in deuterated DMSO (Sigma, United States of America). Hsp90 was buffer exchanged into PBS using Amicon ultracentrifugation 2 ml filters (Ireland). 10 µM Hsp90β and SQA were used and STD NMR was performed in a Bruker Avance III spectrometer. STD experiment was run for 6656 scans with a saturation time of 2s. The protein was saturated at 0.85ppm for on resonance, and 20ppm was used for reference (off resonance). The pseudo 2-d experiment was processed and analysed using the std_split auxiliary program within Topspin 2.1. Following the optimisation of the system, the experiment was performed once.

3.3.9. *Hsp90-Hop conventional pulldown assays*

A total of 1 µg of commercial recombinant hexahistidine-tagged Hop protein was incubated with 10 µl nickel chelated sepharose fast flow bead suspension (GE Healthcare, Sweden) per pulldown assay for 2 hours at 4 °C with gentle rocking. A total of 1 µg commercial Hsp90β was incubated with different concentrations of SQA, GA, NOV or DMSO (vehicle control) at 30 °C for 30 minutes in 100 µl incubation buffer (10 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.01% Nonidet P-40, pH 7.5). GA was selected as a positive control of an N-terminal Hsp90 inhibitor and NOV as a positive control of a C-terminal Hsp90 inhibitor. The pH of the buffer following the addition of the compounds was tested to ensure that the addition of the compounds did not alter the pH of the solution. Compound-treated Hsp90β protein and bead-bound Hop protein were mixed and incubated together for a further 1 hour at 4 °C with gentle rocking. Following incubation beads were collected by centrifugation and washed three times with incubation buffer. The resulting bead suspension was boiled in 30 µl SDS-PAGE sample buffer to release proteins. Protein complexes were resolved by SDS-PAGE (Laemmli, 1970) and analysed by silver staining using the PageSilver™ Silver Staining Kit (Fermentas, EU) according to the manufacturer's instructions. Densitometry analysis was performed using ImageJ software (MacBiophotonics, Canada) with the ratio of Hsp90β to Hop for compound-treated samples expressed relative to the ratio of the two proteins in the DMSO control to allow for the comparison of trends between independent experiments. Three independent experiments were performed for each compound.

3.4. Results and Discussion

3.4.1. *Determination of the effects of SQA, NOV and 17-AAG on Hsp90, Hsp70, Hop and HSF1 localisation in a triple negative breast cancer cell model*

The analysis of the subcellular localisation of proteins allows for the high-throughput screening of compounds that may target a specific protein within cells. The redistribution of the target protein to a different region of the cell could suggest that an interaction between the compound and the protein has occurred. Therefore, confocal microscopy was utilised to study the ability of SQA to modulate the subcellular localisation of Hsp90 (Appendix Figure B.1), Hop (Appendix Figure B.2), Hsp70 (Appendix Figure B.3) and HSF1 (Appendix Figure B.3) in the triple negative MDA-MB-231 breast cancer cell line. Changes in the localisation of the target proteins in response to compound treatment were compared to those caused by treatment with the N- and C-terminal Hsp90 inhibitors 17-AAG and NOV, respectively. Thus, the compounds of interest could be compared to either N- or C- terminal inhibitor to determine which mode of Hsp90 inhibition they more closely resembled. A HS treatment was selected as a positive control to be an indicator of a stress-response within the cells and to provide changes in localisation that were not the result of a direct interaction of a compound with the protein of interest. Cells were treated with each of the compounds for five hours as that time period was found to be optimal to observe changes in localisation at the given concentrations without affecting cell viability. DMSO was used as a vehicle control and the final concentration of DMSO did not exceed 0.1 % in any instance. Nuclei were visualised with Hoechst 33342 and wheat germ agglutinin (WGA) was employed as a stain of the plasma membrane (Gonatas *et al.,* 1979) and Golgi apparatus (Parkkinen *et al*., 1998; Guasch *et al*., 1995). Images were captured on a Zeiss LSM Meta 510 confocal laser scanning microscope (Appendix C) and a quantitative analysis performed. Negative controls were included to determine the background fluorescence and non-specific staining of the secondary antibodies used in this study. Furthermore, the immunofluorescence protocol used in this study has been well validated and both the primary and secondary antibodies selected for use in this study are routinely used within the Biomedical Biotechnology Research Unit (BioBRU) and do not produce non-specific staining.

Cells were classified as showing either cytoplasmic only staining, nuclear only staining or both cytoplasmic and nuclear staining. A quantitative assessment was performed by manually counting the number of cells showing a particular localisation pattern (Figure 3.1). The percentage of cells

displaying each particular localisation in three separate microscope fields, for two independent experiments was calculated.

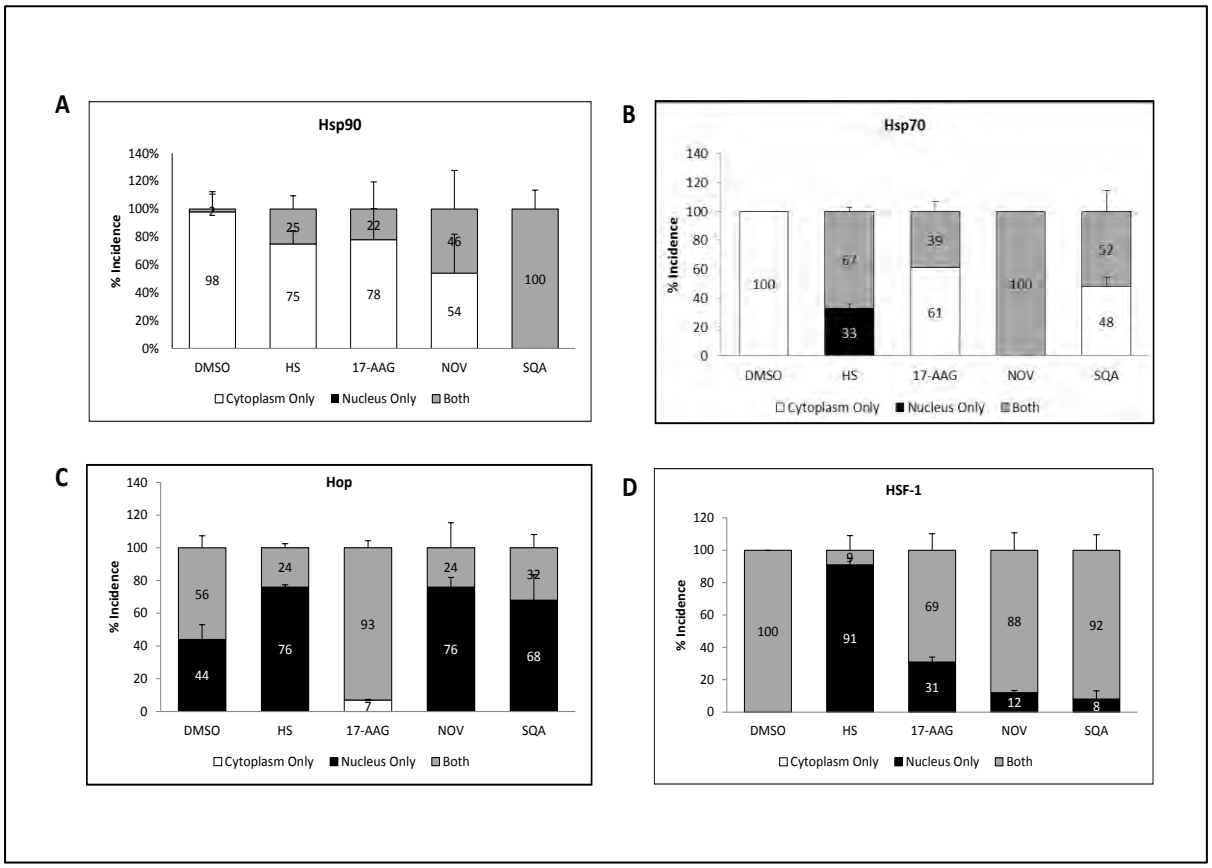


Figure 3.1: Quantitative analysis of the subcellular localisation patterns of (A) Hsp90, (B) Hsp70, (C) Hop and (D) HSF1 following treatment with sargaquinoic acid (SQA), 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) and novobiocin (NOV). The number of cells exhibiting, cytoplasmic only (white), nuclear only (black) or both nuclear and cytoplasmic (grey) staining were quantified by counting the cells in three separate microscope fields for two independent experiments.

The localisation analysis showed that DMSO treatment resulted in a mostly cytoplasmic Hsp90 staining pattern with 98 % of cells showing cytoplasmic staining only, 2 % showing both cytoplasmic and nuclear staining with no cells showing nuclear staining only (Figure 3.1 A). HS, 17-AAG, NOV and SQA treatment resulted in an increase in the percentage of cells showing both cytoplasmic and nuclear staining with treatments showing percentages of 25 %, 22 %, 46 % and 100 % respectively when compared to the 2 % of DMSO treatment (Figure 3.1 A). It was found that Hsp90 inhibition by 17-AAG and NOV resulted in an accumulation of nuclear Hsp90 compared to DMSO treated cells and that this increase in nuclear Hsp90 was similar to HS treatment (Appendix Figure B.1). A punctate Hsp90 nuclear localisation pattern, distinct from HS, 17-AAG and NOV nuclear localisation patterns, was observed in response to SQA treatment (Appendix Figure B.1 B). This SQA-induced nuclear

pattern was suggestive of the presence of Hsp90 within structures described as nuclear speckles. Nuclear speckles are sub-nuclear structures thought to be clusters of interchromatin granules, with functions proposed to include the assembly and/or modification of pre–messenger RNA splicing factors (Spector and Lamond, 2010). Nuclear speckles are formed and seen in cells upon the aggregation of ribonucleoproteins (RNPs) with particles containing protein splicing factors (Bohmann *et al*., 1995). It was thought that SQA treatment may have resulted in a change in gene expression that induced an interaction between Hsp90 and RNPs. Two studies that indicated that Hsp90 was involved in the regulation of the assembly of certain RNPs within nuclear speckles provide basis for this assumption (Boulon *et al*., 2008; Zhao *et al*., 2008). In presenting these data it is acknowledged that further analyses need to be performed to exclude the possibility that this specked pattern is not the result of Hsp90 aggregation within the nuclei of the cells or a highly complexed form of Hsp90.

DMSO treatment resulted in 44 % of the observed cells showing a predominantly nuclear Hop staining and 56 % showing both a cytoplasmic and nuclear staining pattern (Figure 3.1 B). HS, NOV and SQA treatments all resulted in an increase in the incidence of Hop localised within the nuclei only (76 %, 76 % and 68 % respectively) (Figure 3.1 B). This increase in nuclear Hop corresponded with increased nuclear Hsp90 for the same treatments (Figure 3.1 B). Interestingly, treatment with 17-AAG resulted in a decrease in the number of cells showing nuclear Hop only (0 %) and an increase in the number of cells showing cytoplasmic only (7 %) and both cytoplasmic and nuclear Hop (93 %). This indicated that the effects of SQA on the modulation of the localisation of Hop were more similar to that of NOV than of 17-AAG treatment. However, this modulation in the localisation of Hop by SQA was also similar to that caused by HS, which raised the possibility that it may have been as a result of the induction of a stress-response (Appendix Figure B.2).

DMSO treatment resulted in a predominantly cytoplasmic localisation of Hsp70 in 100 % of the examined cells (Figure 3.1 C). An increase in the proportion of cells exhibiting both nuclear and cytoplasmic staining was observed following HS, 17-AAG, NOV and SQA treatments with increases of 67 %, 39 %, 100% and 52 % of cells showing both cytoplasmic and nuclear Hsp70 respectively (Figure 3.1 C). 33 % of cells treated with HS showed Hsp70 exclusively in the nucleus while 17-AAG and SQA maintained a proportion of cells with a cytoplasmic only Hsp70 staining pattern of 61 % and 48 % respectively (Figure 3.3 B). The increases in nuclear Hsp70 for SQA were similar to those of both NOV and 17-AAG, and dissimilar to that caused by HS treatment.

Changes in the localisation of HSF1 in response to SQA were assessed and compared to those caused by DMSO, HS, NOV and 17-AAG treatment. Changes in HSF1 localisation were assessed to confirm that the changes in the localisation of Hsp90, Hsp70 and Hop in response to SQA treatment were more similar to those of 17-AAG and NOV and dissimilar to those of the stress-response caused by HS treatment. Treatment with DMSO resulted in the cytoplasmic and nuclear HSF1 staining of 100 % of cells studied. Treatment with HS, 17-AAG, NOV and SQA resulted in a decrease in the number of cells showing both cytoplasmic and nuclear staining to 8%, 69 %, 88 %, and 92 % respectively (Figure 3.1 D). An increase in the number of cells showing HSF1 localised predominantly to the nucleus was observed from 0 % following DMSO treatment to 91 %, 31 %, 12 % and 8 % following HS, 17-AAG, NOV and SQA treatments respectively. Although an increased number of cells showing nuclear only HSF1 staining was observed following SQA treatment (12 %) compared to DMSO treatment (0 %), this proportion was still less than that following HS treatment (91 %). This suggested that the changes in protein localisation by SQA were distinct from those of the stress-response induced by HS treatment. However, more specific and sensitive techniques such as the staining of inducible-specific Hsp70 isoforms or qualitative RT-PCR to measure HS messages would need to be performed to confirm whether the changes in protein localisation in response to SQA were specific and not caused by a general stress-response. Further to this observation, the data collected indicated that modulation of HSF1 localisation by SQA was most similar to that of the C-terminal inhibitor NOV than that of the N-terminal inhibitor 17-AAG (Appendix Figure B.3).

3.4.2. *Determination of effects of SQA, 17-AAG, NOV on the localisation of the client proteins AKT, STAT3 and pSTAT3 in a triple negative breast cancer cell model*

The ability of 17-AAG, NOV or SQA to modulate the subcellular localisation of the Hsp90 client proteins AKT, STAT3 and pSTAT3 was investigated and compared to the changes in the localisation of the same proteins caused by the N- and C-terminal Hsp90 inhibitors 17-AAG and NOV, respectively. Both N-and C-terminal Hsp90 inhibitors were used to allow for the comparison of the previously untested compounds to the known Hsp90 inhibitors to determine which mode of inhibition the untested compounds more closely resembled. Cells were treated with compounds for the optimal time period of five hours and DMSO was used as a vehicle control. The final concentration of DMSO did not exceed 0.1 % in any instance. A HS treatment was included as a positive control to indicate changes in protein localisation caused by a general stress-response within the cells. Cells were stained separately for AKT and dual stained for STAT3 and pSTAT3 for each experiment and nuclei

visualised with Hoechst 33342. Cells were classified as showing either cytoplasmic only staining, nuclear only staining or both cytoplasmic and nuclear staining. A quantitative assessment was performed by manually counting the number of cells showing a particular localisation pattern (Figure 3.2). The percentage of cells displaying each particular localisation in three separate microscope fields, for two independent experiments was calculated.

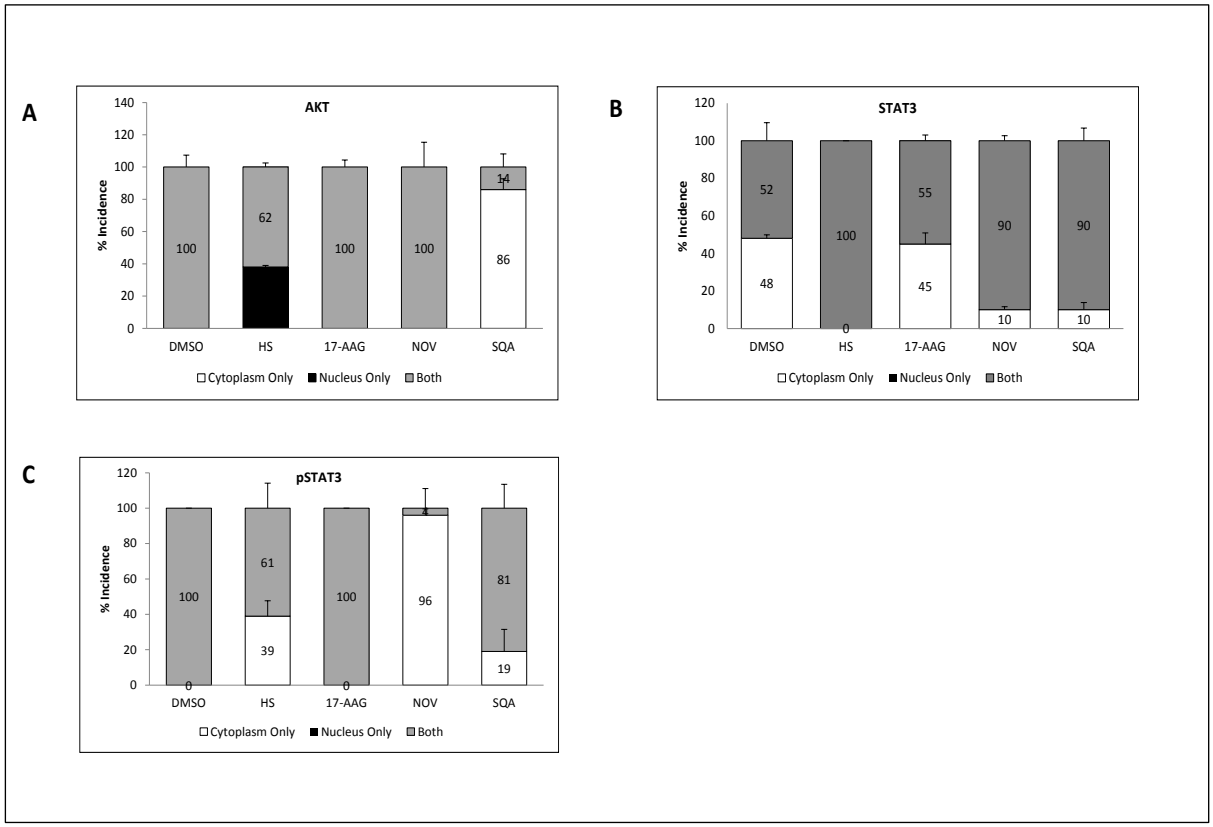


Figure 3.2: Quantitative analysis of the subcellular localisation patterns of (A) AKT, (B) STAT3, (C) pSTAT3 following treatment with sargaquinoic acid (SQA), 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) and novobiocin (NOV). Number of cells exhibiting cytoplasmic only (white), nuclear only (black), or both nuclear and cytoplasmic (grey) staining were quantified by counting the cells in three separate microscope fields for two independent experiments.

Treatment with DMSO, 17-AAG and NOV resulted in both cytoplasmic and nuclear AKT staining pattern in all cells observed (100 %) (Appendix Figure B.6). A total of 38 % of cells showed a primarily nuclear only AKT staining pattern following 17-AAG treatment and 86 % of cells showed a cytoplasmic only staining following treatment with SQA (Figure 3.2 A). Observations also indicated that 38 % of the cells treated with HS showed nuclear only AKT staining and 62 % both cytoplasmic and nuclear staining, compared to DMSO treatment which showed 100 % cytoplasmic staining. The morphology of the cells following HS treatment was found to change and the cells appeared to be

more spindle-like in shape when compared to the characteristic epithelial-like morphology of MDA-MB-231 cells. The localisation of the Hsp90 client protein AKT remained largely unmodulated following 17-AAG and NOV treatments as both treatments produced phenotypes similar to that of the DMSO control with AKT localised to the cytoplasm, nuclei and accumulated to one side of the nuclei. SQA produced a unique phenotype, distinct to that caused by DMSO and HS treatment and resulted in less nuclear AKT and an accumulation of AKT staining to one side of the cells. Anti-pan-AKT antibodies were used to detect all three AKT isoforms and study their function and as such our findings are in agreement with those of Santi and Lee (2010) which showed AKT1 localising to the cytoplasm, AKT2 to the cytoplasm and mitochondria and AKT3 to the nucleus and nuclear membrane. Although this study did not set out to prove that the AKT localised to the sides of the nuclei were localised within the mitochondria, the similarity in the staining pattern shown in this study and of that shown by Santi and Lee (2010) would suggest that this AKT was localised to the mitochondria of cells. Santi and Lee (2010) illustrated how both single and double AKT 1, 2 or 3 isoform knockdown did not alter the localisation of the remaining AKT isoforms in MDA-MB-231 cells. Therefore, as all treatments in this study resulted in cytoplasmic, nuclear and putative mitochondrial staining patterns it would be interesting to perform a similar analysis with antibodies specific to each AKT isoform to determine whether the compounds have greater specificity and efficacy against specific isoforms of AKT. This statement is supported by the apparent decrease in nuclear and putative mitochondrial AKT following SQA treatment.

STAT3 was found to be localised to the cytoplasm (48 %) and to both the cytoplasm and nuclei (52 %) of DMSO treated cells. HS treatment resulted in both cytoplasmic and nuclear staining of STAT3 in all cells studied (Figure 3.2 B). Treatment with SQA produced a STAT3 staining phenotype most similar to that of NOV treatment. Both SQA and NOV treatments resulted in an increase in the number of cells showing both cytoplasmic and nuclear STAT3 staining when compared to the DSMO treated cells. 17-AAG treatment did not result in an observable change in STAT3 localisation when compared to DMSO treated cells. In terms of pSTAT3, DMSO and 17-AAG treatments resulted in similar staining patterns with 52 % and 56 % of cell showing both cytoplasmic and nuclear staining and 48 %and 45 % of cells showing cytoplasmic only staining respectively. Interestingly, NOV and SQA treatments did not produce similar pSTAT3 localisation patterns despite resulting in similar STAT3 patterns. Instead SQA appeared more similar to HS treatment and NOV treated cells appeared unique among the treatments as the only compound to produce predominantly cytoplasm only pSTAT3 staining. Additionally, the presence of putative nucleolar STAT3 staining within the cells was observed (Appendix Figure B.4). This population of STAT3 was localised within distinct foci of the nuclei which excluded the nuclear binding dye Hoechst 33342. The identity of these regions within

the nuclei was confirmed to be nucleoli through the localisation of the nucleoli protein, fibrillarin (Appendix Figure B.5). STAT3 has previously been shown to localise to the cytoplasm and nuclei (Hou *et al*., 2007; Huang, *et al* 2007; Hevehan *et al*., 2002) and nuclear bodies (Hermann *et al*., 2003) and recently been shown to localise to the nucleoli of Jurkat cells (Jarboui *et al*., 2011). The same study performed in Jurkat cells showed that the nucleolar localisation of STAT3 was not constitutive as only 35 % of cells displayed the particular localisation pattern and concluded that this nucleolar localisation may be regulated by factors and conditions such as post-translational modifications, cell cycle stage or cellular stress (Jarboui *et al*., 2011). Interestingly, for all compound treatments in this study pSTAT3 was excluded from the nucleoli (Appendix Figure B.5). Unphosphorylated STAT3 is known to shuttle between the cytoplasm and nuclei of cells (Reich *et al*., 2006), and to play an important role in transcription in response to cytokines and in cancer cells by mechanisms distinct from those underlying pSTAT3 (Yang and Stark, 2008). However, the role of this unphosphorylated STAT3 within in the nucleoli of cells remains to be shown.

3.4.3. *Determination of effects of SQA, NOV and 17-AAG on the expression of Hsp90, Hsp70, Hop and selected client proteins*

As Hsp90 inhibition typically results in the degradation of oncogenic client proteins, the potential of SQA to decrease the expression of AKT, STAT3 and pSTAT3 in a manner similar to either 17-AAG or NOV in a breast cancer cell line was investigated by Western analysis. The ability of SQA to modulate the expression of Hsp90, Hsp70 and Hop in comparison to 17-AAG and NOV was also investigated. Cells were treated with the equivalent volume of DMSO used at the highest compound concentration and as such DMSO treated cells were taken as a negative control to show the presence and level of the selected proteins within untreated cells. The final concentration of DMSO did not exceed 0.1 % in any instance. Cells were treated for 24 hours with DMSO and for 2 and 24 hours with 17-AAG (200 nM), SQA (90 µM) and NOV (260 µM). The treated cells were collected and lysates resolved on a 4 % SDS-PAGE stacking gel pre-cast on top of a 12 % SDS-PAGE resolving gel by SDS-PAGE and Western analysis. Protein levels between DMSO treated cells and compound-treated cells were compared to each other with actin acting as a loading control, to determine the effects of each compound on the expression of the proteins of interest.

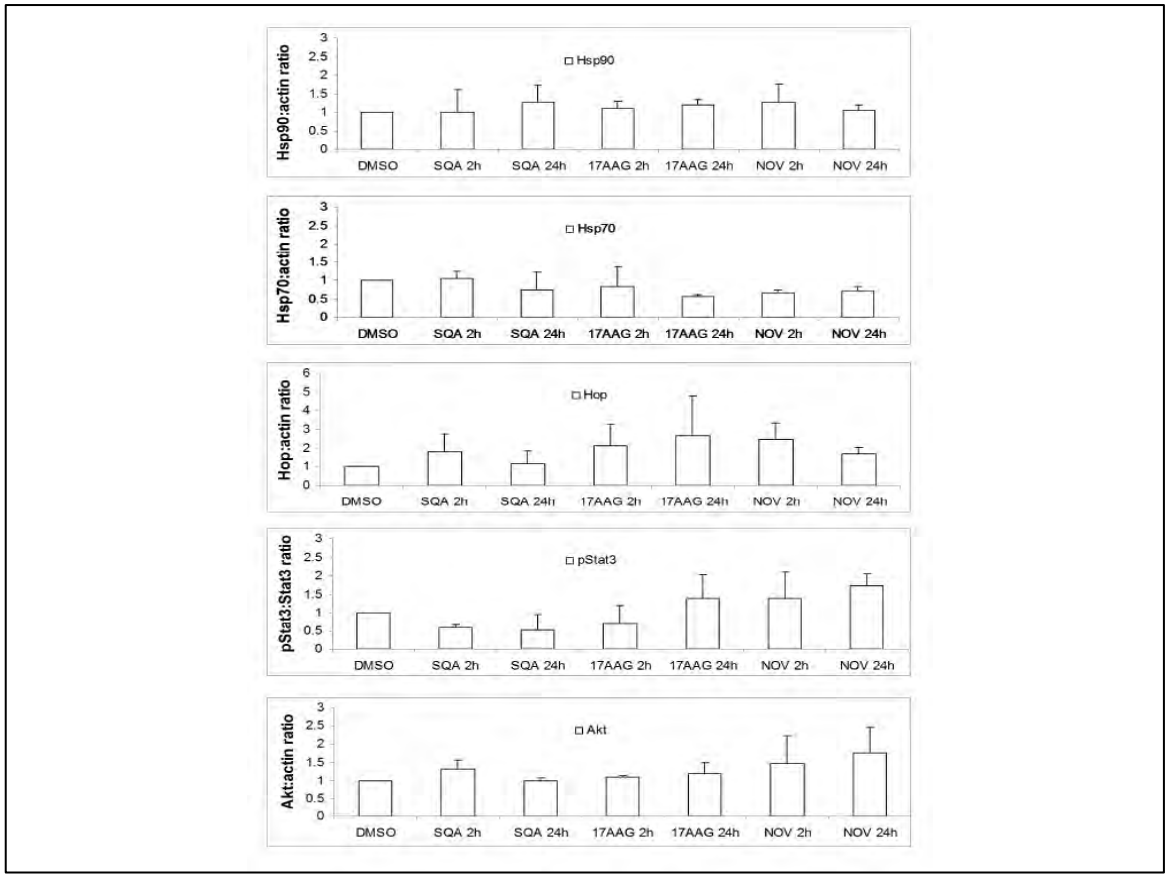


Figure 3.3: Expression levels of proteins of the Hsp90 complex and selected Hsp90 client proteins in cells treated with SQA, 17-AAG and NOV. Equal numbers of MDA-MB-231 cells (1 x 10^6) were treated with 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) (200 nM), sargaquinoic acid (SQA) (90 µM) and novobiocin (NOV) (260 µM) for 2 and 24 hours. The levels of chaperones and client proteins were determined by Western analysis using antibodies indicated to the left of the figure. Actin was used as a loading control to normalise for the protein concentration in all cases, except for pSTAT3 in which case total STAT3 was used as the loading control. The densitometry analysis was performed using MacBiophotonics ImageJ and the intensity of the bands represented as a ratio to those of the loading control (indicated on the y-axis). The ratio of protein to loading control was expressed relative to the DMSO control for each chaperone and client protein to allow comparison of trends between independent experiments. Data shown are an average of three independent experiments. Statistical analysis was performed using ANOVA.

When compared to the DMSO control, Western analysis revealed no significant changes in the Hsp90 expression levels from 2 hours following SQA [F (1, 4) = 1.10, P = 0. 35], 17-AAG [F (1, 4) = 0.03, P = 0. 88] and NOV [F (1, 4) = 0.64, P = 0. 47] treatments to 24 hours following treatment with SQA [F (1, 4) = 0.08, P = 0. 80], 17-AAG [F (1, 4) = 0.64, P = 0. 47] and NOV [F (1, 4) = 0.78, P = 0. 43].

Similarly, no significant changes in the expression levels of Hop were detected from 2 hours SQA [F (1, 4) = 0.84, P = 0. 12], 17-AAG [F (1, 4) = 2.50, P = 0. 19] and NOV [F (1, 4) = 1.40, P = 0. 30] treatments to 24 hours treatments with SQA [F (1, 4) = 3.86, P = 0.12], 17-AAG [F (1, 4) = 4.03, P = 0. 12] and NOV [F (1, 4) = 0. 0087, P = 0. 93].

Western analysis revealed no significant changes in the Hsp70 expression levels from 2 hours following SQA [F (1, 4) = 0.02, P = 0. 89], 17-AAG [F (1, 4) = 0.02, P = 0. 70] and NOV [F (1, 4) = 0.02, P = 0. 61] treatments to 24 hours following treatment with SQA [F (1, 4) = 0.14, P = 0.73], 17-AAG [F (1, 4) = 0.42, P = 0. 55] and NOV [F (1, 4) = 0.40, P = 0. 56] when compared to the DMSO control. As no modulation of Hsp70 expression was observed it was suggested that SQA, NOV and 17-AAG did not induce a generalised stress-response. This finding supported the observation that the effects of the compounds on the localisation of members of the Hsp90 machinery were different to those induced by HS treatment and thus to a general stress-response.

No significant changes in the STAT3 expression levels compared to pSTAT3 expression levels were observed following 2 hour treatments with SQA [F (1, 4) = 1.17, P = 0. 34], 17-AAG [F (1, 4) = 0.24, P = 0. 65] and NOV [F (1, 4) = 0.15, P = 0. 72] or 24 hour treatments with SQA [F (1, 4) = 0.04, P = 0. 84], 17-AAG [F (1, 4) = 0.00021, P = 0. 99] and NOV [F (1, 4) = 0.00010, P = 0. 99]. Similarly, Western analysis revealed no significant changes in the AKT expression levels at 2 hour SQA [F (1, 4) = 0.63, P = 0. 47], 17-AAG [F (1, 4) = 0.28, P = 0. 62] and NOV [F (1, 4) = 0.87, P = 0.40] treatments or 24 hour treatments with SQA [F (1, 4) = 0.43, P = 0. 55], 17-AAG [F (1, 4) = 0.58, P = 0. 49] and NOV [F (1, 4) = 0.98, P = 0. 38] compared to the DMSO control. The ability of Hsp90 inhibitors to reduce Hsp90 client protein expression has been documented (Zhang *et al*., 2008b; Neckers, 2003; Chiosis *et al*., 2001) and the expected client protein degradation profiles were not seen for 17-AAG, NOV or SQA treatments, for AKT or for the ratio of activated pSTAT3 to STAT3 following 2 to 24 hour treatments with the exception NOV treatment in which the increased expression of AKT was noted. It is possible that the inhibitor concentrations used in this study were not sufficient to promote the degradation of Hsp90 clients and as such a dose response study could be conducted in the future. This proposal is supported by the findings that 17-AAG treatment did not result in the overexpression of Hsp70,

which is often seen following treatment with the benzoquinone ansamycin family of compounds (Kaur and Ralhan, 2000). The concentrations used in the literature for the detection of client protein degradation vary with the compound and cell line used with reports of the use of 0.1 (Newman *et al*., 2012) to 0.5 µM 17-AAG (Chen *et al*., 2012) and of up to 50 µM of GA for Hsp90 client protein degradation (Zhang *et al*., 2008b).

3.4.4. *Isothermal titration calorimetry analysis of SQA with Hsp90β*

As SQA was shown to modulate the localisation of proteins of the Hsp90 complex but not the expression of Hsp90 client proteins, it was unclear whether this compound interacted with Hsp90 directly, or whether the observed modulation of Hsp90 localisation was a consequence of an indirect interaction. ITC allows for the label-free analysis of the binding of compounds to proteins and provides data of the binding affinity and thermodynamics of any such interaction. Therefore, the ability of SQA to interact with Hsp90β directly was investigated through ITC. Hsp90β protein was purified from C41 *E.coli* cell lysates by affinity-tag purification followed by ion exchange chromatography to increase the purity of the protein used in the ITC experiments (Appendix A, Figure C.1). Experiments were performed in a VP-ITC MicroCalorimeter (MicroCal Inc., Northampton, Massachusetts, United States of America) with 30 µM purified Hsp90β protein and 700 µM and 450 µM SQA and 17-DMAG in the syringe respectively. 17-DMAG was selected as the positive control as this GA-derivative is water soluble thus eliminating the possibility of compound insolubility issues in the ITC buffer. The 17-DMAG was used at a concentration of 450 µM and was used as a positive control for compound binding to Hsp90β. The N value, which indicates the number of binding sites, was calculated to be 0.956 ± 0.0135. This indicated that the Hsp90β protein was active and that the assay conditions were optimal for compound binding to the protein.

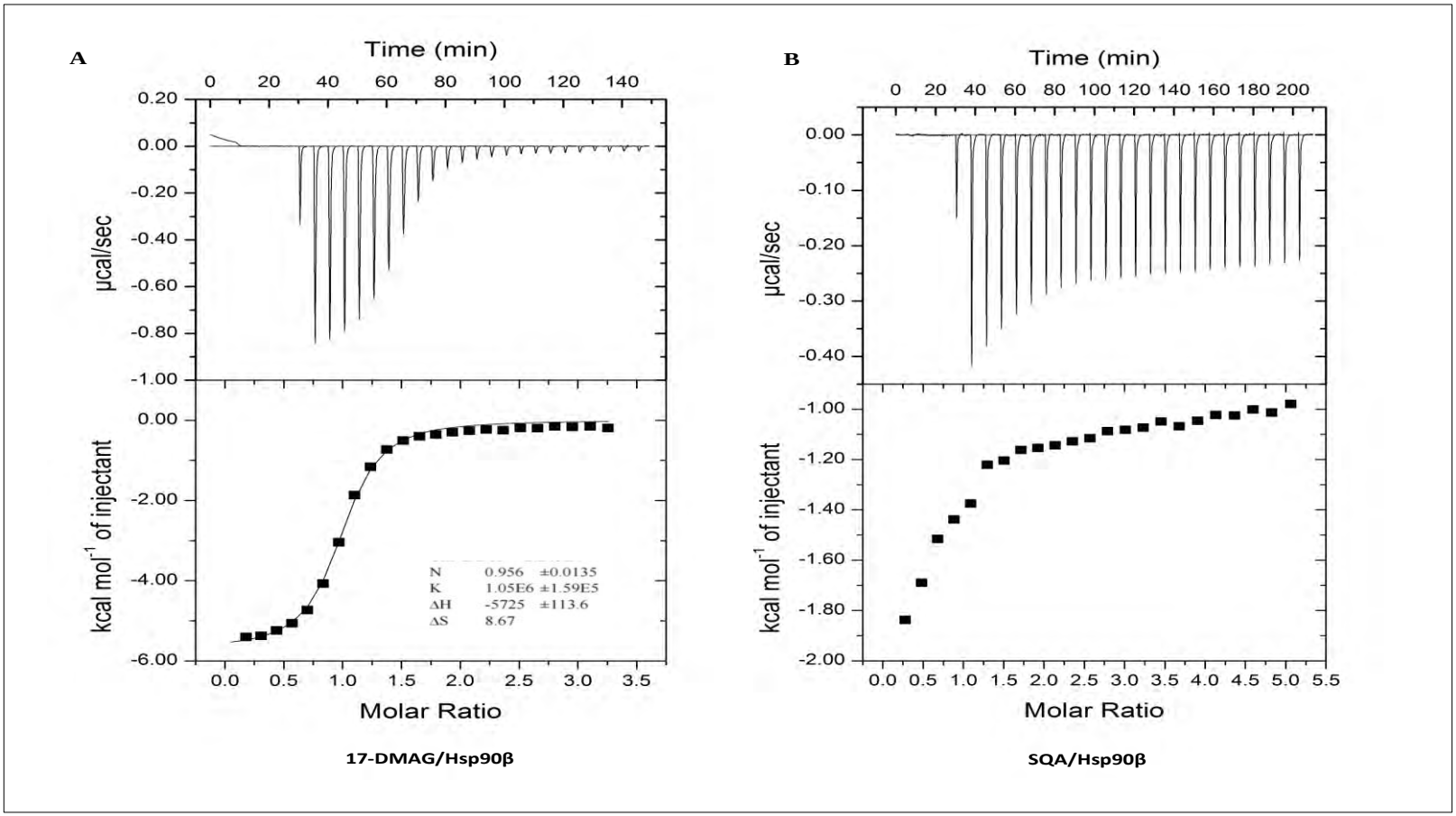


Figure 3.4: SQA may interact with Hsp90β weakly and with a low affinity. (A) 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (450 µM) binding data to Hsp90β (30 µM) (B) Sargaquinoic acid (SQA) (700 µM) binding data to Hsp90β. Raw and fitted data between SQA and Hsp90β equilibrated to 30 °C in 50 mM Tris , 6 mM $MgCl_2$ 20 mM KCl, 1 mM β-mercaptoethanol, pH 7.4. Upper graph represents the raw ITC data in which the incremental heat changes are shown as microcalories per second (µcal.sec^{-1}) plotted against time in minutes over the progression of the experiment, the lower graph shows the normalised integration data in terms of kcal.mol^{-1} of injectant plotted against the molar ratio of the added titrant with the curve fitted to single-site mode. The relationship between the two x-axes allows for the positioning of the integrated area for each peak directly below the associated peak in the raw data. The negative peaks correspond to addition of an aliquot of ligand. The calorimetric output indicated that an exothermic process was occurring in the cell.

For SQA, the DMSO content of the ligand in the syringe was matched by adding equal volumes of DMSO using the same pipette, pipette tip and pipette setting for the compound and for the DMSO added to the reference and sample cells. The ligand was in large excess over the protein to allow for the reaction to reach saturation. Double the concentration of SQA (700 µM) was used than 17-DMAG (450 µM) as no information regarding the nature of SQA-Hsp90 interaction was available. Optimisation was required before any heats of binding were observed. Binding was investigated with Hsp90β at a concentration range of between 5 and 20 µM with the compound at a concentration always twenty times more than that of Hsp90β (Appendix Figure C.7). No heats of binding were detected at the lower protein-compound concentrations but were seen with 30 µM Hsp90 and when the compound concentration was raised to 700 µM (Figure 3.4).The observed negative heats of binding were an indication of an exothermic binding event of the compounds to Hsp90β. The changes in heats following titrations with SQA were much lower than those observed for 17-DMAG and the protein was quickly saturated with compound. The thermodynamic parameters for SQA binding could not be accurately determined as a direct result. However, small changes in heat could still be observed and the change from a sigmoidal plot for the binding of 17-DMAG to a hyperbolic plot of SQA suggested that the interaction between SQA and Hsp90β was weak. The analysis of SQA by ITC proved to be challenging as the compound frequently precipitated out of solution during ITC experiments. The stability of SQA in the ITC buffer is unknown and it is possible that the compound may have partially degraded during the ITC experiment resulting in weak binding to Hsp90β as saturation was observed after the first seven injections at a molar ration of 1.5 as indicated by the isotherms.

The interaction between Hsp90 and SQA was further investigated by STD NMR analysis. The data displayed in Appendix Figure C.8 shows the reference spectrum and the STD NMR spectrum for Hsp90 and SQA. Based on the reduction of the STD signals from the spectrum, SQA was shown to interact to Hsp90β through the methyl group bound to the quinone moiety. Owing to the solubility issues of SQA in PBS containing Hsp90β observed during this experiment, the resolution of this experiment was low. Furthermore, the stability of SQA in PBS for extended periods of time is unknown and this may have affected the quality of the generated results.

3.4.5. *Determination of the ability of SQA, NOV and GA to disrupt the Hsp90-Hop interaction in vitro*

As SQA was shown to interact with Hsp90 weakly through ITC and STD NMR, this study sought to investigate whether SQA could disrupt the association of Hsp90 and Hop as these proteins co-exist in an intermediate complex (Southworth and Agard, 2011). Hop was selected as it is an essential cochaperone of Hsp90, is required for the stabilisation of the Hsp70–Hsp90–client protein complex and transfers client proteins from Hsp70 to Hsp90 during the chaperone cycle (Wegele *et al*., 2006). The effect of SQA (Figure 3.7) on this association was compared to that of the C-terminal Hsp90 inhibitor NOV (Figure 3.6) and the N-terminal Hsp90 inhibitor GA (Figure 3.5). His-tagged pulldown assays were performed in which purified recombinant hexahistidine Hop was incubated with nickel-chelated sepharose beads. Purified Hsp90β protein was incubated with the different concentrations of the compounds of interest before the two proteins were allowed to interact. No ATP was added to assays as Hop binds to Hsp90 in the nucleotide-free or ADP-bound state (Johnson *et al*., 1998). DMSO was used as a vehicle control, with the final concentration not exceeding 0.01 %. Hsp90β (1 µg) and Hop (1 µg) were included as input controls. It was expected that DMSO treatment would not affect the binding of Hsp90β to Hop. A small amount of Hsp90β was expected to bind to the beads non-specifically and therefore Hsp90β protein alone was incubated with the beads as a control to account for any such non-specific binding of Hsp90β to the beads. The amount of Hsp90β bound to the beads non-specifically was subtracted from that bound to DMSO and compound treatments. Hop protein alone was incubated with the beads to show the successful interaction of the recombinant protein through the His tag with nickel-chelated beads in the absence of any external factors. The protein complexes pulled down were analysed following the silver staining of the resultant SDS-PAGE gels.

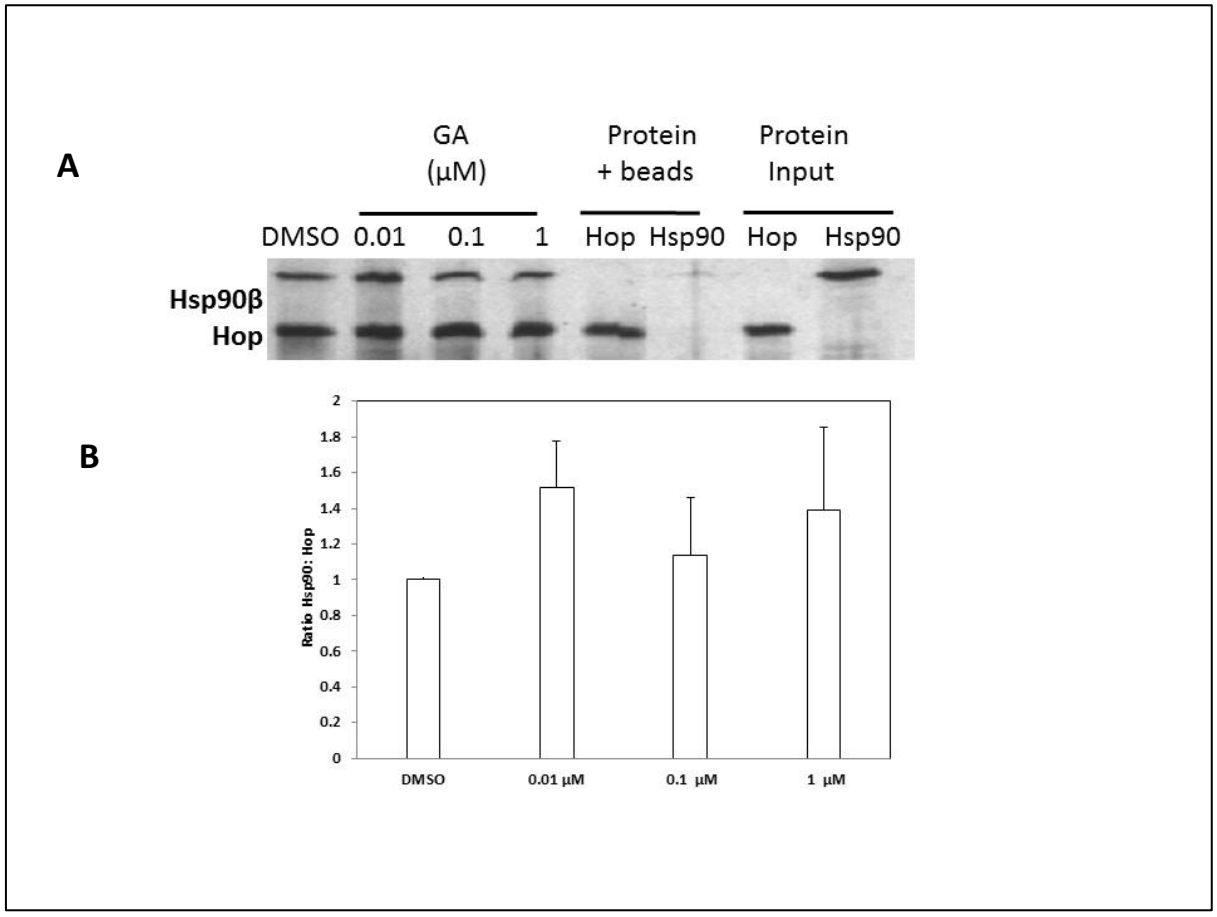


Figure 3.5: Treatment with GA did not disrupt Hsp90-Hop interaction *in vitro.* His-tagged pulldown assays in which Hop protein was incubated with nickel-chelated sepharose beads for 2 hours and then with Hsp90β protein previously incubated with geldanamycin (GA) for 30 minutes. Compounds were used at a concentration range of 0.01 – 1 µM. The Protein + beads lane shows the level of protein interacting non-specifically with beads and the protein input lanes indicated Hsp90β and Hop protein alone. (A) Proteins were resolved on a 4 % SDS-PAGE stacking gel pre-cast on top of a 12 % SDS-PAGE resolving gel and were visualised by silver staining with PageSilver ^{TM} Silver Staining Kit according to the manufacturer's instructions. (B) Densitometry analysis was performed using MacBiophotonics ImageJ and the intensity of the bands of Hsp90 represented as a ratio to those of Hop. The ratio of Hsp90β to Hop was expressed relative to the DMSO control for each treatment at different concentrations to allow for the comparison of trends between independent experiments. Data shown are an average of three independent experiments with similar results. Statistical analysis was performed using ANOVA.

As shown in Figure 3.5, 3.6 and 3.7, Hsp90β was successfully pulled down following treatment with DMSO only for all compound treatments. The ratio of Hsp90β associated with Hop following DMSO treatment was compared to that following treatment with SQA, NOV and GA at 0.01, 0.1 and 1 µM concentrations. Figure 3.5 shows a non-significant increase [$F(1, 4) = 1.30$, $P = 0.31$] in the amount of Hsp90 associated with Hop following treatment with 0.01 µM GA when compared to DMSO treatment. A decrease in the ratio of Hsp90 interacting with Hop was observed following treatment

with GA at concentrations of 0.1 and 1 µM compared to the DMSO only treated sample. Statistical analysis showed that these changes at 0.1 µM [F (1, 4) = 0.0012, P = 0. 97] and 1 µM [F (1, 4) = 0.0034, P = 0. 96] were also not significant as determined by ANOVA. The result showing no disruption of the Hsp90 - Hop association following treatment with GA is in agreement with literature as GA binds to the N–terminal domain of Hsp90 and thus prevents the binding of ATP and the subsequent dissociation of Hop from the Hsp90 complex (Zhang *et al*., 2008b; Söti *et al*, 2003).

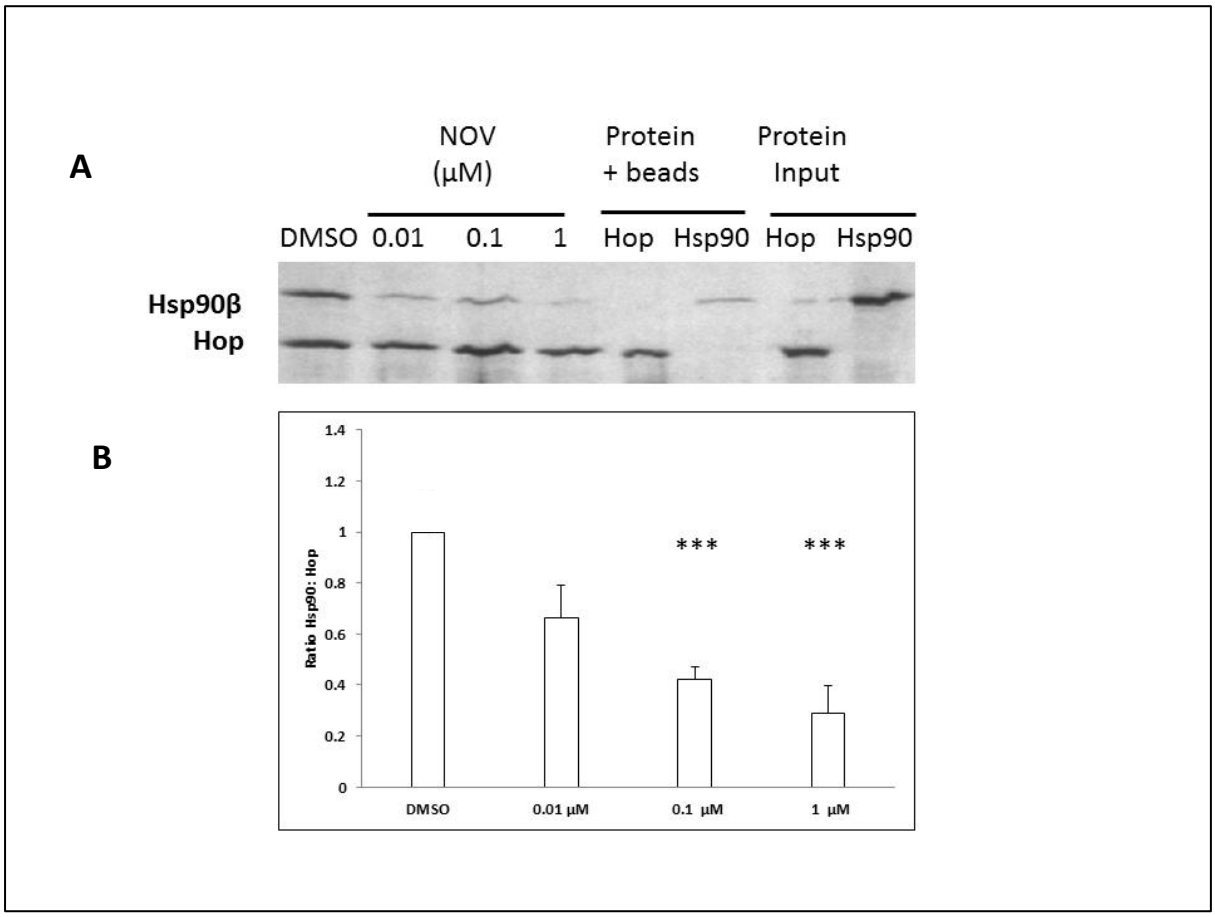


Figure 3.6: Treatment with NOV disrupted the Hsp90-Hop interaction *in vitro*. His-tagged pulldown assays in which Hop protein was incubated with nickel-chelated sepharose beads for 2 hours and then with Hsp90β protein previously incubated with novobiocin (NOV) for 30 minutes. Compounds were used at a concentration range of 0.01 – 1 µM. The Protein + beads lane shows the level of protein interacting non-specifically with beads and the protein input lanes indicated Hsp90β and Hop protein alone. (A) Proteins were resolved on a 4 % SDS-PAGE stacking gel pre-cast on top of a 12 % SDS-PAGE resolving gel and were visualised by silver staining with PageSilver ^{TM} Silver Staining Kit according to the manufacturer's instructions. (B) Densitometry analysis was performed using MacBiophotonics ImageJ and the intensity of the bands of Hsp90 represented as a ratio to those of Hop. The ratio of Hsp90β to Hop was expressed relative to the DMSO control for each treatment at different concentrations to allow for the comparison of trends between independent experiments. Data shown are an average of three independent experiments with similar results. Statistical analysis was performed using ANOVA. *** signifies treatments in which the change in the ratio of Hsp90 to Hop was found to be significant when compared to the DMSO control.

Figure 3.6 shows a dose-dependent decrease in the ratio of Hsp90 interacting with Hop following treatment with NOV (from 0.01 to 1 µM) compared to the DMSO only treated sample. The reduction in Hsp90 associated with Hop, following increasing concentrations of NOV was apparent by visual inspection of the silver stained SDS-PAGE gels and was further shown by densitometry analysis (Figure 3.6). Statistical analysis showed that the reduction in Hsp90 associated with Hop at 0.01 µM [F (1, 4) = 3.86, P = 0.12] was not significant but that the reductions at 0.1 µM [F (1, 4) = 18.3, P = 0.01] and 1 µM NOV [F (1, 4) = 20.3, P = 0.01] concentrations were significant. These results are in accordance with published data that showed that treatment with the C-terminal inhibitor NOV reduces the association of Hsp90 with Hop (Yun *et al*., 2004).

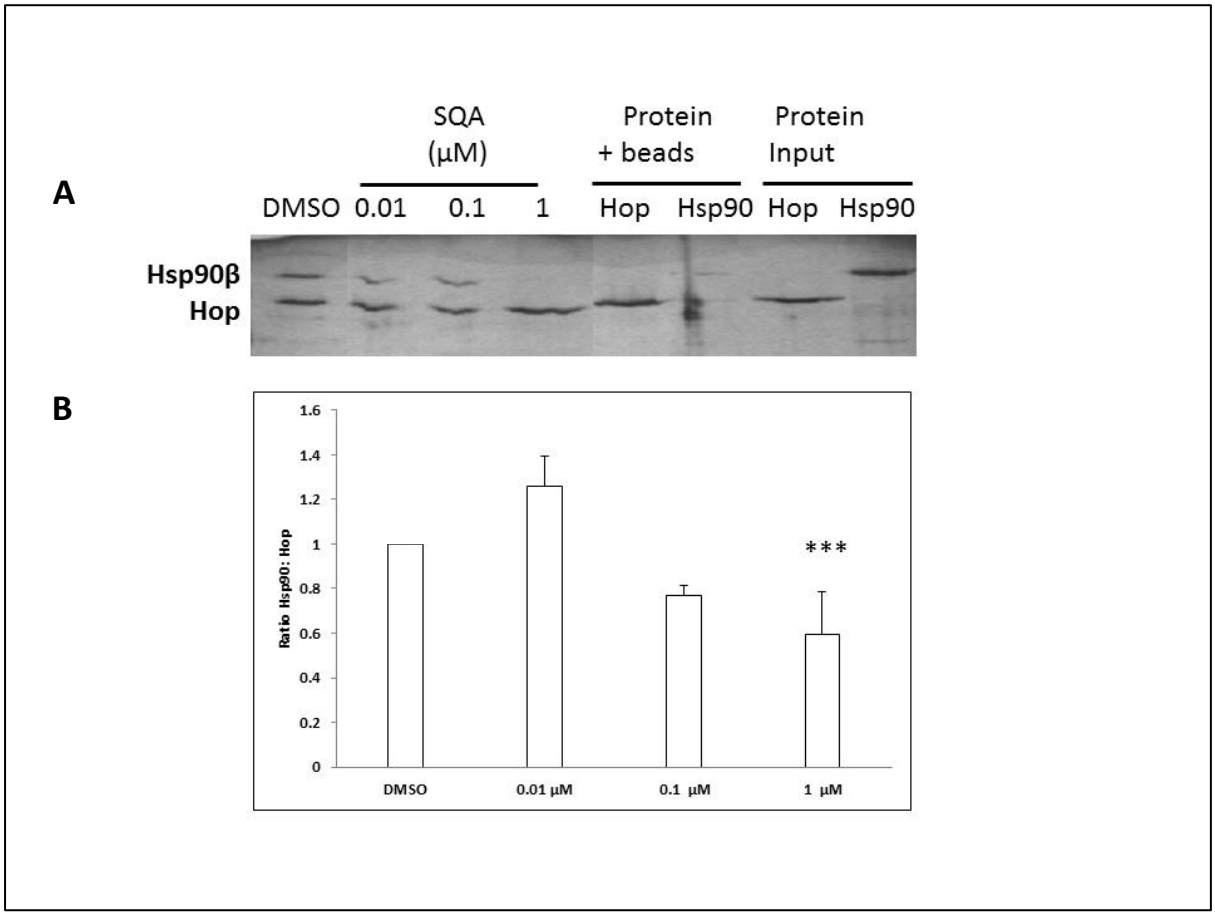


Figure 3.7: Treatment with SQA disrupts Hsp90-Hop interaction *in vitro*. His-tagged pulldown assays in which Hop protein was incubated with nickel-chelated sepharose beads for 2 hours and then with Hsp90β protein previously incubated with sargaquinoic acid (SQA) for 30 minutes. Compounds were used at a concentration range of 0.01 – 1 µM. The Protein + beads lane shows the level of protein interacting non-specifically with beads and the protein input lanes indicated Hsp90β and Hop protein alone. (A) Proteins were resolved on a 4 % SDS-PAGE stacking gel pre-cast on top of a 12 % SDS-PAGE resolving gel and were visualised by silver staining with PageSilver ™ Silver Staining Kit according to the manufacturer's instructions. (B) Densitometry analysis was performed using MacBiophotonics ImageJ and the intensity of the bands of Hsp90 represented as a ratio to those of Hop. The ratio of Hsp90β to Hop was expressed relative to the DMSO control for each

treatment at different concentrations to allow for the comparison of trends between independent experiments. Data shown are an average of three independent experiments with similar results. Statistical analysis was performed using ANOVA. *** signifies treatments in which the change in the ratio of Hsp90 to Hop was found to be significant when compared to the DMSO control.

Figure 3.7 shows that SQA treatment reduced the amount of Hsp90 associated with Hop at the concentrations of 0.1 to 1 µM (Figure 3.10) despite an initial increase in this association at 0.01 µM compared to DMSO. The increase in the amount of Hsp90 associated with Hop at 0.01 µM was found to be non-significant as $F(1, 4) = 3.86$, $P = 0.12$. The decrease in Hsp90 associated with Hop following treatment with 0.1 µM SQA was found to be non-significant as $F(1, 4) = 6.38$, $P = 0.60$ whereas that following treatment with 1 µM was found to be significant as $F(1, 4) = 6.84$, $P = 0.05$. This reduction in the association of Hsp90 and Hop by the addition of SQA is more similar to that following treatment with NOV than treatment with GA. It is also possible that the disruption of this association could be through the interaction of SQA with both Hsp90 and Hop. This proposal could be investigated through pulldown assays in which Hsp90 was bound to the beads and assessing the binding of Hop pre-treated with SQA to Hsp90.

3.5. Conclusions

Immunofluorescence studies showed that SQA modulated the localisation of Hsp90, Hop, STAT3, pSTAT3 and AKT more similarly to NOV than 17-AAG. These changes in localisation in response to SQA treatment suggested that SQA was interacting with Hsp90, although it was later shown that treatment with this compound did not result in the down-regulation of Hsp90 client protein expression. However, this may have been as a result for the need to increase the concentration of the compounds used in the assay, as ITC and STD NMR showed that SQA interacted with Hsp90 weakly. This interaction was shown to be weak and STD NMR suggested that this binding occurred through the methyl group bound to the quinone nucleus of SQA. His-tagged pulldown assays indicated that SQA, like NOV, disrupted the association of Hsp90 with the co-chaperone Hop at a concentration of 1 µM. Taken together the cell biological and biophysical data generated suggested a role for SQA as a novel modulator of Hsp90 activity through a weak interaction with Hsp90. This interaction with Hsp90 was suggested to be more similar to that of NOV than the benzoquinone ansamycin family of Hsp90 inhibitors, GA and 17-AAG. It is also possible that SQA could be a novel modulator of Hop, given the reduction of the protein in association with Hsp90β.

CHAPTER 4

SCREENING OF NAPHTHOQUINONE AND MARINE PYRROLOIMINOQUINONE ALAKOIDS COMPOUNDS FOR NOVEL MODULATORS OF HSP90

4.1. Introduction

This chapter describes the screening and characterisation of compounds with either a naphthoquinone or pyrroloquinoline moiety (in the same manner of that described for SQA in Chapter 3). The compounds explored in this chapter included natural, marine pyrroloiminoquinone alkaloids (DMN, MKM, RIBS-1, RIBS-3, and DSN) and both natural and synthetic 1, 4 and 1, 2 naphthoquinone compounds. As reviewed in Chapter 1, 1, 4 naphthoquinones been shown to exert anti-cancer effects in several cell lines (Chung *et al*., 2004; Kongkathip *et al*., 2003). Although shown to have no antiproliferative activity at concentrations of up to 500 µM in this study (Section 2.3), the naturally occurring 1, 4 naphthoquinone, LAP has known anti-tumour activity in various cancer types (Santana *et al*., 1980). Two (DMN and DSN) of the five marine pyrroloiminoquinone compounds under investigation in this study were found to possess antiproliferative activities while three (MKM, RIBS-1 and RIBS-3) of these compounds did not. Pertinent to this study is that 1, 4 naphthoquinones are thought to represent a new class of Hsp90 inhibitor (Hadden *et al*., 2009) and that cellular structures such as Hsp90 have been identified as targets for quinoid compounds (Asche, 2005). Therefore, this study sought to determine whether LAP, its synthetic derivatives (αLAP, CAL, BBL, HBL and NBL), and the marine pyrroloiminoquinone alkaloid compounds (DMN, MKM, RIBS-1, RIBS-3 and DSN) possessed the potential to modulate Hsp90.

The specific objectives of this study were to investigate the ability of the 1, 4 naphthoquinone and marine pyrolloimoquinone alkaloid compounds to modulate the subcellular localisation and expression levels of Hsp90, Hsp70, Hop, HSF1 and selected Hsp90 client proteins (pSTAT3, STAT3 and AKT) in the MDA-MB-231 breast cancer cell line. The ability of the 1, 4 naphthoquinone compounds to bind to and interact with Hsp90 directly through ITC was determined. Compounds found to interact directly with Hsp90β were analysed for their ability to disrupt the association of Hsp90 and Hop *in vitro.*

4.2. Materials

The MDA-MB-231 (HTB-26) breast cancer cell line was provided by Dr Sharon Prince (University of Cape Town, South Africa). All general reagents were purchased from Sigma-Aldrich (Germany) or Saarchem (Merck, South Africa). Tissue culture reagents (Dulbecco's Modified Eagle Medium [DMEM] with GlutaMAX™-I, FCS, 10 x Trypsin-EDTA and Penicillin/Streptomycin) were from Gibco, Invitrogen (Paisley, United Kingdom) and Biowhittaker (United Kingdom). Tissue culture plasticware

was from Corning Incorporated (United States of America). Hybond nitrocellulose membrane was from Bio-Rad (United Kingdom). Recombinant human Hsp90β protein (SPR-102) and recombinant human Hop protein (SPR-302) were purchased from Stressmarq (Canada). Mouse anti-Hsp90α/β [F-8] (cat no.: sc-13119), mouse anti-Hsp70/Hsc70 [W27] (cat no.: sc-24), rat anti-HSF1 [10H8] (cat no.: sc-13516), rabbit anti-AKT1/2/3 [H-136] (cat no.: sc-9312), rabbit anti-STAT3 [H-190] (cat no.: sc-7179) and mouse anti-pSTAT3 [B7] (cat no.: 8059) antibodies were from Santa Cruz Biotechnology (United States of America). Mouse anti-AKT [pan] [40D4] (cat no.: 2920) was from Cell Signaling Technology, (United States of America). Mouse anti-Hop [STIP, p60] (cat no.: DS14F5) was purchased from Stressgen (United States of America). Rabbit anti-actin (cat no.: A2103) and, goat anti-rabbit IgG (whole molecule)-Peroxidase (cat no.:A9169) and goat anti-mouse IgG (Fab specific)-Peroxidase (cat no.: A2304) were from Sigma Aldrich (Germany). Alexa Fluor® 488 donkey anti-mouse IgG [H+L] (cat no.: A21202), Alexa Fluor® 555 donkey anti-rabbit IgG [H+L] (cat no.: A31572) were from Invitrogen (Paisley, United Kingdom). Hoechst 33342 solution (cat no.: 62249) and Amicon Ultra Centrifugal filters-15 Ultracel® 30K were from Millipore (Ireland). GA (cat no.: 30562-34-6) was purchased from BioMol Int (United States of America) and 17-DMAG (cat no.: D5193-1MG) was purchased from Sigma-Aldrich (Germany). Manufacturers or suppliers of any other specialised reagents are referenced in-text.

4.3. Methods

4.3.1. *Cell culture and maintenance of cell lines*

The MDA-MB-231 cell line was cultured as previously described in Section 2.3.1.

4.3.2. *Immunofluorescence microscopy analyses of proteins of the Hsp90 complex*

The procedure described in Section 3.3.2 was used to determine changes in localisation following treatment with each of the compounds with the exception of the concentrations used. The IC_{50} concentrations of the compounds that showed antiproliferative activity was selected and 7 μM for compounds that showed no antiproliferative activity was selected as it was within the same micromolar range as the compounds displaying antiproliferative activity. The concentrations of each compound used was LAP (7 μM), αLAP (7 μM), CAL (7 μM), NBL (8.78 μM), BBL (7.86 μM), HBL (7.61 μM), DMN (12.4 μM), MKM (7 μM), RIBS-1, (7 μM), RIBS-3 (8.78 μM), or DSN (3.32 μM).

4.3.3. *Harvesting of MDA-MB-231 cells for expression level analysis*

The harvesting of cells for the analysis of the expression level of proteins following treatment with compounds was performed using procedure described in Section 3.3.3. The IC_{50} concentrations of the compounds that showed antiproliferative activity was selected and 7 µM for compounds that showed no antiproliferative activity was selected. 7 µM was selected for compounds that displayed no antiproliferative activity as it was within the same micromolar range as the compounds displaying antiproliferative activity. The concentrations of each compound used was LAP (7 µM), αLAP (7 µM), CAL (7 µM), NBL (8.78 µM), BBL (7.86 µM), HBL (7.61 µM), DMN (12.4 µM), MKM (7 µM), RIBS-1, (7 µM), RIBS-3 (8.78 µM), or DSN (3.32 µM).

4.3.4. *Hsp90-Hop conventional pulldown assays*

Conventional Hsp90-Hop pulldown assays were performed according to the procedure described in Section 3.3.9.

4.4.1 SDS-PAGE analysis of proteins

The procedure described in Section 3.3.4 was used for the separation of proteins by SDS-PAGE analysis.

4.3.5. *Chemiluminescence-based Western analysis of proteins*

The procedure described in Section 3.2.5 was used for the detection of protein by chemiluminescence based Western analysis with the exception of the developing reagent that was used. The Super Signal®West Dura Extended Duration Substrate (cat no.: 34075) was from ThermoScientific (United States of America) was used to detect the protein signals.

4.3.6. *Isothermal titration calorimetry analysis of naphthoquinone compounds and Hsp90β*

The procedure described in Section 3.3.5 was used to analyse the binding of the 1, 4 naphthoquinone compounds to Hsp90β.

4.4. Results and Discussion

4.4.1. *Determination of effects of naphthoquinone and pyrroloiminoquinone compounds on proteins of the Hsp90 complex machinery in triple negative breast cancer cell model*

As described in Chapter 3, the analysis of the subcellular localisation of proteins allows for the high-throughput screening of compounds that may target the proteins under investigation within the cell studied. Therefore, the ability of 1, 4 naphthoquinone and pyrroloiminoquinone compounds to modulate the subcellular localisation of Hsp90 (Appendix Figures B.8, B.9), Hsp70 (Appendix Figures B.10, B.11), Hop (Appendix Figures B.12, B.13) and HSF1 (Appendix Figures B.10, B.11) in the triple negative MDA-MB-231 breast cancer cell line was investigated. Cells were treated with compounds for the optimal time period of five hours and DMSO was used as a vehicle control. As such DMSO treated cells were considered as untreated cells. The final concentration of DMSO did not exceed 0.1 % in any instance. A HS treatment was included as a positive control as an indicator of a stress-response within the cells distinct to that caused by compound treatment. Nuclei were visualised with Hoechst 33342 and WGA was employed as a stain of the plasma membrane (Gonatas *et al.,* 1979) and Golgi apparatus (Parkkinen *et al*., 1995; Guasch *et al*., 1995). Images were captured on a Zeiss LSM Meta 510 confocal laser scanning microscope and a quantitative analysis performed (Figures 4.1 and 4.2).

As described in Section 3.3.2, cells were categorised as showing either cytoplasmic only, nuclear only or both cytoplasmic and nuclear staining. The quantitative assessment was performed by manually counting the number of cells showing each type of localisation (Figure 4.1).

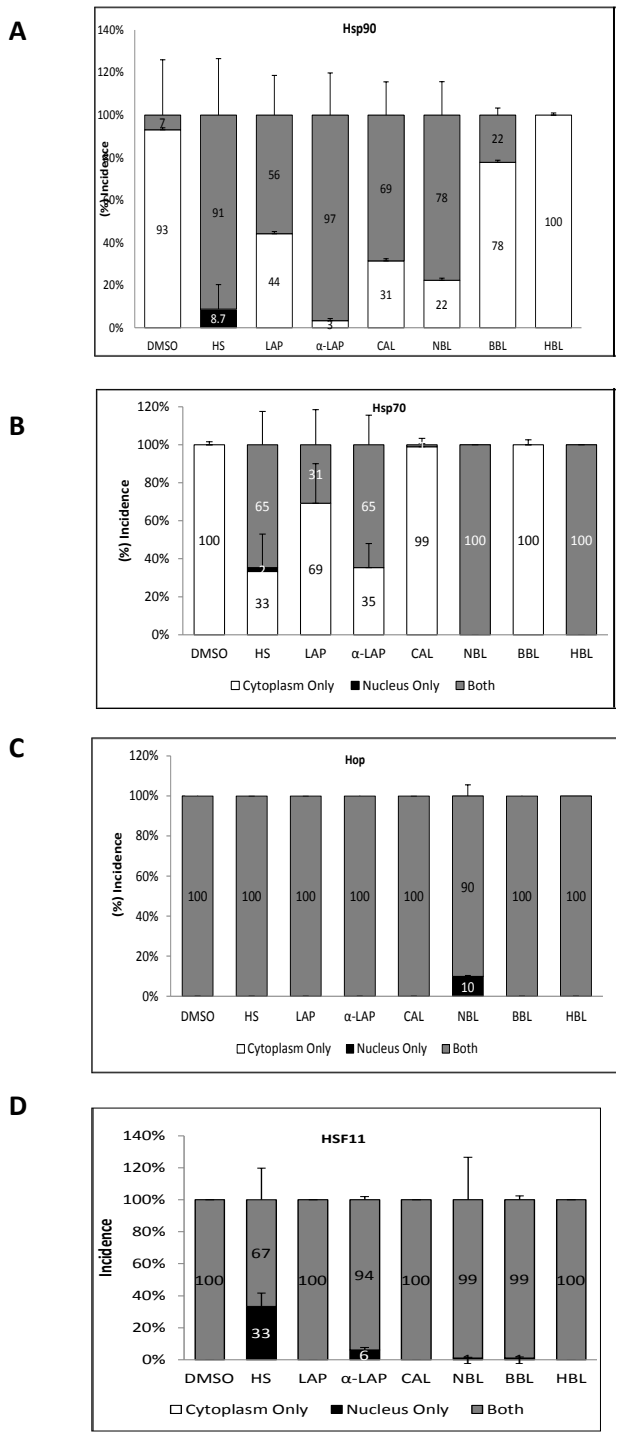


Figure 4.1: Quantitative analysis of the subcellular localisation patterns of (A) Hsp90, (B) Hsp70, (C) Hop and (D) HSF1 following treatment with LAP, αLAP, CAL, BBL, HBL and NBL Number of cells exhibiting nuclear only (black), cytoplasmic only (white) or both nuclear and cytoplasmic (grey) staining were quantified by counting the cells in three separate microscope fields for two independent experiments.

Changes in Hsp90 localisation induced by LAP, αLAP, CAL, NBL and BBL were characterised by an increase in the incidence of cells showing both cytoplasmic and nuclear staining as opposed to the predominately cytoplasmic staining only observed for DMSO treated cells (Figure 4.1 A).These

increases in cells showing both nuclear and cytoplasmic Hsp90 are similar to those seen following treatment with both 17-AGG and NOV and thus suggest the modulation of Hsp90 by these compounds (Figure 3.1). HBL was the exception as treatment with this compound resulted in a similar percentage of nuclear Hsp90 to that of the DMSO only treatment and therefore it would appear that HBL did not modulate the localisation of Hsp90. In addition to the general increase in nuclear Hsp90, NBL treatment also resulted in a punctate nuclear staining pattern of Hsp90 (Appendix Figure B.2 B) similar to that observed for SQA treatment (Appendix Figure B.1 B). Based on the results for SQA described in Chapter 3, it was predicted that NBL may act as a modulator of Hsp90, in a similar way to SQA.

Hop was shown to be localised within both the cytoplasm and the nuclei of DMSO treated cells and upon HS, an accumulation of Hop within the nuclei of the cells was observed (Appendix Figure B.12). Following LAP treatment, Hop appeared to be localised mainly within the perinuclear region of the treated cells as opposed to the nuclei (Appendix Figure B.12). This modulation of Hop by LAP suggested some specificity of LAP over its synthetic derivatives against Hop. As LAP was shown to modulate the localisation of Hsp90, the data also suggested that LAP may modulate of the association of Hsp90 and Hop.

The compounds LAP, αLAP, HBL and BBL were found to modulate the localisation of Hsp70, as treatments with these compound resulted in increases in nuclear Hsp70 (Figure 4.1 C, Appendix Figure B.10). The changes induced by treatment with LAP and αLAP were more similar to those of 17-AAG, while those of HBL and BBL were more similar to those of NOV (Figure 3.1). CAL and NBL did not modulate the Hsp70 localisation and treatment with these compounds produced staining patterns similar to that of the DMSO treated cells. This finding could suggest that that CAL and NBL do not target Hsp70 and could also indicate that these compounds do not induce a stress-response within treated cells. However, the staining of inducible-specific Hsp70 isoforms would have to be performed to confirm or disprove whether these compounds do not induce a general stress-response.

Owing to the modulation of Hsp90, Hsp70 and Hop, it was investigated whether the modulation of Hsp90 and Hop localisation by the naphthoquinone compounds was as a result of the induction of general stress-response within the cells. Visual inspection indicated that DMSO treatment resulted 100 % of cells showing both nuclear and cytoplasmic HSF1 staining and HS was shown to increase the nuclear staining of HSF1. Analysis of the confocal microscopy images indicated that the resultant

changes in cell morphology and increases in nuclear HSF1 staining following compound treatment were different to those caused by HS treatment.

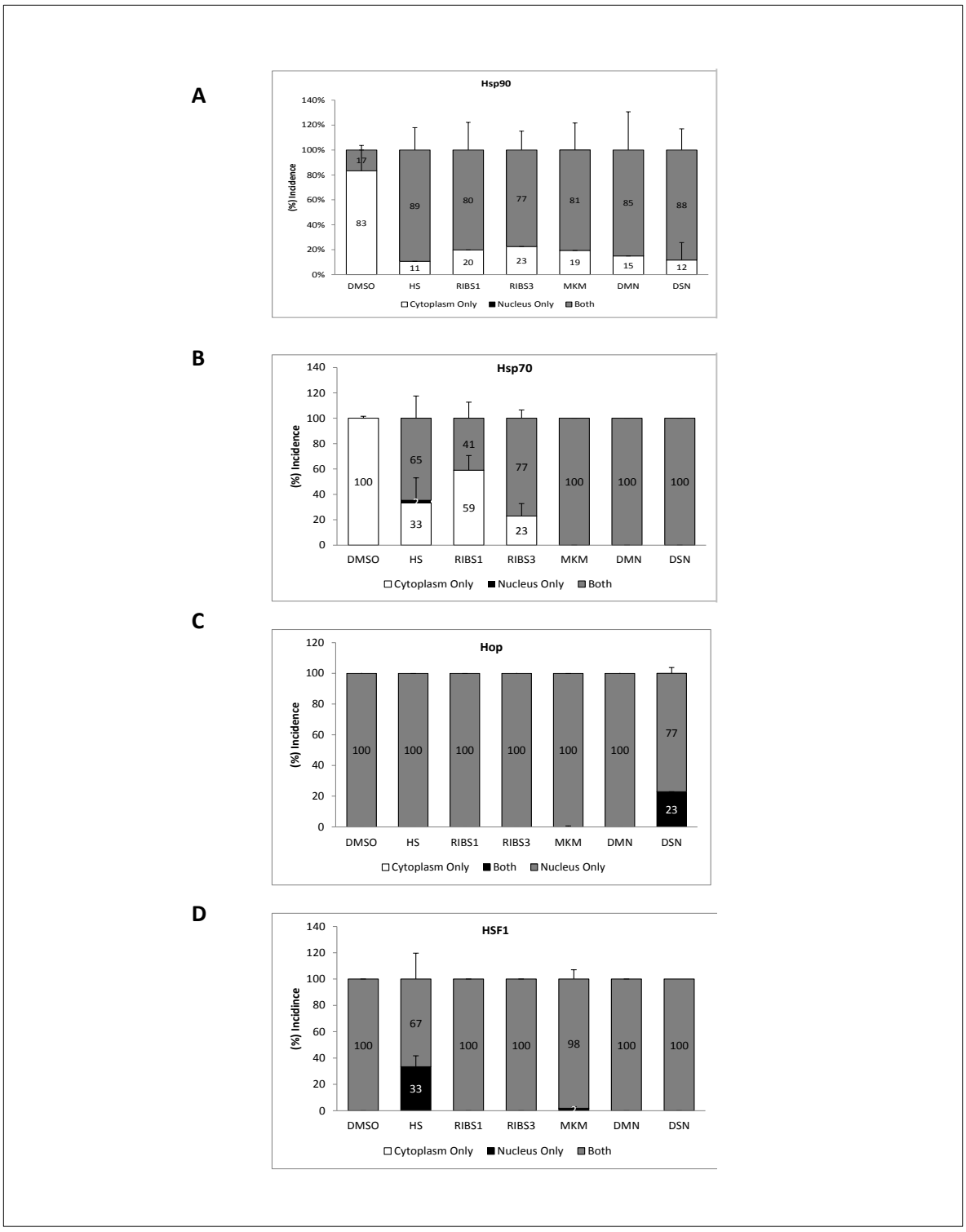


Figure 4.2: **Quantitative analysis of the subcellular localisation patterns of (A) Hsp90, (B) Hsp70, (C) Hop and (D) HSF1 following treatment with damirone C (DMN), makaluvamine M (MKM), *N*-1-β-D ribofuranosyl makaluvamine (RIBS-1), *N*-1-β-D ribofuranosyl makaluvic acid C (RIBS-3) and discorhabdin A (DSN).** The number of cells exhibiting cytoplasmic only (white), nuclear only (black), or both nuclear and cytoplasmic

(grey) staining were quantified by counting the cells in three separate microscope fields for two independent experiments.

As shown in Figure 4.2 A, DMSO treatment resulted in a predominantly cytoplasmic staining of Hsp90. HS treatment was found to increase the localisation of Hsp90 to the nuclei of cells and treatment with compounds of the marine pyrroloiminoquinone alkaloid series resulted in similar increases in nuclear Hsp90. The increase in the number of cells showing both cytoplasmic and nuclear Hsp90 staining was more similar to the HS treatment than it was for either 17-AAG or NOV treatment. It is therefore possible that compounds of the marine pyrroloiminoquinone alkaloid series do not target Hsp90 directly, but through indirect mechanisms as the change in Hsp90 localisation is more similar to changes caused by HS than compound treatment.

Hsp70 was shown to be predominantly localised to the cytoplasm of DMSO treated MDA-MB-231 cells. In cells treated with HS an increase in the percentage of cells showing nuclear only and both cytoplasmic and nuclear Hsp70 staining was observed. Treatment with the marine pyrolloimoquinone alkaloid compounds resulted in an increase in nuclear Hsp70 staining for all compounds when compared to the DMSO control treated cells (Figure 4.3 B). The changes in Hsp70 localisation for RIBS-1 and RIBS-3 were similar to those of HS treatment and 17-AAG (Figure 3.1) while the changes in response to treatment with MKM, DMN and DSN were more similar to those caused by NOV (Figure 3.1). The increases in nuclear Hsp70 following compound treatment were quantified as being higher than that following HS treatment. The data generated from changes in Hsp70 localisation patterns would suggest that treatment with pyrroloiminoquinone alkaloid compounds may have resulted in the induction of a general stress-response within the cells (Figure 4.2 D). This proposal was further investigated through assessing changes in HSF1 localisation in response to the compounds. Visual inspection indicated that DMSO treatment resulted 100 % of cells showing both nuclear and cytoplasmic HSF1 staining and HS was shown to increase the nuclear staining of HSF1.

The ability of pyrolloimoquinone alkaloid series of compounds to modulate the subcellular localisation of the Hop was assessed using the same conditions as those described for the naphthoquinone compounds. All five of the pyrroloiminoquinone alkaloid compounds were found to modulate the localisation of Hop within the MDA-MB-231 cells in comparison to the DMSO control (Figure 4.2 C). All treatments resulted in increased concentration of Hop within the nuclei of the cells with the exception of RIBS-1. DSN, the most toxic compound used in these analyses resulted in the decreased of size of the cells and a smaller cytoplasm volume. Despite this change in cell

morphology, the accumulation of Hop within the nuclei was still apparent. The results from this part of the study suggested that the pyrroloiminoquinone alkaloid compounds were able to alter to subcellular localisation of Hop. The changes in localisation for all the compounds tested did not resemble that caused by HS as the Hop for HS treatment was concentrated within the nuclei of the cells.

4.4.2 Determination of effects of naphthoquinone and pyrroloiminoquinone compounds on selected Hsp90 client proteins

The ability of the naphthoquinone and marine pyrolloimoquinone alkaloid series of compounds to modulate the subcellular localisation of the Hsp90 client proteins AKT (Appendix Figures B18 and B19), STAT3 (Appendix Figures B.14 and B.15) and pSTAT3 (Appendix Figures B.14 and B.15) was investigated to provide an insight into the effect of these compounds on Hsp90 clients. The same conditions described in Section 3.3.2 were used for this part of the study.

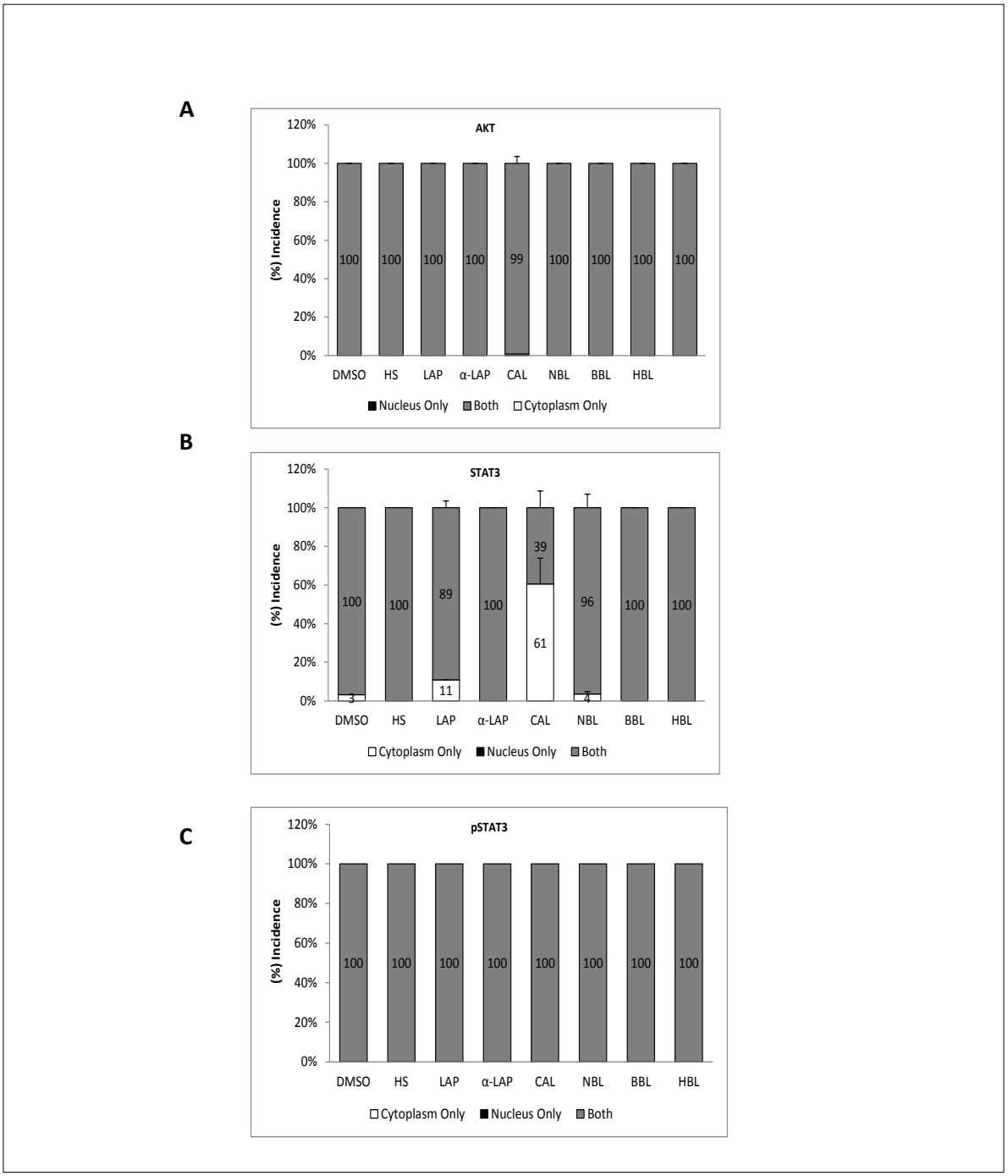


Figure 4.3: **Quantitative analysis of the subcellular localisation patterns of (A) AKT, (B) STAT3 and (C) pSTAT3 following treatment with LAP, αLAP, CAL, BBL,HBL and NBL.** The number of cells exhibiting cytoplasmic only (white), nuclear only (black) or both nuclear and cytoplasmic (grey) staining were quantified by counting the cells in three separate microscope fields for two independent experiments**.**

AKT was shown to localise to both the nuclei and cytoplasm of DMSO treated cells (Figure 4.3 A). HS treatment resulted in the same extent of cytoplasmic and nuclear localisation of AKT as DMSO however; there was an increase in the accumulation of AKT in distinct foci within the cells (Appendix Figure B.16). Changes in the global AKT localisation pattern of cells treated with the naphthoquinone series of were subtly different (Appendix Figure B.16). Cells treated with these compounds appeared

to have slightly more nuclear and less punctate or perinuclear AKT staining compared to the DMSO control treated cells. Changes in cell morphology and the morphology of AKT staining were also evident following treatment with several compounds. DMSO, LAP, CAL, αLAP and BBL treated cells maintained epithelial-like cell morphology of the MDA-MB-231 cells. Cells treated with NBL and HS appeared to be more rounded in appearance. This may have been owing to the stress caused by the HS or may be a consequence of the antiproliferative activity of the NBL. A perinuclear localisation of AKT was seen following treatment with HBL and the presence of AKT accumulated to one side of the cell was more evident (Appendix Figure B.16). These findings indicated that the action of these compounds on the localisation of AKT was similar to both NOV and 17-AAG treatments (Figure 3.1) as treatment with these two compounds also resulted in 100 % of cells showing both cytoplasmic and nuclear staining.

Treatment of cells with DMSO and HS resulted in 100% of cells showing both cytoplasmic and nuclear STAT3 (Figure 4.3 B) and pSTAT3 staining (Figure 4.3 C). Treatment with the naphthoquinone compounds modulated the localisation pattern of the Hsp90 client protein STAT3 and its activated form, pSTAT3, within treated cells (Appendix Figure B.14), although the proportion of cells showing both cytoplasmic and nuclear staining remained the same as for DMSO and HS treatments (Figure 4.3 B and 4.3 C). STAT3 remained localised predominantly to both the cytoplasm and nuclei of the treated cells and changes between treatments were mainly based on increases in nuclear STAT3 and pSTAT3 within treatments (Figure 4.3 B and 4.3 C). Treatment with LAP, CAL and NBL produced a STAT3 and pSTAT3 staining pattern similar to that of 17-AAG whereby a population of cells showing cytoplasmic only and both nuclear and cytoplasmic staining was seen for STAT3, while a 100% of treated cells show a staining pattern of both nuclear and cytoplasmic staining for pSTAT3. αLAP, BBL and HBL show no difference in either STAT3 or pSTAT3 staining when compared to the DMSO control. However, an increase in STAT3 on the membrane of cells was observed across all compound treatments compared to DMSO and HS treated cells (Appendix Figure B.14). The presence of STAT3 localised to what was confirmed to be the nucleoli of the cells was observed (Appendix Figure B.13). The incidence of this staining was independent of treatments and appeared in all cells recorded. However, for HS treatment, this putative nucleolar STAT3 staining pattern was shown to be concentrated around the nucleoli as opposed to inside the nucleoli as observed for other treatments (Appendix Figure B.14). Despite a large increase in nuclear STAT3 staining following HBL treatment, nucleolar STAT3 could still be observed in these cells (Appendix Figure B.14). The cytoplasmic STAT3 staining pattern did not appear to be diffuse for certain naphthoquinone treatments in this study, but appeared to be reticular in the case of HBL and in the case of LAP appeared to be excluded from vesicular structures within the cells. However, without further investigation, the identity of these

structures cannot be determined. Of interest, was the observation that no such nucleolar pSTAT3 was observed with any of the treatments as was shown in Appendix Figure B.14, suggesting once again that this nucleolar STAT3 might not activated within this cell line.

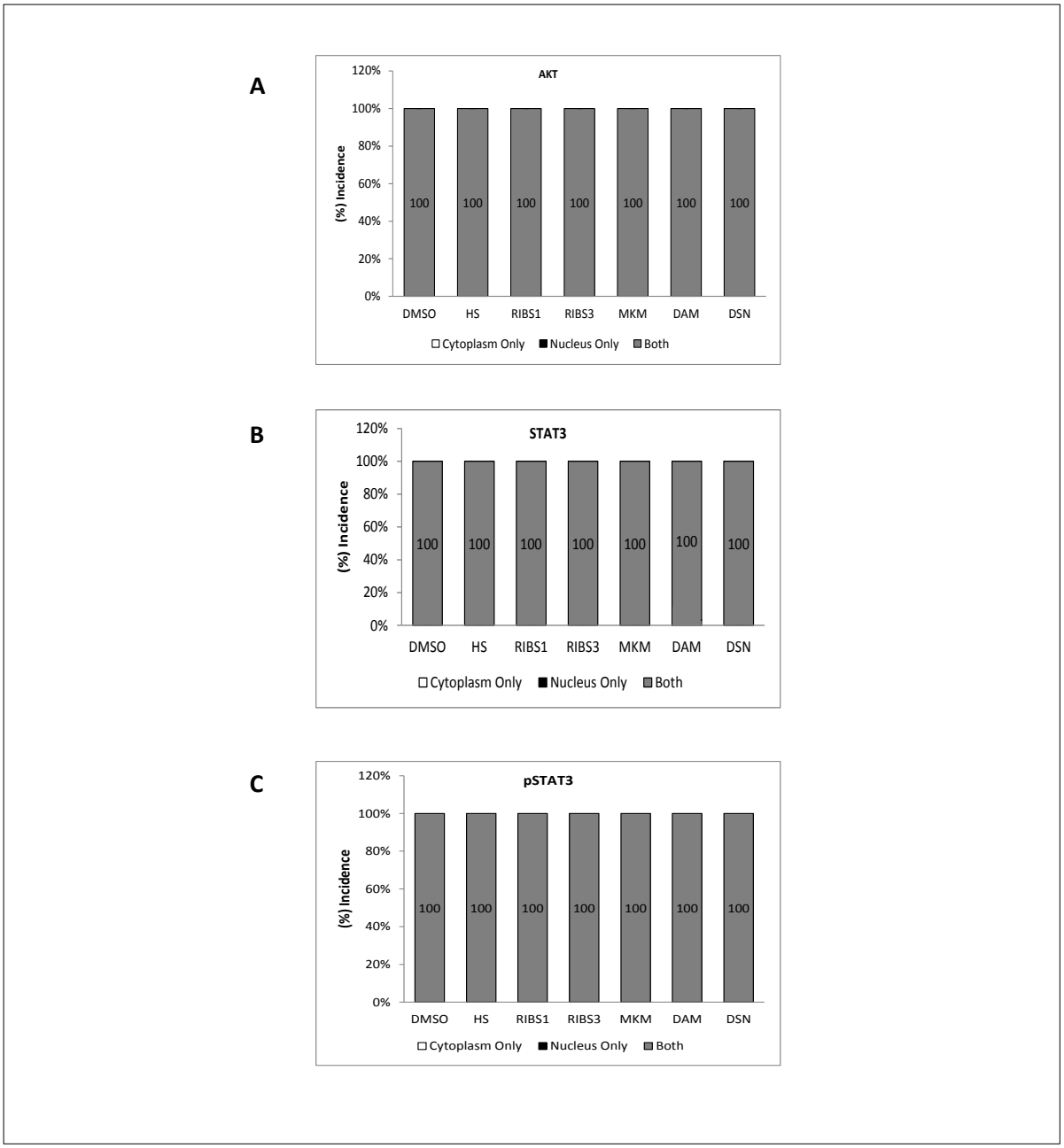


Figure 4.4 Quantitative analysis of the subcellular localisation patterns of (A) AKT, (B) STAT3 and (C) pSTAT3 following treatment with damirone C (DMN), makaluvamine M (MKM), *N*-1-β-D ribofuranosyl makaluvamine (RIBS-1), *N*-1-β-D ribofuranosyl makaluvic acid C (RIBS-3) and discorhabdin A (DSN). The number of cells exhibiting nuclear only (black), cytoplasmic only (white) or both nuclear and cytoplasmic (grey) staining were quantified by counting the cells in three separate microscope fields for two independent experiments.

Similar to treatment with the naphthoquinone compounds, treatment with the pyrroloiminoquinone alkaloid series of compounds did not modulate the localisation pattern of AKT within treated cells (Appendix Figure B.17). The same pattern of AKT localisation was observed following 17-AAG and NOV treatment where 100 % of treated cells showed both cytoplasmic and nuclear AKT staining pattern. However, a change in cell morphology of the cells was evident following treatment with MKM and DSN. While DMSO treated cells maintained an epithelial-like cell morphology, MKM treated cells appeared flatter and more spread out and cells treated with DSN appeared more spindle-like in shape compared to the DMSO control treated cells. The change in cell morphology caused by DSN treatment may be as a consequence of the highly antiproliferative activity of the compound. The changes in cell morphology caused by MKM treatment could be further investigated as Hsp90 is known to maintain cell morphology (Sidera *et al*., 2004) and Hsp90 inhibition has been shown to alter cell migration (Taiyab and Rao*,* 2011). In addition to the changes in cell morphology, treatment with the compounds resulted in cells with different AKT staining patterns. AKT staining in DMSO treated cells appeared diffuse and uniform across the cytoplasm and the nuclei of the cells. HS treatment resulted in an increase in the number of cells showing AKT staining localised to one side of the cells. A similar staining morphology was seen following treatment with DSN and with RIBS-3. Treatment with DMN resulted in the increased presentation of cells showing perinuclear AKT staining while treatment with RIBS-1 resulted in cells showing punctate nuclear AKT staining compared to the DMSO control cells (Appendix Figure B.16).

The lack of modulation of AKT localisation by either the naphthoquinone or pyrroloiminoquinone series of compounds may be a result of the use of a pan AKT antibody in this study. The kinase domain is highly conserved among all AKT isoforms, while the N-terminal pleckstrin homology is less highly conserved (Bellacosa *et al*., 2007). The pleckstrin homology domain is a target for compounds that inhibit AKT directly as it regulates the localisation of AKT to the membrane of cells and controls the kinase domain in an inactive state in the absence of cellular stimuli (Machajewski *et al*., 2005). Therefore, any compound that may have shown specificity towards and modulated the localisation of one isoform of AKT might have not altered the localisation of a different isoform of AKT. Further to this is it unknown whether one isoform of AKT is able to compensate for another in the case of the inhibition of the first AKT isoform in question. A different approach would be the use of AKT 1, 2 and 3 antibodies separately and to determine whether there any changes in localisation on the individual isoforms.

Treatment with the marine pyrroloiminoquinone alkaloid compounds altered the STAT3 and pSTAT3 localisation patterns in compound-treated cells but did not alter the proportion of cells showing both cytoplasmic and nuclear staining as this remained the same as for DMSO and HS treatments. (Figure 4.4 B, 4.4 C). Treatment with compounds was not similar to either 17-AAG or NOV treatment and more closely resembled the DMSO treatment. Both STAT3 and pSTAT3 remained localised predominantly to both the cytoplasm and nuclei of the treated cells and the main difference between treatments was based on increases in intensity of nuclear STAT3 and pSTAT3 within treatments. RIBS-1, MKM and DMN treatments resulted in increased intensities of nuclear STAT3 and pSTAT3 (Figure 4.4 B, 4.4 C). Treatment with all the compounds with the exception of DSN resulted in an increase in STAT3 on the membrane of cells when compared to DMSO only and HS treated cells (Appendix Figure B.15). The presence of nucleolar STAT3 was observed uniformly across all treatments (Appendix Figure B.15) and was independent of treatments.

4.4.3 Determination of effects of naphthoquinone compounds and pyrroloiminoquinone compounds on the expression of Hsp90, Hsp70, Hop and AKT

The potential of the naphthoquinone and marine pyrroloiminoquinone compounds to modulate the expression levels of Hsp90, Hsp70, and Hop and induce the degradation of the Hsp90 client protein AKT in a manner similar either 17-AAG or NOV (Figure 3.7) in MDA-MB-231 metastatic breast cancer cells was investigated. DMSO was used as a vehicle control and as such DMSO treated cells were taken as a negative control. The final concentration of DMSO did not exceed 0.1 % in any instance. Cells were treated with compounds at concentrations equivalent to their IC_{50} values for 8 hours only the number of compounds, collected and lysates resolved on a 4 % SDS-PAGE stacking gel pre-cast on top of a 12 % SDS-PAGE resolving gel by SDS-PAGE and Western analysis (Figure 4.5 and 4.6).

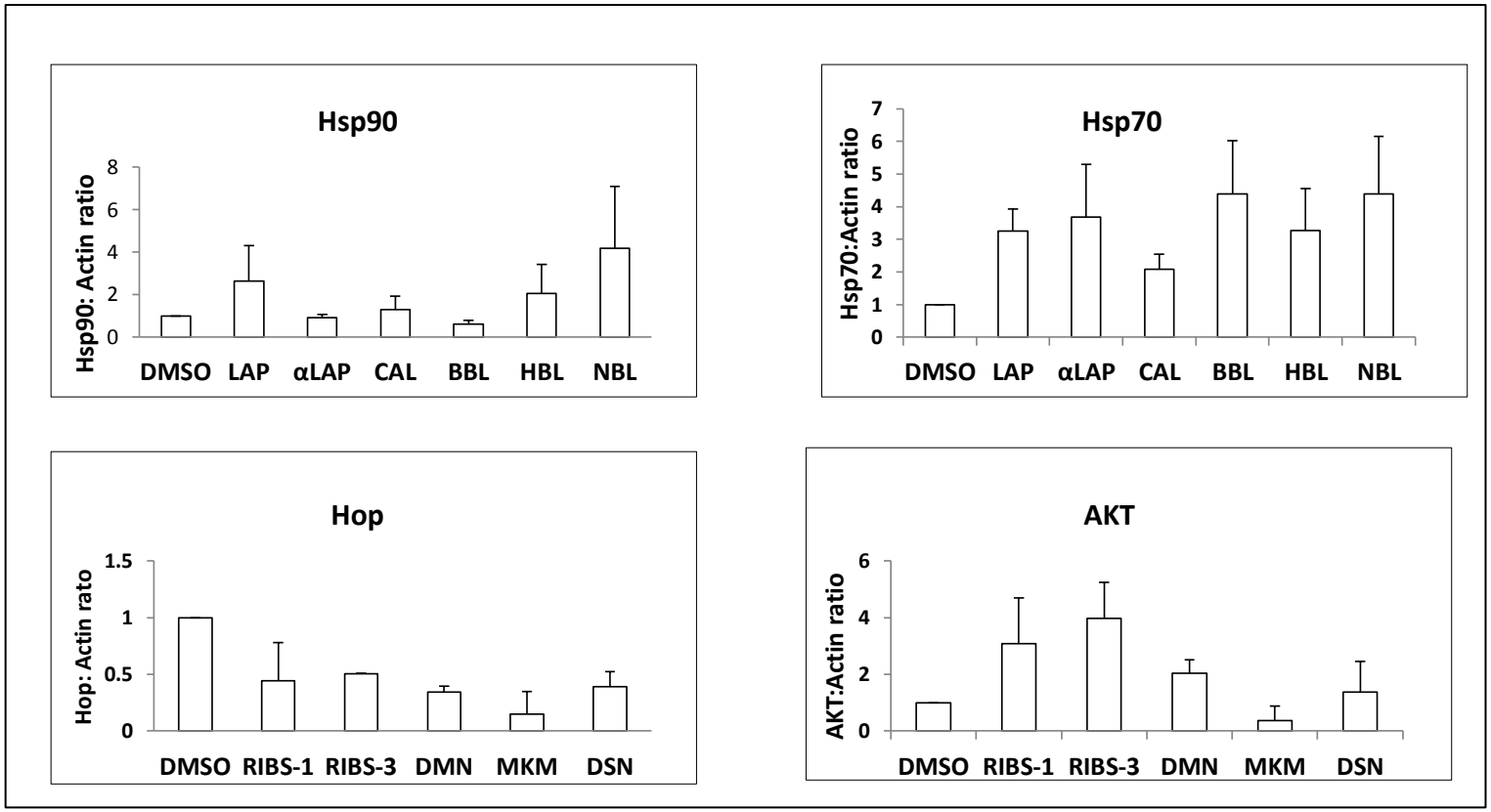


Figure 4.5: Expression levels of proteins of Hsp90 complex and selected client proteins in cells treated with LAP, αLAP, CAL, BBL, HBL and NBL. Equal numbers of MDA-MB-231 cells (1 x 10^6) were treated with DMSO (vehicle control), lapachol (LAP) (7μM), α-lapachona (αLAP) (7 μM), c-alil-lausona (CAL) (7 μM), nor-β-lapachona (NBL) (8.78 μM),bromo-β-lapachona (BBL) (7.86 μM) and hydroxy-β-lapachona (HBL) (7.61 μM) for 8 hours at 37 °C. The levels of chaperones and client proteins were determined by Western analysis using antibodies indicated to the left of the figure. Actin was used as a loading control to normalise for the protein concentration in all cases, except for pSTAT3 in which case total STAT3 was used as the loading control. The intensity of the bands represented as a ratio to those of the loading control (indicated on the y-axis). The ratio of protein to loading control was expressed relative to the DMSO control for each chaperone and client protein to allow comparison of trends between independent experiments. Data shown are an average of three independent experiments. Statistical analysis was performed using ANOVA.

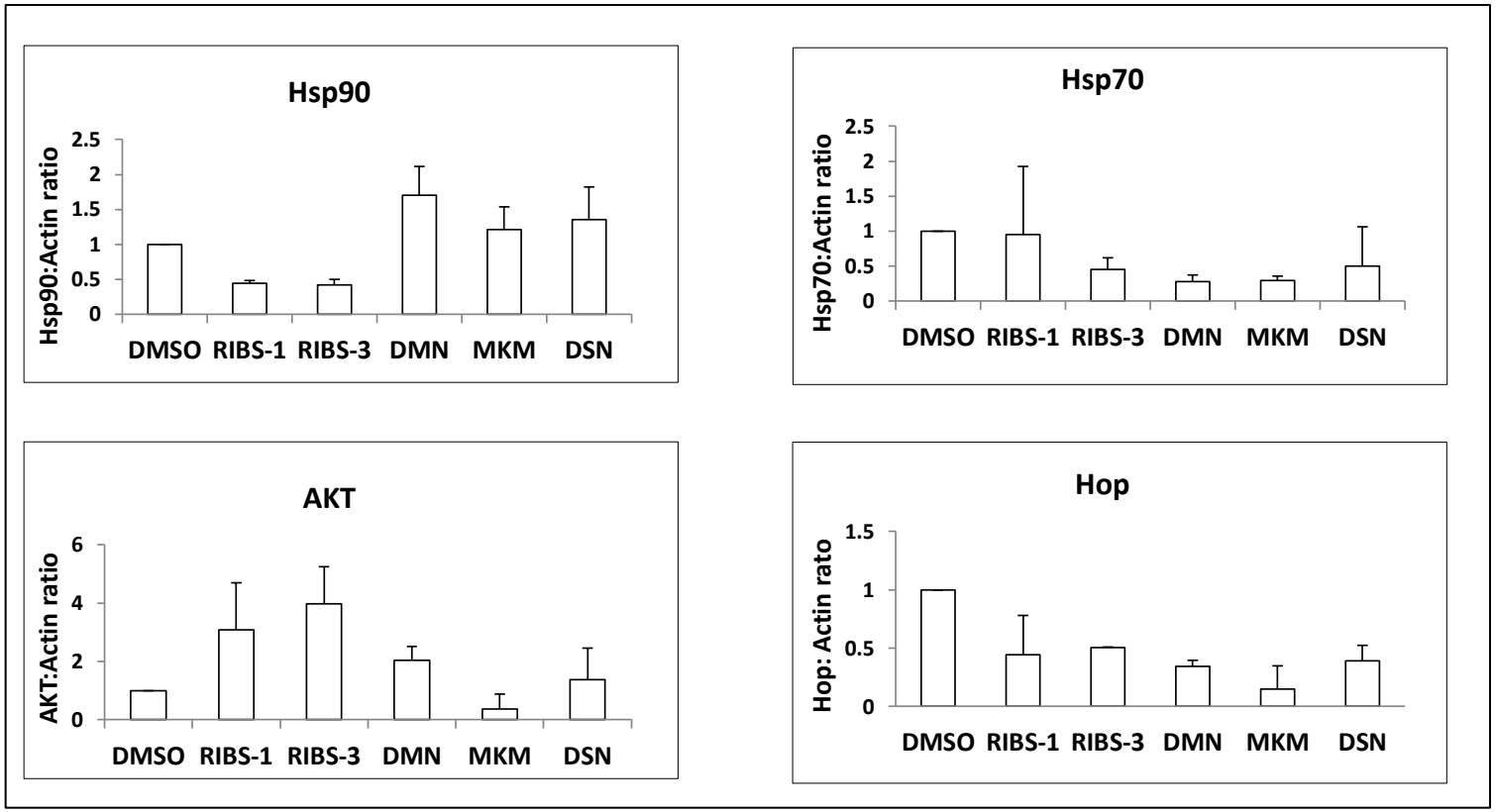


Figure 4.6: Expression levels of proteins of Hsp90 complex and selected client proteins in cells treated with RIBS-1, RIBS-3, DMN, MKM, DSN. Equal numbers of MDA-MB-231 cells (1 x 10^6) were treated with DMSO (vehicle control), damirone C (DMN) (12.4 µM), makaluvamine M (MKM) (7 µM), *N*-1-β-D ribofuranosyl makaluvamine (RIBS-1) (7 µM), *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3) (8.78 µM), discorhabdin A (DSN) (3.32 µM) for 8 hours at 37 °C. The levels of chaperones and client proteins were determined by Western analysis using antibodies indicated to the left of the figure. Actin was used as a loading control to normalise for the protein concentration in all cases was used as the loading control. Densitometry analysis was performed using MacBiophotonics ImageJ and the intensity of the bands represented as a ratio to those of the loading control (indicated on the y-axis). The ratio of protein to loading control was expressed relative to the DMSO control for each chaperone and client protein to allow comparison of trends between independent experiments. Data shown are an average of three independent experiments. Statistical analysis was performed ANOVA.

As indicated in Figure 4.5 when compared to the DMSO control, Western analysis revealed no significant changes in the Hsp90 expression levels following LAP [F (1, 4) =0.16, P = 0. 73], αLAP [F (1, 4) = 2.90, P = 0. 23], CAL [F (1, 4) = 0.11, P = 0. 77], BBL [F (1, 4) = 0.78, P = 0. 43], HBL [F (1, 4) = 0.11, P = 0. 77] and NBL [F (1, 4) = 0.31, P = 0. 64] treatments after an 8 hour treatment. This result was similar to that shown in Figure 3.3 whereby treatment with the N- and C-terminal Hsp90 inhibitors 17-AAG and NOV did not alter the expression levels of Hsp90 even after 24 hours of treatment.

No significant changes were observed in the expression levels of Hsp70 following an 8 hour treatment with αLAP [F (1, 4) = 5.45, P = 0. 15], CAL [F (1, 4) = 10.4, P = 0. 80], BBL [F (1, 4) = 8.55, P = 0. 10] and NBL [F (1, 4) = 7.33, P = 0. 11]. This result was similar to that shown in Figure 3.3 where treatments with either 17-AAG or NOV did not modulate the expression levels of Hsp70. However, a significant decrease was recorded following treatment with LAP [F (1, 4) = 22.5, P = 0. 04]. This finding was unique as the result was dissimilar to all the compounds tested in this study and suggests that LAP may target Hsp70 within treated cells.

The expression levels of Hop were not modulated significantly following an 8 hour treatment with any of the naphthoquinone compounds as indicated by the statistical analyses for the treatments LAP [F (1, 4) = 0.35, P = 0. 61], αLAP [F (1, 4) = 0.68, P = 0.50], CAL [F (1, 4) = 0.54, P = 0.54], BBL [F (1, 4) = 0.18, P = 0. 71], HBL [F (1, 4) = 0.63, P = 0. 51] and NBL [F (1, 4) = 1.09, P = 0. 41]. This result was similar to that shown in Figure 3.3 where treatment with both 17-AAG and NOV did not modulate the expression levels of Hop.

Similarly, no significant changes in the expression levels of AKT were detected following an 8 hour treatment with any of the compounds as indicated by the statistical analyses for the treatments LAP [F (1, 4) = 0.0081, P = 0. 94], αLAP [F (1, 4) = 1. 15, P = 0.40], CAL [F (1, 4) = 0.50, P = 0.55], BBL [F (1, 4) = 0.50, P = 0. 55], HBL [F (1, 4) = 0.63, P = 0. 51] and NBL [F (1, 4) = 0.78, P = 0. 47]. Again, this result was similar to that shown in Figure 3.3 where treatment with both 17-AAG and NOV did not modulate result in the degradation of AKT.

Two outcomes are possible from these findings. The first is that the concentrations of the naphthoquinone compounds used in this may have been too low or the time frame chosen for the assay may have been too short to induce the degradation of AKT. The second possibility would be that the compounds did not interact with Hsp90 or members of the Hsp90 multi-chaperone complex

(with the exception of LAP which degraded Hsp70) and as such no modulation in the expression patterns of these proteins would be expected.

Within pyrroloiminoquinone series of compounds (Figure 4.6), Western analysis revealed no significant changes in the Hsp90 expression levels following MKM [F (1, 4) = 5.82, P = 0.14], DMN [F (1, 4) = 0.91, P = 0. 44] and DSN [F (1, 4) = 1.21, P = 0. 39] treatments after 8 hours when compared to the DMSO control. However, treatment with RIBS-1 [F (1, 4) = 279.0, P = 0.004] and RIBS-3 [F (1, 4) = 110.5, P = 0. 009] resulted in a significant decrease in the expression of Hsp90. The reduction in the expression of Hsp90 by RIBS-1 and RIBS-3 may suggest that the effect of these compounds on Hsp90 may be due to an alternative mechanism as the reduction of Hsp90 expression is not documented within the literature for compounds that inhibit this protein.

Hsp70 expression remained largely unaffected and changes were found to be insignificant following treatment with RIBS-1 [F (1, 4) = 0.005, P = 0. 95] and DSN [F (1, 4) = 1.58, P = 0. 34] after an 8 hour treatment when compared to the DMSO control indicating that these compound did not induce a HS response. Conversely, RIBS-3 [F (1, 4) = 21.9, P = 0.04], MKM [F (1, 4) = 217.0, P = 0.005] and DMN [F (1, 4) = 116.9, P = 0. 008] reduced the expression of Hsp70 significantly.

Western analysis revealed no significant changes in the Hop expression levels following treatments with RIBS-1 [F (1, 4) = 5.38, P = 0. 15], DMN [F (1, 4) = 0.006, P = 0. 95] and DSN [F (1, 4) = 0.06, P = 0. 83] after an 8 hour period when compared to the DMSO control. However, RIBS-3 [F (1, 4) = 9932.5, P = 0.0001], MKM [F (1, 4) = 32.8, P = 0.03] resulted in a significant decrease in the expression levels of Hop.

No significant changes in the AKT expression levels following treatment with marine pyrroloiminoquinone alkaloid compounds RIBS-1 [F (1, 4) = 3.31, P = 0. 21], RIBS-3 [F (1, 4) = 11.0, P = 0.08], MKM [F (1, 4) = 3.14, P = 0.22], DMN [F (1, 4) = 9.90, P = 0. 09] and DSN [F (1, 4) = 0.23, P = 0. 61] after an 8 hour treatment when compared to the DMSO control were observed. This would suggest that these compounds do not target Hsp90 as Hsp90 client protein degradation is a classic indicator of Hsp90 inhibition.

However, as DMN, RIBS-3 and MKM treatment resulted in the decreased expression of Hop and Hsp70, it is possible that these proteins may interact with these two proteins, alternative members

of the Hsp90 complex or perhaps Hsp90. It is possible that a treatment with higher concentration of these compounds may be required to degrade Hsp90 client proteins.

4.4.4 Isothermal titration calorimetry analysis of naphthoquinone compounds and Hsp90β

Cell biological data suggested that certain compounds of the naphthoquinone and marine pyrolloimoquinone alkaloid series may interact with Hsp90 and/or Hsp90 partner proteins based on their ability to modulate the subcellular localisation and expression of selected proteins. Therefore, this study sought to employ the use of ITC to determine whether these compounds were able to interact with Hsp90β directly and to determine the thermodynamic parameters of any such interaction. Compounds of the naphthoquinone series were prioritised for analysis over compounds of the marine pyrroloiminoquinone series of compounds as the naphthoquinone scaffold has been suggested to be an Hsp90 inhibitor scaffold (Hadden, *et al*., 2009) and as ITC is a low throughput technique, requiring high time and material requirements.

Hsp90β was expressed and successfully purified (Appendix A, Figure C.1) from C41 *E.coli* cell lysates by affinity-tag purification followed by ion exchange chromatography to increase the purity of the protein used in the ITC experiments. ITC experiments were performed in a VP-ITC MicroCalorimeter (MicroCal Inc., Northampton, Massachusetts, United States of America) at 30 °C with purified Hsp90β in 50 mM Tris, 6 mM $MgCl_2$ 20 mM KCl, 1 mM β-mercaptoethanol , pH 7.4 in the sample cell, and 700 µM compound injected from syringe with constant mixing. The concentration of DMSO was matched in the reference cell, sample cell and in the syringe. Data analysis performed using Origin 7 software (MicroCal, USA) (Figure 4.14). LAP, αLAP, CAL, BBL, HBL and NBL were analysed for binding to Hsp90β.

The optimisation of the parameters required for successful experiments was performed using Hsp90β at 5, 10 and 20 µM concentrations (Appendix A, Figure C.7) with the concentration of the compounds always twenty times in excess of the protein concentration as no kinetic data for the interaction of the naphthoquinone compounds and Hsp90 was available. The concentration of Hsp90β was increased to 30 µM and the compound concentration to 700 µM, thus increasing the ratio of protein to compound to 1: 23.3. A weak interaction of LAP to Hsp90β was observed under these conditions (Figure 4.7) but the compounds αLAP, CAL, BBL, HBL and NBL showed no association with Hsp90β when tested (Appendix Figures C.2, C.3, C.4, C.5, C.6). The thermodynamic data obtained for the binding of LAP to Hsp90β from the fitted isotherms to the one site model were

N = 5.18E-4 ± 3.62, K = 4.54E3 ± 3.83E3 M^{-1}, ΔH = -9.057E6 ± 6.336E10 $cal.mol^{-1}$ and S = -2.99E4 $cal.mol^{-1}.deg^{-1}$. More accurate data could not be determined owing to the poor fit of the data to the single-site model using Origin 7 software. Despite this, small heat changes with increasing titrations were observed suggesting a weak association of LAP with Hsp90β. As the changes in heat were small (0.00 to 0.12 μcal), the isotherms for Hsp90β and LAP could possibly be improved by increasing the temperature of the sample cell to 35 °C and trying a higher Hsp90β concentration such as 50 μM. One challenge during the isothermal titration calorimetry experiments was maintaining the compound in solution at the required concentrations while maintaining the DMSO level below the cut off value of 5 % as, the compound would precipitate out of solution during the experiment if not maintained at 5 % DMSO in the syringe. Another limitation was the stability of the compound. As the stability of the naphthoquinones was unknown, it could not be determined if the weak association of LAP Hsp90 was caused by the degradation of the compound. Despite these limitations, changes of heat were still observed suggestive of LAP binding to Hsp90β.

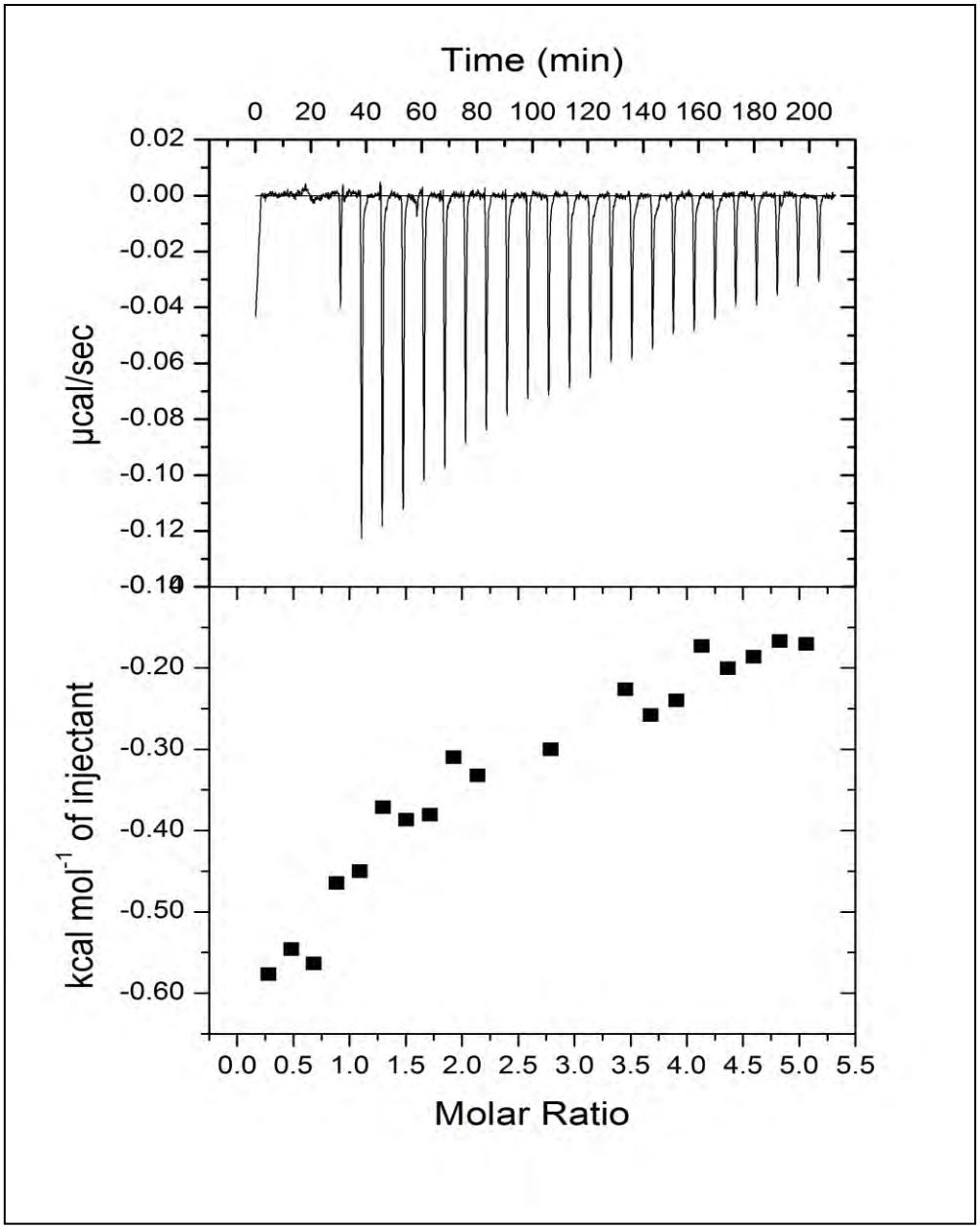


Figure 4.7: Lapachol interacts with Hsp90β with a low affinity. Fitted data between 700 µM lapachol and 30 µM Hsp90β equilibrated to 30 °C in 50 mM Tris, 6 mM $MgCl_2$ 20 mM KCl, 1 mM β-mercaptoethanol, pH 7.4. Upper graph represents the raw ITC data in which the incremental heat changes are shown as microcalories per second ($\mu ca.sec^{-1}$) plotted against time in minutes over the progression of the experiment, lower graph shows the shows normalised integration data in terms of $kcal.mol^{-1}$ of injectant plotted against the molar ratio of the added ligand with the curve fitted to single-site mode. The relationship between the two x-axes allows for the positioning of the integrated area for each peak directly below the associated peak in the raw data. The negative peaks correspond to addition of an aliquot of ligand. The calorimetric output indicated that an exothermic process was occurring in the cell.

4.4.5. *Determination of the ability of LAP to disrupt the Hsp90-Hop interaction in vitro*

As LAP was shown to interact with Hsp90, the effect of LAP on the association of Hsp90 and Hop, using His-tagged pulldown assays was examined as these proteins exist in an intermediate complex (Southworth and Agard, 2011) (Figure 1.2). The effect of LAP on this association was compared to that of NOV and GA This assay was not performed on the other compounds of the 1, 4 naphthoquinone series as these compounds showed no interaction with Hsp90β (Appendix, Figures C2, C3, C.4, C.5, C.6). For these assays purified recombinant hexahistidine Hop was incubated with nickel-chelated sepharose beads and purified Hsp90β was protein incubated with different concentrations of LAP before the two proteins were allowed to interact. DMSO was used as a control with the final concentration not exceeding 0.01 %. Hsp90 alone was incubated with beads as a control and indicated that the Hsp90 associated with Hop was the result of a specific interaction with Hop and not as a result of non-specific binding of Hsp90 to the beads. The protein complexes pulled down were analysed by SDS-PAGE. No ATP was added to assays as Hop binds to Hsp90 in the nucleotide-free or ADP-bound state (Johnson *et al*., 1998).

Hsp90 was successfully pulled down following treatment with DMSO only. The ratio of Hsp90 associated with Hop following DMSO treatment was compared to those following treatment with LAP at 0.01, 0.1 and 1 μM concentrations. The differences between treatments and DMSO were found to be non-significant at 0.01 μM [$F(1, 4) = 0.025$, $P = 0.88$], 0.1 μM [$F(1, 4) = 0.0046$, $P = 0.95$] and 1 μM [$F(1, 4) = 0.022$, $P = 0.89$] by comparing the variance between DMSO and compound-treated samples by ANOVA. This data compared to that of the GA and NOV pulldown assays (Section 3.4.5) which indicated that the effect of LAP on Hsp90β may be similar to that of GA as GA was shown to not disrupt the Hsp90-Hop association. Alternatively, the binding of LAP to Hsp90β may be too weak to disrupt the interaction with Hop. This result could be further validated within a cell model by performing immunoprecipitation experiments in cells would be treated with LAP and the lysates added to Hsp90 bound to the beads to determine which other association of Hsp90 and its partner proteins may be disrupted.

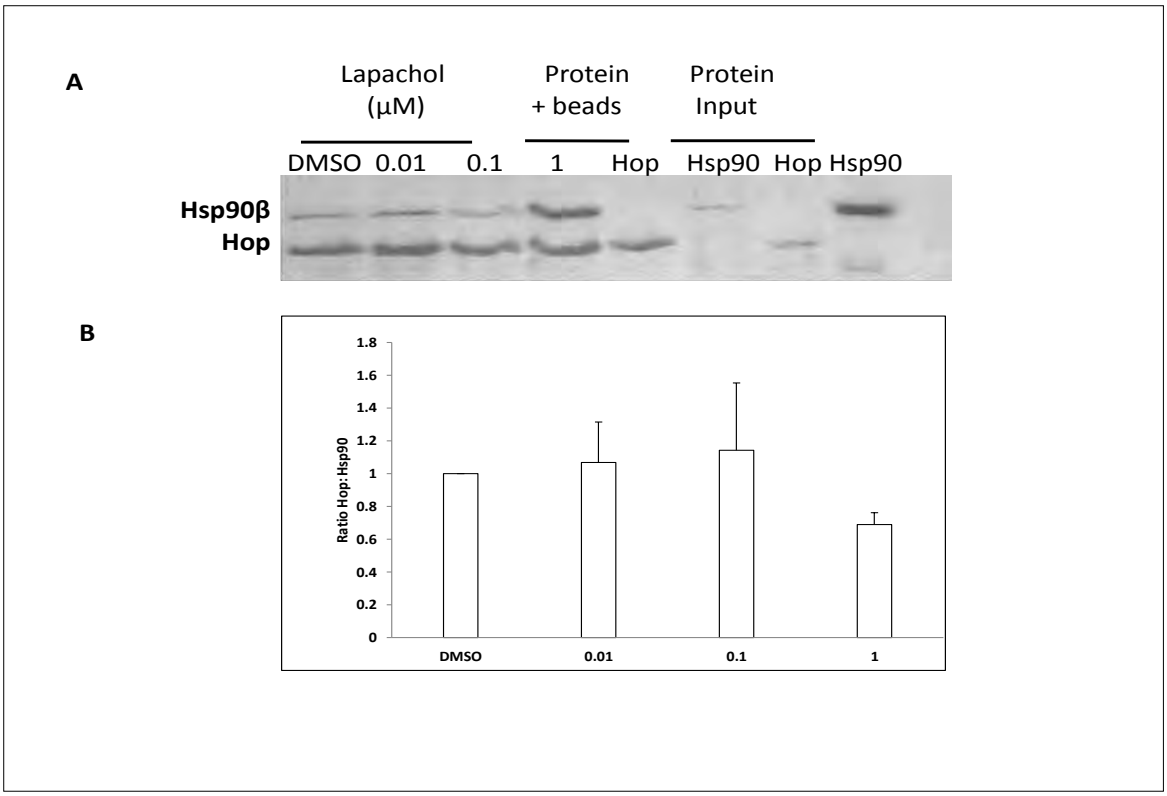


Figure 4.8: Treatment with LAP did not disrupt Hsp90-Hop interaction *in vitro*. His-tagged pulldown assays in which Hop protein was incubated with Ni sepharose beads for 2 hours and then with Hsp90β protein previously incubated with lapachol (LAP) for 30 minutes. Compounds were used at a concentration range of 0.01 – 1 μM. The Protein + beads lane shows the level of protein interacting non-specifically with beads and the protein input lanes indicated Hsp90β and Hop protein alone. (A) Proteins were resolved on a 4 % SDS-PAGE stacking gel pre-cast on top of a 12 % SDS-PAGE resolving gel and were visualised by silver staining with PageSilver ^{TM} Silver Staining Kit according to the manufacturer's instructions. (B) Densitometry analysis was performed using MacBiophotonics ImageJ and the intensity of the bands of Hsp90 represented as a ratio to those of Hop. The ratio of Hsp90β to Hop was expressed relative to the DMSO control for each treatment at different concentrations to allow for the comparison of trends between independent experiments. Data shown are an average of three independent experiments with similar results. Statistical analysis was performed using ANOVA.

4.5. Conclusion

A previous study reported that out of 68 synthetic derivatives of LAP only one compound, 2-(3, 7-dimethyl-2, 6-octadienyl)-3-hydroxy-1, 4-naphthoquinone showed activity against the Walker 256 rat breast carcinoma cell line (Hussain *et al*., 2007). This illustrates the difficulty of identifying compounds specific to or against a single parameter or target. The 1, 4 naphthoquinone series of compounds were screened for their ability to modulate the localisation and expression of Hsp90 and its partner proteins. The data collected initially suggested that several of the compounds possibly interacted with Hsp90, displayed certain selectivity against Hsp90 client proteins as they were able

to modulate the localisation of STAT3 and pSTAT3 but not AKT. The lack of AKT modulation by αLAP, CAL, NBL, HBL and BBL may be as a direct consequence of the lack of binding of these compounds to Hsp90β as indicated by ITC analysis. However, treatment with LAP, BBL, HBL, RIBS-1, and MKM altered AKT subtly and as reflected in the changes in morphology of staining rather than a global shift in the localisation. Although LAP was shown to interact with Hsp90 weakly, it did not disrupt the association between Hsp90β and Hop despite showing low affinity binding to Hsp90β in the ITC analyses. Western analysis further showed that LAP and the derivatives of this compound did not down-regulate the expression of AKT and it was thought that higher concentrations of compounds may be required to induce client protein degradation by LAP. Therefore, as treatment LAP bound to Hsp90, but did not disrupt the association of Hsp90 and Hop, it is possible that this compound may interact with Hsp90 in a manner similar to N-terminal Hsp90 inhibitors. However, the 1, 4 naphthoquinone scaffold has been suggested to bind to Hsp90 in a similar manner as NOV. Therefore, it is also possible that the concentrations used in this study were not sufficient to disrupt the Hsp90 - Hop association.

The compounds RIBS-1, RIBS-3, MKM and DMN of the pyrolloimoquinone series of compounds showed similar promise in terms of modulating the localisation and expression of Hsp90 and its partner proteins. However, owing to restraints on time and resources, these compounds were not screened by ITC to determine their direct binding potential to Hsp90. RIBS-1 reduced the expression of Hsp90 suggesting that the effect of this compound on Hsp90 may be owing to an alternative mechanism as the reduction of Hsp90 expression in response to compound treatment is not documented within the literature. MKM was found to down-regulate Hsp70 expression despite resulting in an increase in nuclear HSF1 following treatment with this compound. RIBS-3 was shown to alter the localisation of members of the Hsp90 chaperone complex and down-regulated the expression of Hop. Treatment with DMN was shown to down-regulate expression of Hop and Hsp70. These findings suggested that these compounds may directly target proteins of the Hsp90 multi-chaperone complex.

CHAPTER 5

FINAL CONCLUSIONS

5.1 Summary of findings

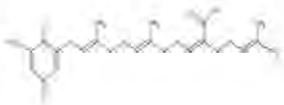
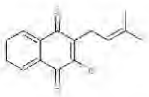
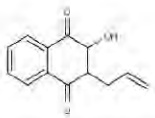
A comparison of structurally similar compounds isolated from South African natural sources was performed during this study to identify potential Hsp90 modulators. The approach of using locally sourced compounds which were structurally similar to each other was favoured and advantageous as a SAR could be developed for the compounds. An alternative approach may have been to identify compounds known to exhibit anti-cancer or anti-tumour effects from chemical biology databases and screen them for Hsp90 modulatory activity. Programs such as Autodock or ArgusLab 4.0.1 could have been utilised to perform *in silico* screening by ligand docking to Hsp90, thus identifying compounds with the potential capacity to bind to Hsp90. These compounds could be identified from numerous open source chemical biology databases such as PubChem[22], ChemSpider[23], ChemBank[24], ChEMBL[25] and ChemBioFinder[26]. The information contained in these databases provides access to chemical and biological findings for millions of small molecules, thus allowing for novel targets to be attributed to previously published compounds or for the modification of existing compounds and/or the synthesis of novel compounds to be developed based on the compounds within these databases. PubChem[22] and ChemBank[24] contain information regarding the chemical structures, biological properties of compounds and siRNA reagents whilst ChEMBL[25] contains data concerning the binding, functional, and ADMET properties of small molecules. ChemSpider[23] and ChemBioFinder[26] are similar to PubChem[22] and ChemBank[24] as they contain information about the physical and chemical properties, molecular structure, spectral data and synthetic methods of compounds. However, the approach used in this study was deemed advantageous as use of locally sourced compounds and small molecules will add to drug discovery research and development in Africa.

Various quinone-bearing compounds have been identified as chemotherapeutic agents (de Paiva *et al*., 2004, Monks and Jones 2002). Therefore, the major aims of the work presented in this dissertation were to screen and characterise compounds bearing the quinone nucleus for their ability to modulate Hsp90 activity within a TNBC cell line model. A TNBC model was selected as this form of the disease is biologically aggressive and as women of African descent have been shown to have a predisposition for this form of breast cancer. Therefore, the identification of any compounds that could have modulated Hsp90 within the TNBC model could further add to the information on the both Hsp90 and TNBC. The compounds examined in this study were divided into three groups,

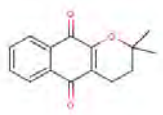
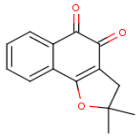
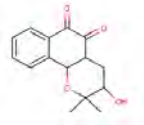
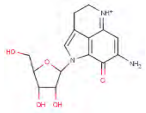
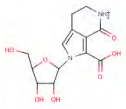
[22] http://pubchem.ncbi.nlm.nih.gov/
[23] http://www.chemspider.com/
[24] http://chembank.broad.harvard.edu/
[25] https://www.ebi.ac.uk/chembl/
[26] http://www.chembiofinder.com

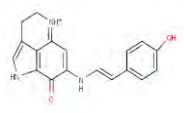
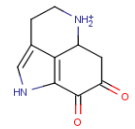
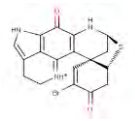
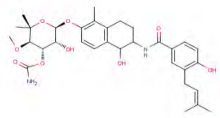
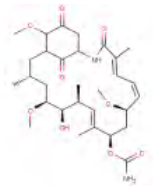
namely; the marine algal compound SQA, the naphthoquinone series and the marine pyrroloiminoquinone alkaloid series of compounds. SQA, a compound shown to exhibit antiproliferative effects against the MDA-MB-231, MCF7 and Hs578T breast cancer lines (de la Mare *et al*., 2012) was selected from a group of marine algal compounds isolated off the coast of South Africa (Afolayan *et al*., 2008) for investigation in this study as it was the only quinone-bearing compound from the algal series of compounds. This compound was shown to have no effect on the cell viability of the non-cancerous MFC12A epithelial breast cell line and was determined to cause cancer cell death through apoptosis (de la Mare *et al*., 2012). The second set of compounds, the naphthoquinone series of compounds, included the 1, 4 naphthoquinones LAP, αLAP, CAL and the 1, 2 naphthoquinones NBL, BBL and HBL all of which were previously untested in any cell biological model within BioBRU. These compounds were of interest as several 1, 4 naphthoquinones have been demonstrated to possess anti-tumour activities (Niculescu *et al*., 2011; Salustiano *et al*., 2010; Kayashima *et al.*, 2009; Lee *et al.*, 2007) and have been hypothesized to bind to Hsp90 (Hadden *et al*, 2009). Additionally, 1, 2 naphthoquinones such as β-lapachone, exhibit strong anti-cancer activities in *in vitro* and *in vivo* systems (Park *et al*., 2005) and induce apoptosis in the MCF-7 breast cancer cell line, xeongraft mouse models (Park *et al*., 2011) and within the MDA-MB-231 breast cancer cell line (Seoane *et al*., 2013). Despite their attractive anti-cancer activities and structural similarity to the 1, 4 naphthoquinones, no reports regarding the Hsp90 binding capacity of the 1, 2 naphthoquinone scaffold have been made. The third group of compounds, the marine pyrroloiminoquinone alkaloid series of compounds were also previously untested in any cell biological model within BioBRU. Compounds from this class of compounds have been shown to exhibit anti-cancer activities in several cell lines (Nag *et al*., 2012; Wang *et al*, 2009; Harayama and Kita, 2005) through their fused-ring structures. These compounds bear some structural similarity to the purine scaffold of Hsp90 inhibitors, thus indicating potential Hsp90 modulatory properties. To the best of our knowledge these compounds have not previously been investigated for Hsp90 binding or modulation properties. The main findings for the compounds analysed in this study are summarized in Table 5.1.

Table 5.1: Summary of the structures, IC_{50} values, localisation patterns, Hsp90 binding and disruption of Hsp90-Hop association of sargaquinoic acid (SQA), lapachol (LAP), c-alil-lausona (CAL), α-lapachona (αLAP), nor-β-lapachona (NBL), bromo-β-lapachona (BBL) and hydroxy-β-lapachona (HBL), *N*-1-β-D ribofuranosyl makaluvamine (RIBS-1), *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3), damirone C (DMN), makaluvamine M (MKM), discorhabdin A (DSN), novobiocin (NOV) and 17-N-allylamino-17-demethoxygeldanamycin (17-AAG)/ 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG)/ geldanamycin (GA). [27]

			Predominant localisation pattern								
Compound	**Chemical structure**	**IC_{50} (μM)**	**Hsp90**	**Hsp70**	**Hop**	**HSF1**	**STAT3**	**pSTAT3**	**AKT**	**Binds Hsp90β**	**Disrupts Hsp90-Hop**
SQA		90.2	Punctate nuclear, pattern and diffuse cytoplasmic	Cytoplasmic	Punctate nuclear, perinuclear, cytoplasmic	Nuclear, cytoplasmic	Cytoplasmic, nuclear, nucleolar	Cytoplasmic, nuclear	Cytoplasmic, nuclear	Yes	Yes
LAP		N.A.A	Diffuse cytoplasmic	Diffuse nuclear and cytoplasmic	Cytoplasmic, perinuclear, nuclear	Punctate nuclear. cytoplasmic	Cytoplasmic, nuclear, nucleolar	Cytoplasmic, nuclear	Cytoplasmic, nuclear	Yes	No
CAL		N.A.A	Diffuse cytoplasmic and nuclear	Diffuse cytoplasmic	Cytoplasmic, perinuclear, nuclear	Punctate nuclear, cytoplasmic	Cytoplasmic, membrane, nuclear, nucleolar, perinuclear	Cytoplasmic, nuclear	Cytoplasmic, nuclear and perinuclear	No	N.D

[27] N.A.A denotes No antiproliferative activity within the concentration range tested
N.D denotes Not determined

αLAP		N.A.A	Membrane, diffuse cytoplasmic and nuclear	Diffuse cytoplasmic, nuclear, perinuclear	Cytoplasmic, increased nuclear	Punctate nuclear, cytoplasmic	Reticular cytoplasmic, membrane, nuclear, nucleolar, perinuclear	Cytoplasmic, nuclear, perinuclear	Cytoplasmic, nuclear and perinuclear	No	N.D
NBL		8.78	Membrane, punctate nuclear, diffuse cytoplasmic	Cytoplasmic and nuclear	Cytoplasmic, nuclear	Punctate nuclear. cytoplasmic	Reticular cytoplasmic, perinuclear, increased membrane, , nucleolar	Cytoplasmic, perinuclear, increase in nuclear	Cytoplasmic, nuclear	No	N.D
BBL		7.86	Membrane, diffuse cytoplasmic and nuclear	Cytoplasmic	Cytoplasmic, nuclear, perinuclear	Punctate nuclear. cytoplasmic	Cytoplasmic, nuclear, nucleolar	Cytoplasmic, per-nuclear and nuclear	Cytoplasmic, nuclear, more perinuclear	No	N.D
HBL		7.61	Membrane, diffuse cytoplasmic, and nuclear staining, membrane	Cytoplasmic and nuclear	Cytoplasmic, nuclear	Punctate nuclear, cytoplasmic	Reticular cytoplasmic, nuclear and membrane	Cytoplasmic, nuclear	Cytoplasmic, nuclear	No	N.D
RIBS-1		N.A	Membrane, diffuse, cytoplasmic and nuclear	Nuclear, cytoplasmic	Cytoplasmic, perinuclear, nuclear	Punctate nuclear. cytoplasmic	Cytoplasmic, membrane, nuclear, nucleolar	Cytoplasmic, nuclear	Cytoplasmic, nuclear and perinuclear	N.D	N.D
RIBS-3		678.4	Membrane, diffuse cytoplasmic and nuclear	Nuclear, cytoplasmic and perinuclear increased	Cytoplasmic, nuclear, perinuclear	Punctate nuclear, cytoplasmic	Cytoplasmic, membrane, nuclear and nucleolar	Cytoplasmic, nuclear	Cytoplasmic, nuclear and perinuclear accumulations to one side	N.D	N.D

MKM		N.A.A	Membrane, diffuse cytoplasmic and nuclear	Cytoplasmic, nuclear, perinuclear	Cytoplasmic, nuclear, perinuclear.	Punctate nuclear, cytoplasmic	Cytoplasmic, membrane, nuclear and nucleolar	Cytoplasmic, nuclear	Cytoplasmic, less nuclear and perinuclear	N.D	N.D
DMN		12.4	Membrane, punctate nuclear, diffuse cytoplasmic	Cytoplasmic, nuclear, perinuclear	Cytoplasmic, nuclear	Punctate nuclear. cytoplasmic	Cytoplasmic, nuclear, cytoplasmic, nucleolar	Cytoplasmic, nuclear	Cytoplasmic, less nuclear and perinuclear	N.D	N.D
DSN		3.32	Diffuse cytoplasmic	Cytoplasmic	Cytoplasmic, nuclear	Nuclear	Perinuclear, nuclear, cytoplasmic, no nucleolar	Cytoplasmic, nuclear	Cytoplasmic, nuclear and perinuclear	N.D	N.D
NOV		260	Nuclear, perinuclear cytoplasmic	Cytoplasmic and nuclear	Punctate nuclear, cytoplasmic	Punctate, nuclear, cytoplasmic	Cytoplasmic	Cytoplasmic	Cytoplasmic and nuclear	Yes	Yes
17-AAG/GA /17-DMAG		0.2	Diffuse nuclear, cytoplasmic	Cytoplasmic, nuclear	Nuclear and cytoplasmic	Punctate nuclear, cytoplasmic	Cytoplasmic and nuclear	Cytoplasmic an nuclear	Cytoplasmic and nuclear	Yes	No

It was found that several compounds possessed antiproliferative effects in the MDA-MB-231 cell line following a 96 hour exposure to the compounds (Figure 2.1). The antiproliferative effects of these compounds could be further assessed in different breast cancer cell lines such as the ZR-75-1 and BT-20 breast cancer cell lines to determine whether the observed antiproliferative activities are limited to the MDA-MB-231 cell line or to the hormone status of the cell line. This differential activity could be extended to non-cancerous epithelial breast cell lines and other cell lines, such as human peripheral blood mononuclear cells, to determine whether the compounds exhibit differential toxicity between cancerous and non-cancerous cell lines (Griffin *et al.,* 2010; Selvendiran *et al*., 2010a; Selvendiran *et al*., 2010b).

In this study, SQA was shown to modulate the localisation pattern of Hsp90 and a nuclear speckled pattern was observed in treated cells, unlike the diffuse nuclear and perinuclear patterns observed following treatment with GA and NOV respectively (Appendix Figure B.1). This nuclear speckled pattern was suggested to be a result of a change in gene expression caused by SQA that induced an interaction between Hsp90 and RNPs as Hsp90 has been suggested to be involved in the regulation of the assembly of certain RNPs within nuclear speckles (Boulon *et al*., 2008; Zhao *et al*., 2008). It was expected that the expression levels of the client proteins analysed in this study would decrease following treatments with the 17-AAG and NOV, as this is a classic indication of Hsp90 inhibition (Fumo *et al*., 2004; da Rocha *et al.*, 2005; Marcu e*t al.*, 2000a). However, it has been documented that the degree of the down-regulation of AKT following treatment with Hsp90 inhibitors varies within cell lines (Theodoraki *et al.,* 2007). Furthermore, a different study showed similar results to those presented here, in that treatment with 17-AAG resulted in no change in the expression of AKT in the MDA-MB-231 cell line (Pimienta *et al*., 2011). In this study STAT3 and pSTAT3 showed a similar resistance to degradation by 17-AAG and NOV. It is therefore possible that SQA may alter the expression of Hsp90 client proteins, but that this change was not observed owing to the Hsp90 client proteins selected for this study, namely AKT, pSTAT3 and STAT3. ITC data (Figure 3.4) suggested that SQA was able to interact with Hsp90β directly, and data collected following STD NMR (Appendix Figure C.8) confirmed this interaction and indicated that SQA interacted with Hsp90 through the methyl moiety attached to the quinone nucleus. Molecular modelling could be performed in which SQA was docked to Hsp90 to confirm the SQA atoms interacting with Hsp90 as determined by STD NMR. ITC data suggested that the interaction between SQA and Hsp90 was of a low affinity but the thermodynamic parameters of this interaction could not be determined accurately (Figure 3.4). However, data from the pulldown assays indicated that the SQA-Hsp90β interaction was within the micromolar range as a decrease in the amount of Hsp90 associated with Hop was observed at a concentration 1 μM (Figure 3.7). The results from the His-tagged pulldown assay following SQA

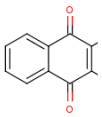
treatment were most similar to those following NOV treatment (Figure 3.6) in which a dose-dependent reduction of Hsp90 associated with Hop was observed. Alternatively, the disruption of the Hsp90-Hop association may have been caused in part by an interaction of SQA with Hop, in addition to a low affinity interaction with Hsp90. ITC experiments in which SQA would be titrated against Hop protein would be required to support this hypothesis. Collectively, these findings suggested that the action of SQA on Hsp90 was more similar to NOV than GA, as treatment with GA (Figure 3.5) did not disrupt the same association.

It was predicted that compounds of the 1, 4 naphthoquinone series would interact with Hsp90 but it was unknown whether compounds bearing a 1, 2 naphthoquinone scaffold would also interact with Hsp90. In this study the 1, 4 naphthoquinone compounds LAP, αLAP and CAL were demonstrated to have no effect on cell viability at concentrations as high as 500 µM in the MDA-MB-231 cell line. The determined IC_{50} values for the 1, 2 naphthoquinones NBL, HBL and BBL fell within 7 and 9 µM. Several biochemical and biophysical techniques were employed to determine whether these compounds could modulate and interact with Hsp90. Localisation studies suggested that all naphthoquinone compounds, with the exception of HBL, were able to modulate the localisation of Hsp90 (Figure 4.1) and selected partner proteins (Appendix Figures B.8, B.10, B.12) with varying degrees as indicated in Table 5.1. However, treatment did not result in the reduced expression of AKT (Figure 4.5). This result was consistent with the findings following treatment with 17-AAG, NOV and SQA on the expression of the same client proteins. ITC binding studies of these compounds to Hsp90 revealed that LAP was able to interact with Hsp90β weakly (Figure 4.7) and that the other compounds of the naphthoquinone series did not show any binding to Hsp90β (Appendix Figures C2, C.3, C.4, C.5 and C.6). All the 1, 4 naphthoquinone compounds did not display any antiproliferative activity in the MDA-MB-231 cell line at the concentration range tested in this study, but functional groups present in the structure of LAP resulted in the binding of this compound to Hsp90 (Figure 5.1). Conversely, all the compounds bearing a 1, 2 naphthoquinone scaffold showed antiproliferative effects within the micromolar range against the breast cancer cell line and none of these compounds showed any binding to Hsp90 (Figure 5.1). As naphthoquinone-scaffold bearing compounds are known Michael acceptors, their antiproliferative effects are thought to occur either through the alkylation of cellular proteins and/or DNA or through the production of resulting in oxidative stress (Bolton *et al*., 2000). The findings from this study suggest that the antiproliferative and cellular effects of the 1, 2 naphthoquinone compounds investigated in this study may be owing to a more general mechanism of naphthoquinones and not to the modulation of Hsp90 function, suggesting that Hsp90 is not the *in vivo* target of these compounds assuming that all the compounds were not

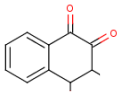
affected with respect to passage through the plasma membrane into the cell. Similarly, ITC experiments indicated that Hsp90β was not the *in vivo* target of the 1, 4 naphthoquinones CAL and αLAP as these compounds did not bind to Hsp90β. Although the cellular effects of these two compounds were found to be independent of Hsp90, other 1, 4 naphthoquinone compounds have been confirmed as interacting with Hsp90 and Grp94 in pulmonary bronchial epithelial cells (Lamé *et al*., 2003). Therefore, the possibility remains that CAL and αLAP may interact with other members of the Hsp90 multi-chaperone complex. This would explain the observed modulation of Hsp90 localisation in this study as an indirect consequence of these compound-Hsp90 partner protein interactions. This hypothesis could be proved initially through molecular modelling of the compounds to various chaperone and co-chaperone proteins. Subsequent binding experiments could be performed to confirm any interactions suggested by the i*n silico* data.

It may be possible that the derivatives of LAP displayed weaker binding to Hsp90β than LAP, and that this weak affinity for Hsp90β combined with the poor solubility of the compounds in the ITC buffer resulted in the appearance of no binding to Hsp90 through ITC. Alternatively, the synthetic derivatives of LAP may show differential binding affinities within Hsp90 isoforms and may bind to Hsp90α, TRAP-1 or Grp94 with a higher affinity than to the Hsp90β protein which was used in the ITC analyses performed in this study. This statement is supported by literature, in which studies have shown that certain inhibitors such as 50-N-ethylcarboxamidoadenosine and PU-H71 have higher affinities towards, or only interact with, certain Hsp90 isoforms (Chan *et al*., 2008; Rosser *et al*., 2000). Furthermore, in the current study, the compounds may have been able to modulate either or both Hsp90α and β protein localisation as an antibody recognizing both isoforms was used. Therefore, these compounds may have been more specific towards and modulated Hsp90αα, monomeric Hsp90α, heterodimers of Hsp90αβ or higher oligomers of Hsp90α, and not Hsp90β as was used for the ITC assays. Thus, the appearance may have been given that these compounds did not interact with Hsp90 protein when in fact they may have not interacted with the Hsp90β isoform but could bind to other isoforms. As LAP was the only naphthoquinone compound found to interact with Hsp90β, the extent to which the function groups within LAP contributed to the Hsp90 binding of the 1, 4 naphthoquinone scaffold could be determined. This analysis could be performed by comparing the binding properties of LAP to that of the 1, 4 naphthoquinone backbone depicted in Figure 5.1 by STD NMR. This would add to the SAR information that has already been determined for these compounds.

His-tagged pulldown assays were employed to further characterise the Hsp90 modulatory properties of LAP and the data obtained indicated that LAP did not disrupt the Hsp90-Hop association (Figure 4.8). A similar result was obtained following treatment with GA (Figure 3.5) in this study, while NOV treatment resulted in a dose-dependent decrease of Hsp90 associated with Hop (Figure 3.6). However, as the 1, 4 naphthoquinone scaffold is predicted to bind to a region of Hsp90 similar to that of NOV (Hadden *et al*., 2009), the inability of LAP to disrupt the association of Hsp90 and Hop may have been as a result of the need to perform this assay with higher concentrations of LAP. This statement is partially supported by the observation that LAP did not show any antiproliferative activity in the MDA-MB-231 cell line when tested at concentration of up to 500 µM and may thus require higher concentrations for any noticeable effects, and by the low affinity binding displayed to Hsp90β by ITC. Furthermore, although the disruption of the Hsp90-Hop interaction was shown between 0.1 and 1 µM for NOV in this study, Yun *et al*., (2004) required NOV concentrations of 10 mM for the disruption of the same interaction. This further supports the hypothesis that a higher concentration of LAP may be required for the disruption of the association between Hsp90 and Hop.



Compound	Functional Group(s)	Required for Hsp90 binding Required for cytotoxicity
LAP		+/- -
αLAP		- -
CAL		- -



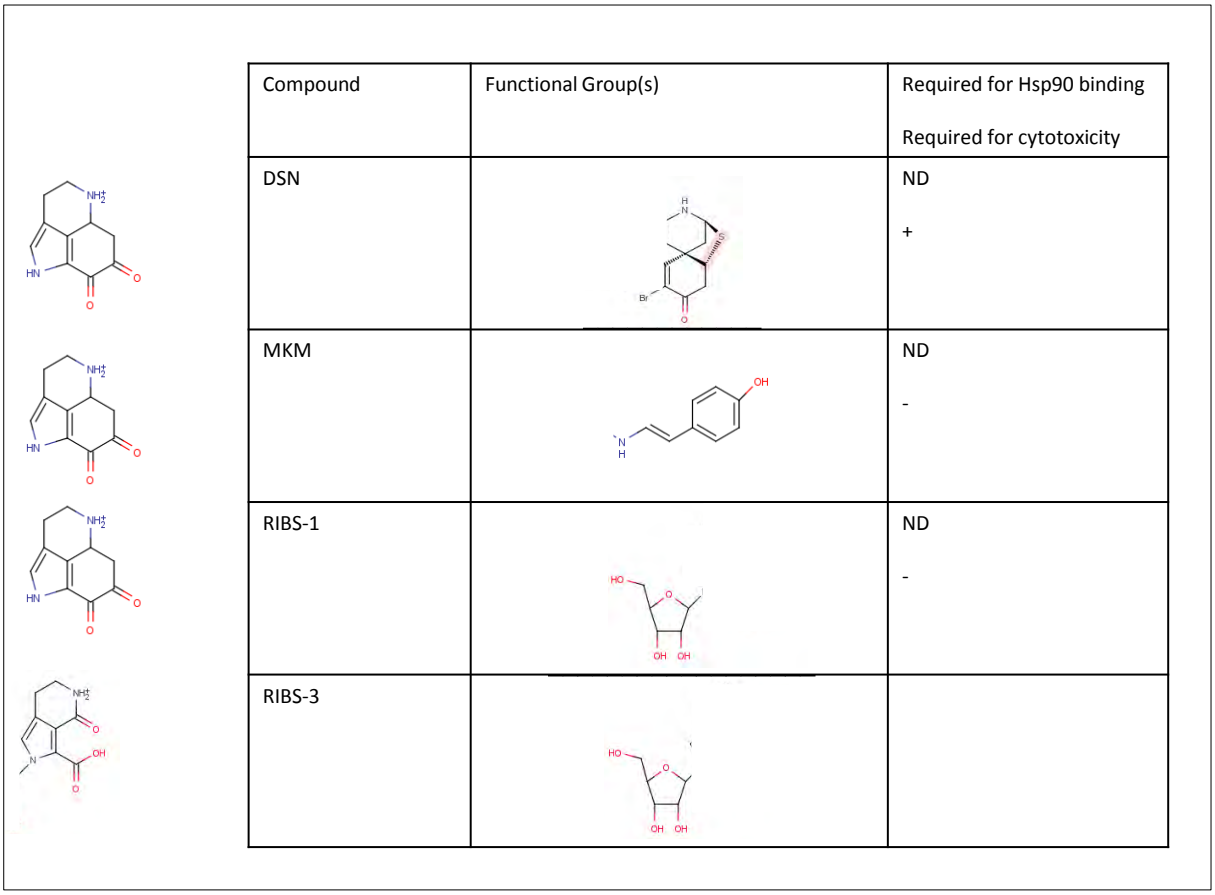
Compound	Functional Group(s)	Required for Hsp90 binding Required for cytotoxicity
BBL		- -
HBL		- -
NBL		- -

Figure 5.1: Structure-activity relationships of compounds lapachol (LAP), α-lapachona (αLAP), c-alil-lausona (CAL), belonging to 1, 4 naphthoquinone scaffold and nor-β-lapachona (NBL), bromo-β-lapachona (BBL) and hydroxy-β-lapachona (HBL) belonging to the 1, 2 naphthoquinone scaffold. Chemical groups thought to be important for antiproliferative activity or for interacting with Hsp90β indicated next to the appropriate scaffold.

The antiproliferative profile of compounds of the pyrroloiminoquinone alkaloid series of compounds indicated that DMN and DSN showed anti-cancer activities within the lower micromolar range in the MDA-MB-231 cell line (Figure 2.1). The addition of further functional groups for the compounds MKM, RIBS-1 and RIBS-3 decreased the antiproliferative effects of the compounds (Figure 5.2) and these compounds were found to have no effect on cell viability at concentrations of up to 500 µM. Further experiments could be performed with the pyrroloiminoquinone alkaloid series of compounds, SQA and those of the naphthoquinone series in which cells would be treated with the

compounds in combination with 17-AAG or NOV as studies have shown that the combination of two modulators of Hsp90 may result in the synergistic enhancement of the antiproliferative effects in cells (Cox *et al*., 2008; Roforth and Tan, 2008; Galabova-Kovacs *et al*., 2006).

Selected compounds of the marine pyrroloiminoquinone alkaloid series were shown to alter the distribution of Hsp90, Hsp70, Hop (Figure 4.2), STAT3 and pSTAT3 (Figure 4.4) within the breast cancer model used in this study. Of interest were the compounds RIBS-1, MKM, RIBS-3 and DMN as treatment with these compounds resulted in the decreased expression of Hsp90, Hsp70 and Hop respectively (Figure 4.6). These changes in protein expression taken together with the modulation of the localisation patterns of proteins of the Hsp90 multi-chaperone complex indicated that these compounds may interact with Hsp90 and/or Hsp90-associated proteins. However, compounds belonging to the marine pyrolloimoquinone series of compounds were not analysed for their ability to interact with Hsp90 directly by ITC or NMR in this study. Therefore, it remains to be seen whether these compounds alter the distribution of proteins of the Hsp90 machinery complex directly or indirectly.



Compound	Functional Group(s)	Required for Hsp90 binding Required for cytotoxicity
DSN		ND +
MKM		ND -
RIBS-1		ND -
RIBS-3		

Figure 5.2: Structure-activity relationships of the pyrolloimoquinone series of compounds of the damirone-based scaffold, discorhabdin A (DSN), makaluvamine M (MKM), *N*-1-β-D ribofuranosyl makaluvamine (RIBS-

1), and modified damirone scaffold compound *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3). Chemical groups thought to be important for antiproliferative activity are indicated. ND denotes compounds for which binding to Hsp90 was not determined.

5.2 FUTURE WORK

This study attempted to develop a screening tool which could be employed to identify and characterise compounds based on the modulation of Hsp90 function. Opportunities for further study would aim to further characterise selected compounds investigated in this study.

5.2.1 Development of LAP and SQA analogues

Approximately 40 % of all compounds identified through various screening methods are estimated to be largely insoluble in water (Lipinski, 2002; Lipinski, 2000). Therefore, it was not surprising that SQA and the other compounds examined in this study lacked substantial solubility in water alone and required initial solubilisation in DMSO. Analogues for the priority compounds, LAP and SQA could be developed to improve the water solubility. Salts of these compounds could be developed to improve the water solubility of the compounds as was performed for 17-AAG resulting in the pharmacologically active hydrochloride salt derivative IPI-504 (Ge *et al*., 2006). Alternatively, polymer-drug conjugates could be produced to improve the solubility of the compounds (Ringsdorf, 1975) through the linkage of the compounds to a hydrophilic polymer through a spacer or linker (Choi and Jo, 2004). This method of increasing the solubility of a compound was successfully demonstrated by the linking of PTX to polyethylene glycol (PEG). This method increased the bioavailability of PEG-PTX to four times of that of PTX alone (Ferguson *et al*., 2001). Micellar systems could also be developed to improve the solubility of the compounds as was done for the 1, 2 naphthoquinone β-lapachone (Blanco *et al*., 2007; Sutton *et al*., 2007). The resultant soluble analogues of these compounds could be assessed for their Hsp90 binding capacities and thus add to the SAR already developed for SQA and the naphthoquinone series of compounds.

5.2.2 Biochemical characterisation of LAP and SQA

This work identified SQA and LAP as modulators of Hsp90 and future work would therefore aim at biochemically characterising these two compounds in terms of modulating Hsp90 ATPase activity and chaperone function. A fluorescent ATPase assay adapted and optimised from Brune et al. (1999)

was attempted but was unsuccessful in a 96-well plate system as sufficient differences in fluorescence were not achieved owing to the limited wavelength options available for use at the time of the study. Aggregation assays using bovine rhodanese, malate dehydrogenase or α-synuclein as the substrate could be performed according to Silberg et al. (1998) to further characterise the compounds in terms of their modulatory effects on Hsp90 chaperone function. Proteolytic footprinting of Hsp90 could also be performed to determine whether LAP or SQA binding to Hsp90 induces a conformational change similar to that of either GA or NOV and thus produce fragmentation patterns similar to either inhibitor as described by Yin et al. (2009).

5.3 CONCLUDING REMARKS

5.3.1 Hsp90 as a drug target

Hsp90 was initially thought to be an unlikely drug target as it was shown to be essential for the functioning of normal cells (Borkovich *et al.*, 1989). However, several studies detailing the anti-cancer properties of Hsp90 inhibitors have led to this protein being considered a promising drug target (Cheung *et al*., 2005 ; Schulte, 1998; Supko *et al*., 1995; Whitesell *et al*., 1994). Hsp90 is an attractive drug target as the inhibition of this chaperone would result in the targeting of multiple oncogenic signal transducers (Zhang and Burrows, 2004). However, concerns over the safety of inhibiting Hsp90 have arisen based on the potential of Hsp90 inhibitors to disrupt other ATP-binding interactions within cells. This concern was addressed by the findings that very few known proteins have been shown to have same left handed β-α-β Bergerat fold found within the Hsp90 ATP binding site (Bergerat, 1997) and therefore, the potential to disrupt other ATP-protein interactions may be limited (Dutta and Inouye, 2000). At present, no drug-resistant Hsp90 mutations have been detected in cancer patients, even after long-term treatment with Hsp90 inhibitors (Whitesell and Lin., 2012). It is possible that drug resistance to certain classes of Hsp90 inhibitors may occur in the future. It has been found that a reduction in the expression of the oxidoreductase NQO1 may result in resistance to the N-terminal targeted benzoquinone ansamycin Hsp90 inhibitors; but the same observation has not been made for other Hsp90 inhibitors (Gaspar *et al*., 2009). A mutation in *Humicola fuscoatra* fungi has resulted in resistance to the resorcinol class of Hsp90 inhibitors (Prodromou *et al*., 2009), but such a change is yet to be reported in humans (Whitesell and Lin, 2012).

An additional Hsp90 inhibition problem faced by traditional N-terminal targeted inhibitors is the activation of HSF1 resulting in the overexpression of several chaperones which protect the cells from

the effects of the Hsp90 inhibitors (Didelot *et al*., 2006; Garrido *et al*., 2006; McCollum *et al*., 2006). However, the induction of HSF1 may be prevented through the identification and development of inhibitors that target both Hsp90 and Hsp70 simultaneously (Holzbeierlein *et al*., 2010; Donnelly and Blagg, 2008; Brodsky and Chiosis, 2006). Therefore, the ability of the naphthoquinone, pyrroloiminoquinone alkaloid compounds and SQA investigated in this study to interact directly with either Hsp70 or Hsp90 co-chaperones could be probed as this will increase the understanding of the mechanism of action of these small molecules. Although more specific for highly complexed forms of Hsp90 in cancer cells (Kamal *et al*., 2003), Hsp90 inhibitors have been shown to have deleterious effects in non-cancerous cells and have been shown to alter dendritic cell function (Bae *et al*., 2007) and glomerular filtration (Ramirez *et al*., 2008). Furthermore, Hsp90 inhibitors such as 17-AAG have been shown to promote bone metastases (Yano *et al*., 2008; Price *et al*., 2005) indicating the need for novel compounds identified as potential Hsp90 inhibitors to be screened for cell migration with methods such as wound healing assays.

These challenges faced by Hsp90 inhibitors further validate the need for the development of novel Hsp90 scaffolds. Different Hsp90 inhibitor scaffolds have been shown to exhibit different efficacy profiles and may therefore be beneficial to certain cancer types, or may be useful when administered in combination with inhibitors belonging to a different class owing to the synergetic or additive effects of the compounds in the cells (Biamonte *et al*., 2010; Janin *et al*., 2010; Kim *et al*., 2009). The development of Hsp90 inhibitors targeting specific Hsp90 isoforms including Grp94 (Duerfeldt *et al*., 2012), TRAP1 and membrane-associated Hsp90 may overcome some of the issues associated with compounds that target all Hsp90 isoforms of Hsp90 in cells and will further add to the understanding of the roles of the different forms of Hsp90 (Duerfeldt and Blagg, 2010).

5.3.2 Conclusions of the study

In conclusion, several biochemical and biophysical techniques were established as a screening tool for the identification of 'hit' compounds that may act as modulators of Hsp90. These methods may be used in the future to screen small libraries of compounds which may lead towards the identification of further modulators of Hsp90 and, therefore, potential inhibitors and the rational design of novel compounds that may inhibit Hsp90. The systematic analysis of LAP and the synthetic derivatives of this compound performed during this research contributed to the overall understanding of the structure-activity relationships that exist within the naphthoquinone class of compounds. Furthermore, the two 'hit' compounds SQA and LAP were identified as showing

promising Hsp90 modulatory functions from a group of twelve compounds selected based on the presence of a quinone nucleus. In addition to their Hsp90 modulatory functions, the two compounds, LAP and SQA, were shown to interact weakly with Hsp90. The domains with which these compounds interact and the mechanisms by which LAP and SQA modulate Hsp90 require further investigation.

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APPENDIX A: COMMON PROTOCOLS OF STANDARD MOLECULAR BIOLOGY TECHNIQUES

Preparation of competent *E. coli* cells

A transformant of the strain of interest was inoculated into 2 x YT broth culture and grown for 16 hours with shaking at 37 °C. The culture was inoculated into fresh 2 x YT broth and grown to the exponential growth phase when the absorbance at 600 nm was between 0.6 and 0.8 AU. Cells were harvested by centrifugation at 3829 x *g* for 10 minutes, 4 °C using JA-14 rotor on an Avanti^{TM} J-26XP Centrifuge. The pelleted cells were resuspended in ice-cold RF 1 solution (100 mM KCl, 50 mM $MnCl_2$, 30 mM CH_3COOK, 10 mM $CaCl_2$, 15 % glycerol, pH 5.8). Following a 20 minute incubation at 4 °C the cells were pelleted by centrifugation as before and were resuspended in RF 2 solution (10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2$, 15 % glycerol, pH 6.8). The cells were aliquoted, snap frozen in liquid nitrogen and stored at – 80 °C.

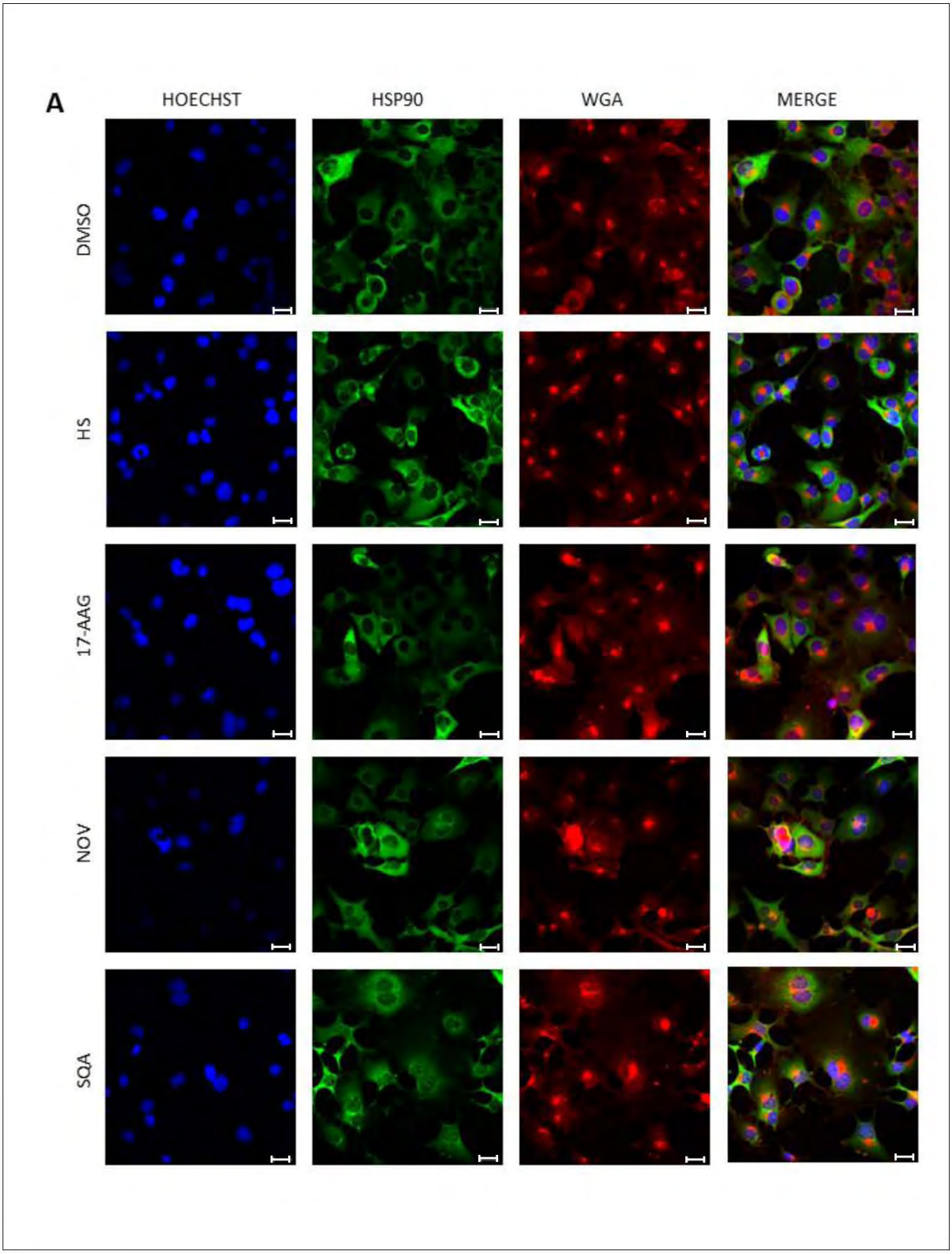
Transformation of competent *E. coli* cells

100 µl competent *E. coli* cells were incubated with 100 ng plasmid DNA at 4 °C for 20 minutes. Cells in which the plasmid DNA was replaced by with sterilised, distilled water were included as a negative control. Cells were subjected to heat shock at 42 °C for 45 seconds followed by incubation at 4 °C for 2 minutes. Cells were diluted 1:10 in 2 x YT media pre-warmed to 37 °C, and were incubated for 45 minutes with shaking at 37 °C. 100 µl of the bacterial suspension was plated onto 2 x YT plates supplemented with 100 $\mu g.ml^{-1}$ ampicillin. The plates were incubated at 37 °C for not more than 16 hours.

Agarose gel electrophoresis

0.8 % agarose gels were prepared using molecular grade agarose in 0.5 x TBE buffer (45 mM borate, 1 mM EDTA, 45 mM Tris pH 8.3) with 0.5 $\mu g.ml^{-1}$ ethidium bromide. DNA samples of interest were mixed with 6 x DNA gel loading buffer (0.25 % [w/v] bromophenol blue, 30 % [v/v] glycerol) loaded into the wells of the gel and run at 100 V for approximately one hour. Lambda (λ) DNA digested with *Ps1*I restriction enzyme was used as a molecular weight marker. Samples were visualised ultraviolet light with a Chemidoc EQ system (Biorad, United Kingdom).

APPENDIX B: CONFOCAL MICROSCOPY FIGURES



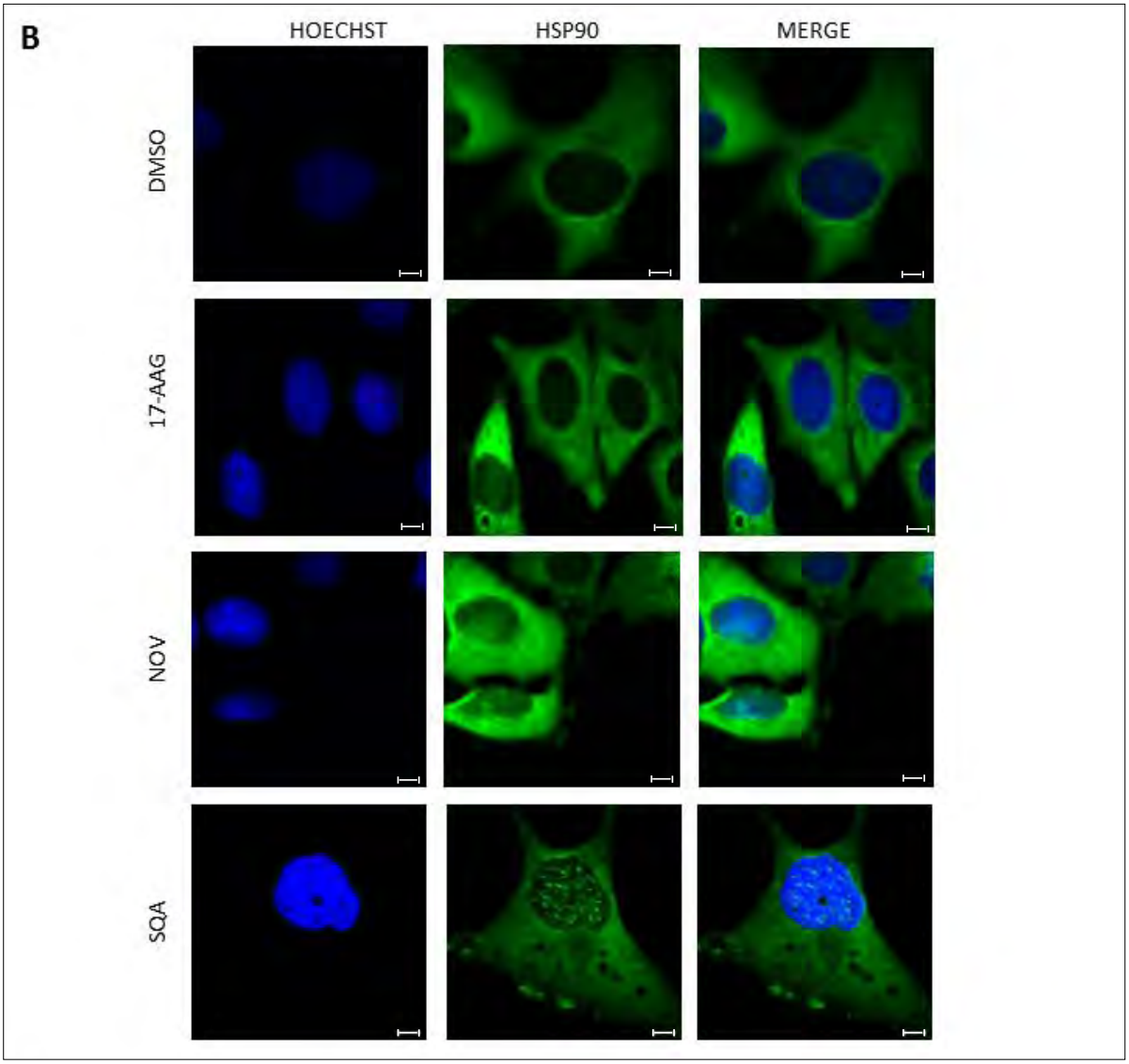


Figure B.1: **Subcellular localisation pattern of Hsp90 following SQA treatment is distinct to that of 17-AAG and NOV**. **(**A) MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C), 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) (200 nm), novobiocin (NOV) (260 µM) and sargaquinoic acid (SQA) (90 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-HSP90α/β antibody (1:100) followed by anti-mouse AlexaFluor 488 conjugated secondary antibody and AlexaFluor 555 conjugated WGA (1 $\mu g.ml^{-1}$). Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images were captured using the Zeiss 510 Meta laser scanning confocal microscope using 40x objectives and were analyzed using AxioVision LE Rel 4.8 (Zeiss, Germany). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm (B) SQA treatment resulted in punctate nuclear staining pattern distinct to that of NOV and 17-AAG using 40x objectives and 4 x magnification. Scale bars 10 µm.

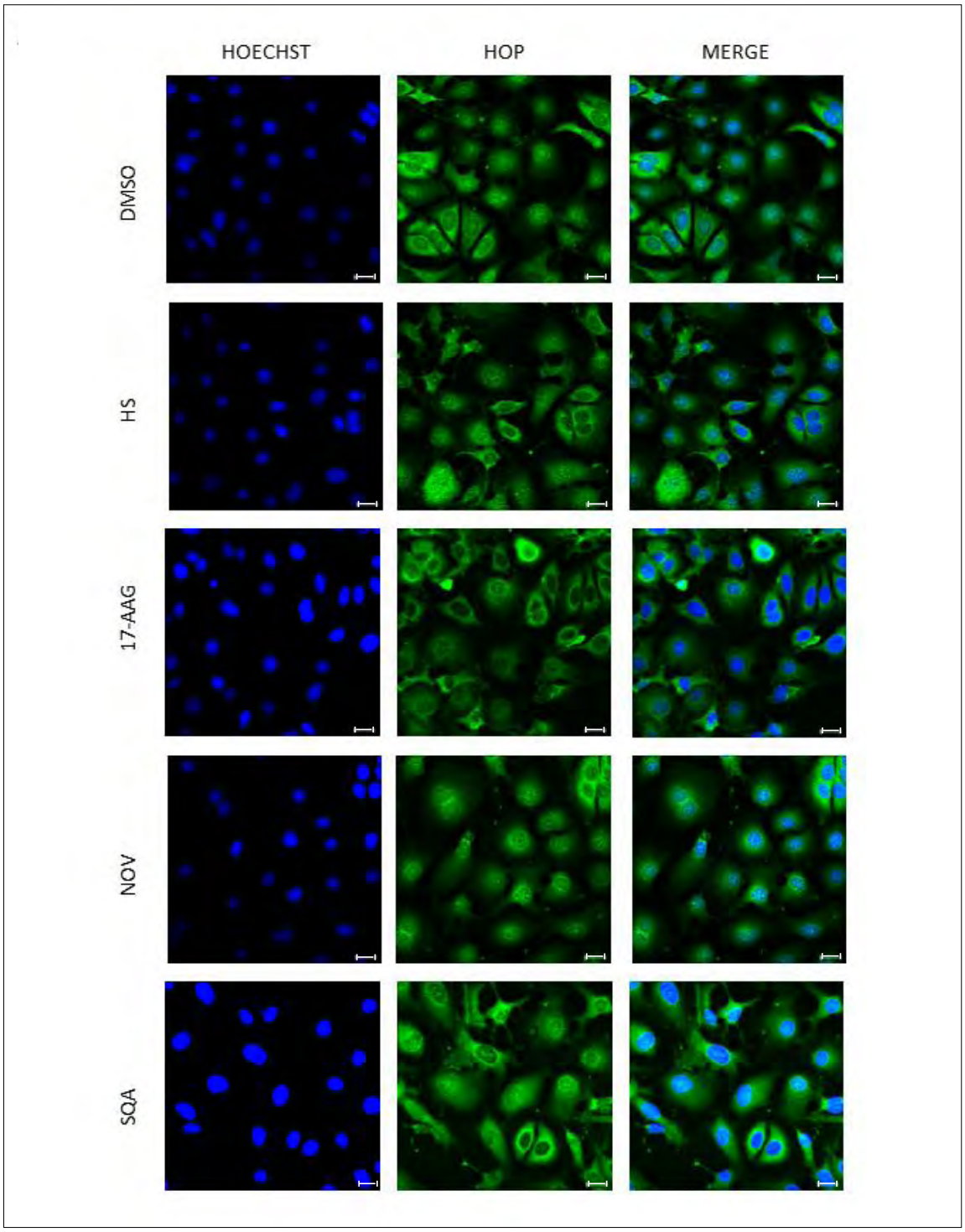


Figure B.2: **Subcellular localisation of Hop in SQA treated cells is similar to cells treated with NOV**. MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C), 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) (200 nm), novobiocin (NOV) (260 µM) and sargaquinoic acid (SQA) (90 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-Hop antibody (1:100) followed by anti-mouse AlexaFluor 488 conjugated secondary antibody. Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm. Images were captured using the Zeiss 510 Meta laser scanning confocal microscope using the 40x objective and were analyzed using AxioVision LE Rel 4.8 (Zeiss, Germany).

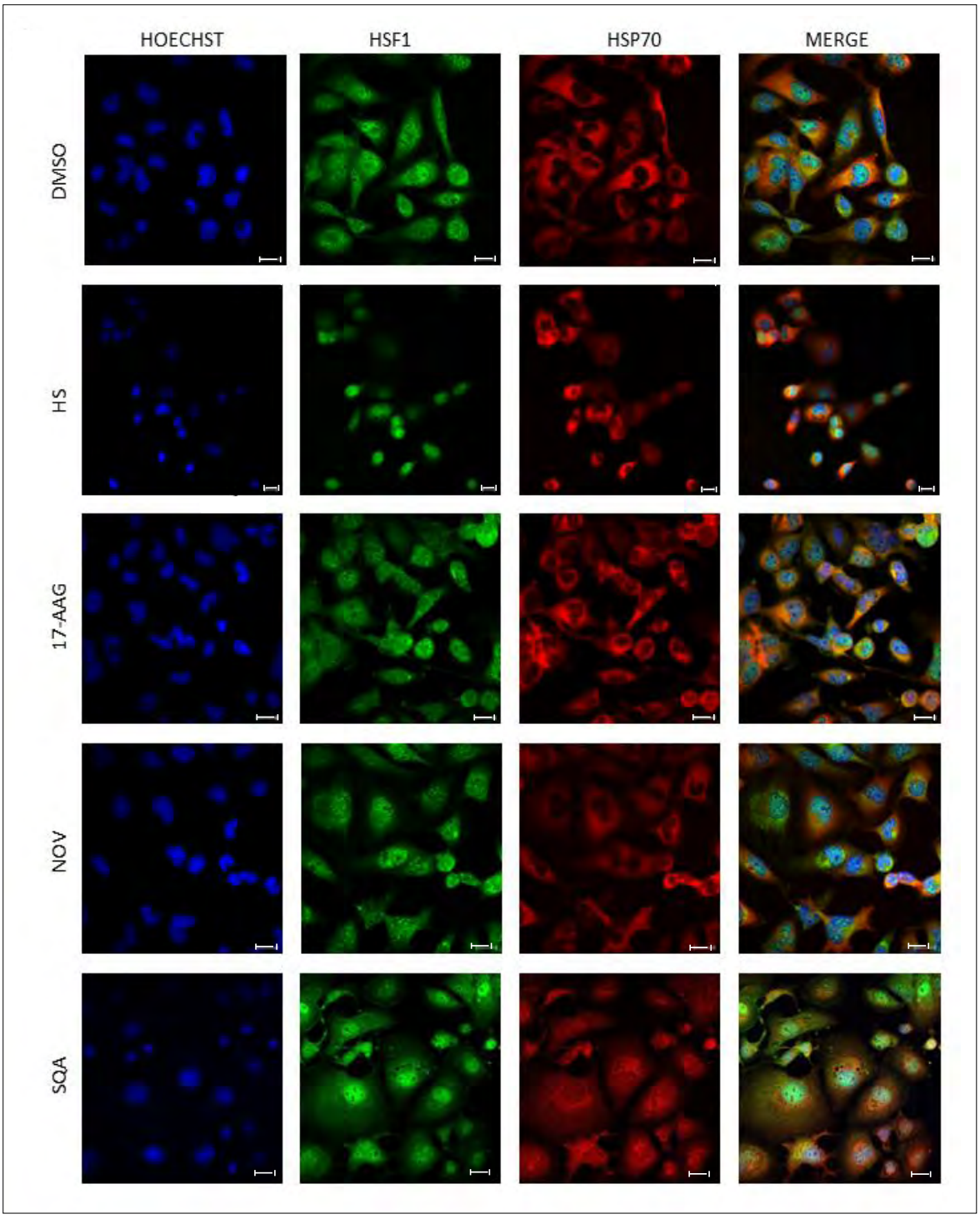


Figure B.3: SQA does not induce general heat shock response similar to that following treatment with heat shock. MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C), 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) (200 nm), novobiocin (NOV) (260 µM) and sargaquinoic acid (SQA) (90 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-Hsp70 antibody (1:100) and rabbit anti-HSF1 (1:100) followed by anti-rabbit AlexaFluor 488 conjugated secondary antibody and anti-mouse AlexaFluor 555 conjugated secondary antibody. Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images were captured using the Zeiss 510 Meta laser scanning confocal microscope using the 40x objective and were analyzed using AxioVision LE Rel 4.8 (Zeiss, Germany). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm.

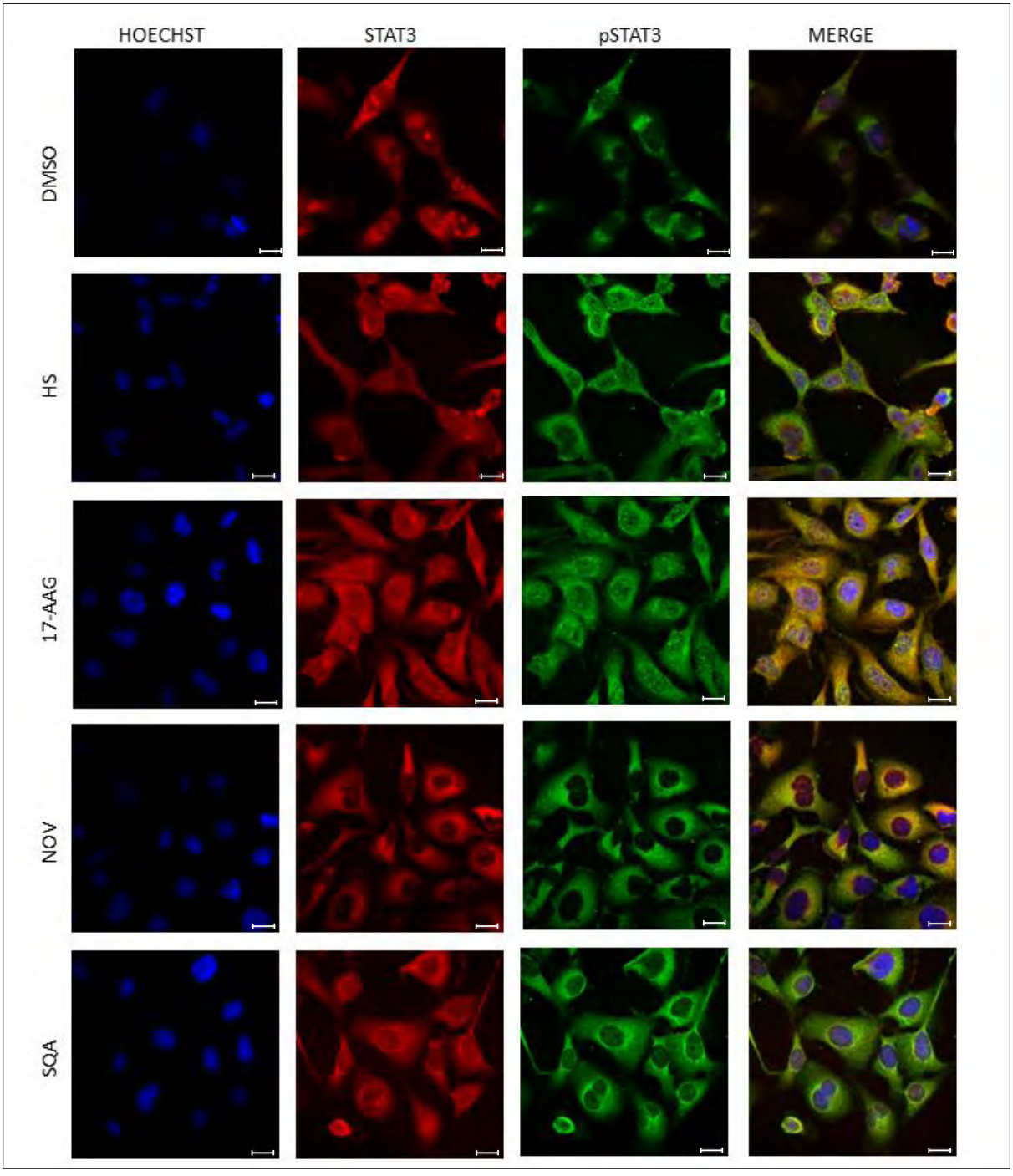


Figure B.4: SQA and NOV treated cells shown increased nuclear STAT3 and pSTAT3. MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C), 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) (200 nm), novobiocin (NOV) (260 µM) and sargaquinoic acid (SQA) (90 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-pSTAT3 antibody (1:100) and rabbit anti-STAT3 antibody (1:100) followed by anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 555 secondary antibodies. Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm. Images were captured using the Zeiss 510 Meta laser scanning confocal microscope using the 40x objective and were analyzed using AxioVision LE Rel 4.8 (Zeiss, Germany).

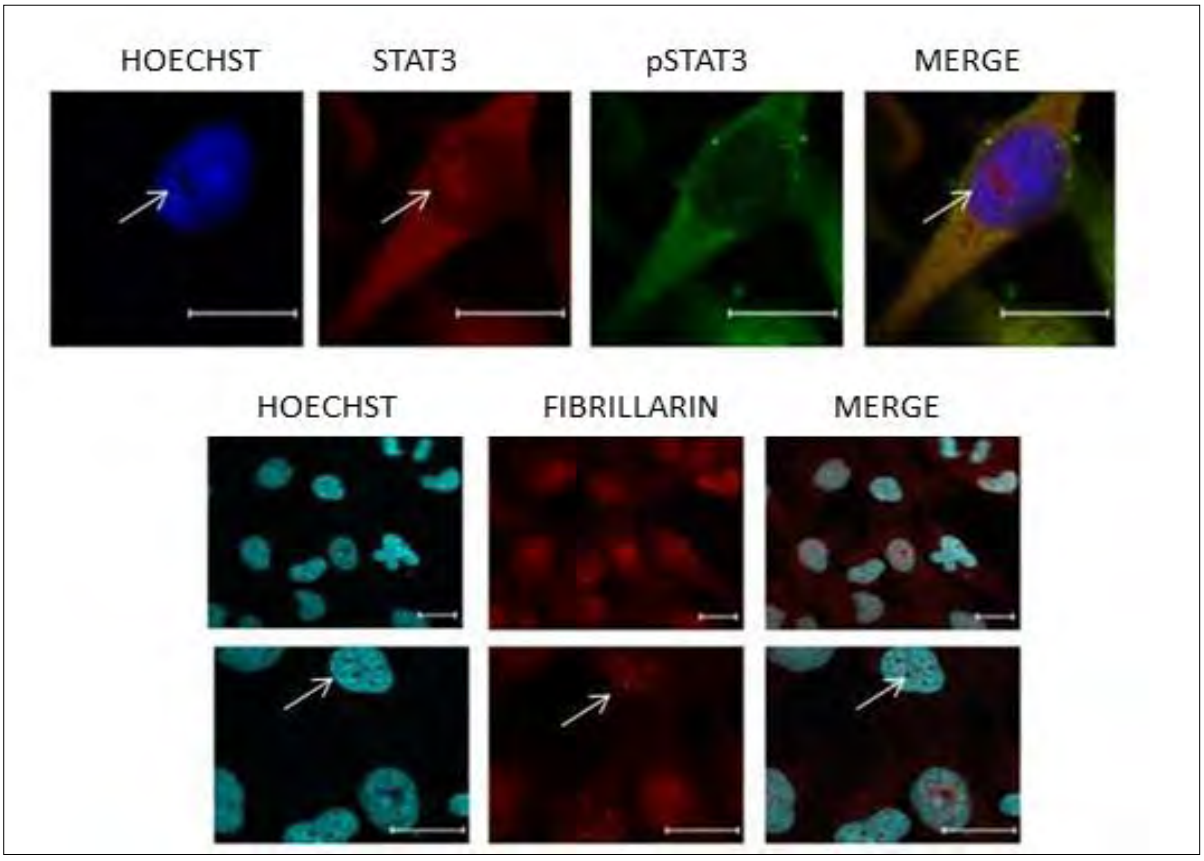


Figure B.5: Hsp90 client protein STAT3 was localized within distinct foci in the nucleoli of both treated and untreated cells. (A) Areas in which STAT3 was specifically localized within the nucleoli are indicated by the white arrows in the panels. Scale bars: 20 µm. (B) To identify the Hoechst negative regions of the nuclei as nucleoli, untreated MDA-MB-231 cells were fixed and stained with mouse anti-fibrillarin followed by anti-mouse AlexaFluor 488 secondary antibody. Nuclei were counterstained blue with Hoechst 33342 (1 µg.ml^{-1}). Images shown are representative of two independent experiments.

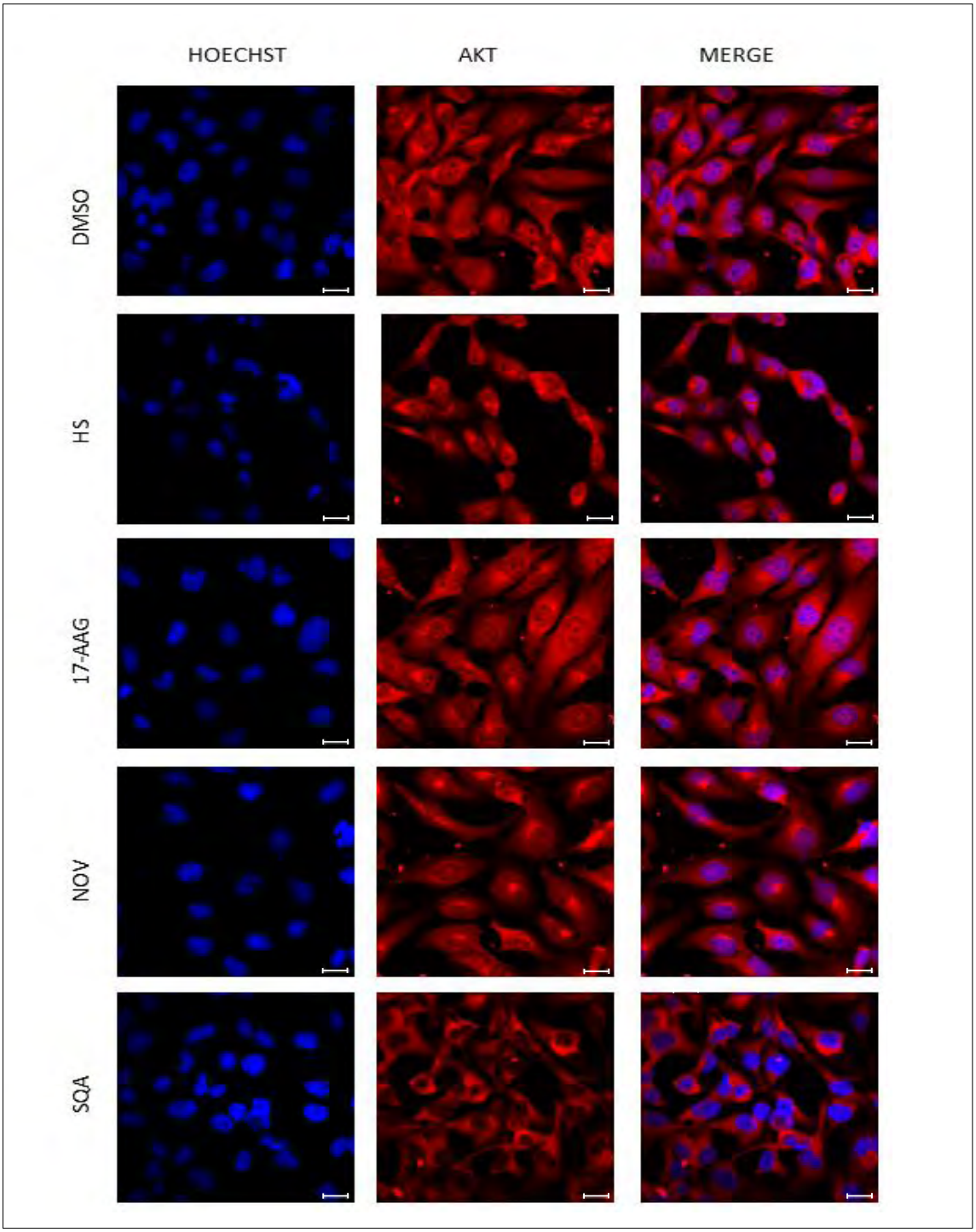
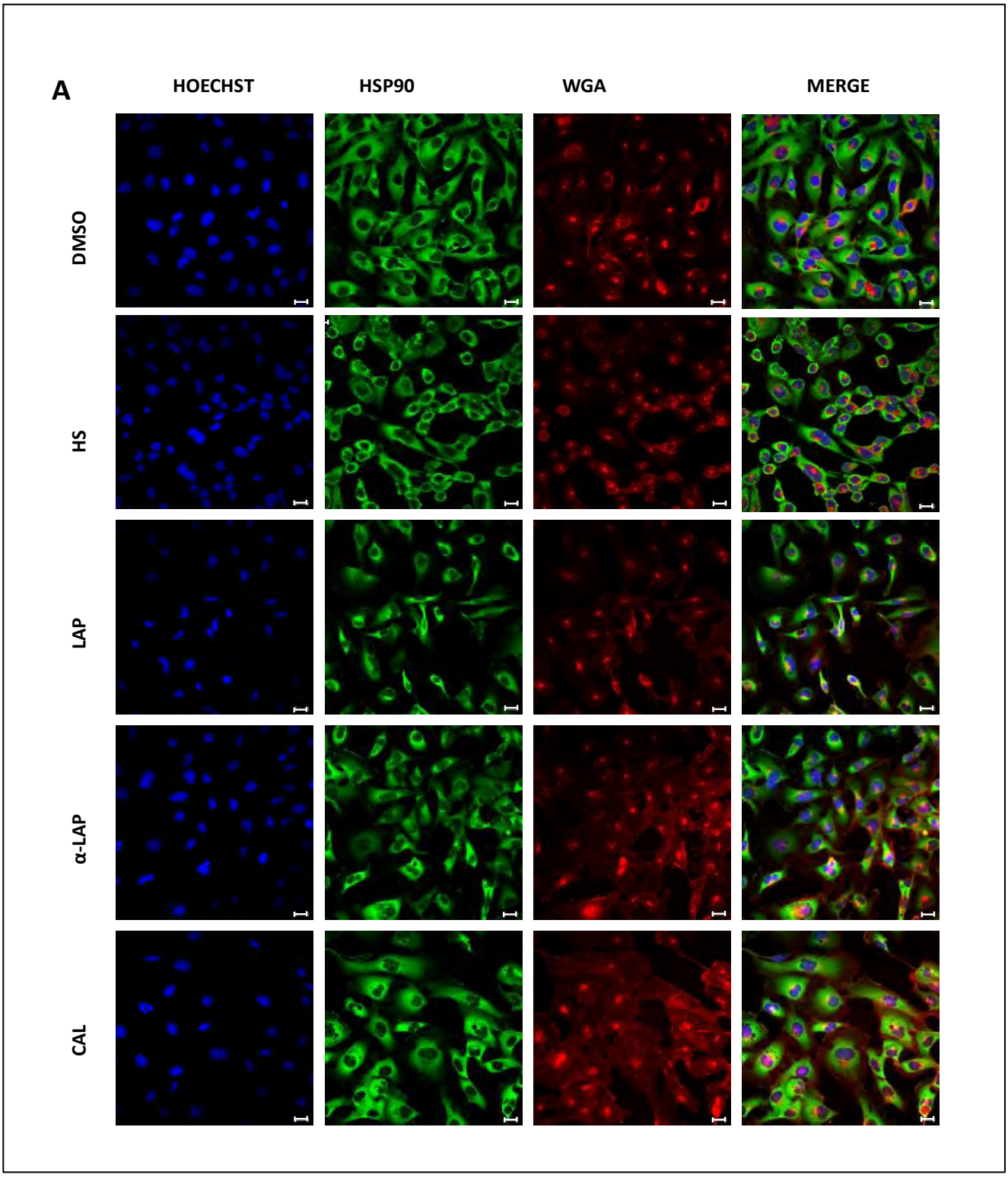


Figure B.6: SQA reduces incidence nuclear AKT in treated cells but Hsp90 inhibitors 17-AAG and NOV do not influence AKT localisation. MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C), 17-N-allylamino-17-demethoxygeldanamycin **(**17-AAG) (200 nm), novobiocin (NOV) (260 µM) and sargaquinoic acid (SQA) (90 µM) for 5 hours at 37 °C. Cells were fixed and stained with rabbit anti-AKT antibody (1:100) followed by anti-rabbit AlexaFluor 555 conjugated secondary antibody. Nuclei were counterstained blue with Hoechst 33342 (1 µg.ml^{-1}). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Images were captured using the Zeiss 510 Meta laser scanning confocal microscope using the 40x objective and were analyzed using AxioVision LE Rel 4.8 (Zeiss, Germany). Scale bars: 20 µm.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WGA
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LAP
α-LAP
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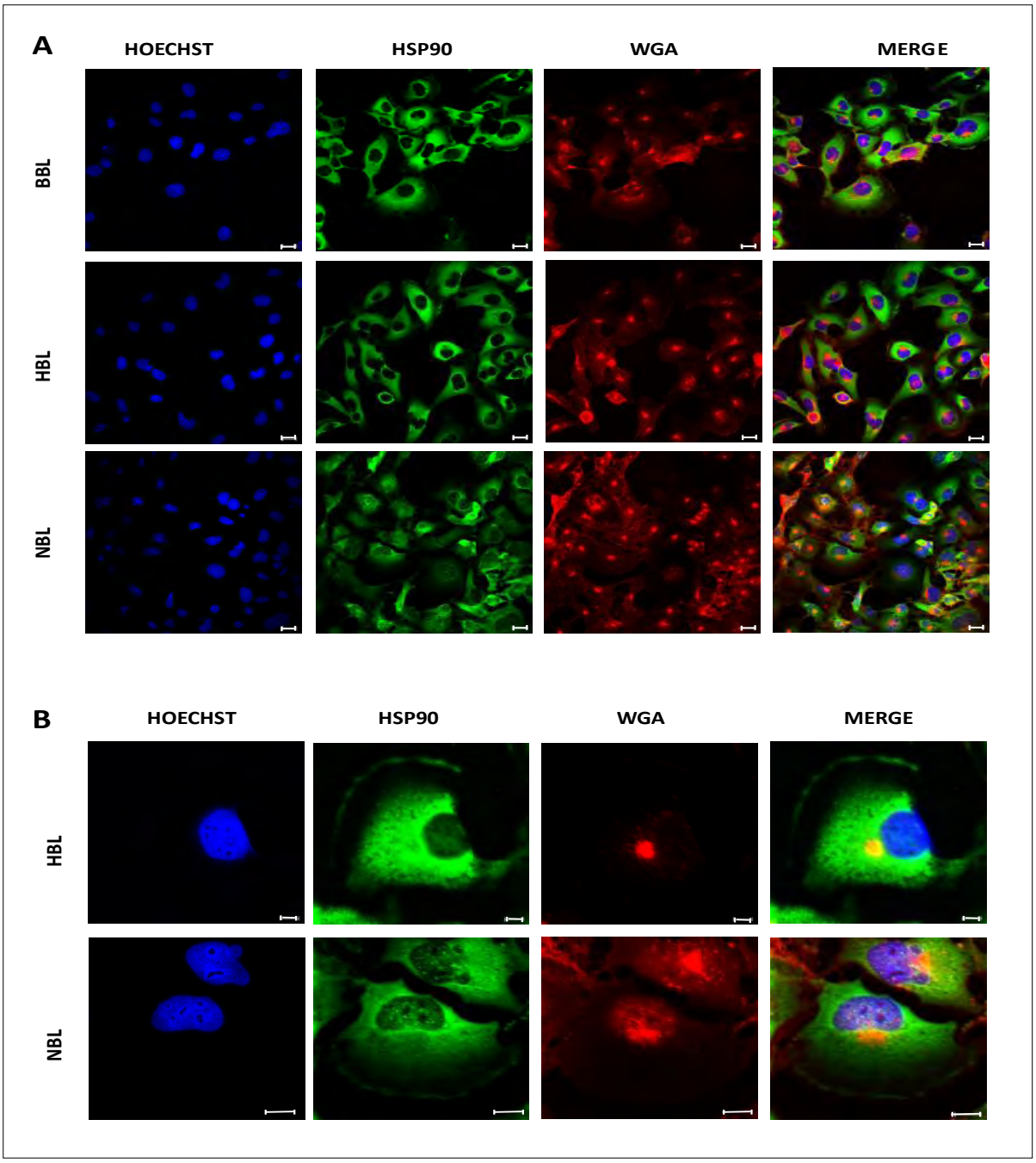
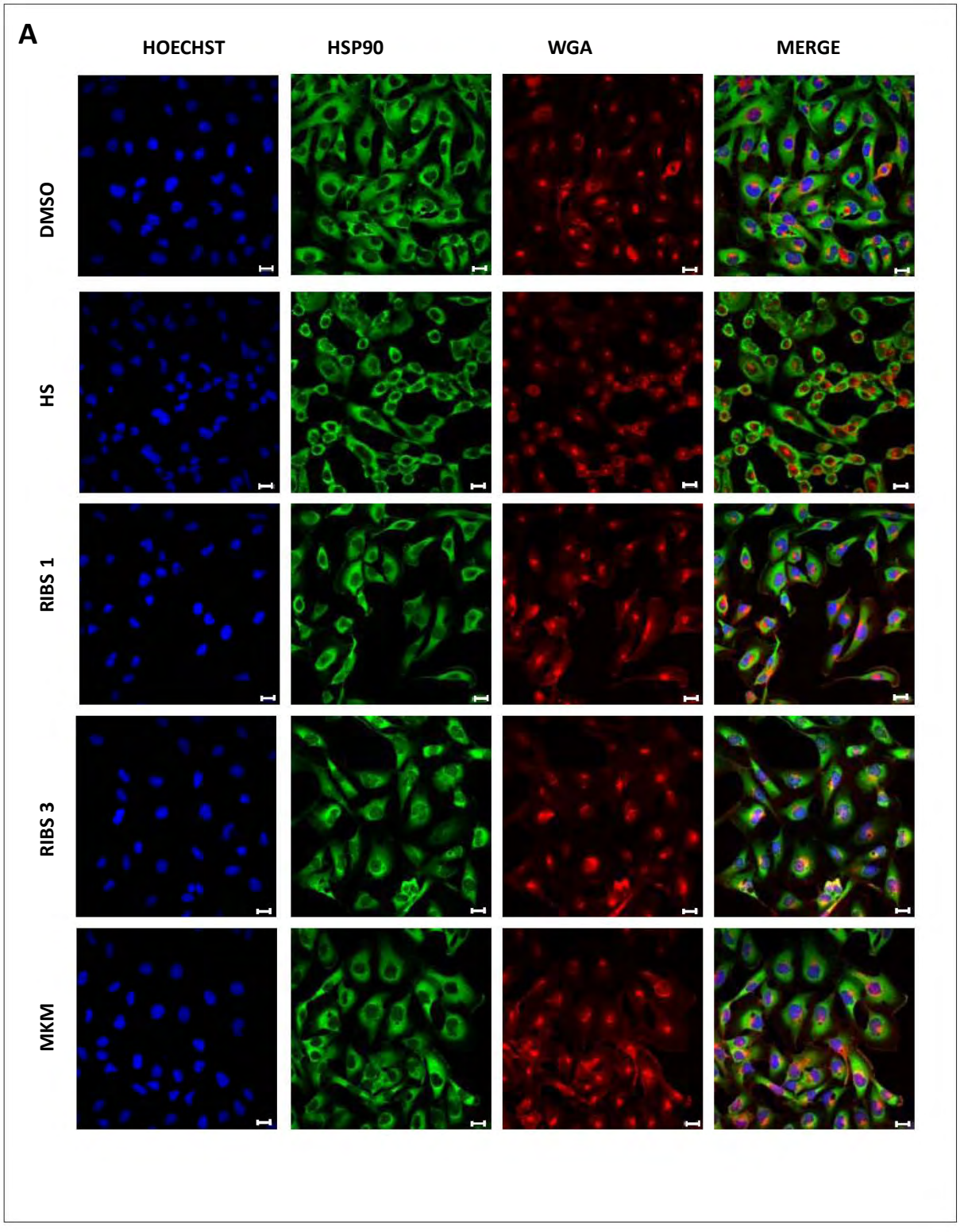


Figure B.7: **Screening of 1, 4 naphthoquinone compounds for the ability to modulate Hsp90 subcellular localisation in treated cells.** (A) MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C), lapachol (LAP) (7 µM), α-lapachona (αLAP) (7 µM), c-alil-lausona (CAL) (7 µM), nor-β-lapachona (NBL) (8.78 µM), bromo-β-lapachona (BBL) (7.86 µM) and hydroxy-β-lapachona (HBL) (7.61 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-Hsp90 antibody (1:100) followed by anti-mouse AlexaFluor 488 conjugated secondary antibody and AlexaFluor 555 conjugated WGA (1 $\mu g.ml^{-1}$). Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm. Images were captured using the Zeiss 510 Meta laser scanning confocal microscope using the 40x objective and were analyzed using AxioVision LE Rel 4.8 (Zeiss, Germany) (B) HBL treatment resulted in Hsp90 localized to the membrane of cells, whilst NBL treatment resulted in a punctate nuclear Hsp90 staining.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WGA
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DMSO
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RIBS 1
RIBS 3
MKM

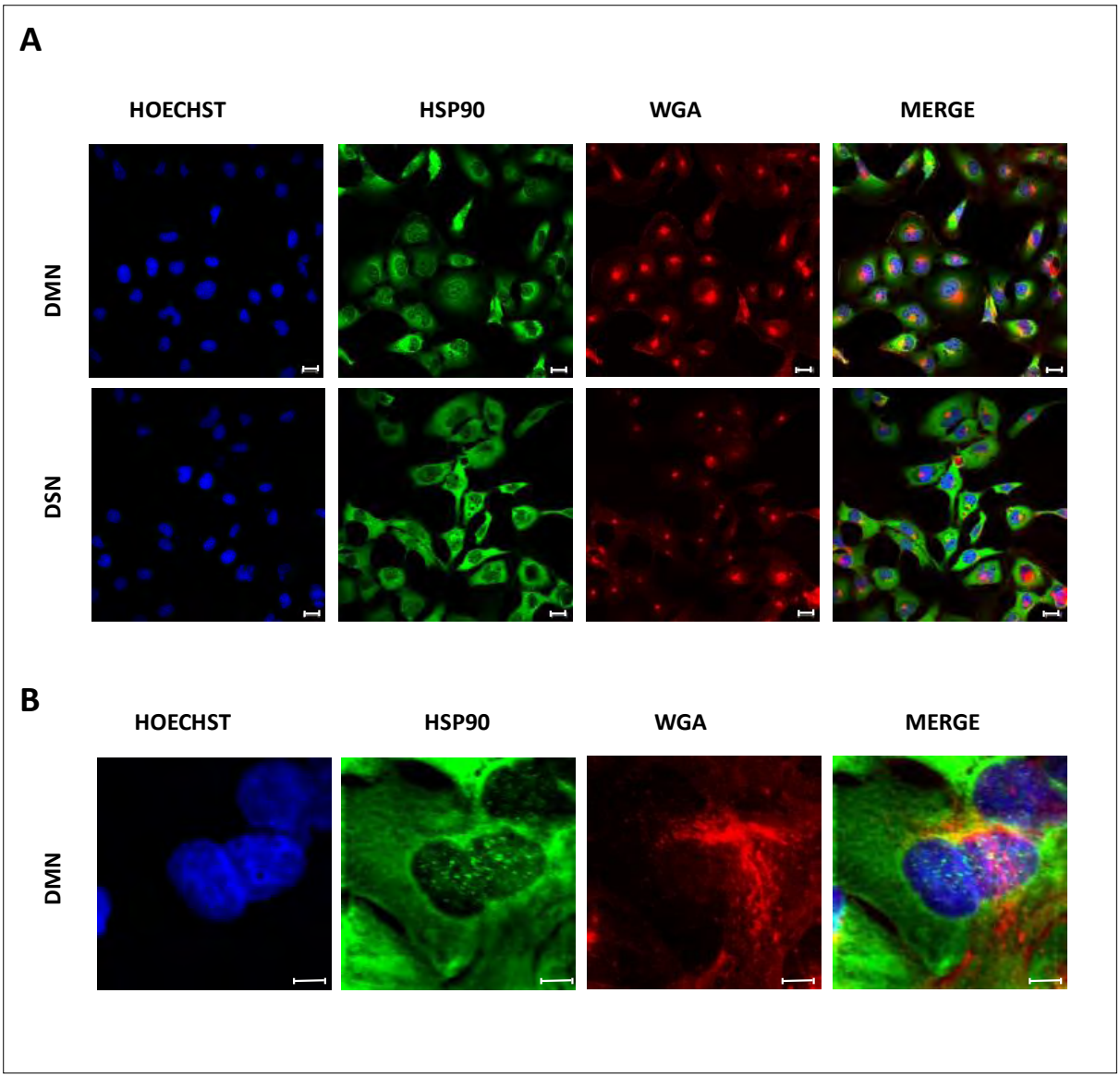
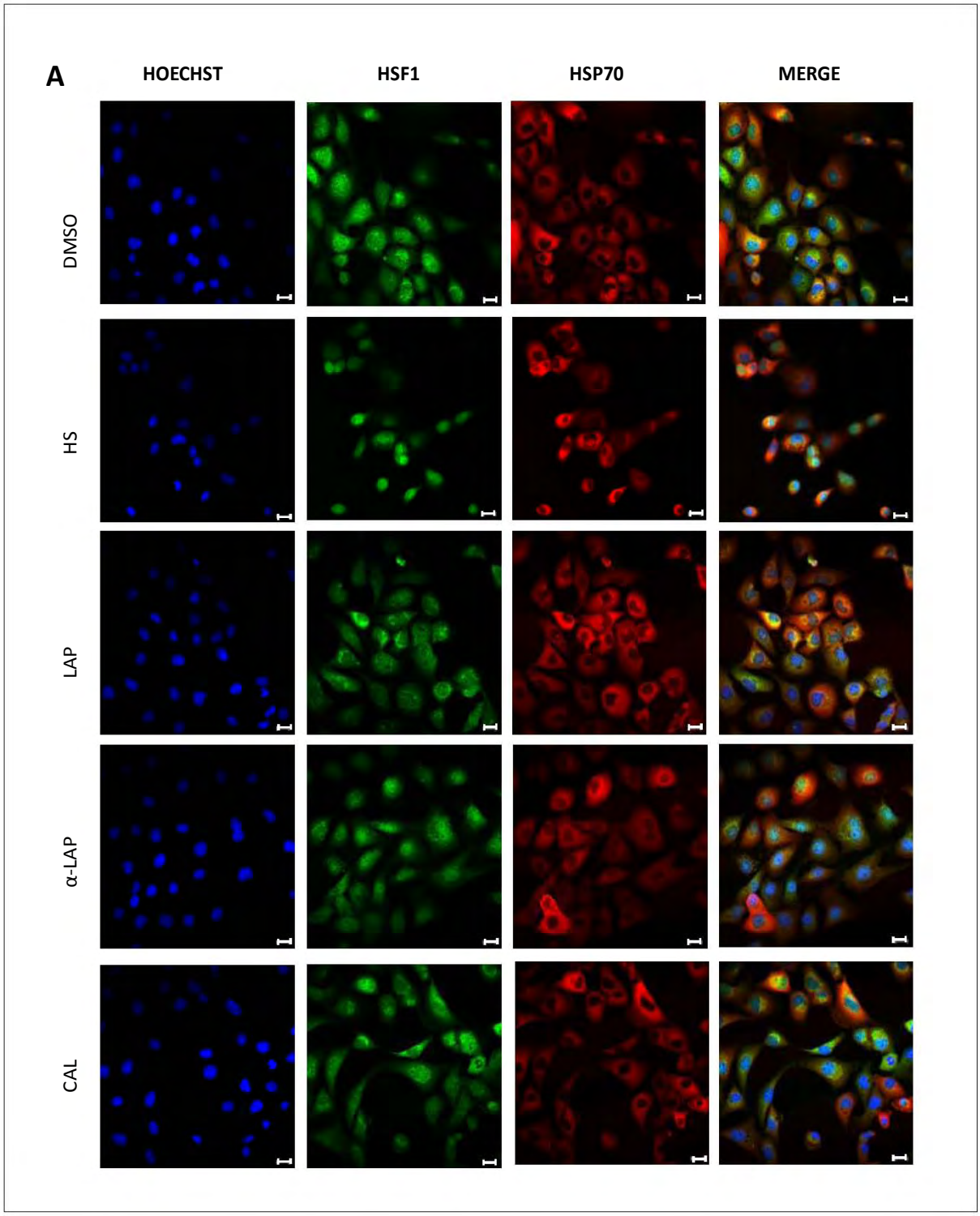


Figure B.8: Screening of marine pyrroloiminoquinone alkaloid compounds for the ability to modulate Hsp90 subcellular localisation in treated cells. (A) MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C), damirone C (DMN) (12.4 µM), makaluvamine (MKM) (7 µM), *N*-1-β-D ribofuranosyl makaluvamine (RIBS-1) (7 µM), *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3) (8.78 µM), discorhabdin A (DSN) (3.32 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-Hsp90 antibody (1:100) followed by anti-mouse AlexaFluor 488 conjugated secondary antibody and AlexaFluor 555 conjugated WGA (1 $\mu g.ml^{-1}$ l). Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm. Images were captured using the Zeiss 510 Meta laser scanning confocal microscope using the 40x objective and were analyzed using AxioVision LE Rel 4.8 (Zeiss, Germany). (B) DMN treatment resulted in peri-nuclear and punctate nuclear Hsp90 staining.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LAP
α-LAP
CAL

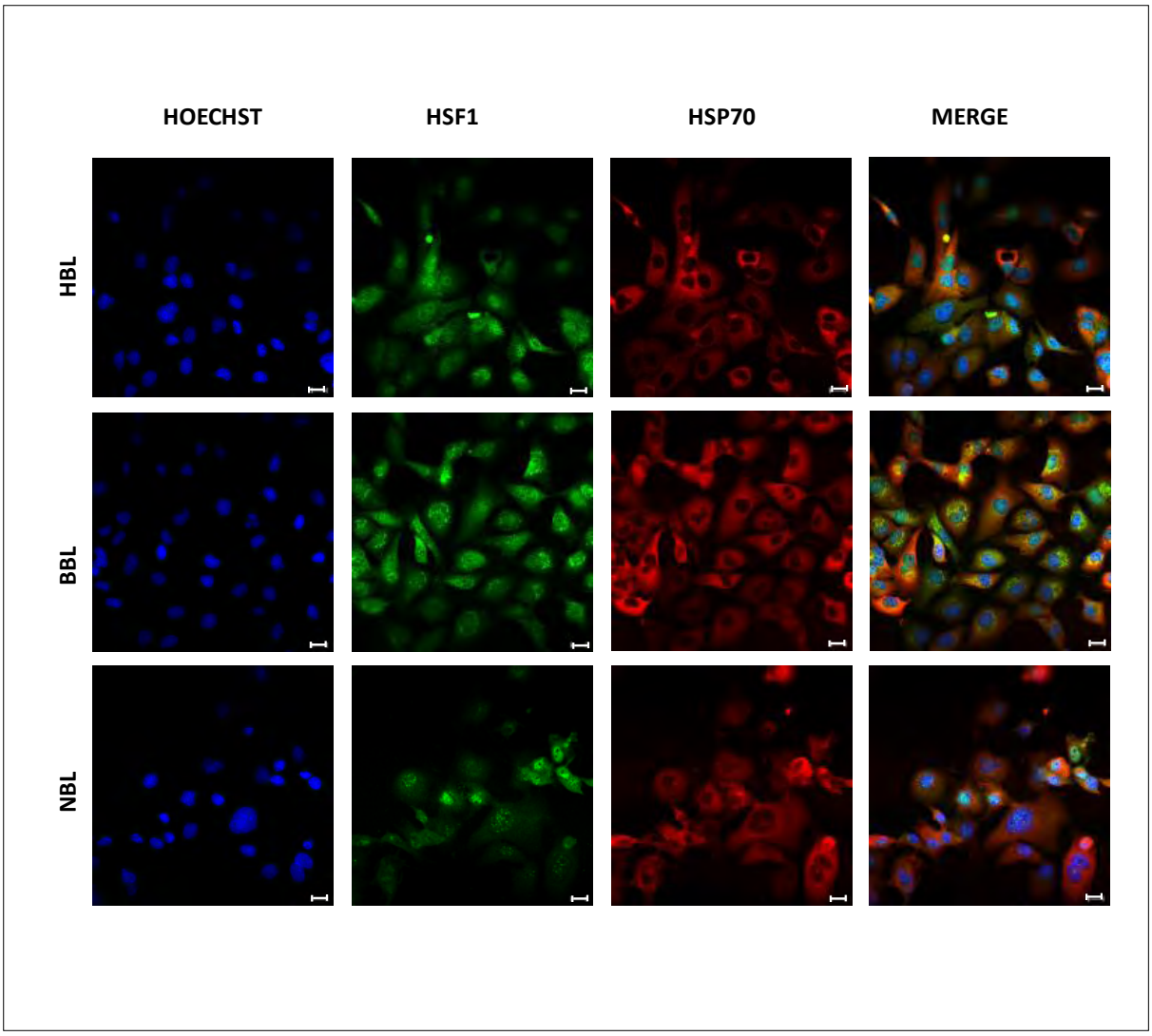
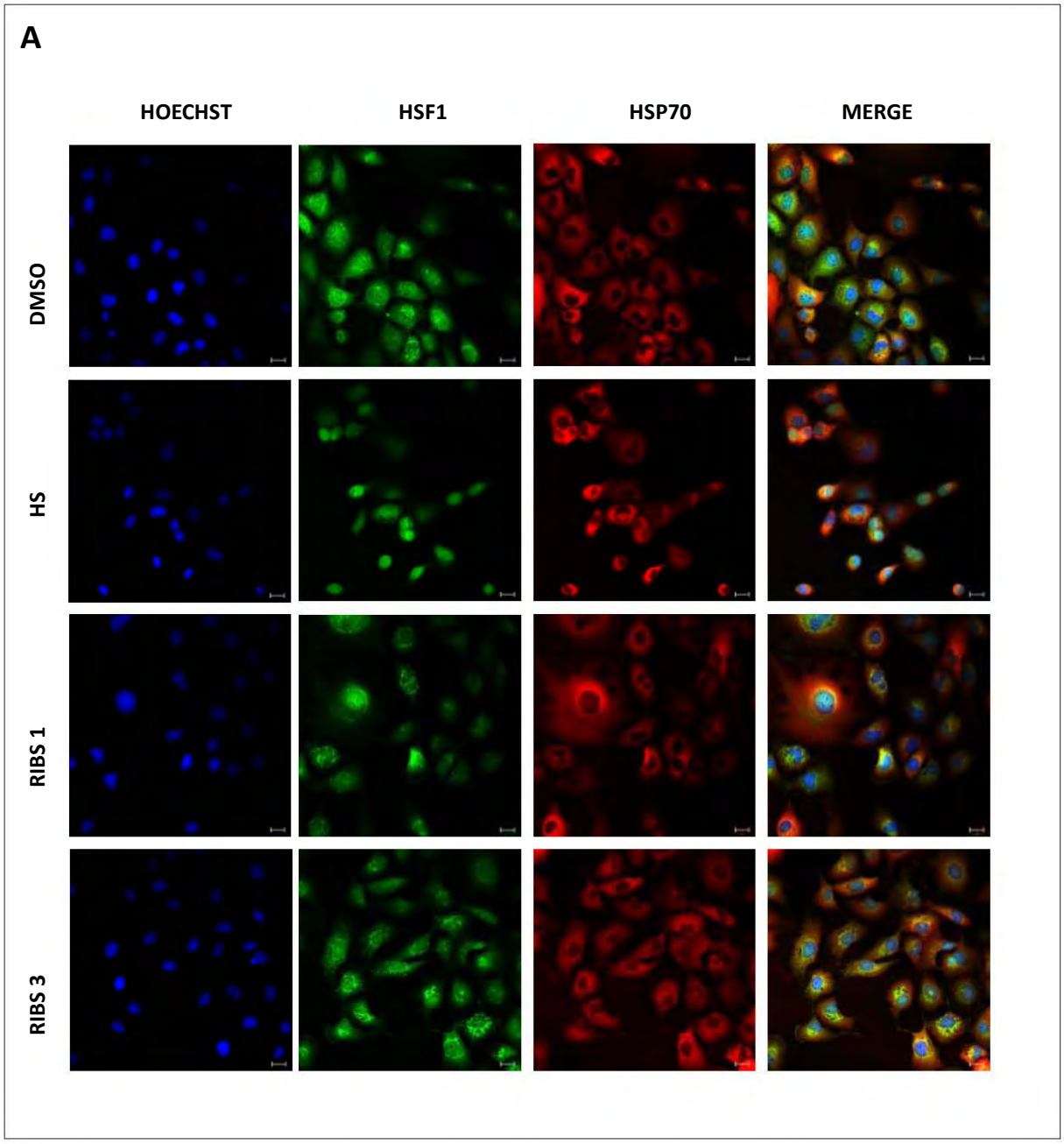


Figure B.9: Treatment with lapachol and its derivatives modulates Hsp70/Hsc localisation may induce general heat shock response. MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C), lapachol (LAP) (7 µM), α-lapachona (αLAP) (7 µM), c-alil-lausona (CAL) (7 µM), nor-β-lapachona (NBL) (8.78 µM), bromo-β-lapachona (BBL) (7.86 µM) and hydroxy-β-lapachona (HBL) (7.61 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-Hsp70 antibody (1:100) and anti-HSF1 antibody (1:100) followed by anti-mouse AlexaFluor 488 conjugated secondary antibody and anti-rabbit AlexaFluor 555 conjugated secondary antibody (1:100). Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm. Images were captured using the Zeiss 510 Meta laser scanning confocal microscope using the 40x objective and were analyzed using AxioVision LE Rel 4.8 (Zeiss, Germany).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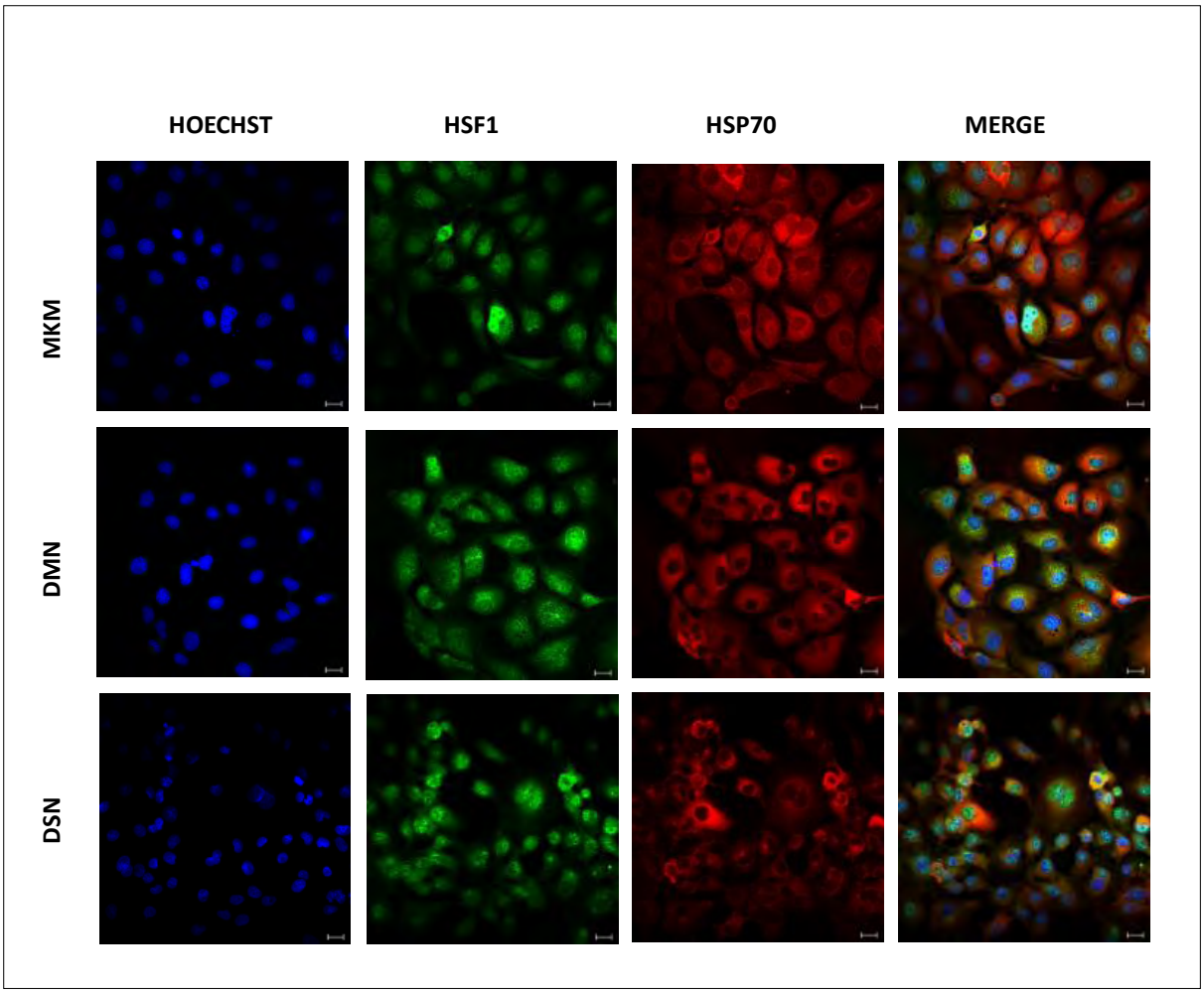
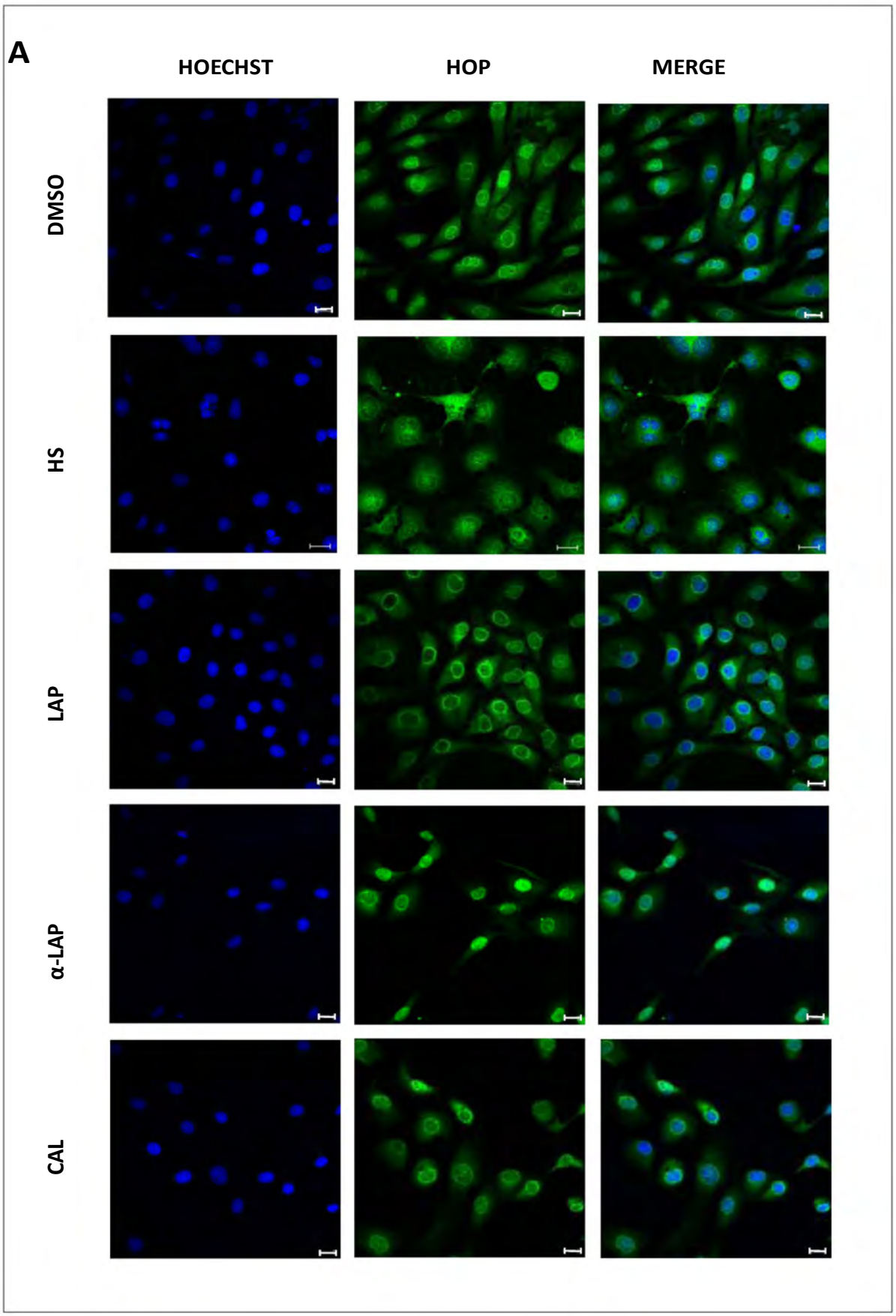


Figure B.10: **Marine pyrolloimoquinone series of compounds modulate Hsp70/Hsc localisation and possibly induce general heat shock response.** MDA-MB-231 cells were treated DMSO (vehicle control), heat shock (HS) (42°C), damirone C (DMN) (12.4 µM), makaluvamine M (MKM) (7 µM), *N*-1-β-D ribofuranosyl makaluvamine (RIBS-1) (7 µM), *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3) (8.78 µM), discorhabdin A (DSN) (3.32 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-Hsp70 antibody (1:100) and anti-HSF1 antibody (1:100) followed by anti-mouse AlexaFluor 488 conjugated secondary antibody and anti-rabbit AlexaFluor 555 conjugated secondary antibody (1:100). Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm. Images were captured using the Zeiss 510 Meta laser scanning confocal microscope using the 40x objective and were analyzed using AxioVision LE Rel 4.8 (Zeiss, Germany).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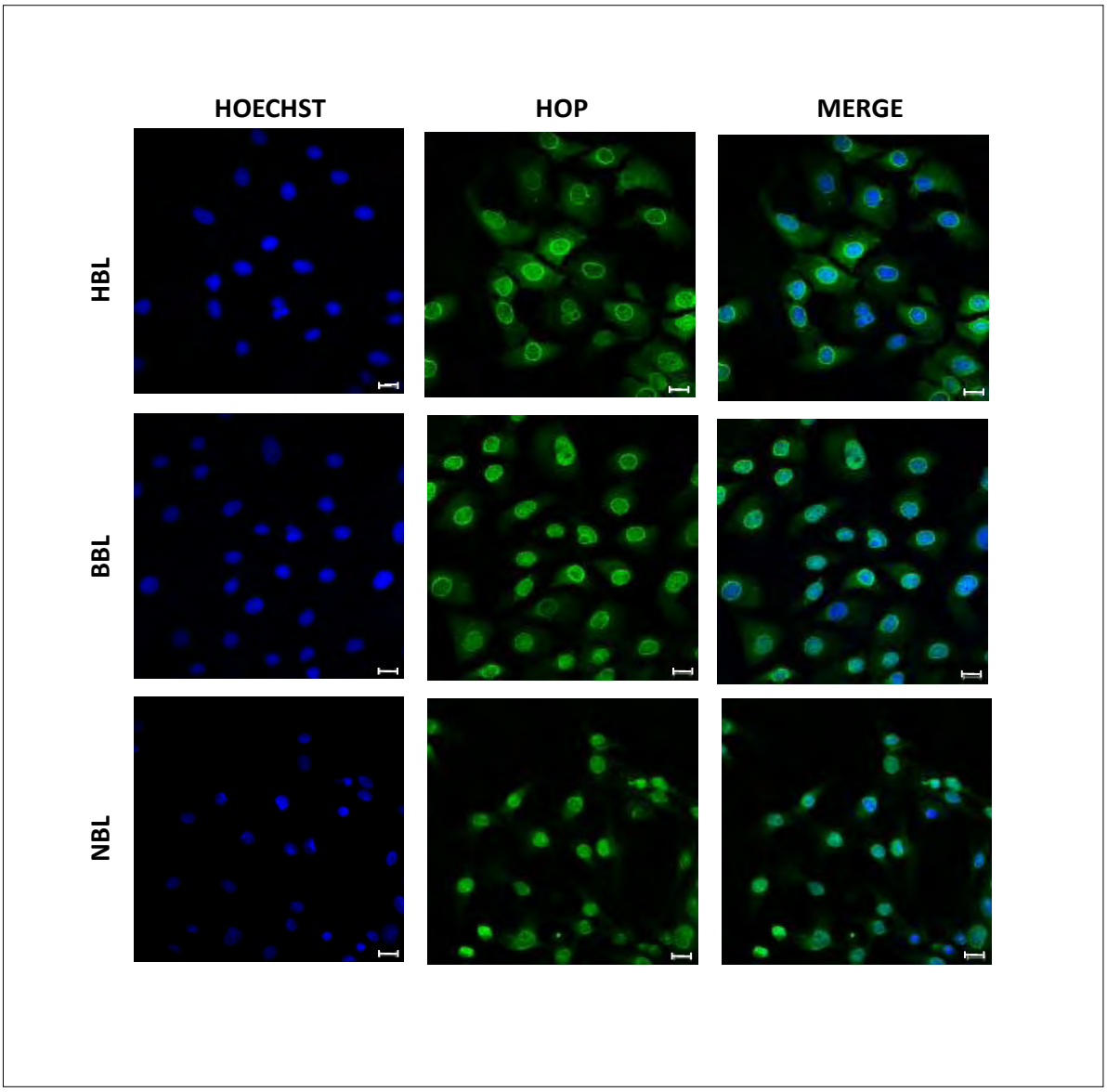
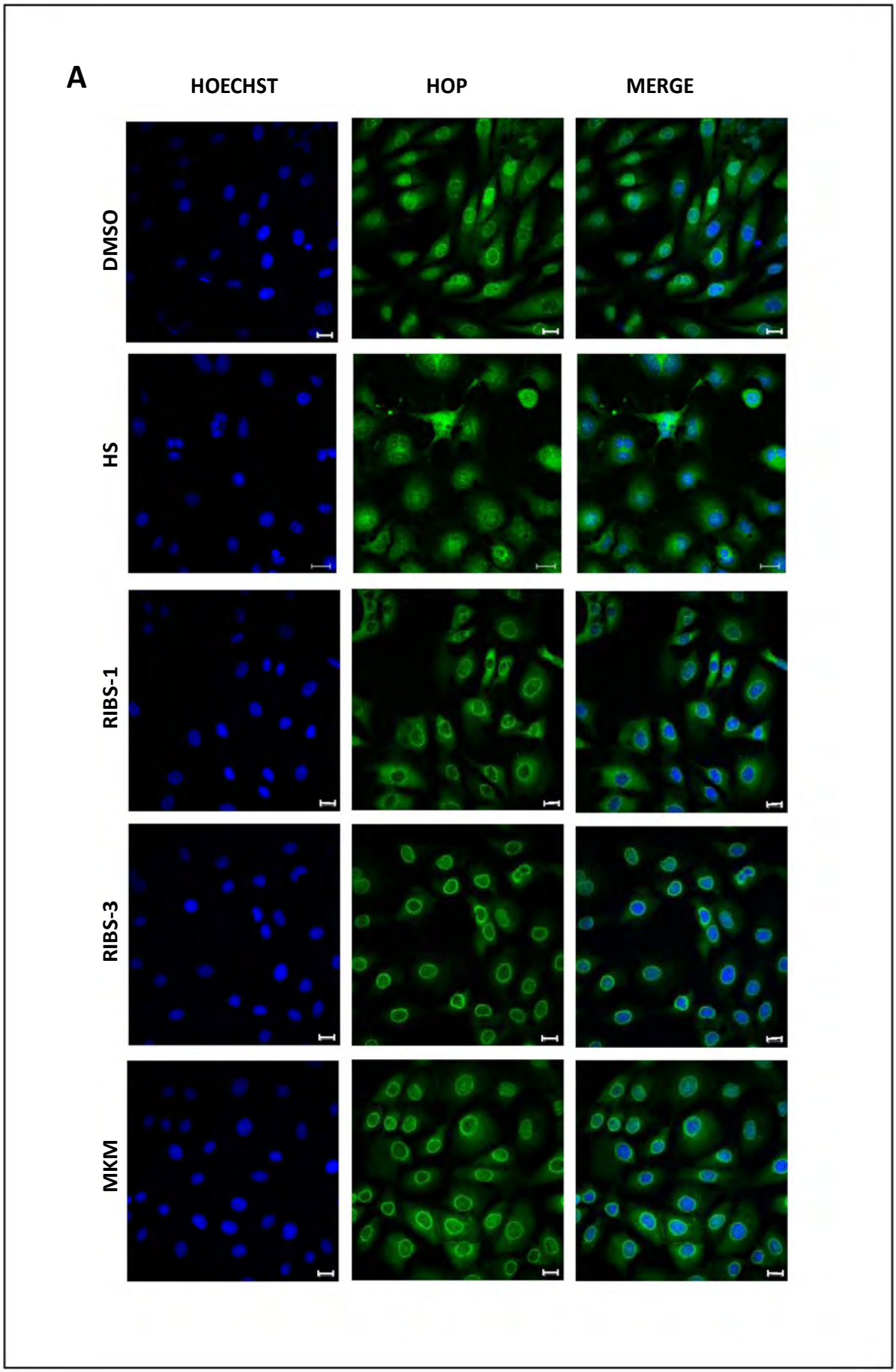


Figure B.11: **Naphthoquinone series of compounds excluding lapachol resulted in concentration of Hop in the nuclei of treated cells.** MDA-MB-231 cells were treated DMSO (vehicle control), heat shock (HS) (42 °C), lapachol (LAP) (7 µM), α-lapachona (αLAP) (7 µM), c-alil-lausona (CAL) (7 µM), nor-β-lapachona (NBL) (8.78 µM), bromo-β-lapachona (BBL) (7.86 µM) and hydroxy-β-lapachona (HBL) (7.61 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-Hop antibody (1:100) followed by anti-mouse AlexaFluor 488 secondary antibody. Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images were captured using the Zeiss 510 Meta laser scanning confocal microscope, 40x objectives and were analyzed using AxioVision LE Rel 4.8 (Zeiss, Germany). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm.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HOP
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HS
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MKM

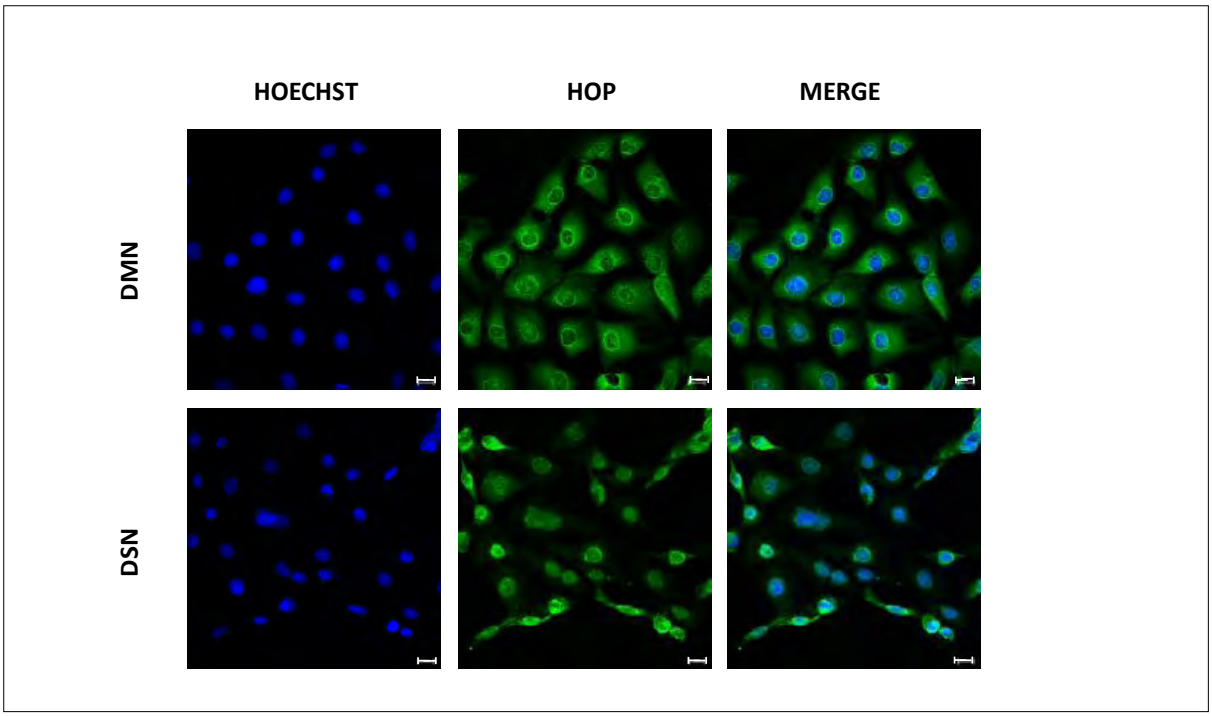
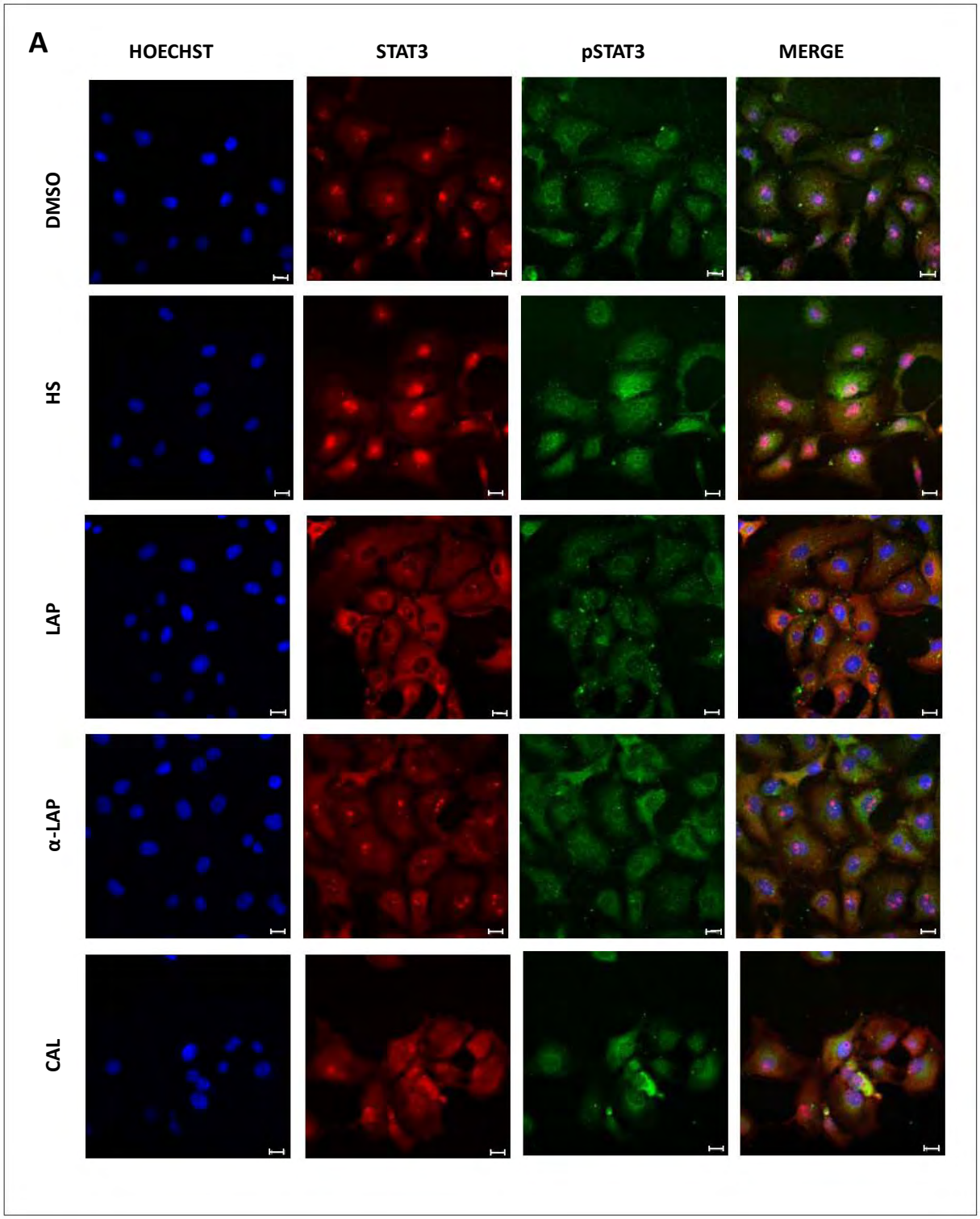


Figure B.12: **DMN, RIBS-1, RIBS-3 and MKM treated cells increase perinuclear Hop and DSN treatment increases nuclear Hop in cells.** MDA-MB-231 cells were treated DMSO (vehicle control), heat shock (HS) (42°C), damirone C (DMN) (12.4 µM), makaluvamine M (MKM) (7 µM), *N*-1-β-D ribofuranosyl makaluvamine (RIBS-1) (7 µM), *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3) (8.78 µM), discorhabdin A (DSN) (3.32 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-Hop antibody (1:100) followed by anti-mouse AlexaFluor 488 secondary antibody. Images were captured using the Zeiss 510 Meta laser scanning confocal microscope, 40x objectives and were analyzed using AxioVision LE Rel 4.8 (Zeiss, Germany). Nuclei were counterstained blue with Hoechst 33342 (1 µg.ml^{-1}). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm.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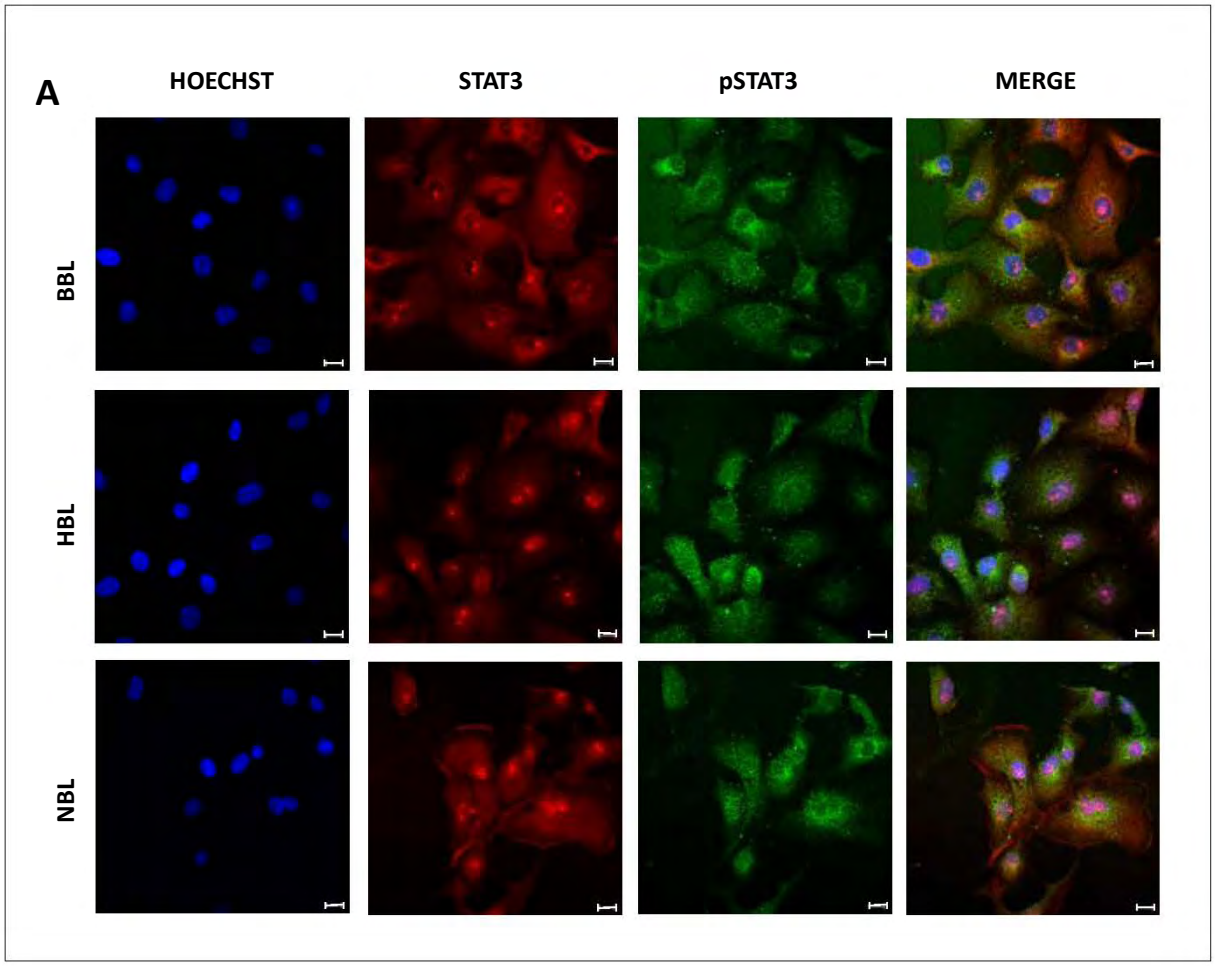
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NBL

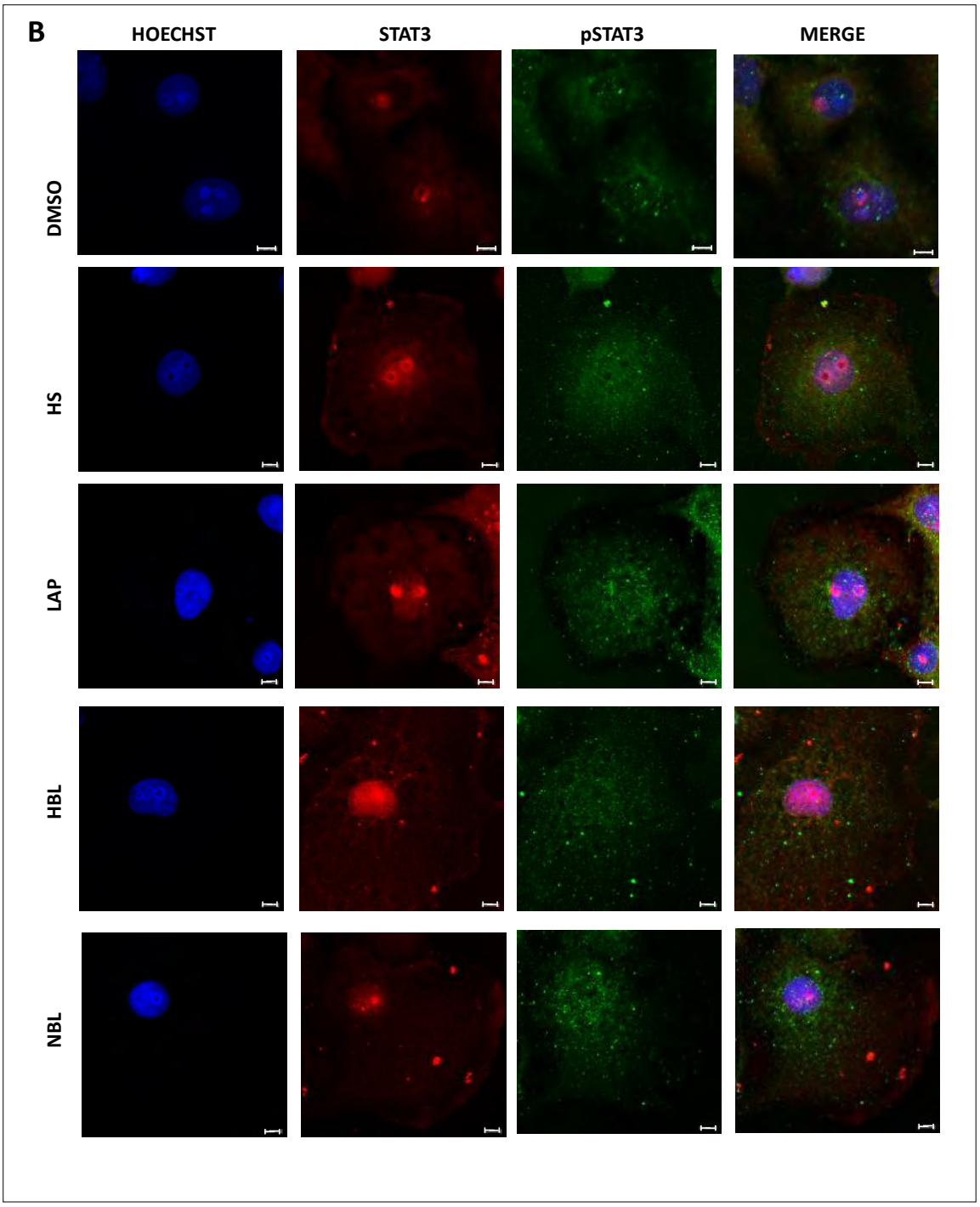
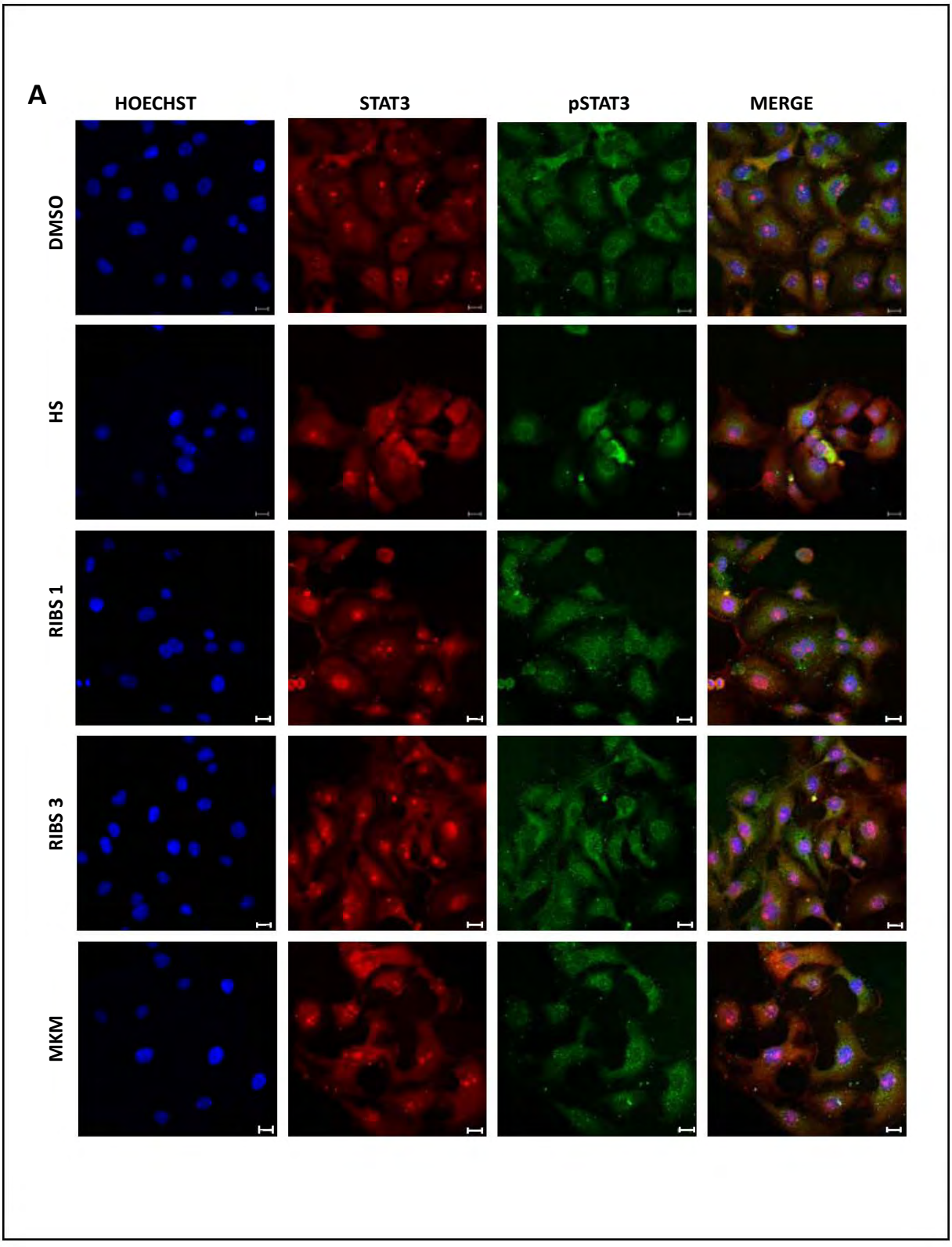


Figure B.13: Lapachol and its derivatives influence STAT3 and pSTAT3 localisation in treated cells. (**A**) MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C) lapachol (LAP) (7 µM), α-lapachona (αLAP) (7 µM), c-alil-lausona (CAL) (7 µM), nor-β-lapachona (NBL) (8.78 µM), bromo-β-lapachona (BBL) (7.86 µM) and hydroxy-β-lapachona (HBL) (7.61 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-pSTAT3 antibody (1:100) and rabbit anti-STAT3 antibody (1:100) followed by anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 555 secondary antibodies. Nuclei were counterstained blue with Hoechst 33342 (1 µg.ml^{-1}). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm. (B) The Hsp90 client protein STAT3 was localized within distinct foci in the nucleoli in both treated including DMSO, heat shock (HS) (42°C), lapachol (LAP) (7 µM) and hydroxy-β-lapachona (HBL) treatments.


A
HOECHST
STAT3
pSTAT3
MERGE
DMSO
HS
RIBS 1
RIBS 3
MKM

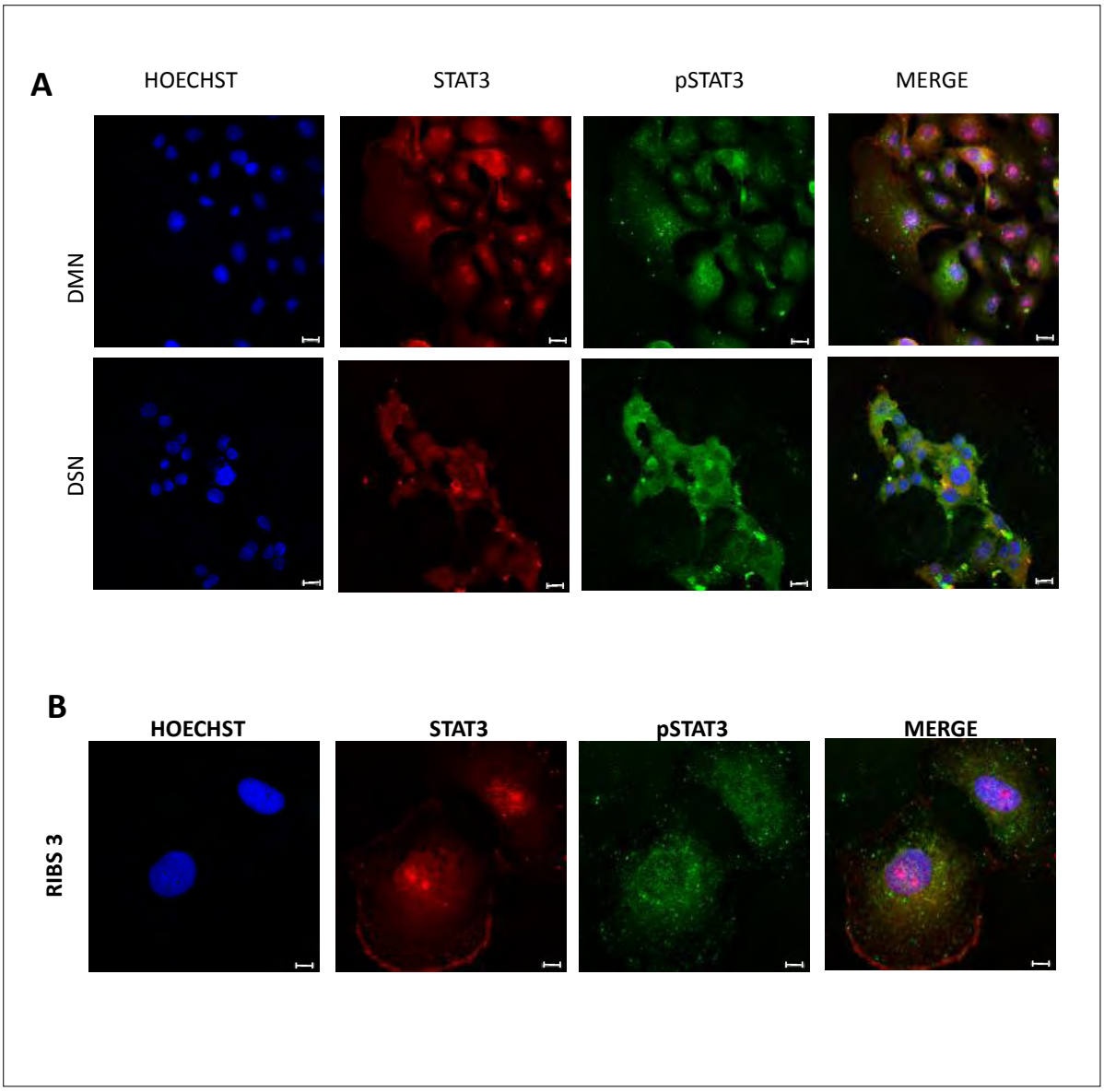
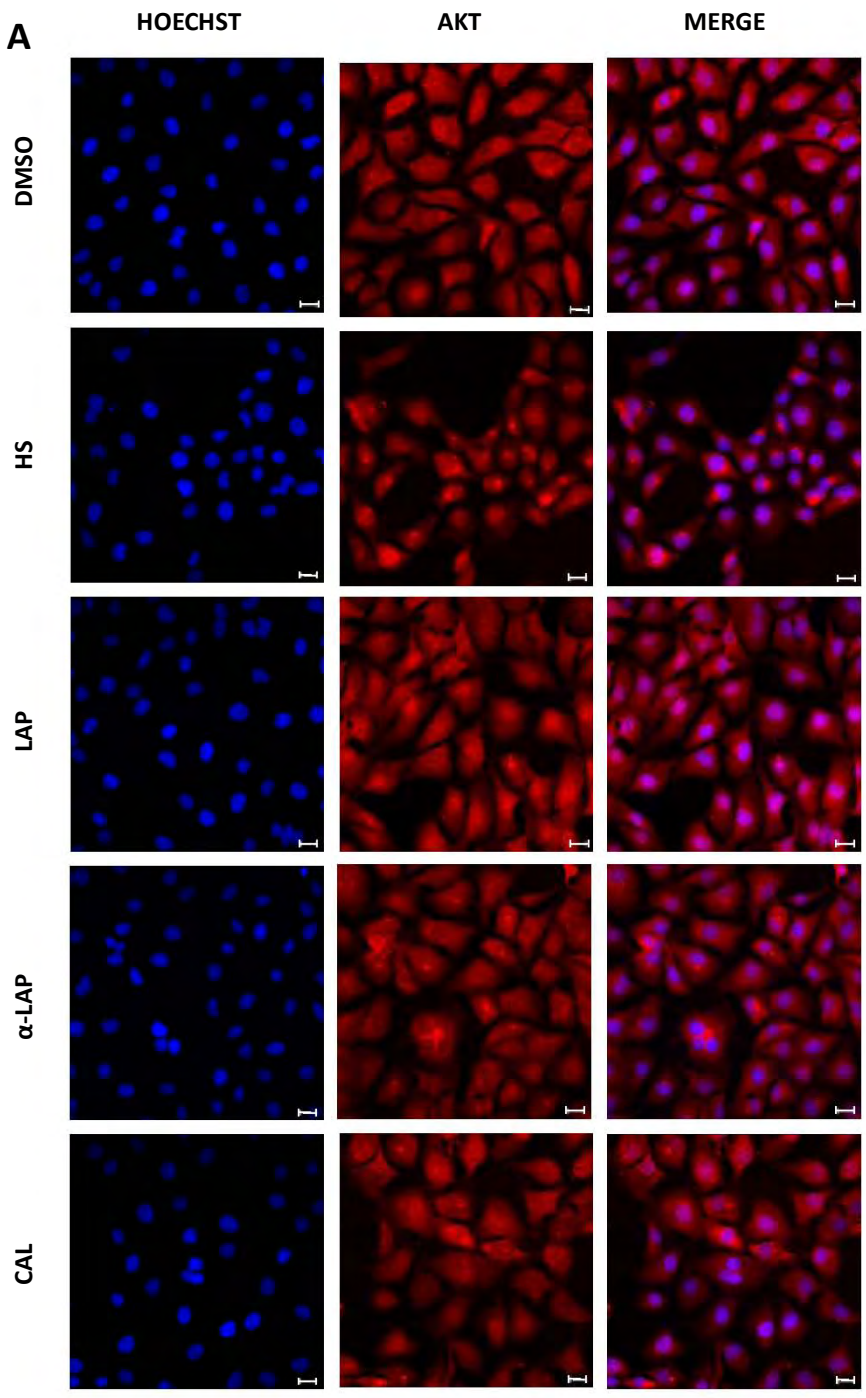


Figure B.14: Marine pyrolloimoquinone compounds influence STAT3 or pSTAT3 localisation in treated cells (A**)** MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C) damirone C (DMN) (12.4 µM), makaluvamine M (MKM) (7 µM), N-1-β-D ribofuranosyl makaluvamine (RIBS-1) (7 µM), *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3) (8.78 µM), discorhabdin A (DSN) (3.32 µM) for 5 hours at 37 °C. Cells were fixed and stained with mouse anti-pSTAT3 antibody (1:100) and rabbit anti-STAT3 antibody (1:100) followed by anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 555 secondary antibodies. Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm. (B) The Hsp90 client protein STAT3 was localized to the cytoplasm, nucleus, membrane and nucleoli of cells following treatment with *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3) (8.78 µM).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HOECHST
AKT
MERGE
DMSO
HS
LAP
α-LAP
CAL

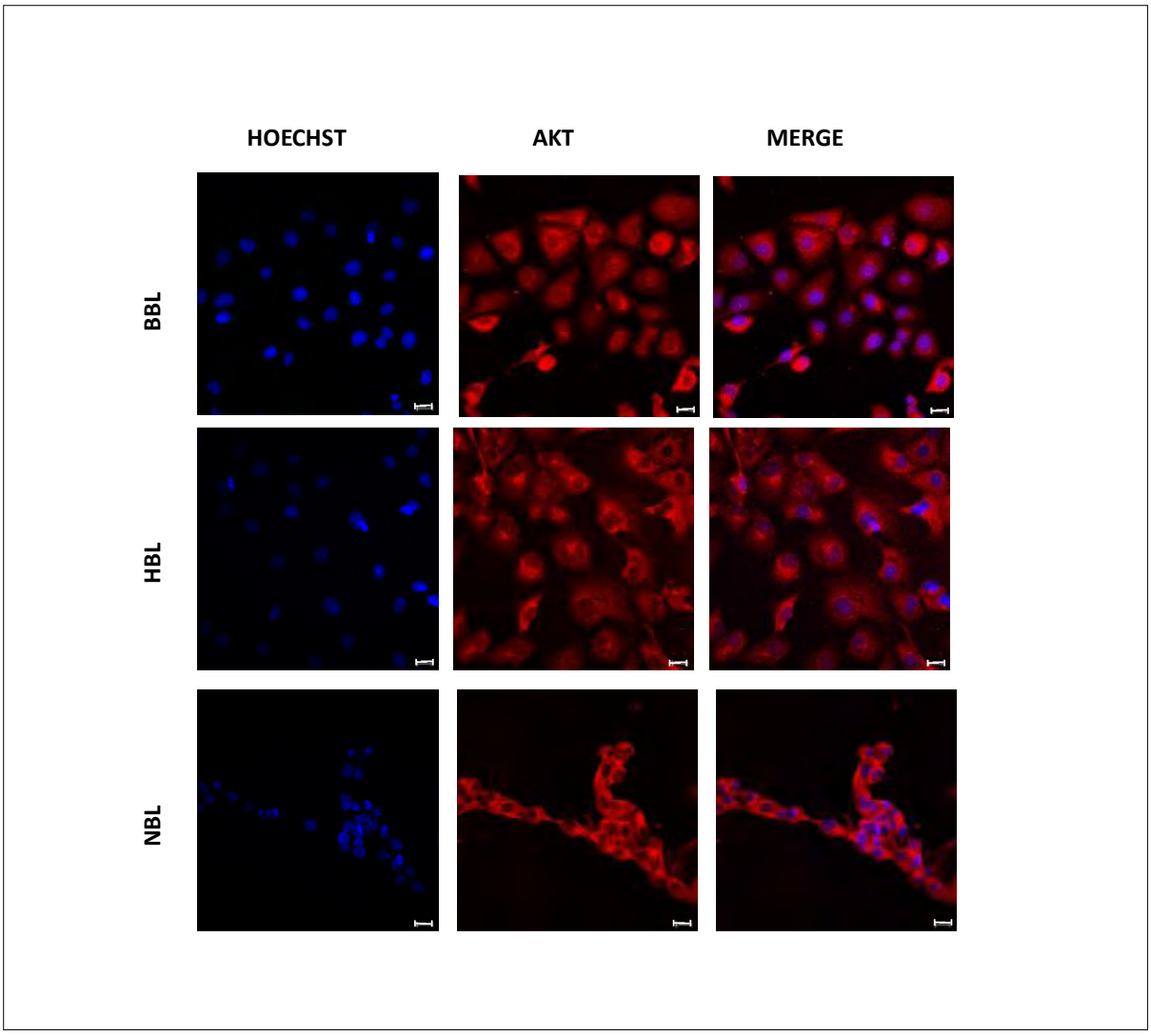
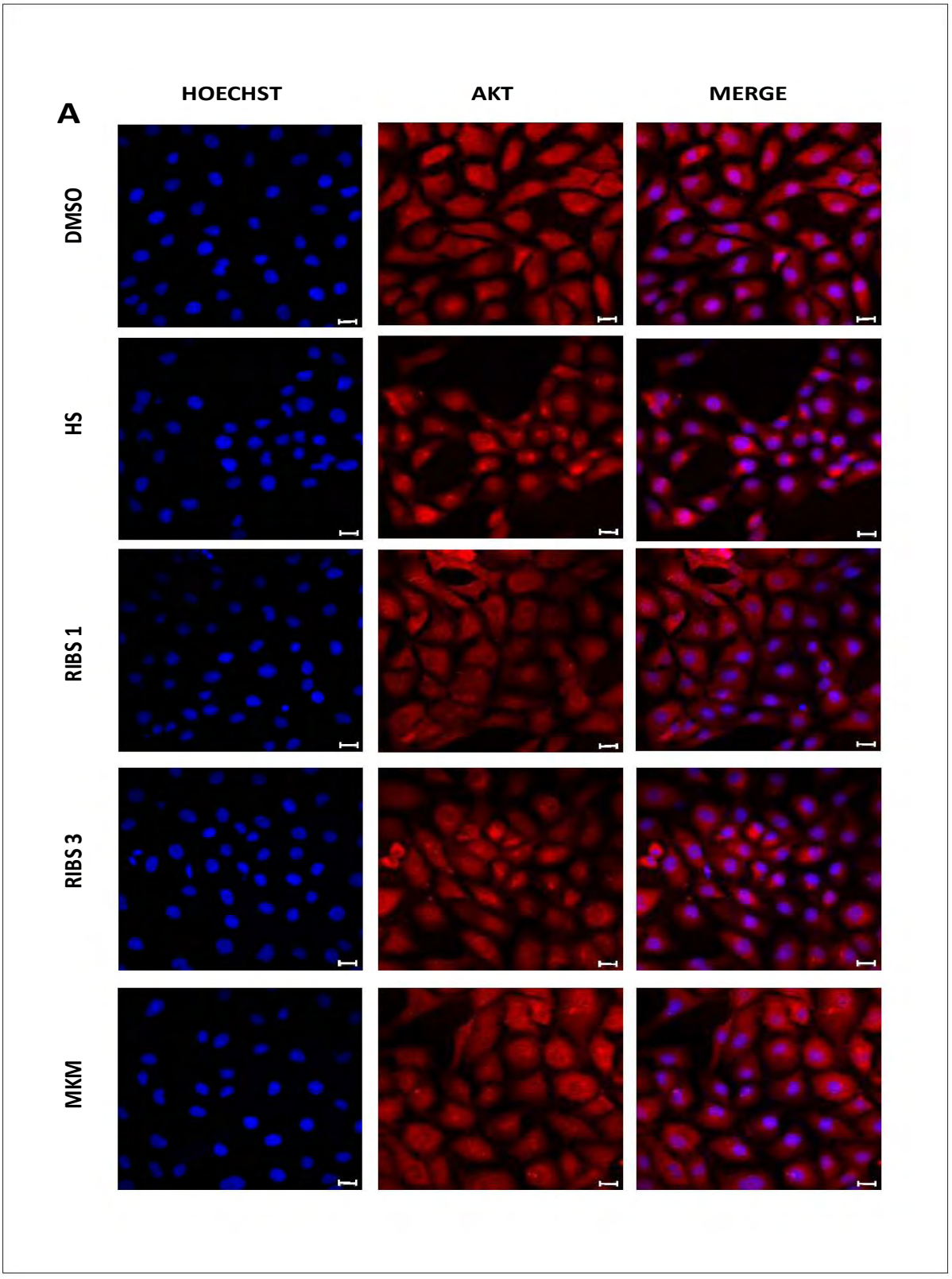


Figure B.15: 1, 4 Naphthoquinone compounds do not modulate AKT localisation in treated cells. MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C), lapachol (LAP) (7 µM), α-lapachona (αLAP) (7 µM), c-alil-lausona (CAL) (7 µM), nor-β-lapachona (NBL) (8.78 µM), bromo-β-lapachona (BBL) (7.86 µM) and hydroxy-β-lapachona (HBL) (7.61 µM) for 5 hours at 37 °C. Cells were fixed and stained with rabbit anti-AKT antibody (1:100) followed by anti-rabbit AlexaFluor 555 secondary antibody. Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm.


A
HOECHST
AKT
MERGE
DMSO
HS
RIBS 1
RIBS 3
MKM

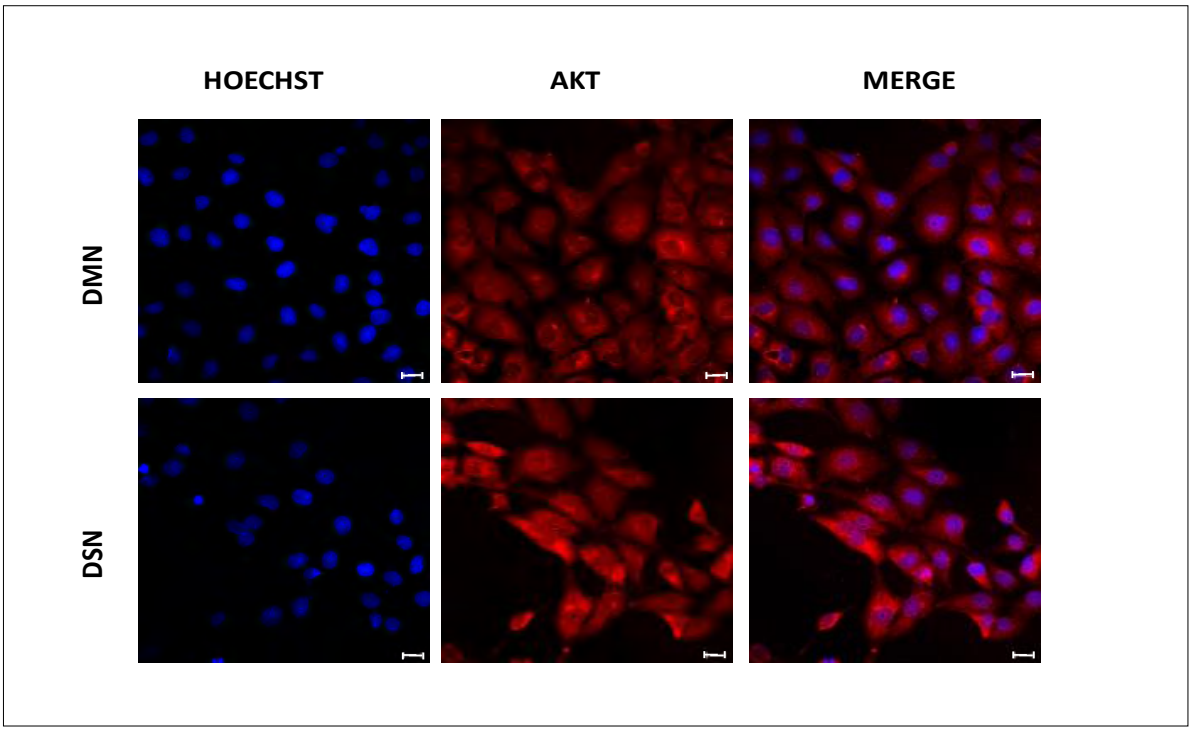


Figure B.146: Marine pyrolloimoquinone compounds do not influence do not influence AKT localisation in treated cells. MDA-MB-231 cells were treated with DMSO (vehicle control), heat shock (HS) (42 °C), damirone C (DMN) (12.4 µM), makaluvamine M (MKM) (7 µM), *N*-1-β-D ribofuranosyl makaluvamine (RIBS-1) (7 µM), *N*-1-β-D-ribofuranosyl makaluvic acid C (RIBS-3) (8.78 µM), discorhabdin A (DSN) (3.32 µM) for 5 hours at 37 °C. Cells were fixed and stained with rabbit anti-AKT antibody (1:100) followed by anti-rabbit AlexaFluor 555 secondary antibody. Nuclei were counterstained blue with Hoechst 33342 (1 $\mu g.ml^{-1}$). Images shown are representative of two independent experiments in which the subcellular localisation of pattern was counted in three separate microscope fields. Scale bars: 20 µm.

APPENDIX C: ADDITIONAL SUPPORTING FIGURES

Heterologous Expression and Hsp90β Purification

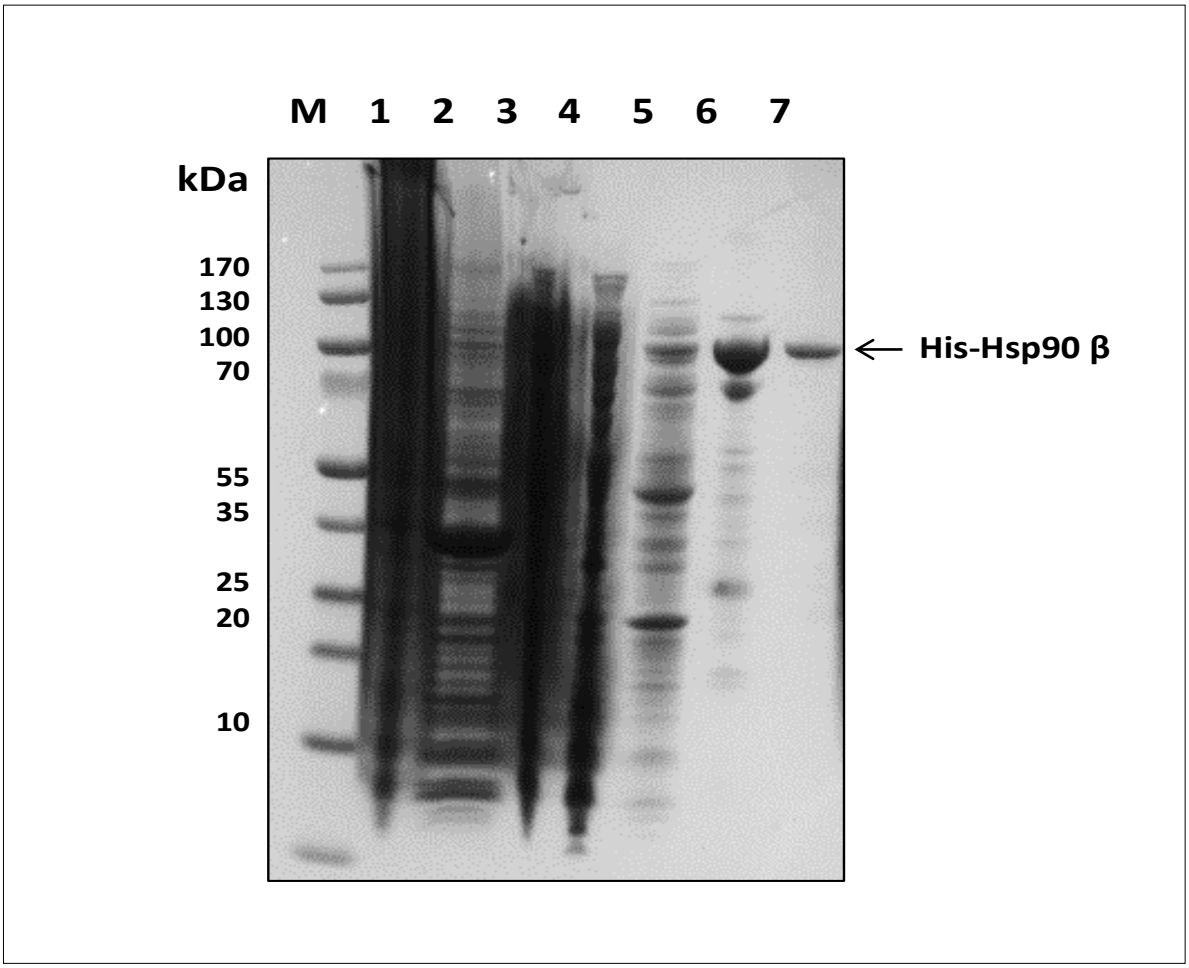


Figure C.1: Coomassie stained SDS-PAGE gel of the heterologous expression and purification of recombinant hexahistine-tagged Hsp90β protein. Proteins were expressed from a pET14b-based plasmid in *E.coli* C41 cells at 25 °C following induction with 0.5 mM IPTG in 2 x YT media supplemented with 100 ug.ml^{-1} ampicillin. Protein was purified by Nickel affinity chromatography on an ÄKTA Basic FPLC^{TM} through 3 x 5ml His-Trap^{TM} columns followed by ion exchange chromatography on an ÄKTA Basic FPLC^{TM}. Proteins were resolved on a precast 12 % SDS-PAGE gel and a representative gel shown. Lanes: 1 – *E.coli* whole cell lysate post overnight induction, 2 - *E.coli* pellet fraction following centrifugation, 3 – clarified supernatant following centrifugation, 4 - non-target proteins unbound to nickel column, 5 – non-target proteins washed off nickel column with 50 mM Imidazole, 6 – protein eluted from His-Trap^{TM} columns with 300 mM Imidazole, 7 – protein eluted by ion exchange chromatography on MonoQ 10/10 column with 0.1 – 1M NaCl gradient.

Determination of the binding of small molecules to Hsp90β by ITC

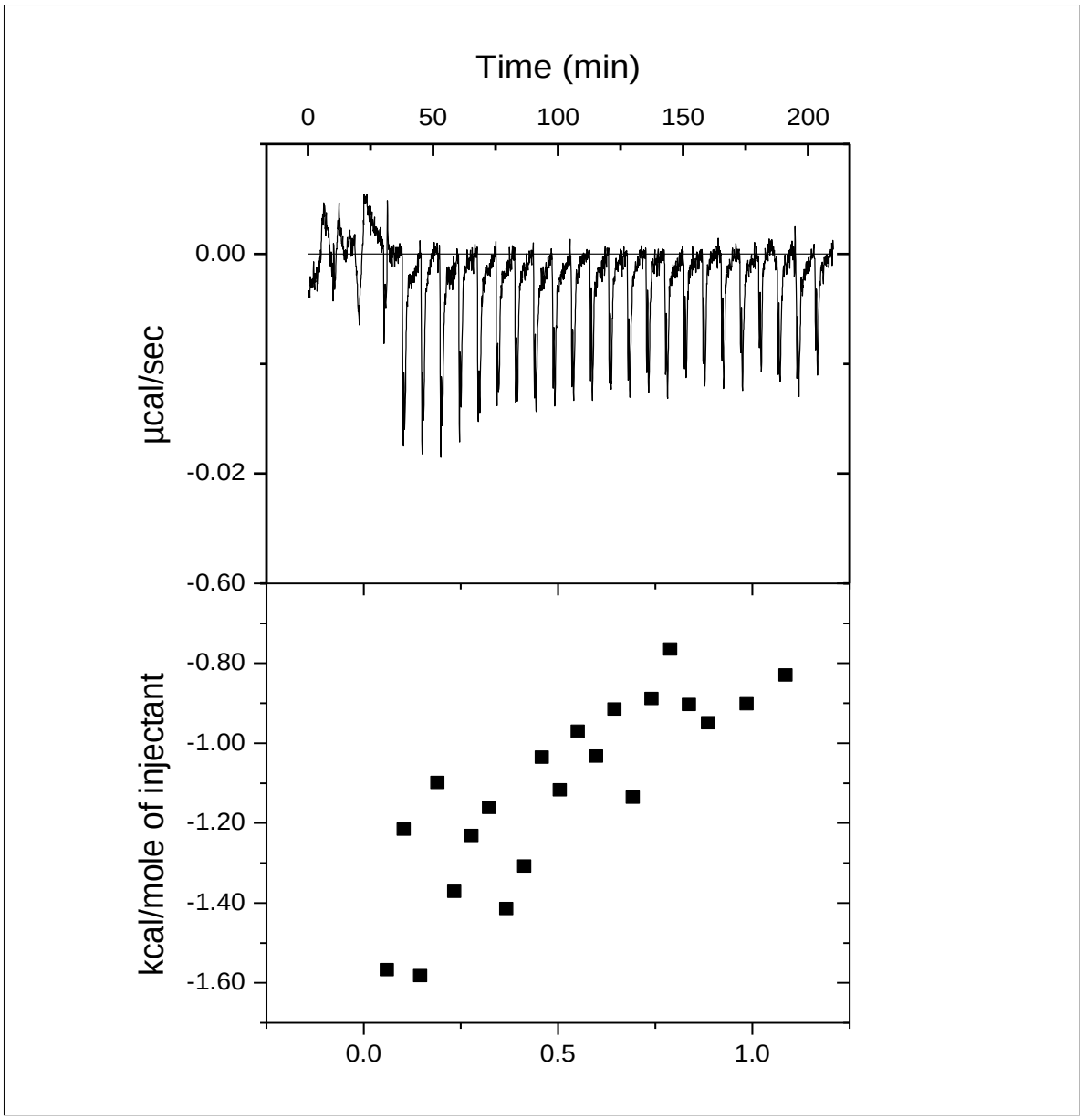


Figure C.2: αLAP does not interact with Hsp90β. Raw and fitted data between α-lapachona (αLAP) (700 µM) binding to Hsp90β (30 µM) equilibrated to 25 °C in 50 mM Tris (pH7.4), 6 mM $MgCl_2$ 20 mM KCl, 1 mM β-mercaptoethanol. Upper graph represents the raw ITC data in which the incremental heat changes are shown microcalories per second ($\mu cal.sec^{-1}$) plotted against time in minutes over the progression of the experiment, lower graph shows the normalised integration data in terms of kilocalories per mole ($kcal.mol^{-1}$) of injectant plotted against molar ratio of the added titrant with the curve fitted to single-site mode. The relation between the two x-axes allows for the positioning of the integrated area for each peak directly below the associated peak in the raw data. The negative peaks correspond to addition of an aliquot of ligand, and the calorimetric output indicated that an exothermic process was occurring in the cell.

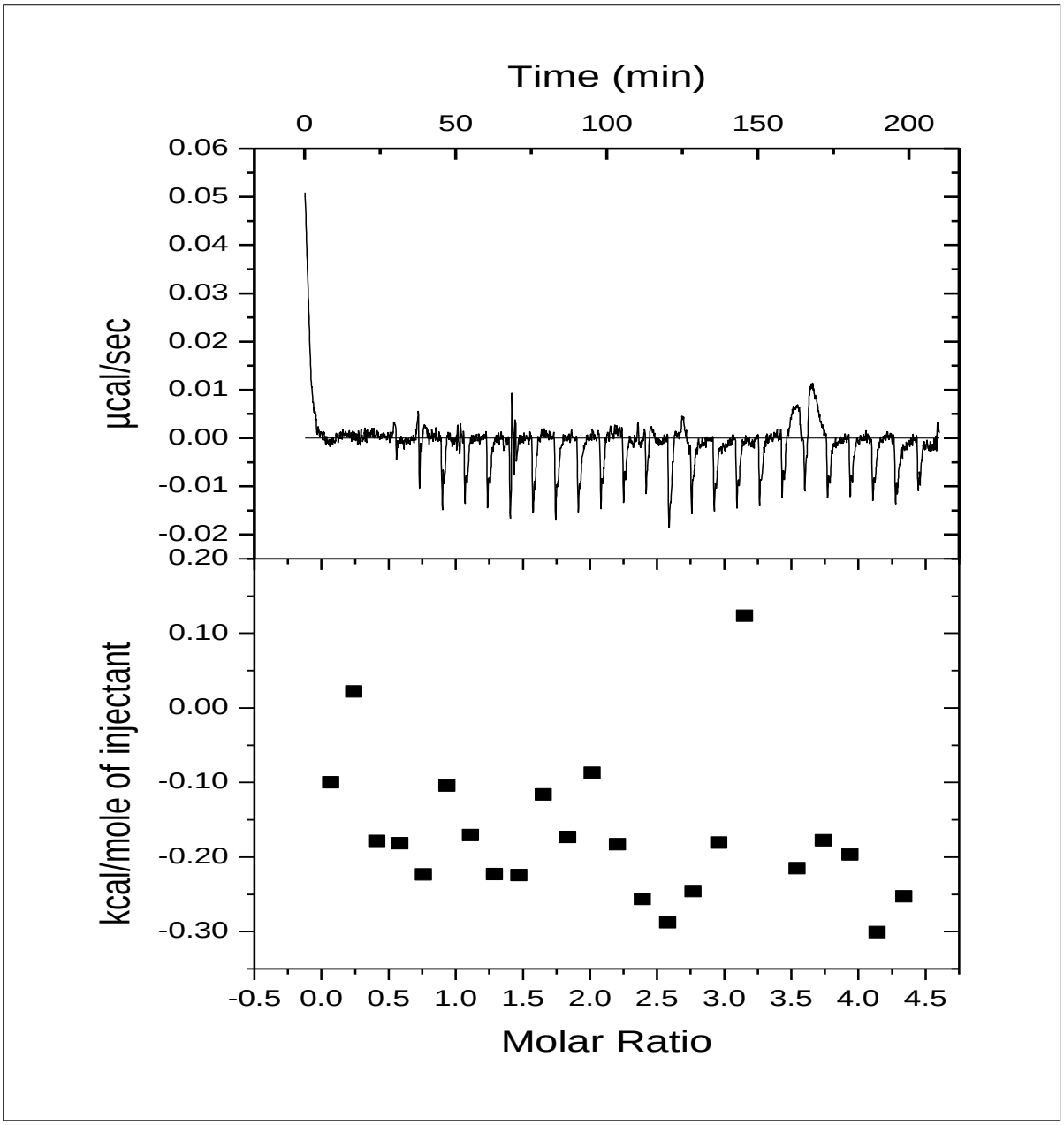


Figure C.3: CAL does not interact with Hsp90β. Raw and fitted data between c-alil-lausona (CAL) (700 µM) binding to Hsp90β (30 µM) equilibrated to 25 °C in 50 mM Tris (pH7.4), 6 mM $MgCl_2$ 20 mM KCl, 1 mM β-mercaptoethanol. Upper graph represents the raw ITC data in which the incremental heat changes are shown microcalories per second ($\mu cal.sec^{-1}$) plotted against time in minutes over the progression of the experiment, lower graph shows the normalised integration data in terms of kilocalories per mole ($kcal.mol^{-1}$) of injectant plotted against molar ratio of the added titrant with the curve fitted to single-site mode. The relation between the two x-axes allows for the positioning of the integrated area for each peak directly below the associated peak in the raw data. The negative peaks correspond to addition of an aliquot of ligand, and the calorimetric output indicated that an exothermic process was occurring in the cell.

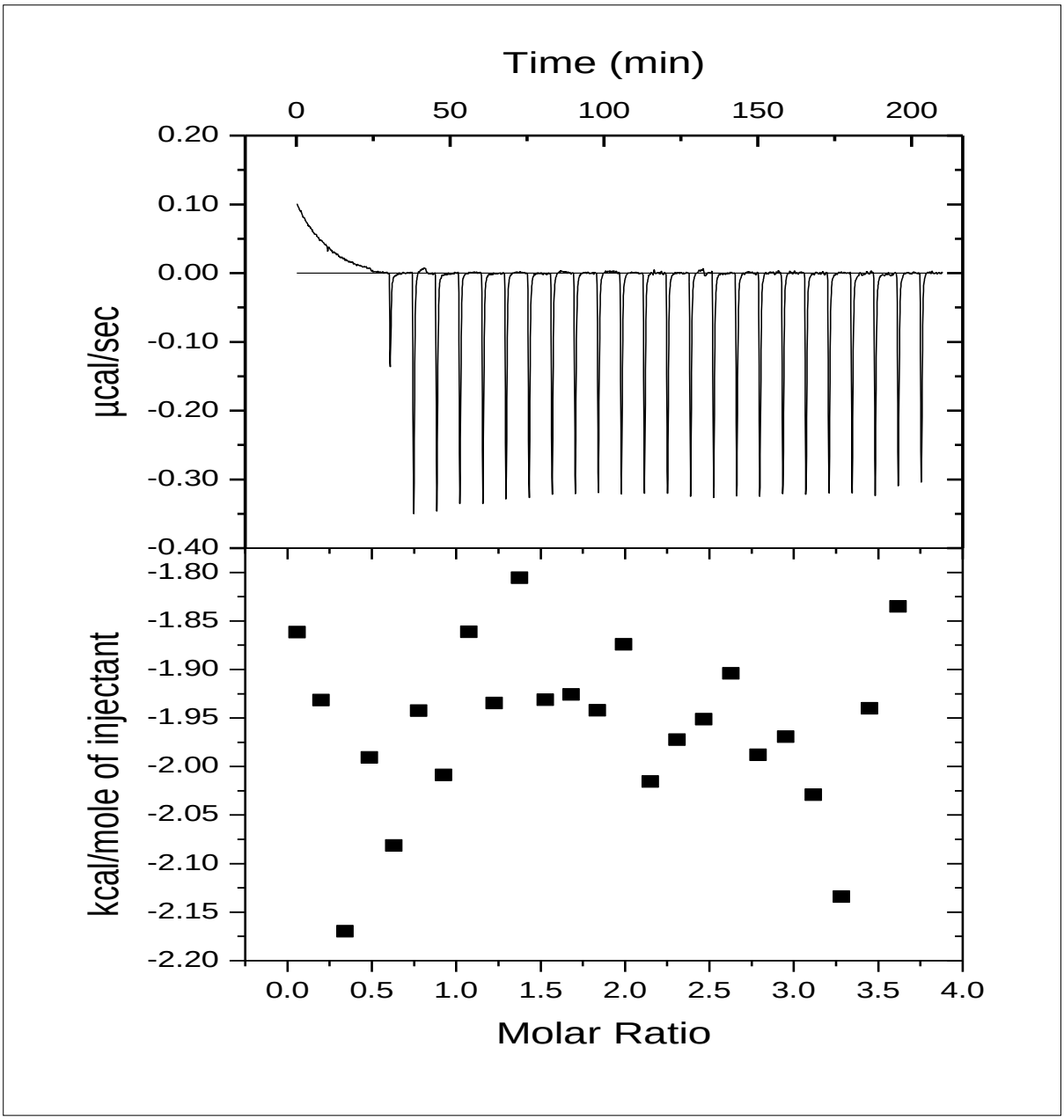


Figure C.4: **BBL does not interact with Hsp90β.** Raw and fitted data between bromo-β-lapachona (BBL) (700 µM) binding to Hsp90β (30 µM) equilibrated to 25 °C in 50 mM Tris (pH7.4), 6 mM $MgCl_2$ 20 mM KCl, 1 mM β-mercaptoethanol. Upper graph represents the raw ITC data in which the incremental heat changes are shown microcalories per second ($\mu cal.sec^{-1}$) plotted against time in minutes over the progression of the experiment, lower graph shows the normalised integration data in terms of kilocalories per mole ($kcal.mol^{-1}$) of injectant plotted against molar ratio of the added titrant with the curve fitted to single-site mode. The relationship between the two x-axes allows for the positioning of the integrated area for each peak directly below the associated peak in the raw data. The negative peaks correspond to addition of an aliquot of ligand, and the calorimetric output indicated that an exothermic process was occurring in the cell.

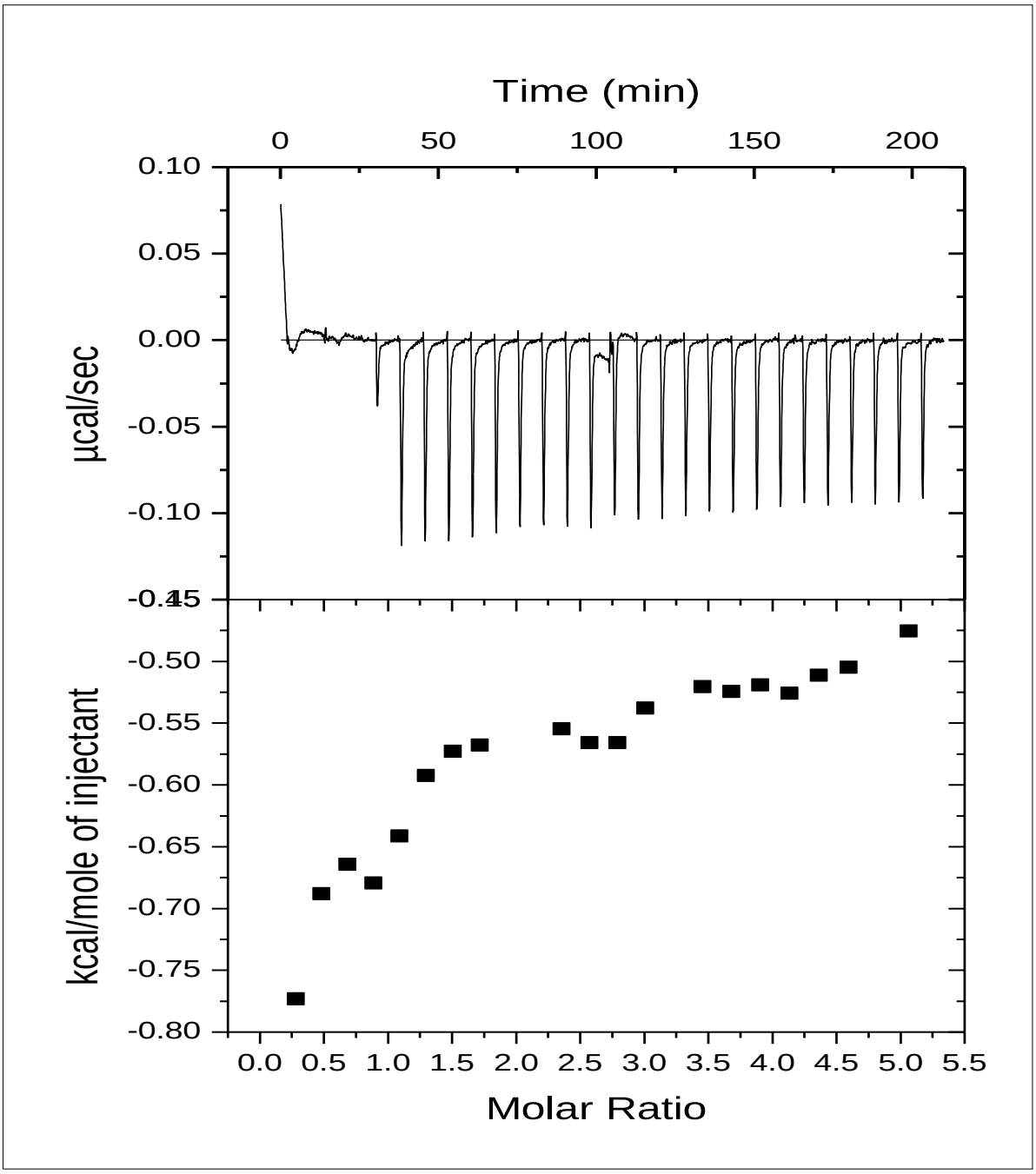


Figure C.5: **HBL does not interact with Hsp90β**. Raw and fitted data between hydroxy-β-lapachona (HBL)(700 µM) binding to Hsp90β (30 µM) equilibrated to 25 °C in 50 mM Tris (pH7.4), 6 mM $MgCl_2$ 20 mM KCl, 1 mM β-mercaptoethanol. Upper graph represents the raw ITC data in which the incremental heat changes are shown microcalories per second ($\mu cal.sec^{-1}$) plotted against time in minutes over the progression of the experiment, lower graph shows the normalised integration data in terms of kilocalories per mole ($kcal.mol^{-1}$) of injectant plotted against molar ratio of the added titrant with the curve fitted to single-site mode. The relationship between the two x-axes allows for the positioning of the integrated area for each peak directly below the associated peak in the raw data. The negative peaks correspond to addition of an aliquot of ligand, and the calorimetric output indicated that an exothermic process was occurring in the cell.

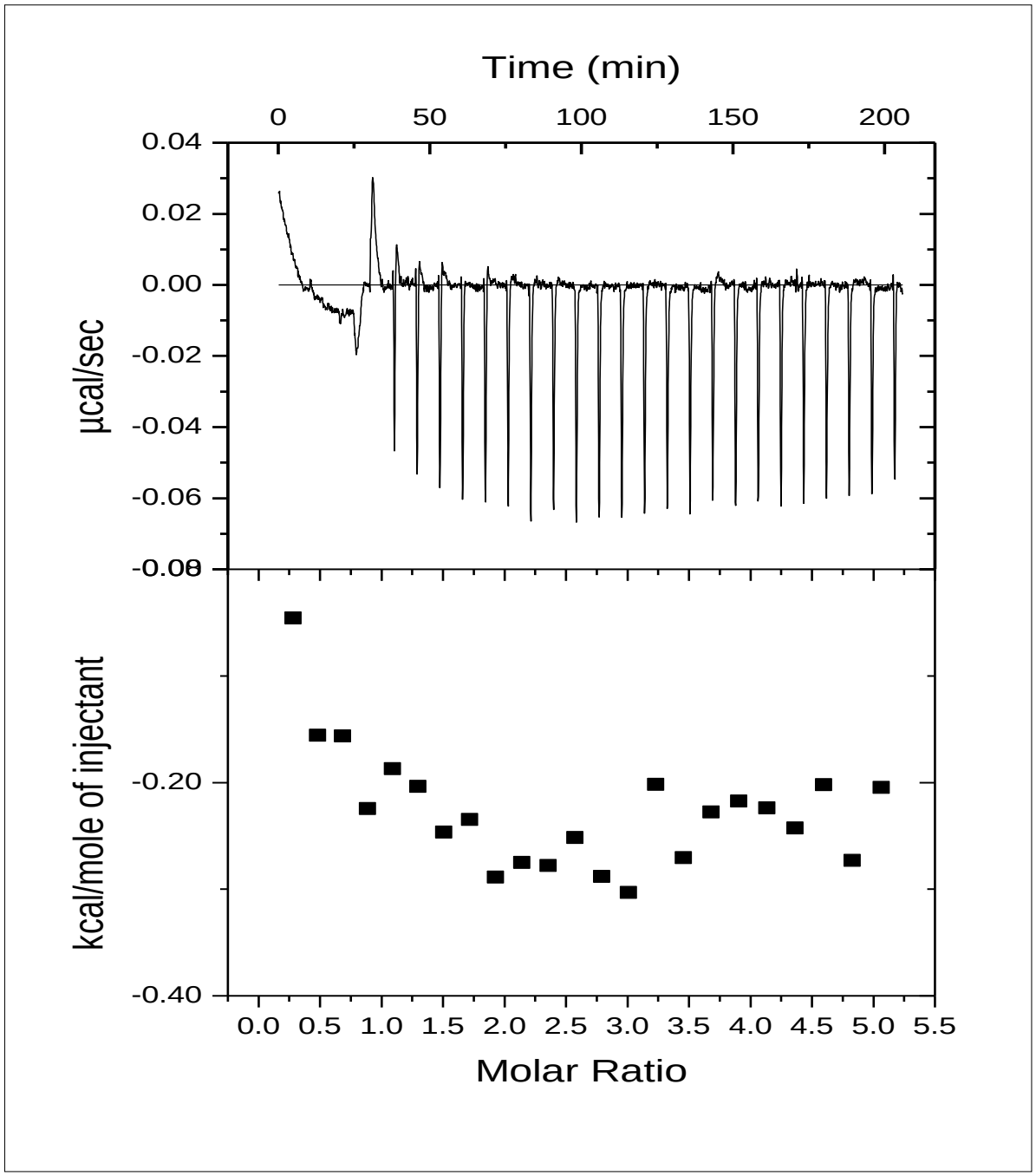


Figure C.6: NBL does not interact with Hsp90β. Raw and fitted data between nor-β-lapachona (NBL) (700 µM) binding to Hsp90β (30 µM) equilibrated to 25 °C in 50 mM Tris (pH7.4), 6 mM $MgCl_2$ 20 mM KCl, 1 mM β-mercaptoethanol. Upper graph represents the raw ITC data in which the incremental heat changes are shown microcalories per second ($\mu cal.sec^{-1}$) plotted against time in minutes over the progression of the experiment, lower graph shows the shows normalised integration data in terms of kilocalories per mole ($kcal.mol^{-1}$) of injectant plotted against molar ratio of the added titrant with the curve fitted to single-site mode. The relationship between the two x-axes allows for the positioning of the integrated area for each peak directly below the associated peak in the raw data. The negative peaks correspond to addition of an aliquot of ligand, and the calorimetric output indicated that an exothermic process was occurring in the cell.

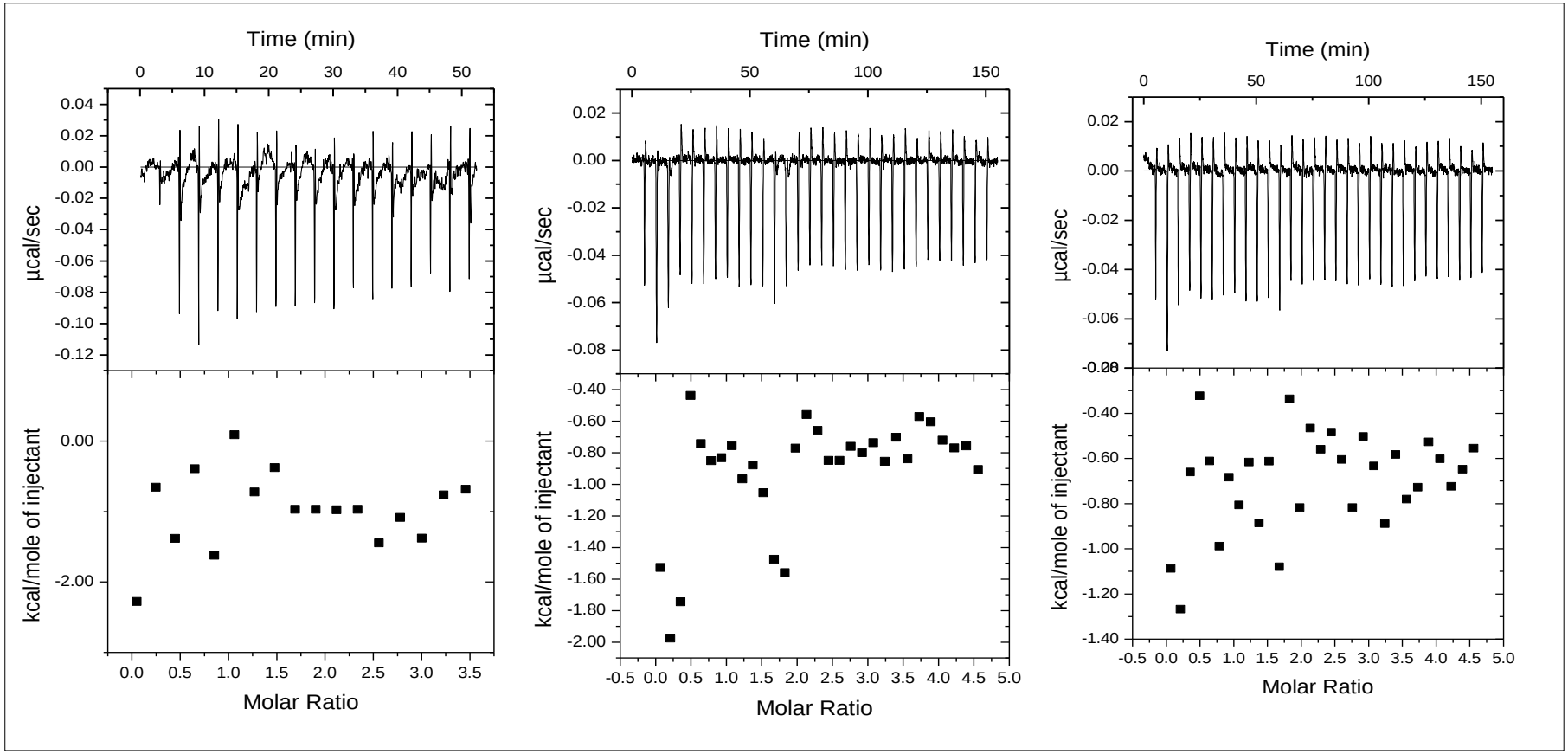


Figure C.7: Optimisation of SQA binding to Hsp90β. Sargaquinoic acid (SQA) did not interact with Hsp90β at the lower protein concentrations of (A) 5 µM (B) 10 µM and C (20 µM). Raw and fitted data of SQA binding data to Hsp90β equilibrated to 25 °C in 50 mM Tris (pH7.4), 6 mM $MgCl_2$ 20 mM KCl, 1 mM β-mercaptoethanol. Upper graph represents the raw ITC data in which the incremental heat changes are shown microcalories per second ($\mu cal.sec^{-1}$) plotted against time in minutes over the progression of the experiment, lower graph shows the shows normalised integration data in terms of kilocalories per mole ($kcal.mol^{-1}$) of injectant plotted against molar ratio of the added titrant with the curve fitted to single-site mode. The relationship between the two x-axes allows for the positioning of the integrated area for each peak directly below the associated peak in the raw data. The negative peaks correspond to addition of an aliquot of ligand, and the calorimetric output indicated that an exothermic process was occurring in the cell.

Determination of the binding of SQA to Hsp90β by STD NMR

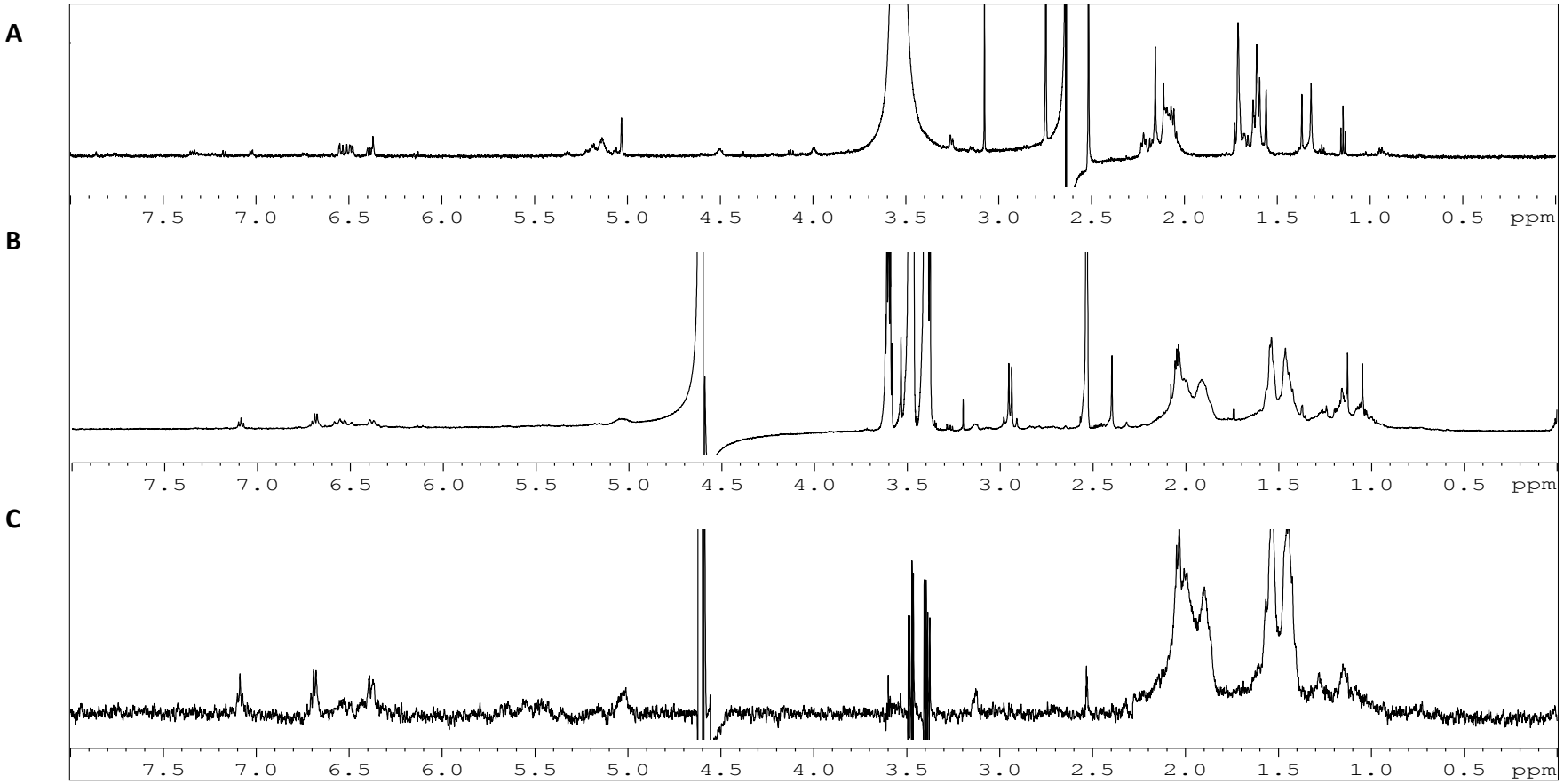


Figure C.8: STD NMR of Hsp90β with sargaquinoic acid. (A) Sargaquinoic acid (SQA) alone, (B) STD reference SQA + Hsp90, (C) STD. Signal at 5.08ppm present in the STD spectrum is from 8-CH_2 of SQA and indicates a protein-ligand interaction. It is difficult to observe other signals (which must be present) owing to spectral crowding and the difference in conditions (solvent, buffer) between the raw ligand and the ligand with protein. The slight differences cause deviations in chemical shift.