UNIVERSITY OF ZULULAND

Investigation of Interaction between Heat shock protein 70.14 (Hsp 70.14) and RBBP6 RING Finger Domain

Dissertation submitted in partial fulfillment of the requirement for the degree

MASTERS IN BIOCHEMISTRY

Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, KwaDlangezwa, KwaZulu Natal, South Africa.

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DECLARATION

I, **Paul Chukwudi Ikwegbue** (Student No: **201329989**) declare that "**Investigation of the putative interaction between Hsp70.14 and RING finger domain of RBBP6**" is my own unaided work and has not been submitted for any degree or examination in any other university. Additionally, I have acknowledged and quoted all the sources used and correctly referenced them.

Candidate

Paul Chukwudi Ikwegbue

Abstract

Despite improvement in cancer treatment modalities, cancer remains one of the leading causes of death worldwide. Studies show that in 2012, about 14.1 million new cancer cases resulting in 8.2 million deaths were reported worldwide according to the WHO. Over the years, several unsuccessful attempts to completely eradicate cancer diseases from human life has led to variety application of therapies (such as immunotherapy, targeted therapy, antibody based therapy, chemotherapy, surgery) aimed at finding treatment and radical cure for this disease. Although, there have been major improvements in the application of these therapies, however, challenges of non-specificity, as well as several side effects, resulting from killing of normal cells by chemotherapeutic agents are still widespread. This has led to urgent and continuous need for alternative therapies aimed at finding alternative drugs, or improving the current available treatment modalities. In the same vein, researchers have exploited therapeutic potential of heat shock proteins (Hsps) as an alternative intervention against cancer because of their ever-presence in the pathology of different facets of cancer development. Studies have shown that tumour cells require Hsp chaperonin activities for survival and proliferation than normal cells, because most oncoproteins in invading cells are often unfolded, therefore require chaperonage protection of Hsps for survival. Hsp70.14, alternatively known as HSP70L1, is an Hsp70 variant that lacks the c-terminal domain but contains substrate binding domain and the ATPase domain. The postulated interaction of this protein with the RING finger domain of RBBP6, suggested their role in protein quality control system and chaperone-mediated ubiquitination. Therefore, this study aimed to recombinantly express, purify, characterize and investigate the interaction between Hsp70.14 and RING finger domain of RBBP6. *In silico* analysis using homology modelling and subsequent validation by Ramachandran plot revealed that modelled structures were of good quality. Molecular docking studies show a strong interaction between the two proteins with a high binding score (K_D) of 16038. The recombinant proteins were successfully expressed in BL21 cells and subsequently purified

using GSH-agarose and on cobalt-recharged Nikel affinity chromatography columns. Structural characterization of the proteins using far UV, CD spectroscopy, tryptophan fluorescence and SE-HPLC showed that proteins were properly folded, predominantly α-helices, absorbs light at 280 nm and contain no impurities or protein aggregates. ANS binding studies reveals more hydrophobic patches in Hsp70.14 than RING and complex. The binding results obtained from this study using molecular docking, size exclusion high pressure liquid chromatography and ANS binding assay revealed there was indeed an interaction between Hsp70.14 and RING finger domain of RBBP6, which holds a major therapeutic potential towards the development of alternative cancer drugs.

DEDICATION

This desertation is dedicated to the Almighy God; He alone deserves all the glory. Also, to my sponsor: Dr Joseph Nnaemeka Ikwegbue.

ACKNOWLEDGEMENT

I would like to express my unalloyed gratitude to my main supervisor Prof Abidemi Paul Kappo for his wonderful support, advice, motivation and guidance throughout the course of this project. I would also like to express my heartfelt gratitude to my noble co-supervisor Dr Ikechukwu Achilonu for his massive contributions, advice and guidance throughout the course of my stay at Wits University, your efforts are highly appreciated.

My profound gratitude to these bioinformatics Gurus: Oga Rapheal Taiwo Aruleba and Baba Tayo Alex Adekiya, for their assistance with the bioinformatics studies. Also, I would like to extend my gratitude to Mr Sbonelo Khanyile, Philisiwe Molefe and all the BSB group members at University of Zululand, for their immensive contributions. My gratitude goes to members of Protein-Structure Function Research Unit, Wits, most especially Blessing Oyiogu, Thulo Monare, Roland Wort, for making my stay at Wits a successful story.

Lastly, my deepest gratitude to my brother and guidiance Dr Joseph Nnaemeka Ikwegbue and his wife Mrs Genevive Ifeanyinwa Ikwegbue and all the entire Ikwegbue Ejor family, most especially my mother, Mrs Elizebirth Ikwegbue and my brothers: Sunday Ikwegbue, Ike Ikwegbue and Aloysius Ikwegbue, your contributions in my life are well appreciated. My undiluted gratitude goes to Gloria Ezinne Chukwu (Doctor in making) for her support.

Finally, I thank the Almighty Chukwu for leading me through all the obstacles and turbulent phases of my life. Also, for the good health and gift of life and existence bestowed upon me, all glory and honour be unto your holy name.

LIST OF ABBREVIATION

5-HT	5-hydroxytryptamine
AIDS	Acquired Immune Deficiency Syndrome
AMPs	Antimicrobial Peptides
APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
cAMP	Cyclic adenosine Monophosphate
DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ExPASY	Expert Protein Analysis System
FTIR	Fourier Transform Infrared Spectroscopy
GDP	Guanosine-5'-diphosphate
GTP	Guanosine Triphosphate
GRAVY	Grand Average of Hydropathicity
HIV	Human Immunodeficiency Virus
IPTG	Isopropyl β-D-1-thiogalactopyranoside

ITC	Isothermal Titration Calorimetry
NCBI	National Centre for Biotechnology Information
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
Phyre2	Protein Homology/analogy Recognition Engine 2
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SPR	Surface Plasmon Resonance
SSA	Sub-Saharan Africa
STDs	Sexually Transmitted Diseases
TB	Tuberculosis
WHO	World Health Organization

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Cancer is a group of diseases that cause an increase in abnormal cell growth and have the ability to proliferate to other parts of the body. Cancer has been named as one of the leading causes of death and disease worldwide. Out of the 14.1 million new cases that occurred in the year 2012 alone, 7.4 million cases (53%) occurred in males while 47% (6.7 million) of the cases were diagnosed in females (Ferlay *et al.*, 2013). Hsp70.14 like other classes of Hsp70 has been hypothesized to be up-regulated in response to stress signals like in the onset of tumours. The up-regulation of Hsp70.14 in tumours has been linked to its interaction with cancer genes like the RING finger domain of RBBP6; hence it would be interesting to study the molecular basis of these proteins.

Heat shock proteins (Hsps) which are primarily grouped into two according to their molecular weight are molecular chaperones released by cells in response to stressful signals such as heat: cytokines, acute or chronic infection, intense exercise (Asea, 2003), metabolic disruption, nutrient deprivation, the presence of oxygen radicals and viral infection (Searle *et al.*, 1993). Hsp70, which is one of the high molecular weight chaperones is primarily known for its roles in protein folding, translocation of organellar across membranes, as well as facilitating the degradation of misfolded proteins through chaperone mediated protein quality control, thereby playing a vital role in maintaining protein cellular homeostasis (Mayer and Bukau, 2005; Shiber and Ravid, 2014).

Retinoblastoma binding protein 6 (RBBP6), through its conserved cysteine residue, is known to interact with many biologically important proteins including: tumour suppressor proteins (p53 and RB), as well as Yb-1 through the ubiquitin-mediated pathway (Chibi *et al.,* 2008; Kappo *et al.,* 2012). This interaction together with RBBP6's up-regulation in esophageal cancer, overexpression in lung cancer and human carcinomas, suggests its function in oncogenesis, and presents it as a good therapeutic candidate for cancer drugs (Ntwasa, 2008; Mbita, et al., 2011; Motadi *et al.,* 2011; Yoshitake *et al.,* 2004). RING finger domains are small cysteine-rich domains which are known to adapt "cross brace" topology by coordinating two zinc ions and are classified according to their zinc ion coordinating pattern (Freemont, 2000; Kappo *et al.,* 2012). RING finger domains which are grouped based on the length of their secondary structural elements, zinc ion coordination, and sequence homology, have been identified to adapt a number of common motifs, including C3HC4, C3HHC3, C2H2C4 and C4C4, with the C4C4 being the most common (Dominguez *et al.,* 2004; Kappo *et al.,* 2012). This study focuses on expressing, purifying and characterizing Hsp70.14 and ascertaining its association with the RING finger domain of RBBP6, as well as their role in the protein quality control system as previously suggested by Kappo *et al.,* 2012.

1.2 Literature Review

1.2.1 Cancer

Cancer remains one of the leading causes of death worldwide, in both developed and developing countries (Torre *et al.,* 2012). Several reports suggest that this could be due to the increasing prevalence of associated factors such as obesity, smoking, unhealthy life style and diet, alcohol and lack of exercise. Studies have statistically shown that in 2012 about 14.1 million new cancer cases and a resulting 8.2 million deaths were reported worldwide (WHO

GloboCan, 2015). Though cancer cases in South Africa are poorly documented due to the lack of nationwide surveillance in the country, studies have reported prostate cancer as the most common among men and boys and breast cancer among women and girls (Singh *et al.*, 2015). Lung and breast cancer have been reported as the leading causes of death worldwide in males and females respectively (WHO GloboCan, 2015).

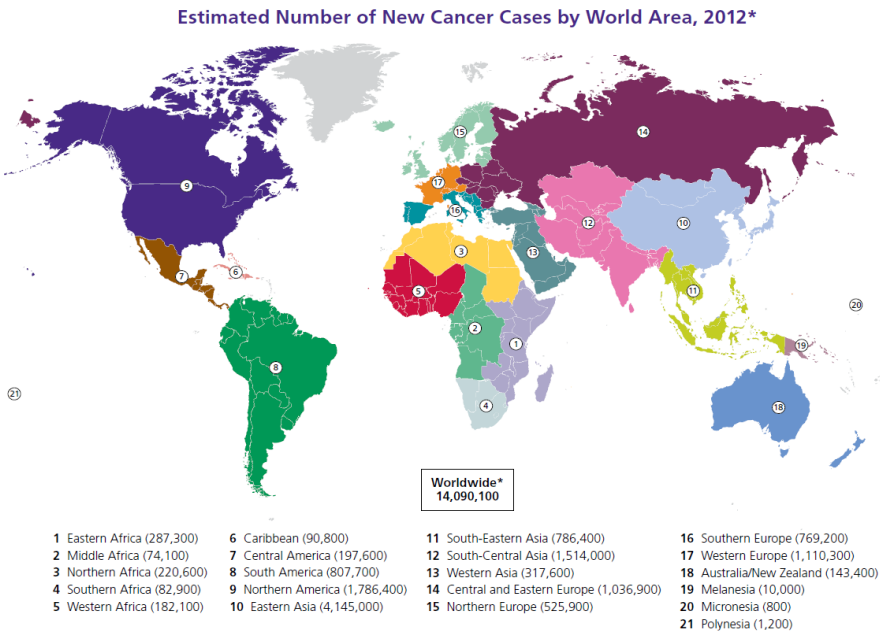

Figure 1.1: Chart estimating the number of new cancer cases in different regions of the world by the year 2012. Taken from WHO, GloboCan (2015).

Various researchers have attempted over the years to explore ways of combating the effects and spread of cancer with some but still unsatisfactory success. Understanding structural and functional proteins such as heat shock protein 70.14 and its interactions with RBBP6 RING finger that plays biologically important roles in the formation of tumours would be an interesting aspect in cancer drug design, as well as in the early detection of tumours.

1.2.2 Heat shock proteins (Hsps)

Heat shock proteins (Hsps) are proteins usually produced by cells to protect themselves from any form of environmental stress both internal and external; and such stressors can be heat, cytokines, acute or chronic infection, intense exercise, metabolic disruption, nutrient deprivation, the presence of oxygen radicals and viral infection (Asea, 2003; Searle *et al.*, 1993). These responses enable cellular protection against protein denaturation and possibly degradation of misfolded proteins, which may in turn result in protein aggregation and

apoptosis (Sharma *et al.,* 2012). Though heat shock proteins are mostly produced upon thermal induction, some Hsps are constitutively expressed and are termed heat shock cognates (Searle *et al.,* 1993; Zügel and Kaufmann, 1999); these are crucial for cell survival during unfavorable conditions (Sung and MacRae, 2011).

Hsps are primarily grouped into two families according to their molecular weight, amino acid composition and the specific functions they perform; these are known as the high-molecular-weight Hsps and the small molecular weight Hsps (Garbuz *et al.,* 2011; Lanneau *et al.,* 2010). The high molecular ones include: Hsp90, Hsp70, and Hsp60 (Lanneau *et al.,* 2010). Binding and folding of nascent proteins through their ATP-dependent allosteric organization is the main function of these families, though they differ in molecular structure and how they operate (Yik *et al.,* 2013). The high molecular weight Hsps are ATP-dependent chaperones, whereas the small molecular weight Hsps are ATP independent (Lanneau *et al.,* 2010). The Hsps interact with the 26S proteasome degradation system in order to ensure that protein refolding and degradation is tightly controlled; this interaction certifies that protein quality-control mechanisms are maintained at all times (Lanneau *et al.,* 2010; Shiber and Ravid, 2014). The failure of cells to maintain protein homeostasis due to environmental stressors that disrupt the protein quality-control (PQC) system results in improper folding and aggregation of proteins which in turn results in apoptosis. Such protein conformational changes can cause conformational diseases such as Alzheimer's, Parkinson's, and Huntington's, which highlights the importance of the protein quality-control (PQC) system that is mediated by Hsps (Shiber and Ravid, 2014). This protein quality-control mechanism enables cell survival during unfavorable or harsh conditions.

Since the discovery of heat shock proteins in 1962 by Ritossa in the salivary glands of the *Drosophila* larvae, many studies have demonstrated the important functions of heat inducible proteins in response to various stressful signals including human cancer (Zhang *et al.,* 2007) and cystic fibrosis, thus several research papers have been published on heat inducible proteins (Daugaard *et al.,* 2007; Singh *et al.,* 2008). Initially, it was thought that heat shock proteins are only produced in response to heat stress, however, subsequent studies have shown that heat induced proteins can also be produced in response to many signals such as degradation of improperly folded proteins by the U-box proteasome-system, protecting the aggregation of misfolded proteins by enhancing their recognition to U-box ligation, and shuttling ubiquititylated substances to proteasome degradation (Shiber and Ravid, 2014).

1.2.2.1 Heat shock protein 70 (Hsp70)

Hsp70s are molecular chaperones that are primarily involved in the folding of nascent proteins and degradation of improperly or misfolded proteins (Lanneau *at el.,* 2010). Hsp70 is the most studied and most conserved protein in evolution in all cells (Searle *et al.,* 1993; Shiber and Ravid, 2014; Sung and Mac Rae, 2013). Hsp70 which is encoded by the HSP-1A gene is the most produced heat shock protein upon thermal induction (Sharma *et al.,* 2012). Its expression can be used as a stress indicator upon adaptation during unfavorable environmental conditions (Patir and Upadhyay, 2010).

Hsp70 which weighs 70kDa is one of the high molecular weight Hsps that share almost 50% amino acid similarities with the DnaK homolog of Hsc70 (heat shock cognate 70) that is found in prokaryotic cells (Daugaard *et al.,* 2007; Lanneau *et al.,* 2010). The families of Human Hsp70 have no less than eight unique gene products that are different from each other in amino composition, sub-cellular localization, and expression level; with the exception of

Hsp70-5 and Hsp70-9 which are found in the lumen of the endoplasmic reticulum and mitochondria, respectively; the other six are confined to the nucleus and cytosol where they perform their particular cellular functions (Daugaard *et al.*, 2007).

Hsp70 is known to help a broad spectra of proteins in folding processes into three dimensional structures; these helper functions include: assembling and folding of nascent proteins, refolding of improper or aggregated proteins (Bukua *et al.*, 2003; Hartl and Hayer-Hartl, 2002), regulating the functions of regulatory proteins, and translocation of organellar across membranes (Ryan and Pfanner, 2002). Hsp70 is also involved in housekeeping activities, signal transduction pathways, protein quality control systems and in the chaperone-mediated ubiquitination pathway. Hsp70 has the ability to proofread, identify, and repair the misfolded or improperly folded proteins (Bukua *et al.*, 2003; Mayer and Bukau 2005). Reports suggest that Hsp70 assists approximately 15% of *de novo* folding of all bacterial proteins in relation to its size (Bukua *et al.*, 2000; Hartl and Hayer-Hartl, 2002).

The domain organization of Hsp70 shows three structural and functional domains: an N-terminal 45kDa ATPase region (conserved ATPase), 15kDa peptide-binding domain (substrate binding site), and a C-terminal 10kDa region (Daugaard *et al.*, 2007; MacAry *et al.*, 2004). The N-terminal 44kDa ATPase region which contain 1-358 amino acids and a site for nucleotide binding, is responsible for the ATPase activity of Hsp70, while the substrate binding domain (359-494 amino acids) binds Hsp70 to substrate, and the C-terminal (495-609 amino acids) contains an EEVD-motif for binding co-chaperones and other heat shock protein related families (Daugaard *et al.*, 2007; Sung and MacRae, 2013).

1.2.3 Substrate binding domain of hsp70

Though its functional differences are poorly understood, the substrate binding domain sequence is highly conserved among the hsp70 family and most information related to the structure of this domain is found in prokaryotic DnaK (specifically *E. coli*), a homolog of hsp70 (Mayer and Bukau, 2005). A study showed that substrate binding residues generate a sandwich of 2 helices and 2 β-sheets when the peptide binding domain X-structure was solved at 2.0Â resolution together with a heptameric peptide substrate (Mayer and Bukau, 2005; Zhu *et al.,* 1996). The same study showed that the two β-sheets contain four loops that protrude upwards, and the two α-helices (A and B) are packed against loop $L_{1,2}$ and loop $L_{4,5}$ inside the complex as shown in Figure 1.2 (Zhu *et al.,* 1996).

The β-sheets (1 and 2) and loops ($L_{1,2}$ and $L_{3,4}$) form the cavity of the substrate binding domain of hsp70. The opening and closing of this cavity is controlled through a salt bridge and hydrogen bonding between the outer loops ($L_{3,4}$ and $L_{5,6}$) and helix B, the lid of the cavity (Mayer and Bukua, 2005). The binding of ATP to the DnaK homolog of Hsp70 in *E. coli* actually opens the lid that closes the cavity, resulting in the release of the substrate. Co-crystallization of the substrate domain DnaK with substrate peptide revealed two types of interactions over a stretch of five residues (Zhu *et al.,* 1996).

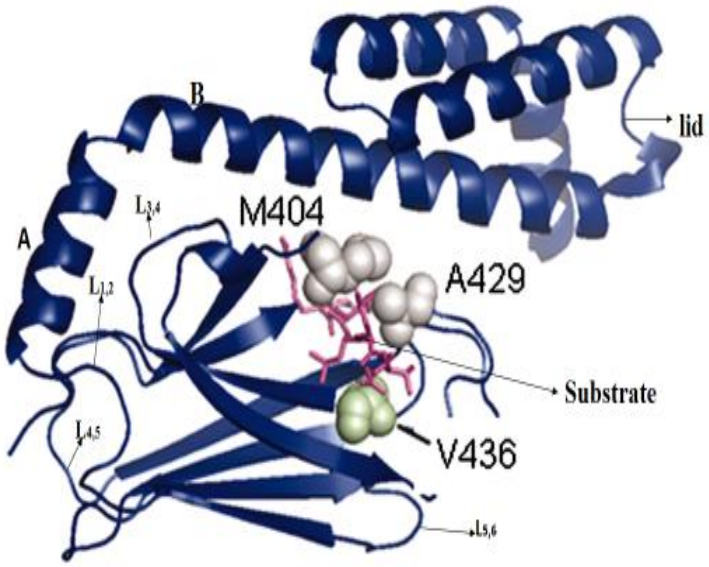

Figure 1.2: **The substrate domain organization of Hsp70.14.** The domain consists of two α-helices, four β-sheets pointing upwards, a substrate binding site, a lid that controls the opening and closing of the cavity, and numerous loops that form the cavity. **Figure taken from Cegielska and Georgopoulos (1989).**

The hydrogen bonding between the backbone of the cavity give rise to two loops ($L_{1,2}$ and $L_{3,4}$) and the substrate mediated backbone which recognizes the conformation of the extended peptide. According to Figure 1.2, the affinity of DnaK towards the hydrophobic peptide residues is shown by the van der Waal interactions of hydrophobic side chains in proximity to the substrate binding cavity, as well as the side chains of the substrate (Zhu *et al.,* 1996). Furthermore, Rüdiger and colleagues (2001) reported that DnaK has different preferences for authentic or inverse peptides even though they have similar composition of amino acids (Rüdiger *et al.,* 2001). This means that DnaK has low affinity for the peptide in authentic sequence than in inversed sequence's peptide, and cannot go back and bind to the other side of the N-terminus of bacterial Hsp70 homolog, DnaK (Mayer and Bukua, 2005; Rüdiger *et al.,* 2001). Therefore, DnaK peptide interactions follow a specific direction of the structural backbone. Recent studies have shown that Hsp70 and HscA specifically bind to the substrate peptide in a reverse direction though the evolution of the Hsp70 family suggests that

peptide binding to the cavity is in a forward orientation manner (Cupp-Vickery *et al.,* 2004; Tapley and Vickery, 2004).

The central hydrophobic pocket, the *α*-helix and the arch enclosing the substrate peptide, are the three structural components of the cavity. These structural components of the cavity of the substrate binding domain are crucial for the functioning of this domain. The affinity of DnaK to bind the protein substrate and peptide reduces by 3 to 7-fold by the removal of the α-helix lid within the complex helix B, thereby increasing the dissociation rates (Buczynski *et al.,* 2001; Mayer *et al.,* 2000; Pellecchia *et al.,* 2000; Slepenkov and Witt, 2002). More so, DnaK's affinity for substrates reduces by 2 to 4-fold by simple substitution of Ala429 for Trp or Met404 for Ala within the arch in ADP state, and this alteration has a greater effect on the rates of dissociation (Mayer *et al.,* 2000; Mayer and Bukua, 2005). The affinity of DnaK to bind the substrate decreases because the alteration of amino acids within the arch results in additional conformational changes in the specificity of DnaK, making it difficult to be recognized by the peptide substrates (Mayer *et al.,* 2000; Mayer and Bukua, 2005). The central hydrophobic pocket contributes to the majority of binding affinity of DnaK to the substrates. Interestingly, studies have shown that the equilibrium dissociation rate of DnaK and the substrates increase by 20-and 40-fold respectively, due to the alteration of Val436 to Phe within the hydrophobic pocket. Studies have also shown that although the rate of dissociations in both ADP and ATP form remain the same over wild-type DnaK, the decrease in the affinity of Dnak to bind substrate could be generally caused by a decrease in the rate of dissociations (Mayer *et al.,* 2000; Mayer and Bukua, 2005). The ability of the substrates to bind the protein's mutant in the same away that it binds wild-type DnaK suggests the possibility of the substrates binding to DnaK in an induced fit-like fashion and high degree of flexibility of hydrophobic pocket, as previously suggested using nuclear magnetic resonance

(Pellecchia *et al.*, 2000; Wang *et al.*, 1998). Studies have shown that the introduction of amino acids with bulky side chains to the hydrophobic pocket impairs the functions of Hsp70 chaperones *in vivo* and *in vitro* despite the high structural flexibility of the hydrophobic pocket, even at high concentrations of DnaK as observed in *luciferase* species grown at 40°C (Mayer *et al.*, 2000).

1.2.3 Heat shock protein 70.14 (Hsp70.14)

Hsp70.14 is a variant of Hsp70 (HSPA1A) that lacks the 10kDa C-terminal domain that is common to the Hsp70 family, but contains an N-terminal ATPase domain and substrate binding domain (Kappo *et al.*, 2012). Although the function of Hsp70.14 is poorly understood, Searle and co-workers (1993) have suggested, based on work done on mitochondrial Hsp70 from *Trypanosoma Cruzi,* that both Hsp70.14 and Hsp70 molecules may perform similar functions within the same subcellular compartment in the trypanosomatid bacterial species (Searle *et al.*, 1993). Even though Hsp70.14 is smaller in size as compared to Hsp70, both possess the same structural and functional similarities. However, Hsp70.14 has been reported to exhibit differences with Hsp70 when interacting with dendritic cells due to the fact that it elevates dendritic cell maturation and stimulation to secrete cytokines and chemokine (Wan *et al.*, 2004).

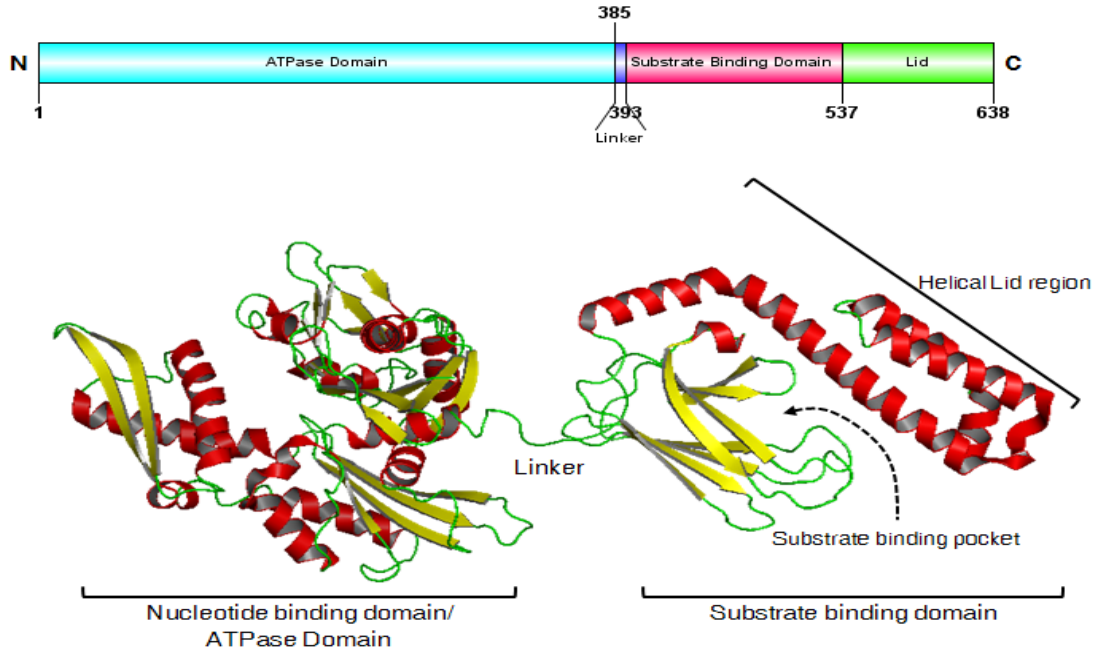

Figure 1.3: structural organization of Hsp70.14: ATPase Domain and Substrate binding domain. The two domains of Hsp70.14 are linked to each other by a connector called "linker" through which the signal passes to the substrate binding domain whenever ATP binds to the ATPase domain. **Taken from Nagarajan *et al.*, (2012).**

1.3 Retinoblastoma binding protein 6 (RBBP6)

Retinoblastoma binding protein 6 (RBBP6) is a multi-domain, multi-functional nuclear protein that weighs about 250kDa and has been identified in all eukaryotes; however, none have been identified in prokaryotes so far (Kappo *et al.*, 2012, Mbita *et al.*, 2012; Pugh *et al.*, 2006). RBBP6 has been reported to interact with tumour suppressor proteins pRb and p53 directly, thereby suggesting its crucial role in tumour formation (Kappo *et al.*, 2012; Mbita *et al.*, 2012). This interaction has been reported to enhance p53 ubiquitination and degradation through the MDM2-mediated ubiquitination pathway since RBBP6 promotes the activity of MDM2 which is the major cellular repressor of p53 (Li *et al.*, 2007; Ntwasa, 2008). SNAMA, a homologue of RBBP6 found in *Drosophila*, has been characterized to perform an important function in embryo development and apoptosis though it lacks p53 binding ability (Mather *et al.*, 2005). Another homologue, Mpe1, which is found in yeast, forms part of the

polyadenylation complex and is known to play a role in pre-mRNA processing (Vo *et al.*, 2001). RBBP6, like other proteins, that contains the SR domain which interacts with nuclear antigens-SmG in the nuclear speckles. This interaction is additional evidence that suggests its function in mRNA-splicing (Simons *et al.*, 1997).

RBBP6, which is mapped to the chromosome 16p11.2-p12 encoding three protein products in humans, was reported to have protein degradation function because of its conserved N-terminal RING finger domain (Motadi *et al.*, 2011; Pugh *et al.*, 2006; Yoshitake *et al.*, 2004). Work done by Pugh and colleagues (2006) demonstrated that the presence of the conserved Domain With No Name (DWNN) suggests that RBBP6 may act as a novel ubiquitin-like modifier of many proteins through the ubiquitin-mediated pathway (Pugh *et al.*, 2006). RBBP6 is involved in a variety of cellular functions, including mRNA metabolism (Shi *et al*., 2009; Vo *et al*., 2001; Witte and Scott, 1997), control of the cell division cycle (Gao and Scott, 2002; Scott *et al.*, 2003), oncogenesis (Yoshitake, 2004), and progression of cancer (Li *et al.*, 2007).

RBBP6 has been implicated in invasive esophageal cancer, suggesting its possible function in the promotion of tumour cell proliferation as well as low rate of cancer patient survival due to the over-expression of P2P-R (Proliferation Potential-Related Protein) in cancer cells (Yoshitake *et al.*, 2004). P2P-R is a homolog of RBBP6 that has been reported in many studies to interact with the p53-Hdm2 complex and enhance degradation of p53 through Hdm2-mediated degradation, though its exact effects on p53-Hdm2 complex is poorly understood (Motadi *et al.*, 2011; Yoshitake *et al.*, 2004). This observation presents RBBP6 as a good remedy for immunotherapy against diseases (Yoshitake *et al.*, 2004). RBBP6 through its number of conserved domains has been shown to be involved in protein degradation,

programed cell death and cell cycle control (Kappo *et al.*, 2012; Ntwasa, 2008; Pugh *et al.*, 2006). The alteration of tumour suppressor proteins normally results in tumour formation which will eventually lead to cancer, since RBBP6 has been reported to regulate the level of tumour suppressor proteins p53 and Rb. This interaction could be targeted for anti-cancer drug design (Ntwasa, 2008).

The structure alignment of RBBP6 reveals that RBBP6 consists of an N-terminal ubiquitin-like domain (DWNN), Zinc knuckle, SR domain, Rb binding domain, p53 domain and the RING finger-like domain (Pugh *et al.*, 2006).

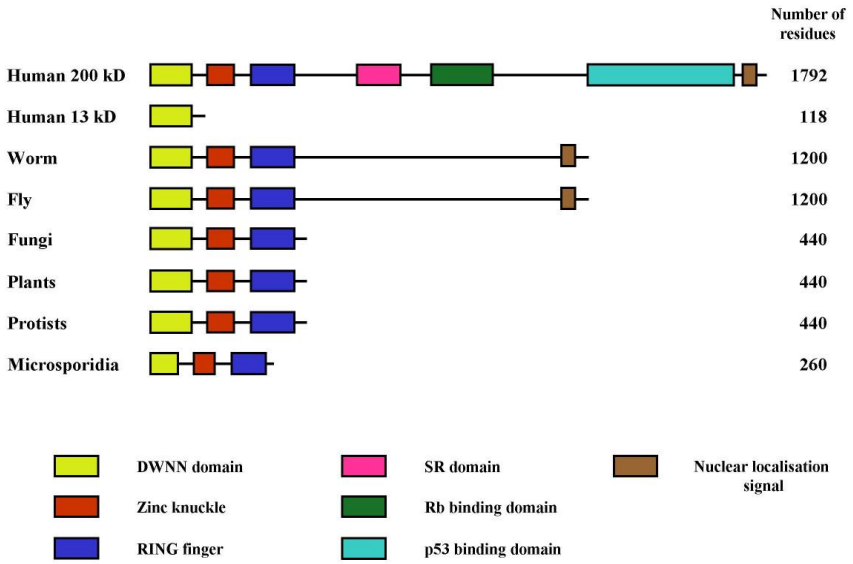

Figure 1. 4: The domain structure of the RBBP6 family of proteins. RBBP6 homologues containing a DWNN domain, a zinc knuckle and a RING finger are found in all complete eukaryotic genomes analyzed to date, including the single celled parasite *E. cuniculi*, in which it is very much reduced in size. In vertebrates and insects, the protein includes a long C-terminal extension containing p53 and Rb-interaction domains in human and mouse. A short form consisting of the DWNN domain and a poorly conserved C-terminal tail is also found in vertebrates. **Figure taken from Pugh** *et al.*, 2006.

1.3.1 Domain with no name (DWNN)

DWNN, which was discovered while screening the components of the genetic novel antigen through MHC class I (major histocompatibility), is the N-terminal domain of RBPP6 which has been reported to act as "novel ubiquitin-like modify" of other proteins (George, 1995; Pugh *et al.,* 2006). The functions of DWNN of the RBBP6 protein in the ubiquitination pathway are largely unknown; however, literatures have reported that it shares almost 22% similarity folds with ubiquitin. These structural fold similarities suggest its function in cell cycle control, programmed cell death induced by camptothecin, as well as regulating cellular protein turn-over (Gao *et al.,* 2002; Gao and Scott, 2003; Pretorius 2007). RBBP6, through its conserved cysteine residues, is known to initiate ubiquitination of Yb-1 protein by binding and blocking its transactivational activity, thereby decreasing its cellular level (Chibi *et al.,* 2008). Mbita *et al,* (2011), in support of work done by Li and colleagues (2007), suggest that the ability of the RBBP6 protein to regulate the cellular levels of tumour suppressor proteins (pRb and p53) is through its conserved RING domain in an ubiquitin-dependent manner (Li *et al.,* 2007; Mbita *et al.,* 2011;). Li and co-workers (2007) reported the involvement of RBBP6 in regulating the cellular level of p53 in ubiquitin-mediated degradation to be anti-apoptotic. However, work by Chibi and colleagues (2008) a year later contradicted this observation, suggesting a pro-apoptotic function of RBBP6 in the degradation of Yb-1, an anti-apoptotic protein that enables cell survival (Chibi *et al.,* 2008). However, more research is needed in this area to validate these claims.

1.3.2 RING finger domain

RING (Really Interesting New Gene) finger domains are small cysteine-rich domains of approximately 70 residues in length that coordinate two zinc ions in a "cross braced" fashion. The Zn^{2+} are coordinated by four pairs of cysteine or histidine residues, meaning that both Zn^{2+} ions are coordinated by the first and third, as well as the second and fourth cysteine or histidine respectively (Kappo *et al.,* 2012). RING fingers, which are folded independently with the aid of zinc ions, are grouped according to the pattern of the coordinating zinc ions. The RING finger motif can be written as a series of conserved cysteine and histidine residues: Cys-$X_{(2)}$-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys-$X_{(2)}$-Cys-$X_{(4-48)}$-Cys-$X_{(2)}$-Cys, where X represents any amino acid. The sequence was found in the human gene RING1 which is located closer to the histocompatibility region on chromosome 6 (Freemont, 2000; Kappo *et al.,* 2012). A number of common motifs have been identified, including C3HC4, C3HHC3, C2H2C4 and C4C4, with the C4C4 being the most common (Dominguez *et al.,* 2004; Kappo *et al.,* 2012). RING finger domains are grouped based on the length of their secondary structural elements, zinc ion coordination, and sequence homology. The RBBP6-cysteine-rich domain is grouped as a RING finger domain because of the conserved cysteine residues but the presence of conserved hydrophobic residues suggests an alternative classification as U-box family (Kappo *et al.,* 2012). RING finger domain from RBBP6, which adapts C4C4 motif in coordinating two zinc ions due to the presence of conserved eight cysteine-rich residues, consists of a central α-helix and the anti-parallel β-sheets connected by the long zinc ion stabilizing loops (Dominquez *et al.,* 2004; Kappo *et al.,* 2014). Similar folds are found in the U-box family, though they coordinate using salt bridges and hydrogen bonds to stabilize themselves rather than zinc ions (Ohi *et al.,* 2003). MDM2 RING finger belongs to a novel C2H2C4 which has been shown to be different from other classical RING finger domains but

well adapted to perform E3 ubiquitin ligase on p53 by controlling its level, activity, and stability (Kostic *et al.,* 2006). The RING finger domain is found in proteins that have been implicated in the following cellular processes such as apoptosis, viral infections, oncogenesis and ubiquitination (Borden, 2000). Other domains, other than the RING finger domain have also been demonstrated to adapt cross-brace topology in coordinating zinc ion-like RING fingers and these domains include U-box domains, PHD, FYVE and LIM, which coordinate zinc ions in zinc finger topology instead of cross brace fashion (Aasland *et al.,* 1995; Capili *et al.,* 2001; Stenmark *et al.,* 2002).

1.3.3 Other RING finger related proteins

1.3.3.1 U-box family

The U-box family is a group proteins, which possess similar folds and structural similarities to the RING finger, but coordinate salt bridges and hydrogen bonds instead of zinc ions that are more common to RING finger domains (Ohi *et al.,* 2003). The primary function of these two proteins is to recruit protein to the E2 ligase and then transfer them to E3 ubiquitin for proteasomal degradation. This plays an important role in the protein quality control mediated pathway. Furthermore, U-box was originally considered an E4 ligase or elongation factor because it does not initiate *de novo* synthesis of ubiquitination chain but catalyses the elongation of already existing polyubiquitination chains (Cyr *et al.,* 2002).

1.3.3.2 PHD (Plant Homeodain)

The plant homeodain, also called leukemia associated protein (LAP) which contains about 60 amino acid residues, is a small domain that is found in many eukaryotic cells and is localised in the nucleus. PHD domains are known to associate with the maintenance of chromatin

structures and protein-protein interactions. PHD domain which was first discovered in homeodomain protein, is capable of E3 ligase activity on cellular kinase MEKK1 and is also involved in the development of plant roots where it got its name (Bienz, 2006; Bottomley *et al.,* 2005; Capili *et al.,* 2001; Dul *et al.,* 2007). PHD adapts C4HC3 topology in cordinating zinc ions (Dominguez *et al.,* 2004).

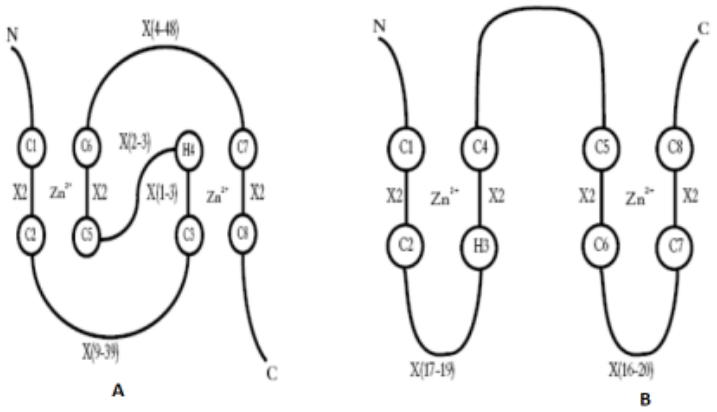

Figure 1.5: Representations of the zinc coordinating pattern of RING finger domain (A) and LIM (B) respectively. RING coordinates the zinc ion in a cross topology, meaning that 1,3 His/Cyst and 2,4 His/Cys coordinate first and second zinc ion respectively. Figure taken from Borden and Freemont, 1996.

1.4 The Ubiquitination Pathway and Proteasomal Degradation

The ubiquitin proteasome system is highly regulated to quickly and efficiently remove specifically targeted proteins from cells. This function is accomplished through the action of numerous important enzymes that tag target proteins to the 26S proteasome complex for degradation. The enzymes involved include ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2, ubiquitin ligating enzyme E3 and sometimes ubiquitin extension factor E4 (Koegl *et al.,* 1999; Pant and Loranzo, 2014). Ubiquitin which contains about 76 amino acids is present in eukaryotic cells but absent in all prokaryotic cells including bacteria

and archaea (Sorokin *et al.*, 2009). Ubiquitin weighs 8kDa contains di-glycine at position 76 and this feature enables it to conjugate side chains of lysine residues found on target proteins in a process known as ubiquitination or mono-ubiquitination.

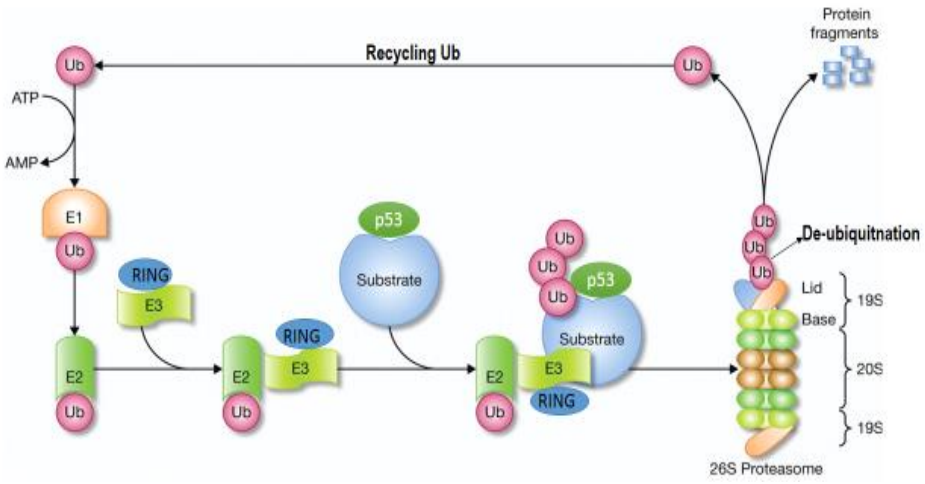

Figure 1. 6: Schematic representation of the ubiquitination pathway system. E1 activating enzyme activates ubiquitin in an ATP-dependent reaction, and transfers it to E2 conjugating enzyme. E2 then transfers the ubiquitin to E3-ubiquitin ligase, which facilitate the transfer of ubiquitin to the substrate. The polyubiquitin chain is formed by repeated linkage of ubiquitin moieties. This chain is then recognized by 26S proteasome for degradation. The ubiquitin molecules are recycled by DUPs enzymes while the substrate is degraded. Figure taken from Rahimi, 2012**.**

Typically, Lysine 48 and 63 link poly-ubiquitin chains to form polyubiquitination chains. Ubiquitination has been shown to give different results in response to proteasome degradation signals such as sorting into specific cellular compartments and endocytosis of receptors (Woelk *et al.,* 2007). Ubiquitination can be reversed in the presence of iso-peptidase called de-ubiquitination enzymes or DUBs in the cells. These enzymes control the rate of ubiquitination by removing ubiquitin tags from proteins before degradation in the proteasome, thereby facilitating the recycling of ubiquitin molecules (Woelk *et al.*, 2007). The main function of the E3 ligase is to recognize the substrate and bind it to the E2 conjugating ubiquitin enzyme, thus containing both E2 binding and substrate binding domains. In contrast, E1 only recognizes E2 and ubiquitin and is therefore expected to have

fewer numbers per cell. More so, E2 recognizes a huge number of E3s and little of E1 and is therefore expected to have moderate number per cell in humans (Metzger *et al.,* 2014; van Wijk and Timmers, 2010). The binding of E2 to ubiquitin plays an important role in the RING finger domain which acts as an adapter molecule that binds substrate and E2 together to facilitate the transfer of ubiquitin to the substrate (Metzer *et al.,* 2014).

1.4.1 Hsp70 in Protein Quality Control and Ubiquitin-mediated Pathway

The Hsp70 molecular chaperone is known for its diverse functions in assisting the *de novo* nascent protein folding, refolding of misfolded proteins and proteasome degradation of proteins that cannot be folded. Hsp70 accomplishes these functions through its ATPase domain that provides needed energy as well as substrate binding, which it uses to bind to the exposed hydrophobic core of the substrates or targeted protein. Repetitive binding and dissociation of Hsp70 with substrates, subsequent hydrolysis of ATP leads to the release of ADP and inorganic phosphate, thus enabling the folding of the protein to continue until biological function of the particular protein is obtained. However, if the protein cannot be folded, this protein together with other misfolded proteins, are targeted for proteasomal degradation (Shiber and Ravid, 2014). Hsp40 together with nuclear exchanger factor (NEF) and sometimes Hsp90 and Hsp60, are co-chaperones that assist Hsp70 in protein folding and in degradation of improperly folded proteins. The function of these co-chaperones of Hsp70 especially Hsp40, is to hydrolyse ATP to ADP so as to energize protein folding and degradation reactions respectively (Hartl *et al.,* 2011).

Although the mechanism by which E3 ligase enzymes and hsp70 interact with the protein quality control (PQC) system during protein degradation is poorly understood, the nature of interaction between Hsp70 and E3 ligase are suggested to be highly specific, since most E3

ligase enzymes in PQC work hand-in-hand with Hsp70 during protein proteolysis (Kriegenburg *et al.,* 2012). Recognition of misfolded protein by E3 ligase is the first step of the ubiquitin-mediated pathway (Ravid and Hochstrasser, 2008; Shiber and Ravid, 2014).

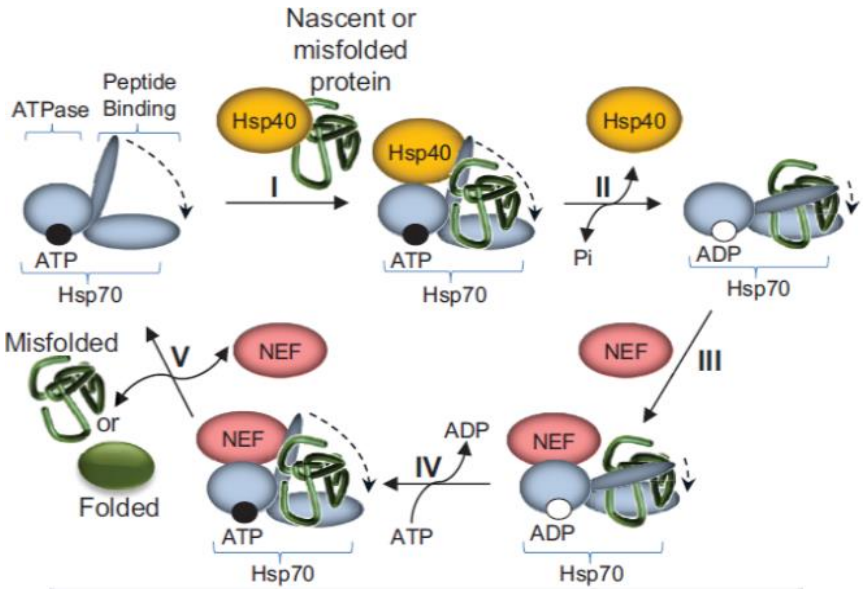

Figure 1.7: The schematic representation of Hsp70.14 roles in protein and ubiquitin-mediated degradation of misfolded proteins. In step I, Hsp70 identifies the hydrophobic side of misfolded protein and bind to it and keep it in soluble form. Step II, Hsp40 binds to the ATPase causing hydrolysis of ATP, release of inorganic sulphate (Pi), as well as conformational change at substrate binding domain (ATP to ADP state). In step III, nuclear exchange factor (NEF) binds to the complex reducing the affinity of ADP-bound to the complex. In step IV, ATP binds again to the complex causing the release ADP and regaining of ATP state. In step V, a protein is either folded or misfolded protein will be recognized by Hsp70 so the process will continue over and over until correct conformation is obtained**.** Diagram taken from Shiber and Ravid, 2014.

Before the ubiquitination pathway actually begins, Hsp70 recognizes and binds to the hydrophobic core of the misfolded protein, solubilizes it, and prevents it from forming insoluble aggregates, since the targeted protein must be in non-aggregate and soluble form during protein folding or degradation. The binding of Hsp70 to the exposed hydrophobic core of the substrate targets it for possible ubiquitination. Hsp70 then facilitates the transfer of the targeted protein to the proteasome complex and at the same time protects the ubiquitin-mediated sequestration of the targeted proteins (Shiber and Ravid, 2014). BiP/Kar2, the yeast

homolog of Hsp70 localized in the lumen of endoplasmic reticulum, has been found to keep secretory proteins in a soluble form, thereby promoting their folding as well as endoplasmic reticulum-associated proteasomal degradation (ERAD) (Nishikawa *et al*., 2005; Plemper *et al.,* 1997). Interestingly, inhibition of BiP/Kar2 activities leads to the aggregation of mutated carboxypeptidase yscY and *pro-α-factor* (non-glycosylated form) under stressful conditions like high temperatures. In addition, during the above mentioned conditions, the functions of endoplasmic reticulum associated-proteasomal degradation are seriously impaired (Nishikawa *et al.,* 2005). This observation suggests that ERAD cannot function properly in the absence of functional BiP/Kar2, thus highlighting their importance in the PQC system (Venkatraman *et al.,* 2004).

Work done by Nakatsukasa and co-workers (2008) showed that *SSA* (a Hsp70 homolog in yeast) is involved in folding and PQC mediated protein degradation, by keeping targeted substrates in a soluble form in various cell compartments such as in the cytosol, nuclear membrane and other membrane-embedded PQC systems (Nakatsukasa *et al.,* 2008). Even though *SSA*-dependent degradation pathways are found in many cell compartments, the major *SSA*-pathways are located in cytosol. These cytosolic *SSA*-dependent degradations are mediated by E3 ligase Ubr1 and ER embedded E3 Doa10 respectively (Huyer *et al.,* 2004; Metzger *et al.,* 2008). The mechanism by which these cells switch from refolding to degradation of misfolded proteins is largely unknown, thus a possible explanation could be exposure of the hydrophobic residues in the misfolded proteins (Shiber and Ravid, 2014).

1.4.2 The role of E3 ligase-RING finger domain in ubiquitination pathway

Generally, the ubiqiutination pathway involves three important enzymes namely: E1 ubiquitin-activating enzyme, E2 ubiquitin conjugating enzyme, E3 ubiquitin ligase, and

sometimes E4 or elongation factor (Koegl *et al.,* 1999). E3 ligase-RING finger plays intermediate role in the attachment of ubiquitin to the substrates as well as the activation of ubiquitin moiety. Basically, it is thought that the specificity of ubiquitin binding to the substrates is mediated by E3 ligase, which interacts with both E3 ubiquitin conjugating enzyme and substrate, thereby, facilitating the transfer of Ubiquitin from E2 enzyme to the substrate. In this way, the E3 ligase binds to the lysine residues of the targeted protein substrate and forms an isopeptide bond before transferring it to the ubiquitin molecule for proteasomal degradation (Chibi, PHD, 2008). There are two major classes of E3 ligase enzymes: the RING finger domain and HECT domain proteins. E3 ligase enzymes are grouped based on their interaction with the substrates or the targeted proteins. According to reports, E3-containing RING finger domains are by far the biggest members of the E3 ligase family and over 600 RING-related proteins are encoded in the human genome (Li *et al.,* 2008). All known RING finger domains that form four molecular structures such as single chain, multi-component, homodimer and heterodimer, associate directly with the E2-ubiquitin conjugating enzyme. The RING finger domain-E3 which is alternatively classified as U-box family due to the presence of hydrophobic residues binds to the substrate's nucleophilic lysine residue and facilitates its transfer to the ubiquitin moiety. Furthermore, additional structural elements in the RING finger domain and U-box could play essential roles in their interactions with substrates. For example, the ability of the RING finger domain to form homo or hetrodimeric structures is attributed to elements close to them which control their activity and specificity to the substrates (Brzovic *et al.,* 2001; Buchwald *et al.,* 2006; Linker *et al.,* 2009; Zhang *et al.,* 2005).

HECT family which has been characterized to show C-terminal amino acid similarity with that of C-terminal E6-AP and just like E1 and E2, form thio-ester bonds with the C-terminal

of activated ubiquitin due to the presence of its conserved cysteine residues. This implies that ubiquitin is first transferred to the E3 from E2, before transferring it to the substrate's lysine side chain (Huibregtse *et al.*, 1995; Zheng, 2003). E6-AP is a human papillomavirus-interacting cellular protein which performs its E3 activity by repressing the cellular level of tumour suppressor protein p53 through the ubiquitin mediated pathway. The inability of this enzyme (E6-AP) to carry-out its cellular activity is the major cause of a neurological disorder called Angelman's syndrome, (Zheng, 2003). E6-AP like HECT has been characterized to form thio-ester intermediates with ubiquitin through its C-terminal domain in the presence of E2 conjugating enzyme subfamilies such as UBC4/UBC5 and human UbcH5. Similarly, the formation of thio-ester intermediates by the RSP5, a family of HECT in yeast *Saccharomyces cerevisiae*, suggests the E3 activity of HECT (Nuber and Scheffner, 1999).

1.5 Interaction between the substrate binding domain of Hsp70.14 and RING finger domain of RBBP6

Work by Kappo and co-workers (2012) using yeast-2-hybrid system (Y2H) suggested that Hsp70.14 putatively interacts with the N-terminal domain of RBBP6-RING finger domain and that this interaction suggests possible function in the degradation of aggregated, denatured and misfolded proteins through the protein quality control and chaperone mediated ubiquitination system (Kappo *et al.*, 2012).

1.6 Biophysical characterization of Hsp70.14 and human RING finger of RBBP6

Biophysical characterization of proteins has become a subject of interest in the biopharmaceutical field for understanding protein molecular structure and conformation which aid industries or researchers in designing new drugs. The characterization of proteins gives information that enables biopharmaceutical industries to understand different physio-

chemical parameters and look out for the best interacting partners. In this way, the protein of interest can actually be targeted for drug design, since the biophysical properties of the protein are known. The following methods will be used in this study to characterize the substrate binding domain of Hsp70.14 and RING finger domain of RBBP6, circular dichroism (CD), Far-UV spec, SE-HPLC, molecular docking (PatchDock), homology modelling.

1.7 PROBLEM STATEMENT

Despite all efforts and improvement in cancer management and treatment, cancer remains one of the major global health threats of this century according to the World health organization. In fact, about 14.1 million new cancer cases and a resulting 8.2 million deaths were reported in 2012 (Torre *et al.,* 2012). So far, the problem associated with the current drugs and other methods of treatment and management therapies or strategies are: they very expensive, non-specific, not easily accessible, and thus subjected to several healths concern as well as placing more burden in the world economy. Therefore, there is urgent and fast need to find alternative means of treatment, management or cure for cancer.

Like other Hsps, Hsp70.14 have been shown to be upregulated during different stages of cellular stress including the onset of tumours, therefore, hypothesized to play a crucial role during cancer development due to its unique ability to elevate dendritic cells and provoke $CD8^+$ CTL specific epitope response. On the other hand, RBBP6 through its RING finger domain have also been hypothesized to play a crucial role in tumour development by controlling the level of tumour suppressor proteins p53 and RB. Using yeast-2-hybrid system, these proteins have been hypothesized to show putative interaction *in vivo*. Therefore understanding the molecular structure and interactions between these two tumorigenic

proteins will not only serve as a therapeutic target, but will also aid in the treatment and management of cancer as well as novel vaccine candidate or improve the current cancer therapies efficacy.

1.8 RESEARCH QUESTION

Using yeast-2-hybrid system, Kappo and colleagues postulated the putative interaction between Hsp70.14 and RING finger domain of RBBP6, and their possible role in protein control mediated ubiquitination pathway. Hence this study seeks to determine if there is interaction between the two proteins and what is the strength of the interaction?

1.9 AIM

This study focused on the expression, purification and characterization of Hsp70.14, as well as its interaction with RING finger domain from RBBP6, with the aim of providing answers to questions relating to the involvement of these two tumorigenic proteins in tumour formation.

1.10 OBJECTIVES

The aim of this study will be followed through by these objectives:

1. To perform homology and molecular modelling studies using SWISS model and PatchDock respectively.

2. To heterologously express recombinant GST-RING and 6xHis-Hsp70.14, and purify them using GST agarose and Nikel affinity chromatography.

3. To determine the secondary structure of the proteins using far-UV circular dichroism

4. To determine the light absorbance and subsequent ANS binding affinity using intrinsic tryptophan fluorescence and extrinsic 8-analino-1-napthalene sulfonate fluorescence spectroscopy

5. To quantify the size of the proteins using size exclusion high pressure liquid chromatography (SE-HPLC).

6. To determine the interaction using molecular docking (PatchDock), SE-HPLC and ANS binding essay.

REFERENCES

Aasland R, Gibson TJ and Stewart AF (1995). The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends in Biochemical Sciences* **20**: 56-59.

Asea A (2003). Chaperokine-induced signal transduction pathways. *Exercise Immunology Review* ***9*:** 25.

Bienz M (2006). The PHD finger, a nuclear protein-interaction domain. *Trends in Biochemical Sciences* **31**: 35-40.

Borden KL (2000). RING domains: master builders of molecular scaffolds? *Journal of Molecular Biology* **295**: 1103-1112.

Bottomley MJ, Stier G, Pennacchini D, Legube G, Simon B, Akhtar A, Sattler M and Musco G (2005). NMR Structure of the First PHD Finger of Autoimmune Regulator Protein (AIRE1) Insights into Autoimmune Polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) disease. *Journal of Biological Chemistry* **280**: 11505-11512.

Brzovic PS, Rajagopal P, Hoyt DW, King MC and Klevit RE (2001). Structure of a BRCA1–BARD1 heterodimeric RING–RING complex. *Nature Structural & Molecular Biology* **8**(10): 833-837.

Buchwald G, van der Stoop P, Weichenrieder O, Perrakis A, van Lohuizen M and Sixma TK (2006). Structure and E3-ligase activity of the Ring–Ring complex of Polycomb proteins Bmi1 and Ring1b. *The EMBO Journal* **25**(11): 2465-2474.

Buczynski G, Slepenkov SV, Sehorn MG and Witt SN (2001). Characterization of a lidless form of the molecular chaperone DnaK: deletion of the lid increases peptide on- and off-rate constants. *Journal of Biological Chemistry* **276:** 27231–27236.

Bukau B, Deuerling E, Pfund C and Craig EA (2000). Getting newly synthesized proteins into shape. *Cell* **101:** 119–122.

Capili AD, Schultz DC, Rauscher FJ and Borden KL (2001). Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING and LIM zinc-binding domains. *The EMBO Journal* **20**: 165-177.

Cegielska A and Georgopoulos C (1989). Functional domains of the Escherichia coli dnaK heat shock protein as revealed by mutational analysis. *Journal of Biological Chemistry* **264**(35): 21122-21130.

Chibi M, Meyer M, Skepu A, Rees DJG, Moolman-Smook JC and Pugh DJ (2008). RBBP6 interacts with multifunctional protein YB-1 through its RING finger domain, leading to ubiquitination and proteosomal degradation of YB-1. *Journal of Molecular Biology* **384**(4): 908-916.

Cupp-Vickery JR, Peterson JC, Ta DT and Vickery LE (2004). Crystal structure of the molecular chaperone HscA substrate binding domain complexed with the IscU recognition peptide ELPPVKIHC. *Journal of Molecular Biology* **342(**4): 1265-1278.

Cyr DM Höhfeld J and Patterson C (2002). Protein quality control: U-box-containing E3 ubiquitin ligases join the fold. *Trends in Biochemical Sciences* **27(**7): 368-375.

Daugaard M, Kirkegaard-Sørensen T, Ostenfeld MS, Aaboe M, Høyer-Hansen M, Ørntoft TF, Rohde M and Jäättelä M (2007). Lens epithelium-derived growth factor is an Hsp70-2 regulated guardian of lysosomal stability in human cancer. *Cancer Research* **67**(6): 2559-2567.

Dominguez C, Bonvin AM, Winkler GS, van Schaik FM, Timmers HTM and Boelens R (2004). Structural model of the UbcH5B/CNOT4 complex revealed by combining NMR, mutagenesis, and docking approaches. *Structure* **12**(4): 633-644.

Dul BE and Walworth NC (2007). The plant homeodomain fingers of fission yeast dystrophy (APECED) disease. *Journal of Biological Chemistry* **280:** 11505-11512.

Freemont PS (2000). Ubiquitination: RING for destruction? *Current Biology* **10**: R84-R87.

Gao S and Scott RE (2003). Stable overexpression of specific segments of the P2P-R protein in human MCF-7 cells promotes camptothecin-induced apoptosis. *Journal of Cellular Physiology* **197**(3): 445-452.

Gao S, Witte MM and Scott RE (2002). P2P-R protein localizes to the nucleolus of interphase cells and the periphery of chromosomes in mitotic cells which show maximum P2P-R immunoreactivity. *Journal of Cellular Physiology* **191**(2): 145-154.

Garbuz DG, Astakhova LN, Zatsepina OG, Arkhipova IR, Nudler E and Evgen'ev MB (2011). Functional organization of hsp70 cluster in camel (Camelus dromedarius) and other mammals. *PloS One*, **6**(11): p.e27205.

George AE (1995). *A new method for isolating genes involved in the processing and presentation of antigens to cytotoxic T cells* (Doctoral dissertation, University of Oxford).

Hartl FU, Bracher A and Hayer-Hartl M (2011). Molecular chaperones in protein folding and proteostasis. *Nature **475***(7356): 324-332.

Huibregtse JM, Scheffner M, Beaudenon S and Howley PM (1995). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proceedings of the National Academy of Sciences* **92**(7): 2563-2567.

Huyer G, Piluek WF, Fansler Z, Kreft SG, Hochstrasser M, Brodsky JL and Michaelis S (2004). Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. *Journal of Biological Chemistry* **279**(37): 38369-38378.

Kappo MA, Eiso AB, Hassem F, Atkinson RA, Faro A, Muleya V, Mulaudzi T, Poole JO, McKenzie JM, Chibi M and Moolman-Smook JC (2012). Solution structure of RING finger-like domain of retinoblastoma-binding protein-6 (RBBP6) suggests it functions as a U-box. *Journal of Biological Chemistry* **287**: 7146-7158.

Koegl M, Hoppe T, Schlenker S, Ulrich HD, Mayer TU and Jentsch S (1999). A Novel Ubiquitination Factor, E4, Is Involved in Multiubiquitin Chain Assembly. *Cell* **96**: 635–644.

Kostic M, Matt T, Martinez-Yamout MA, Dyson HJ, Wright PE (2006). Solution structure of the HDM2 C2H2C4 RING, a domain critical for ubiquitination of p53. *Journal of Molecular Biology* **363**: 433–450.

Kriegenburg F, Ellgaard L and Hartmann-Petersen R (2012). Molecular chaperones in targeting misfolded proteins for ubiquitin-dependent degradation. *Febs Journal* **279**(4): 532-542.

Lanneau D, Wettstein G, Bonniaud P and Garrido C (2010) Heat Shock Proteins: Cell Protection through Protein Triage. *The Scientific World Journal* **10:** 1543–1552.

Li L, Deng B, Xing G, Teng Y, Tian C, Cheng X, Yin X, Yang J, Gao X, Zhu Y, Sun Q, Zhang L, Yang X and He F (2007). PACT is a negative regulator of p53 and essential for cell growth and embryonic development. *Proceeding of National Academy of sciences U.S.A.* **104**: 7951–7956.

Li W, Bengtson MH, Ulbrich A, Matsuda A, Reddy VA, Orth A, Chanda SK, Batalov and Joazeiro CA (2008). Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. *PloS one* **3**(1): p.e1487.

Linke K, Mace PD, Smith CA, Vaux DL, Silke J and Day CL (2008). Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. *Cell Death and Differentiation* **15(**5): 841-848.

MacAry PA, Javid B, Floto RA, Smith KG, Oehlmann W, Singh M and Lehner PJ (2004). HSP70 peptide binding mutants separate antigen delivery from dendritic cell stimulation. *Immunity* **20(**1): 95-106.

Mayer MP and Bukau B (2005). Hsp70 chaperones: cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences* **62**(6): 670.

Mayer MP, Schröder H, Rüdiger S, Paal K, Laufen T and Bukau B (2000). Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nature Structural and Molecular Biology* **7:** 586–593.

Mbita Z, Meyer M, Skepu A, Hosie M, Rees J and Dlamini Z (2012). De-regulation of the RBBP6 isoform 3/DWNN in human cancers. *Molecular and Cellular Biochemistry* **362(**1-2): 249-262.

McConkey BJ, Sobolev V and Edelman M. (2002). The performance of current methods in ligand–protein docking. *Current Science* 845-856.

Metzger MB, Maurer MJ, Dancy BM and Michaelis S. (2008). Degradation of a cytosolic protein requires endoplasmic reticulum-associated degradation machinery. *Journal of Biological Chemistry* **283**(47): 32302-32316.

Metzger MB, Pruneda JN. Klevit RE and Weissman AM (2014). RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. *Biochimica et Biophysica Acta* **1843**: 47–60.

Motadi LR, Bhoola KD and Dlamini Z (2011). Expression and function of retinoblastoma binding protein 6 (RBBP6) in human lung cancer. *Immunobiology* **216(**10): 1065-1073.

Nagarajan NS, Arunraj SP, Sinha D, Rajan VBV, Esthaki VK and D'Silva P (2012). HSPIR: a manually annotated heat shock protein information resource. *Bioinformatics* **28**(21): 2853-2855.

Nakatsukasa K, Huyer G, Michaelis S and Brodsky JL (2008). Dissecting the ER-associated degradation of a misfolded polytopic membrane protein. *Cell* **132**(1): 101-112.

Nishikawa, S.I., Brodsky, J.L. and Nakatsukasa, K. (2005). Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD). *Journal of biochemistry* **137**(5): 551-555.

Ntwasa M. (2008). The retinoblastoma binding protein 6 is a potential target for therapeutic drugs. *Biotechnology and Molecular Biology Reviews* **3**(2): 24-31.

Nuber U and Scheffner M (1999). Identification of determinants in E2 ubiquitin-conjugating enzymes required for hect E3 ubiquitin-protein ligase interaction. *Journal of Biological Chemistry* **274**(11): 7576-7582.

Ohi MD, Vander Kooi CW, Rosenberg JA, Chazin WJ and Gould KL (2003). Structural insights into the U-box, a domain associated with multi-ubiquitination. *Nature Structural & Molecular Biology* **10**: 250-255.

Pant V and Lozano G (2014). Limiting the power of p53 through the ubiquitin proteasome pathway. *Genes & development* **28**: 1739–1751.

Patir H and Upadhyay RC (2010). Purification, characterization and expression kinetics of heat shock protein 70 from Bubalus bubalis. *Research in Veterinary Science* **88**(2): 258-262.

Pellecchia M, Montgomery DL, Stevens SY, Vander Kooi CW, Feng HP, Gierasch LM and Zuiderweg ER (2000). Structural insights into substrate binding by the molecular chaperone DnaK. *Nature Structural & Molecular Biology* **7**(4): 298-303.

Plemper RK, Böhmler S, Bordallo J, Sommer T and Wolf DH (1997). Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature* **388**(6645): 891-895.

Pretorius A (2007). *Functional analysis of the mouse RBBP6 gene using interference RNA* (Doctoral dissertation, Faculty of Science, University of the Western Cape).

Pugh DJ, AB E, Faro A, Lutya PT, Hoffmann E and Rees DJ (2006). DWNN, a novel ubiquitin-like domain, implicates RBBP6 in mRNA processing and ubiquitin-like pathways. *BMC Structural Biology* **6:** 1

Rahimi N (2012). The Ubiquitin-Proteasome System Meets Angiogenesis. *Molecular Cancer Therapeutic* **11**: 538–48.

Ravid T and Hochstrasser M (2008). Diversity of degradation signals in the ubiquitin–proteasome system. *Nature Reviews Molecular Cell Biology* **9**(9): 679-689.

Rüdiger S, Schneider-Mergener J and Bukau B (2001). Its substrate specificity characterizes the DnaJ co-chaperone as a scanning factor for the DnaK chaperone. *The EMBO Journal* **20**(5):1042-1050.

Ryan MT and Pfanner N (2001). Hsp70 proteins in protein translocation. *Advances in Protein Chemistry* **59**: 223-242.

Schasfoort RB and Tudos AJ (2008). *Handbook of surface plasmon resonance*. Royal Society of Chemistry

Scott RE, Giannakouros T, Gao S and Peidis P (2003). Functional potential of P2P-R. A role in the cell cycle and cell differentiation related to its interactions with proteins that bind to matrix-associated regions of DNA? *Journal of Cellular Biochemistry* **90:** 6–12.

Searle S, McCrossan MV and Smith DF (1993). Expression of a mitochondrial stress protein in the protozoan parasite Leishmania major. *Journal of Cell Science* **104**(4): 1091-1100.

Sharma G, Nath A, Prasad S, Singh N, Dubey P and Saikumar G (2012). Expression and Characterization of Constitutive Heat Shock Proteins 70.1 (HSP A-1A) Gene in In Vitro produced and In Vivo-Derived Buffalo (Bubalis) Embryos. *Reproduction in Domestic Animals* **47:** 975-979.

Shi Y, Di Giammartino DC, Taylor D, Sarkeshik A, Rice WJ, Yates JR3rd, Frank J and Manley JL (2009). Molecular architecture of the human pre-mRNA 3′-processing complex. *Molecular Cell* **33:** 365–376.

Shiber A and Ravid T (2014). Chaperoning proteins for destruction: diverse roles of Hsp70 chaperones and their co-chaperones in targeting misfolded proteins to the proteasome. *Biomolecules* **4**(3): 704-724.

Simons A, Melamed-Bessudo C, Wolkowicz R, Sperling J, Sperling R, Eisenbach L and Rotter V (1997). PACT: cloning and characterization of a cellular p53 binding protein that interacts with Rb. *Oncogene* **14**(2): 145-155.

Singh E, Ruff P, Babb C, Sengayi M, Khoali L, Kellett P and Underwood JM (2015). Establishment of a cancer surveillance programme: *The South African Experience. The Lancet Oncology* **16**: 414-421.

Singh OV, Pollard HB and Zeitlin PL (2008). Chemical rescue of ΔF508-CFTR mimics genetic repair in cystic fibrosis bronchial epithelial cells. *Molecular & Cellular Proteomics* **7**(6): 1099-1110.

Slepenkov SV and Witt SN (2002). Kinetic analysis of interdomain coupling in a lidless variant of the molecular chaperone DnaK: DnaK's lid inhibits transition to the low affinity state. *Biochemistry* **41:** 12224–12235.

Sorokin AV, Kim ER and Ovchinnikov LP (2009). Proteasome system of protein degradation and processing. *Biochemistry (Moscow)* **74**: 1411-1442.

Stenmark H, Aasland R and Driscoll PC (2002). The phosphatidylinositol 3-phosphate-binding FYVE finger. *FEBS Letters* **513**: 77–84.

Sung YY and MacRae TH (2013). Heat shock proteins and disease control in aquatic organisms. *Journal of Aquaculture Research & Development* (S2): 1.

Tapley TL and Vickery LE (2004). Preferential substrate binding orientation by the molecular chaperone HscA. *Journal of Biological Chemistry* **279**(27): 28435-28442.

Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jermal A (2015). Global cancer statistics 2012. *A Cancer Journal for Clinician* **65**: 187-108

Van Wijk SJL and Timmers HTM (2010). The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. *Federation of American Societies for Experimental Biology* **24**: 981–993.

Venkatraman P, Wetzel R, Tanaka M, Nukina N and Goldberg AL (2004). Eukaryotic proteasomes cannot digest polyglutamine sequences and release them during degradation of polyglutamine-containing proteins. *Molecular Cell* **14**(1): 95-104.

Vo N and Goodman RH (2001). CREB-binding protein and p300 in transcriptional regulation. *Journal of Biological Chemistry* **276**(17): 13505-13508.

Wan T, Zhou X, Chen H, An H, Chen T, Zhang W, Lui S, Jiang Y, Yang F, Wu Y and Cao X (2004). Novel heat shock protein Hsp70L1 activates dendritic cells and acts as a Th1 polarizing adjuvant. *Blood* **103:**1747-1754.

Wang H, Kurochkin AV, Pang Y, Hu W, Flynn GC and Zuiderweg ERP (1998). NMR solution structure of the 21 kDa chaperone protein DnaK substrate binding domain: a preview of chaperone-protein interaction. *Biochemistry* **37:** 7929–7940.

Witte M and Scott R (1997). The proliferation potential proteinrelated (P2P-R) gene with domains encoding heterogeneous nuclear ribonucleoprotein association and Rb1 binding shows repressed expression during terminal differentiation. *Proceeding of National Academy of sciences USA* **94:**1212–1217.

Woelk T, Sigismund S, Penengo L and Polo S (2007). The ubiquitination code: a signalling problem. *Cell division* **2**: 11.

Yik Sung Y and MacRae T (2013). Heat Shock Proteins and Disease Control in Aquatic Organisms. *Journal of Aquaculture Research and Development*

Yoshitake Y, Nakatsura T, Monji M, Senju S, Matsuyoshi H, Tsukamoto H, Hosaka S, Komori H, Fukuma D, Ikuta Y and Katagiri T (2004). Proliferation potential-related protein,

an ideal esophageal cancer antigen for immunotherapy, identified using complementary DNA microarray analysis. *Clinical Cancer Research* **10**(19): 6437-6448.

Zhang H, Liu R and Huang W (2007). A 14-mer peptide from HSP70 protein is the critical epitope which enhances NK activity against tumor cells in vivo. *Immunological Investigations* **36**(3): 233-246.

Zhang M, Windheim M, Roe SM, Peggie M, Cohen P, Prodromou C and Pearl LH (2005). Chaperoned ubiquitylation—crystal structures of the CHIP U box E3 ubiquitin ligase and a CHIP-Ubc13-Uev1a complex. *Molecular Cell* **20**(4): 525-538.

Zheng N (2003). A closer look of the HECTic ubiquitin ligases. *Structure* **11**(1): 5-6.

Zhu X, Zhao X, Burkholder WF, Gragerov A, Ogata CM, Gottesman M (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272:** 1606–1614.

ZüGEL U and Kaufmann E (1999). Role of Heat Shock Proteins in Protection from and Pathogenesis of Infectious Diseases. *Clinical Microbiology Reviews* **12:** 19-

CHAPTER TWO

Roles of Heat Shock Proteins in Apoptosis, Oxidative Stress, Human Inflammatory Diseases, and Cancer

Published Manuscript: Pharmaceuticals 2018, ***11,*** 2; doi: http://doi.org.10.3390/ph11010002

Review

Roles of Heat Shock Proteins in Apoptosis, Oxidative Stress, Human Inflammatory Diseases, and Cancer

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Received: 23 October 2017; Accepted: 17 November 2017; Published: date

Abstract: Heat shock proteins (HSPs) play cytoprotective activities under pathological conditions through the initiation of protein folding, repair, refolding of misfolded peptides, and possible degradation of irreparable proteins. Excessive apoptosis, resulting from increased reactive oxygen species (ROS) cellular levels and subsequent amplified inflammatory reactions, is well known in the pathogenesis and progression of several human inflammatory diseases (HIDs) and cancer. Under normal physiological conditions, ROS levels and inflammatory reactions are kept in check for the cellular benefits of fighting off infectious agents through antioxidant mechanisms; however, this balance can be disrupted under pathological conditions, thus leading to oxidative stress and massive cellular destruction. Therefore, it becomes apparent that the interplay between oxidant-apoptosis-inflammation is critical in the dysfunction of the antioxidant system and, most importantly, in the progression of HIDs. Hence, there is a need to maintain careful balance between the oxidant-antioxidant inflammatory status in the human body. HSPs are known to modulate the effects of inflammation cascades leading to the endogenous generation of ROS and intrinsic apoptosis through inhibition of pro-inflammatory factors, thereby playing crucial roles in the pathogenesis of HIDs and cancer. We propose that careful induction of HSPs in HIDs and cancer, especially prior to inflammation, will provide good therapeutics in the management and treatment of HIDs and cancer.

Keywords: apoptosis; cancer; heat shock proteins; inflammation; reactive oxygen species; tumour necrosis factor-α

1. Introduction

Although some heat shock proteins (HSPs) are constitutively produced, most are molecular chaperones that are normally over-expressed by cells in response to inducible signals that may lead to protein denaturation [1]. These stressors include heat, nutrient deficiency, oxidative stress, acute or chronic inflammatory diseases, viral infections, ischemia, heavy metals, exercise, gravity, and bacterial infections [2–5]. These responses enable cellular protection against protein denaturation and possible degradation of misfolded proteins, which may, in turn, result in protein aggregation and cancer [6]. Some of these constitutively-expressed heat shock polypeptides are involved in protein folding and translocation of organelles across cellular membranes, prompting many authors to label them "molecular chaperones" [5–8].

Since the discovery of heat shock proteins in 1962 by Ritossa in the salivary glands of the *Drosophila* larvae, the important functions of heat shock proteins in response to various stressful signals including human cancer and cystic fibrosis, has been well elucidated [9–11]. Upon discovering

these proteins, it is not surprising that HSPs have made a very large impact in various areas of research, including medical and biological fields, because of their diverse functions in both pathological and normal conditions [12,13].

Molecular chaperones, which are found in all living cells and form part of the defence system against both internal and external stressors, are primarily grouped into two major groups according to their amino acid composition, molecular weight, as well as their specific cellular function as the high molecular weight and the small molecular weight HSPs [14,15]. The high molecular weight HSPs which range from 60 to 110 kDa are ATP-dependent and their primary cellular function is binding and folding of nascent proteins through ATP-dependent allosteric organization, even though assembling, transportation, vaccination against cancer metastasis, and degradation of improperly-folded peptides have also been reported [16,17]. Small molecular weight HSPs or heat shock protein β (HspBs), which range from 15 to 43 kDa, are ATP-independent molecular chaperones, of which their functions have been documented in embryo developmental processes, formation of respiratory organs, like cardiac muscles, as biomarkers for tumour formation, in exercise-induced stress, as well as in protein folding [5,15,18]. The classification, localization, and roles of HSPs are highlighted as shown (see Table 1).

Table 3. 1 Classification of heat shock protein families.

Classification	Location	Cellular Function	Reference
Hsp10	Mitochondria	Serves as biomarker in endometrial cancer and helps protein folding	[19,20]
Hsp27	Cytosol, endoplasmic reticulum & nucleus	Facilitates refolding of denatured proteins (chaperoning activity) and serves as a biomarker in many cellular diseases such as cancer	[21]
Hsp40	cytosol	Assists HSP70 in protein folding (co-chaperoning with HSP70)	[22]
HSP60	Cytoplasm & mitochondria	Assists in protein folding, prevents protein aggregation and assembling of unfolding proteins via the formation of the hetero-oligomeric complex	[23]
Hsp70	Cytoplasm & nucleus	Aids protein assembling, protein folding, degradation of improperly folded peptides and translocation of organelles	[5]
Hsp90	Cytoplasm	Assists in protein folding, refolding and degradation. It also facilitates signal transduction and important roles in cancer and sarcomere formation as well as in myosin folding	[5,24]
Hsp100	Cytoplasm	Complexes with other HSPs to refold aggregated or misfolded proteins	[25]
Hsp110	Cytosol & nucleus	Helps immune response and complexes with HSP70 to promote protein refolding and cell survival under stress	[26]

The roles of HSPs in the pathology of many diseases including human inflammatory diseases (HIDs), has been well documented. Hsp70 and Hsp60 in particular have been reported to form part of the auto-antigen complex capable of eliciting immunoregulatory cascades, thus suppressing the immune response which is commonly observed in various HIDs, such as Type 1 diabetes, atherosclerosis, rheumatoid arthritis, asthma, and allergies [27]. Hsp functions in the immunology of HIDs could be attributed to their diverse properties: (1) their ability to assemble immune system apparatus to infectious sites; (2) they are capable of interacting with antigen-presenting cells and

initiating CD8+ immune responses and are, therefore, seen as potential cancer vaccines; (3) their ability to refold denatured proteins, including most immune cells, thus promoting their survival under stressful conditions [28,29]. In this review, we discuss in detail the interplay between HSPs, apoptosis, reactive oxygen species (ROS) and inflammatory diseases, as well as possible roles and potential target HSPs hold in HIDs and cancer.

2. Role of HSPs in Apoptosis

Apoptosis, which is alternatively called programmed cell death or "cellular suicidal", is a process by which cells are selectively killed without deteriorating neighbouring tissues [30]. This process is normally induced during embryo development, cell division, aging, as well as maintenance of cellular homeostasis, although it has also been reported to form part of the immune defence mechanism in response to cellular damage including the onset of cancer, and neurodegenerative and human inflammatory diseases (HIDs) [31–33]. Inappropriate stimulation of apoptosis has been associated with a variety of human diseases, such as ischemic damage, neurodegenerative disorders, autoimmune diseases, as well as cancer, making it a good therapeutic candidate against human diseases [32].

Studies have shown that apoptosis is mediated following the activation of caspases (a group of aspartate-specific cysteine proteases) that catalyses the addition or removal of specific cysteine or aspartic acid residues from target substrates, thereby activating or inhibiting the action of the targeted substrate [34,35]. These endoproteases are produced in an inactive state as zymogenes but upon activation, they play a central role in controlling apoptosis-mediated cell death, pyroptosis, necroptosis, as well as inflammatory reactions [36]. Caspases are broadly classified according to their roles in biological processes: apoptotic caspases (-7,-8, and -9) and inflammatory caspases (-1, -4, -5, and -12) in human and (-1, -11, and -12) in mice, respectively. The pro-inflammatory cytokines (such as interleukin 17, IL1β, TNF-α, IL-8, among others) and other inflammatory mediators are up-regulated as a result of the activation of caspases that are involved in inflammatory responses resulting in the innervation of innate immunity responses to cellular insults [36,37].

Caspase mediation of apoptosis falls into two broad categories: intrinsic and extrinsic mechanisms. Intrinsic or mitochondrial apoptotic pathway is a highly regulated and active pathway that cells use to antagonize mitochondrial stimulations as a result of stressors such as DNA damage, hypoxia, growth factor deprivation, as well as the accumulation of misfolded proteins. In response to cell death signals which activate the diverse functions of Bcl-2 families, intrinsic apoptosis is known to induce mitochondrial-membrane permeability through the opening of the permeability transition pore (PTP), thus allowing the release of cytochrome *c* (a pro-apoptotic factor that plays an important role in intrinsic apoptosis) into the cytosol. Inside the cytosol, cytochrome *c* complexes with apoptotic protease activating factor-1 (Apaf-)—an adaptor peptide—to recruit and activate pro-caspase-9, thereby forming a complex called apoptosome. The active caspase-9, on the other hand, triggers the activation and release of downstream 'executioner' caspase-3, which facilitates the degradation of the targeted substrate [34,36,38].

The extrinsic apoptosis pathway is characterised by interactions between Fas receptors (TNFR1, DR3 or death receptor 3, TRAIL-R1 or DR4, and Fas or CD95) and Fas ligands (TNF, Apo2-L, and FasL) on the surface of lymphocytes in response to suicidal signals. The binding of Fas ligand to FADD adaptor protein causes the dimerization of Fas associated death domains (FADDs) found in both Fas receptor and FADD adaptor proteins. This interaction allows the death effector domain (DED) to relate with pro-caspase-8 resulting in the formation of a complex called the death inducing signalling complex (DISC). The subsequent innervation of pro-caspase-8, which then triggers the activation of other pro-caspases, which then leads to the suicidal execution of cells [31–34,36,39,40].

Cells respond to numerous stressors, ranging from external to internal stressors, by expressing highly-regulated proteins upon thermal induction called heat shock proteins (HSPs). These highly-

conserved proteins are known for their diverse functions including protein folding, translocation of organelles across membranes, assembling and disassembling of proteins, signalling transduction, degradation of misfolded proteins, as well as ROS generation in the mitochondria capable of inducing apoptosis [41]. Overwhelming evidence has shown that HSPs have a wide array of functions in apoptosis, which in most cases, leads to the suppression of apoptotic pathways. Interestingly, the same stress signals that trigger apoptosis also stimulates the expression and release of HSPs. However, induction of HSPs represses apoptosis through inhibition of pro-apoptosis factors, such as p53, Bax, Bid, Akt, Apaf-1, and other Bcl-2 families. So far, numerous mechanisms of how HSPs incite cytoprotective effects against apoptosis has been proposed; one of them being the ability of Hsp27 to interact with cytochrome *c* and block its dimerization with Apaf-1, hence preventing the formation of apoptosome complex, which is the hallmark of mitochondrial cell suicide [36,42].

Work by Rane and colleagues have shown that Hsp27 relates directly with the serine/threonine (Akt) signalling pathway and this association inhibits neutrophil-mediated apoptosis in a phosphorylation-dependent manner [43]. Although the exact mechanism is still obscure, subsequent studies have shown that another molecular chaperone, Hsp70, directly associates with Apaf-1, blocking the production of apoptosome in an ATPase-dependent manner rather than its chaperoning activity [34,38,44]. Goto and co-workers and Beere reported that Hsp70 together with its co-chaperone Hsp40 homologs, inhibits nitric oxide mediated apoptosis by blocking the mitochondrial translocation of Bax, a pro-apoptotic member of Bcl-2 family in both ATPase and chaperoning dependent-fashion [34,45]. In spite of all the negative functions of HSPs in attenuating apoptosis, members of Hsp60 located in the mitochondria, in complexes with Hsp10, are involved in the signalling complex that results in pro-caspase-3 activation in cytochrome *c*-dependent apoptosis. In addition, several studies have shown that cytosolic Hsp60 associates with Bcl-2 proapoptic protein, Bax, leading to its activation, as well as its mediated apoptosis [46,47]. This observation suggests HSPs' roles in apoptosis to be complex, complicated, and controversial [34,38].

3. HSPs and Oxidative Stress

Due to continuous mitochondrial oxidative respiratory reactions and other cellular and non-cellular processes, including phagocytosis, inflammatory reactions, ionizing radiation, air pollutants, exercise, cigarette smoking, and ozone [48–50], cells frequently generate reactive oxygen species (ROS) and reactive nitrogen species (RNS), which disturb normal oxidant and antioxidant cellular homeostasis, leading to oxidative stress [51,52]. These oxygen-containing compounds can be broadly categorized according to their oxygen-containing capacity: superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), alkoxyl radical ($RO\cdot$), peroxyl radical ($HOO\cdot$), nitric oxide radical ($NO\cdot$), nitrogen oxide ($NO_2\cdot$), as well as potent non-radicals, such as hydrogen perioxide (H_2O_2), ozone (O_3), and oxygen singlet ($^1O_2$) [53,54]. Both ROS and RNS in accumulated levels are very reactive and more potent than normal oxygen and nitrogen, thus causing deleterious effects to the living system.

In spite of all negativities associated with accumulated cellular ROS, several studies have shown that, at low or moderate levels of unknown concentration, ROS perform important cellular beneficial roles, including acting as secondary messengers in signal transduction, in immune defence, in antibacterial infections in the phagosome and vascular tone, as well as in ROS-induced programmed cell death in cancer cells [52,54–58]. The hydroxyl radical ($\cdot OH^-$) which is the most reactive and most dangerous radical, is regenerated when H_2O_2 (a product of enzymatic reactions) decomposes slowly in the presence of Fe^{2+} in a process called the Fenton reaction and through other cellular reactions, including one between NO and O_2^- to form a perioxynitrite intermediate which immediately decomposes to OH^- [59]. Upon OH^- formation, OH^- is capable of abducting electrons from biomolecules, especially lipids (polyunsaturated fatty acids), thereby inflicting DNA, carbohydrate, protein, and lipid oxidation and, most importantly, resulting in oxidative stress when the antioxidant system is supressed [58]. The body uses the antioxidant system to neutralise free radical cellular

damage by converting them to less harmful substances under physiological conditions; however, this balance can be disrupted upon cellular stress leading to the accumulation of cellular levels of free radicals, especially ROS, thus activating many inflammatory cascades which have been implicated in various human inflammatory diseases (HIDs), such as arthritis, asthma, stroke, atherosclerosis, trauma, hepatitis, and in cancer [52,60–62].

The deteriorating cellular effects of free radicals in accumulated levels has been well documented; however, it is not surprising that biological systems protect themselves by increasing the expression level of highly-regulated proteins termed "heat shock proteins" in response to these reactive species (ROS) that may otherwise lead to oxidative stress. HSPs are known for their cytoprotective activities in response to a variety of cellular insults through their chaperoning activities ranging from polypeptide folding, assembling, and translocation of organelles across membranes, to conducting repairs, and the degradation of irreparable peptides [8,63]. Nevertheless, DNA fragmentation has been observed in cells undergoing ROS-mediated genotoxicity, but this effect has been rescued with the addition of the Hsp70 family, thereby suggesting that the cytoprotective effects of HSPs could be by protecting DNA breaks in response to ROS-induced insults [64]. Interestingly, HSPs have been reported to work hand-in-hand with the antioxidant system to inhibit or neutralise the cellular effects of ROS [65,66]. Accumulated ROS levels are said to induce apoptosis and are associated with a variety of inflammatory reactions, which is the hallmark of human inflammatory disease (HID) pathogenesis [53]. Hence, it is tempting to suggest that HSPs could play cytoprotective roles in the pathogenesis of HIDs and could be targeted as drug candidates for immunotherapy against HIDs.

4. HSPs in Human Inflammatory Diseases (HIDs) and Cancer

Inflammation, which forms part of the human first line of defence in response to stressful insults (such as pathogen invasion, oxidants, and cell damage), is characterized by swelling, pain, heat, and redness in the infected area [67]. This response enables cellular injury repair as well as elimination of any sign of necrotic cells, thereby activating innate immunity [68]. The inflammatory reaction is considered beneficial to humans in response to cellular insults because of the fact that it helps to clear and repair damaged cells and tissues. However, long-term unregulated inflammation may result in chronic inflammatory reactions marked with massive tissue and cell destruction, and this has been reported to play central role in the pathogenesis of many human inflammatory diseases (HIDs) [53]. HSPs have been reported to prevent inflammation through the inhibition of pro-inflammatory cytokines including tumour necrosis factor-α (TNF-α). The following sections in this review will hereby focus on the roles of HSPs in adult respiratory distress syndrome, rheumatoid arthritis, asthma, and cancer.

4.1. Acute Respiratory Distress Syndrome

Adult Respiratory Distress Syndrome (ARDS), which is alternatively called acute respiratory distress syndrome, is a lung inflammatory disorder characterized by Diffuse Alveolar Damage (DAD) as a result of the influx of liquid into the alveoli sacs (the site of blood-oxygenation), as well as the imbalance between pro-and-anti-inflammatory cytokines (interferon, TNF-α, interleukins, platelets derived growth factor) which, in most cases, leads to severe hypoxemia, stiffness of the lungs, pulmonary infiltration and organ failure, without causing cardiogenic pulmonary oedema [69,70]. Although ARDS mortality depends on several factors such as age, critical illness and other medical complications, ARDS has been estimated according statistical reports, to affect approximately 50 in every 100,000 people, resulting in almost 40% of deaths in infected patients worldwide [71,72]. So far, the actual causes of ARDS are obscured but, in most cases, ARDS has been reported in trauma or

critically ill patients. Age and other unhealthy lifestyles, like smoking and chronic alcoholism, have also been documented as predisposing factors associated with ARDS cases [73].

In response to severe DAD cases, alveoli sac permeability of lung membranes is increased; this allows for the influx of neutrophils, tumour necrosis, macrophage inhibitory factor, together with platelet activation and sequestration, which are believed to be the centre stage for the development, progression, and pathogenesis of ARDS. However, elimination of activated inflammatory cytokines that cause tissue and cellular destructions in inflamed area have been reported to decrease morbidity and deaths in HIDs [74]. Nevertheless, heat shock proteins are known for their cytoprotective effects in response to cellular insults including inflammatory diseases; these proteins are said to be up-regulated during this stage of infection. Wesis and colleagues demonstrated that Hsp70 has the ability to suppress inflammatory responses by initiating the refolding of protein aggregates, thereby preventing the cellular damage and destruction observed in the pathology of ARDS and sepsis [75]. In support of this finding, other studies have shown that decreased mortality rates immediately after heat shock protein administration to endotoxin may mark the events of ARDS after several hours of development, as previously observed in rats [72,76]. Overwhelming evidence has shown that loss of pulmonary cells promotes cell division which contributes massively to the pathogenesis of ARDS and heat shock proteins, Hsp70 in particular, has been previously shown to limit inflammation in HIDs, by inhibiting the pathway that leads to nuclear factor (NF)-kB activation as observed in pneumocytes [75]. Although the mechanism of Hsp-cytoprotective action in ARDS is largely unknown, it has been reported to follow the same mechanism as previously observed in other lung inflammatory diseases, like sepsis and pneumocystis [77].

4.2. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a long-term autoimmune-inflammatory disease where, instead of the immune system defending the body, it attacks synovial fluid-membranes normally found in the wrist, hand, or joints of the feet. RA, like other inflammatory diseases, is characterised by stiffness, swelling, and warmness and pain of the joints which, if left untreated long-term, may result in severe inflammation, deformity, and several functional disabilities [78,79]. Severe inflammation is said to attract numerous immune cytokines, chemokine, lymphocytes, and other immune components to the area of the infection (normally in the joints), causing redness, warmness, and painful discomfort, which are the symptoms, observed in rheumatoid arthritis infection. To date, the exact cause of RA is poorly understood and there is no pronounced cure. However, RA has been reported to arise as a result of family history or genetics (people that are genetically predisposed to RA) and other predisposing factors, such as environmental effects, educational background, and low socio-economic status, as well as unhealthy lifestyles, such as smoking and lack of exercise [78,80–82].

In spite of all the efforts in treatment, different medications and improvement in lifestyle with added exercise and healthier nutrition, RA remains one of the leading inflammatory autoimmune diseases worldwide. According to statistical reports, 24.5 million people were effected by RA in 2015, with a rate of 100, 000 people every year [78]. RA, which occurs more in middle-aged females than in males, and has shown 10% mortality increase between 1990 and 2013, making it one of the prevalent health concerns according to the National Institute of Health (NIH).

In response to RA infection which is characterized by severe inflammation, biological systems always increase the synthesis of heat shock proteins especially Hsp70, the most inducible protein upon stress. Hsp70 exerts its anti-apoptotic activities by inhibiting pro/inflammatory signals or factors that lead to apoptosis, inflammatory pathways, such as activation of caspases, JNK (Jun N-terminal) signalling pathway, the release of cytochrome *c*, and the formation of apoptosome, which is the hallmark of apoptosis and inflammation progression [34]. More so, it is not surprising that Hsp70 is, therefore, up-regulated in the synovial membrane during rheumatoid arthritis fibroblast-like synoviocyte (RA-FLSs) infection to modulate the effects of T-cells, as well as to control inflammation

via inhibition of pro-inflammation signals [83]. Surprisingly, Kang and colleagues reported that repression of Hsp70 by siRNA in an in vitro experiment decreased inflammation by protecting RA-FLSs from nitric oxide mediated-programmed cell death, although the actual function of Hsp70 in the RA in vivo experiment is still not yet clear [84]. This observation suggests the pro-apoptotic and negative roles of Hsp70 in the pathogenesis of RA FLSs infection; thus, inhibition of Hsp70 expression in RA could be one of the mechanisms of controlling severe inflammation observed in rheumatoid arthritis. In addition, van Room and co-workers showed that T-cells taken from RA patients were able to react with human or self-Hsp60 and inhibit the activation of TNF-α (a pro-inflammatory factor) through the activation of Th2 cytokine regulator, whereas there were no regulatory effects observed in Hsp65 isolated from *Mycobacterium tuberculosis* [85,86]. Consistent with this finding, several studies have shown that T-cell response to self-Hsp70 and Hsp60 through production of interleukin-4 and interleukin-10 regulatory cytokines suppresses arthritis diseases in many animal models. These observations suggest that cross-reactivity of HSPs with T-cells could be one of the ways of controlling human inflammatory diseases, like RA, under stressed physiological states [86,87]. Taken together, the self-Hsp60 reactivity observed in Lewis rats and adjuvant arthritis [88] makes it easy to speculate that human Hsp60 and mycobacterial Hsp60 could be used as potential vaccines against autoimmune inflammatory diseases since they are capable of eliciting immune responses.

4.3. Asthma

Asthma is a multigenic and multifactorial bronchial chronic inflammatory disorder characterised by airway obstruction, bronchospasm and remodelling of the bronchial wall, thus resulting in thickness of airflow walls and difficulties in breathing [89]. The symptoms of asthma which can be caused by genetic or environmental factors (allergens and air pollutions), or both, range from chest tightness and wheezing sounds when breathing, down to shortness of breath; all these symptoms may vary in individuals. The various associations of different cellular networks, such as smooth muscle, macrophages, fibroblasts, eosinophil and epithelial cells may result in airway remodelling and inflammation [90]. This process of remodelling promotes further thickening of bronchial walls and narrowing the airflow, leading to the breathing difficulties commonly observed in the pathogenesis of asthmatic patients [91].

Statistics have shown that asthma affects 358 million people worldwide which has, so far, resulted in 397,100 deaths in 2015 alone, as compared to the 183 million people who were affected in 1990 [92,93]. According to the Global Initiative for Asthma (GINA), South Africa remains the fourth leading country, globally, and the first on the African continent with the highest mortality resulting from asthma attacks [94]. Furthermore, an estimated 3.9 million South Africans were affected by asthma in 2012, accounting for 1.5% of deaths in this country every year [95]. Taking into account the cytoprotective roles of HSPs in response to cellular insults, several authors have reported the over-expression of Hsp70 in asthmatic patients [96,97]. Surprisingly, recent studies have suggested that intracellular synthesis of Hsp70 chaperones in airways and alveolar sacs of asthma patients correlate with the deleterious effects and severity of the disease, probably by forming part of the inflammatory complex, where it may up-regulate THP-1 synthesis by inducing CD23 expression in the Th2 environment [98–100]. Nevertheless, initial studies have proposed its autoprotective activities in response to asthma and lung complications by inhibiting TNF-α mediated-inflammation [96,101]. In support of this finding, many studies have conclusively stated with evidence that the interaction between anti-Hsp70 and anti-Hsp60 correlates with progression, poor prognosis, and severity observed in asthmatic patients, as previously suggested by Shingai and colleagues in a patient with autoimmune liver disease [102] and *Mycobacterium* Hsp65 in chronic human atherosclerosis [103]. Interestingly, serum Hsp70 circulation is said to increase in pregnant asthmatic patients and this elevated Hsp70 level correlates with foetal and maternal complications such as low birth weight in infants, pre-eclampsia and preterm delivery, and these result in an almost 35% increased death rate in

asthmatic pregnant women [104]. Other factors such as smoking and maternal obesity, on the other hand, have also been reported to promote perinatal death [105].

The exact mechanism of Hsp70 activity during asthma development remains to be seen, although evidence has it that Hsp70 mostly targets T-cells and humoral immunity in response to infectious agents, and this may provide a link between T-lymphocyte cross-reactivity-induced autoimmunity and immune responses to infectious diseases [106]. Another possible explanation is the ability of Hsp70 to interact with antigen presenting cells (APCs) and amplify its activities, which plays a crucial role in the modulation and initiation of asthmatic attacks in chronic asthma patients [96,104]. Additional studies have shown the up-regulation of Hsp90 and Hsp72 in response to ROS-induced asthma attacks in young children. Even though the exact functions of Hsp90 and Hsp72 remain unknown, it has been suggested that the elevated levels of these heat shock proteins could be to refold denatured proteins that may result from ROS induced-oxidative stress [107], thereby preventing protein aggregates, cellular deteriorations, and further complications. Tong and Luo showed that elevated Hsp70 levels in peripheral blood mononuclear cells (PBMCs) suggest Hsp involvement in the pathogenesis of asthma infection [108]. Through these observations we, therefore, propose that enhancing Hsp inhibitors in asthmatic patients could reduce the severity in this disease and will become another interesting aspect in the management of asthma and other lung inflammatory diseases, nonetheless more research is needed in this area of study.

4.4. Cancer

Cancer is a term given to the group of diseases characterized by the abnormal invasion and proliferation of cells as a result of uncontrolled cell divisions or mutated tumour suppressor genes [109]. Cancer remains one of the major sources of global morbidity and mortality and persists as the leading cause of death in individuals below 85 years old in the United States despite recent improvements in treatment modalities [53,110]. This exponential increase in cancer cases could be due to increasing prevalence in related factors, such as obesity, unhealthy life styles (smoking, lack of physical activity and imbalance diet), as well as genetic predisposition [31].

Hanahan and Weiberg proposed that the mechanisms by which cancerous cells survive in the human body are by increased resistance to anti-proliferative signals and apoptosis induced-suicidal or abnormal cell death [111]. Interestingly, several studies have reported that this resistance to apoptosis and antiproliferative signals observed in cancerous cells are actually induced in the presence of HSPs, which favour the refolding of denatured peptides through Hop (Hsp70/Hsp90 organizing protein) chaperoning related activities instead of peptide degradation [112,113]. Previous studies have shown that HSPs inhibit both pro-inflammatory and pro-apoptotic caspases, by binding and blocking their activation, thus increasing cancer cell survival [114]. Overexpression of Hsp27 in prostrate, ovarian and bladder cancers have been shown to correlate with down-regulation of p53 induced-stimulation of the p21 gene and up-regulation of matrix metalloproteins (MMPs), proteins that are known to pave the way for cancer cell migration, invasion, proliferation, and metastasis, thereby inhibiting p53 mediated senescence, apoptosis and cell cycle arrest [115]. These observations are simple indications of the roles of Hsp27 in prognosis, angiogenesis, proliferation, as well as metastasis of cancer cells.

In line with this, Hsp27 has been reported to promote epithelial-to-mesenchymal transition (EMT) in prostate cancer patients. EMT is an important event that occurs during organ morphogenesis and embryogenesis, characterized by the actin cytoskeleton remodelling, loss of apico-basolateral polarity and cell-cell junction dissolving, thus resulting in proliferation and mobility of cancer cells [116]. Down-regulation of Hsp27 using OGX-427 (an antisense therapy) decreases cancer cell invasion, migration, phosphorylation of LL-6-dependent STAT3 (a major mediator of EMP in many cancer types) and nuclear translocation, as well as matrix metalloproteinase, thereby

reversing EMT activity. This observation suggests that Hsp27 is needed for interleukin LL-6 to induce EMP possibly through the modulation of the STAT3 signalling pathway [117].

Similarly, overexpression of Hsp70 corresponds with an increased proliferation of malignant cells and knockdown of Hsp70 experiments in various malignant tumours have been shown to increase the susceptibility of cancer cells to certain chemotherapy agents, suggesting the negative roles of Hsp70 in the invasiveness and proliferation of cancerous cells [118]. In addition, Hsp70 also acts as a cancerous cell-surviving factor by inhibiting TNF-α mediated-apoptotic cell death, thus promoting carcinogenesis and cancer cell oncogenic potential through the mechanism of escape immunology [115]. Furthermore, studies have reported that either Hsp90 or Hsp70 can bind and block the activation of apoptotic protease activating factor 1 (Apaf-1) and indirectly inhibit pro-caspase activation, apoptosis, as well as enhance abnormal cell survival [119]. Nevertheless, Hsp60 has been reported to show pro-carcinogenic activity through the inhibition of dusterin in neuroblastoma cells, cyclophilin D mediated-mitochondrial cell death, and promotion of cell survival via nuclear factor-kB activation [120,121].

Conversely, previous in vivo experiments by Chalmin and colleagues showed that the down-regulation of Hsp70 correlates with cancer cell survival due to the reduced immune killing of cancer cells [122]. Consistent with this finding is that Hsp40 homolog-DNAJA3 has been shown to inhibit squamous cell carcinoma invasion, migration, growth, recurrence and proliferation in both in vivo and in vitro experiments [115], possibly through induction of mitochondrial apoptosis, as previously observed in MCF-7 breast cancer cells [123]. These findings suggest that Hsp70 could suppress tumour cells when co-expressed with DNAJA3 in the absence of other molecular chaperones, like Hsp90, via CHIP (C-terminal HSP70 interacting protein)-mediated protein degradation.

5. Association between Heat Shock Proteins, Oxidative Stress, Apoptosis, Human Inflammatory Diseases (HIDs), and Cancer

Although accumulated levels of ROS have been proposed to cause deleterious effects to biomolecules, such as lipids, carbohydrates, proteins, and nucleic acids, ROS at moderate levels of unknown concentrations have been documented to play significant roles, such as acting as secondary messengers in signal transduction, immune defence, antibacterial infections in the phagosome, as well as ROS-induced programmed cell death in cancer cells [52,54]. Biological systems employ antioxidants (reduced glutathione, catalase, superoxide dismutase) to nullify the negative effects of free radicals, as well as to prevent ROS-mediated cellular damages and functional impairments. However, this antioxidant system can be supressed under severe cellular stress conditions as a result of elevated ROS levels, resulting in oxidative stress and amplification of inflammatory reactions, as well as activation of immune system cascades, a proposed central stage in progression and pathogenesis of several HIDs [53,124,125].

Inflammation is said to be part of the first line of the innate immunity complex that responds to cellular damage caused by infectious agents or xenobiotics [53]. Onset of stress has been established to attract several inflammatory cells, such as cytokines, macrophages, and chemokines, at the site of damaged cells, which is a process mediated upon toll-like receptor (TLRs) activation. The aim of this TLR mediated-inflammatory response is to eliminate detrimental cells and promote cellular repair via the mechanism of apoptosis. However, long-term un-regulated inflammatory reaction may lead to excessive and amplified apoptosis, resulting in chronic inflammation characterized by massive tissue and cellular destruction, commonly seen in many chronic and neurodegenerative diseases [62,126].

The roles of ROS induced-apoptosis in inflammatory reactions can be viewed as a double-edged sword. ROS-induced apoptosis under normal cellular conditions performs beneficial roles in suicidal killing of cells via mitochondrial apoptosis, but the abnormal stimulation of this mechanism under stressful conditions could result in excessively amplified intrinsic apoptosis, leading to the massive cellular destructions observed in the aetiology of several HIDs [127,128]. In line with this, elevated

levels of ROS in the airways of asthmatic and ARDS patients suggests their roles in the pathogenesis and progression of HIDs by inducing severe inflammation [129,130]. HSPs, on the other hand, are mostly induced upon heat stress and, therefore, it is not surprising that HSPs are highly expressed in the inflamed area, possibly to refold denatured peptides caused by ROS-induced reperfusion injury on the inflammation sites identified in rheumatoid arthritis [131]. Heat is also one of the major characteristics of inflammation, and excessive inflammation leads to HIDs. Although some HSPs perform pro-inflammatory/apoptotic functions, most HSPs are known for their anti-apoptotic/inflammatory potential, including the inhibition of pro-inflammatory/apoptotic factors or pathway capabilities such as through the nuclear factor (NF-kB), activation of caspases and c-Jun NH2-terminal kinase pathway [132]. Suppression of HSP expression levels, therefore, leads to worse inflammation cases, which can be linked to the severe inflammation seen in several HIDs.

6. Future Direction in the Use of HSPs as Therapeutic Candidates

From this point, it can be proposed that carefully regulation of inflammatory responses, induction of apoptosis and endogenous generation of ROS would definitely help in the management or treatment of HIDs. In many studies, HSPs have been reported to play crucial roles in the pathogenesis of HIDs and cancer due to their modulating effects in inflammation cascades that lead to the endogenous generation of ROS and apoptosis [27,34,133], possibly via their chaperoning activities of refolding misfolded proteins, or via inhibition of pro-inflammatory cytokines under pathological conditions. Under stressful conditions, HSPs has been suggested to play a prominent role by binding to the lipid rafts inside lipid membranes, thus maintaining lipid membrane stability, physical orderliness, as well as preventing lipid membrane functional impairments. Altered membrane functionality has been associated with cancer, neurodegenerative diseases, and diabetes, suggesting the possible role of HSPs as therapeutic targets in the management of these diseases [134].

Up–regulation of HSPs in cancerous cells has been well documented and has been associated with poor prognosis, proliferation, cell differentiation, invasion, progression, and metastasis [135,136]. Among others, Hsp90, Hsp70, and Hsp27 in particular, have been reported by many studies to increase tumour cell survival via inhibition of pro-inflammatory cytokines and ROS-mediated apoptosis [119,136,137]. Chauhan and co-workers demonstrated that Hsp27 can promote the survival of malignant tumours by conferring resistance to the inflammatory drug dexamethasone (a drug for treating HIDs such as rheumatoid arthritis, skin inflammation, and cancer) in myeloma cell lines via the inhibition of SMAC (mitochondrial release of second mitochondrial-derived activator of caspases) and cytochrome *c*, both of which are masters of intrinsic apoptosis mediators [138].

Hsp27 can also act as an anti-apoptotic factor by promoting the activities of nuclear factor-kB (NF-kB) while blocking apoptosis pathways mediated by NF-kB inhibitor (IkBα), thereby promoting cancer cell proliferation and survival [135]. Hsp27 involvement in cancer could be through phosphorylation at three-serine residues mediated by MAPKAPK2 (mitogen-activated protein kinase activated protein kinase). This phosphorylation enables the Hsp27 to form oligomers up to 100 kDa, making it ideal in preventing protein aggregation by refolding denatured peptides in an ATP-independent manner [119]. Similarly, Hsp70 and Hsp90 function in cancer cell survival has been previously elucidated. Work by Nylandsted and colleagues showed that selective inhibition of Hsp70 in breast cancer lines increased the susceptibility of cancer to chemotherapy and sensitized them to caspase-mediated apoptotic death [139]. Hsp90 is known to play a crucial role in cancer cell survival and has been reported as a drug target in many cancer types. Inhibition of Hsp90 in leukaemia, colorectal, breast, lung, melanoma, and bladder cancer correlates with decreased invasion, motility, and prognosis of cancer, as well as an increase in the susceptibility of cancer cells to therapy [115]. This may be possible via the inhibition of signalling pathways that confer resistance to chemotherapy. In view of this, Zuninga and Shonhai previously postulated that Hsp70 and Hsp90 are the most druggable HSPs due to the fact that most Hsp inhibitors either mimic or target their ATPase activity

[140]. This could be via the inhibition of Hsp70/Hsp90 organizing protein (Hop), which favours the refolding of aberrant peptides while blocking CHIP-mediated peptide degradation. Interestingly, subset studies have shown that physical exercise induces Hsp expression [141–143], and is one of the ways of managing HIDs and cancer. Furthermore, one can then speculate that one of the health benefits of exercise is to induce Hsp expression, which is known for its cellular cytoprotective activities, probably by forming part of the immune protective system against infection.

The onset of stress increases ROS generation, as well as inflammatory reactions. The generated ROS and inflammation activates the immune response and induces apoptosis, which aims at fighting off the infectious agent. However, if this mechanism is not well stimulated, it could degenerate to chronic inflammation. Interestingly, heat shock factor-1 (HSF-1) is also upregulated during this stage of infection. HSF-1 increases the synthesis of protective HSPs, which stops inflammatory reactions and massive cellular destruction through apoptosis, as well as further ROS generation, possibly via inhibition of pro-inflammatory factors and activation of the immune system, thus preventing chronic inflammation, HIDs, and cancer progression, as proposed in Figure 1.

HSPs have been reported to perform beneficial cytoprotective effects when induced prior to inflammation and deleterious effects after propagation of pro-inflammation reactions [132]. We, therefore, proposed that induction of HSPs prior to inflammation and carefully regulation of ROS, inflammation and apoptosis through the induction of HSPs as well as the inhibition of HSPs in cancer and certain HIDs (asthma and ARDS) and enhancement of HSP activities in RA may, and will, serve as future study references as proposed in the model (Figure 1), which highlights the possible roles of HSPs in HIDs and cancer.

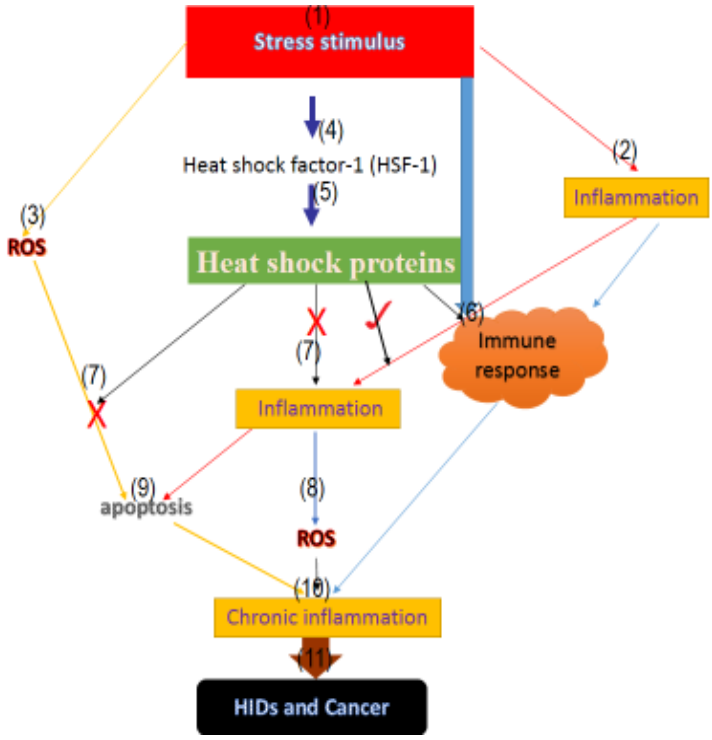

Figure 2.1: Model proposing the roles of heat shock proteins in HIDs and cancer. The above model represents the proposed roles that heat shock proteins play in human inflammatory diseases and cancer. (1) the onset of the stress signal; (2) stress activates inflammatory reactions which aims at repairing the damage caused by the stress; (3) generation of ROS from the infected area; (4) activation of the heat shock factor-1 (HSF-1), which increases the synthesis of the cytoprotective heat shock proteins; (5) activation of heat shock proteins; (6) stress, as well as inflammation and heat shock proteins, activate the immune response and form part of the

innate immune response (7); cytoprotective heat shock proteins inhibit further generation of ROS, as well as inflammation, thus blocking excessive ROS and inflammation mediated-apoptosis via the inhibition of pro-inflammatory and pro-apoptotic factors; (9) excessive apoptosis mediated by ROS; (8) accumulated level of ROS leading to oxidative stress; (10) immune response, inflammatory reaction, accumulated ROS level and excessive apoptosis mediated by ROS as a result of antioxidant suppression, leading to oxidative stress and chronic inflammation marked with massive cellular and tissue destruction; and (11) long-term uncontrolled chronic inflammation degenerates to HIDs and cancer.

7. Conclusions

The search for new drugs for the treatment of HIDs and cancer continues, and new studies are now focused on discovering drugs that will have minimal side effects. Recently, HSPs have attracted a great deal of research interest because of their ever-present occurrence in a variety of human diseases, including HID-tested patients, even though their action in some HIDs is still unclear. From our perspective as proposed in the model (Figure 1). We, therefore, suggest that targeting HSPs in HIDs will serve as good potential candidates towards the treatment and management of many HIDs, as well as early detection of these diseases.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Srivastava, P. Roles of heat-shock proteins in innate and adaptive immunity. *Nat. Rev. Immunol.* **2002**, *2*, 185.
2. Asea, A. Chaperokine-induced signal transduction pathways. *Exerc. Immunol. Rev.* **2003,** *9,* 25–33.
3. Searle, S.; McCrossan, M.V.; Smith, D.F. Expression of a mitochondrial stress protein in the protozoan parasite Leishmania major. *J. Cell Sci.***1993,** *104,* 1091–1100.
4. Zügel, U.; Kaufmann, E. Role of Heat Shock Proteins in Protection from and Pathogenesis of Infectious Diseases. *Clin. Microbiol. Rev.* **1999**, *12*, 19–39.
5. Jee, H. Size dependent classification of heat shock proteins: A mini-review. *J. Exerc. Rehabil.* **2016**, *12*, 255–259.
6. Sharma, G.; Nath, A.; Prasad, S.; Singh, N.; Dubey, P.; Saikumar, G. Expression and Characterization of Constitutive Heat Shock Proteins 70.1 (HSPA-1A) Gene in In Vitro produced and In Vivo-Derived Buffalo (Bubalis) Embryos. *Reprod. Domest. Anim.* **2012**, *47*, 975–983.
7. De Maio, A. Heat shock proteins: Facts, thoughts, and dreams. *Shock* **1999**, *11*, 1–12.
8. Shiber, A.; Ravid, T. Chaperoning proteins for destruction: Diverse roles of HSP70 chaperones and their co-chaperones in targeting misfolded proteins to the proteasome. *Biomolecules* **2014**, *4*, 704–724.
9. Daugaard, M.; Kirkegaard-Sørensen, T.; Ostenfeld, M.S.; Aaboe, M.; Høyer-Hansen, M.; Ørntoft, T.F.; Rohde, M.; Jäättelä, M. Lens epithelium-derived growth factor is an HSP70-2 regulated guardian of lysosomal stability in human cancer. *Cancer Res.* **2007,** *67,* 2559–2567.
10. Singh, O.V.; Pollard, H.B.; Zeitlin, P.L. Chemical rescue of ΔF508-CFTR mimics genetic repair in cystic fibrosis bronchial epithelial cells. *Mol. Cell. Proteom.* **2008**, *7*, 1099–1110.
11. Zhang, H.; Liu, R.; Huang, W.A. 14-mer peptide from HSP70 protein is the critical epitope which enhances NK activity against tumor cells in vivo. *Immunol. Investig.* **2007**, *36*, 233–246.
12. De Maio, A. Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: A form of communication during injury, infection, and cell damage. *Cell Stress Chaperones* **2011**, *16*, 235–249.
13. De Maio, A.; Santoro, M.G.; Tanguay, R.M.; Hightower, L.E. Ferruccio Ritossa's scientific legacy 50 years after his discovery of the heat shock response: A new view of biology, a new society, and a new journal. *Cell Stress Chaperones* **2012**, *17*, 139–143.
14. Garbuz, D.G.; Astakhova, L.N.; Zatsepina, O.G.; Arkhipova, I.R.; Nudler, E.; Evgen'ev, M.B. Functional organization of *hsp70* cluster in camel (*Camelus dromedarius*) and other mammals. *PLoS ONE* **2011,** *6*, 27205.
15. Lanneau, D.; Wettstein, G.; Bonniaud, P.; Garrido, C. Heat Shock Proteins: Cell Protection through Protein Triage. *Sci. World J.* **2010**, *10*, 1543–1552.

16. Sung, Y.Y.; MacRae, T.H. Heat shock proteins and disease control in aquatic organisms. *J. Aquac. Res. Dev. S* **2011**, *2*, doi:10.4172/2155-9546.
17. Wan, T.; Zhou, X.; Chen, G.; An, H.; Chen, T.; Zhang, W.; Liu, S.; Jiang, Y.; Yang, F.; Wu, Y.; et al. Novel heat shock protein HSP70L1 activates dendritic cells and acts as a Th1 polarizing adjuvant. *Blood* **2004**, *103*, 1747–1754.
18. Juo, L.Y.; Liao, W.C.; Shih, Y.L.; Yang, B.Y.; Liu, A.B.; Yan, Y.T. HSPB7 interacts with dimerized FLNC and its absence results in progressive myopathy in skeletal muscles. *J. Cell Sci.* **2016**, *129*, 1661–1670.
19. Dubé, V.; Grigull, J.; DeSouza, L.V.; Ghanny, S.; Colgan, T.J.; Romaschin, A.D.; Siu, K.M. Verification of endometrial tissue biomarkers previously discovered using mass spectrometry-based proteomics by means of immunohistochemistry in a tissue microarray format. *J. Proteom. Res*. **2007**, *6*, 2648–2655.
20. Meyer, A.S.; Gillespie, J.R.; Walther, D.; Millet, I.S.; Doniach, S.; Frydman, J. Closing the folding chamber of the eukaryotic chaperonin requires the transition state of ATP hydrolysis. *Cell* **2003**, *113*, 369–381.
21. Vidyasagar, A.; Wilson, N.A.; Djamali, A. Heat shock protein 27 (HSP27): Biomarker of disease and therapeutic target. *Fibrogenesis Tissue Repair* **2012**, *5*, 7, doi:10.1186/1755-1536-5-7.
22. Li, J.; Qian, X.; Sha, B. Heat shock protein 40: Structural studies and their functional implications. *Protein Pept. Lett.* **2009**, *16*, 606–612.
23. Belles, C.; Kuhl, A.; Nosheny, R.; Carding, S.R. Plasma membrane expression of heat shock protein 60 in vivo in response to infection. *Infect. Immun*. **1999**, *67*, 4191–4200.
24. Tuttle, J.A.; Castle, P.C.; Metcalfe, A.J.; Midgley, A.W.; Taylor, L.; Lewis, M.P. Downhill running and exercise in hot environments increase leukocyte Hsp72 (HSPA1A) and Hsp90α (HSPC1) gene transcripts. *J. App. Physiol.* **2015**, *118*, 996–1005.
25. Krobitsch, S.; Brandau, S.; Hoyer, C.; Schmetz, C.; Hübel, A.; Clos, J. Leishmania donovani heat shock protein 100 characterization and function in amastigote stage differentiation. *J. Biol. Chem*. **1998**, *273*, 6488–6494.
26. Zuo, D.; Subjeck, J.; Wang, X.Y. Unfolding the role of large heat shock proteins: New insights and therapeutic implications. *Front. Immunol*. **2016**, *7*, 75.
27. Van Eden, W.; Van der Zee, R.; Prakken, B. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat. Rev. Immunol.* **2005**, *5*, 318.
28. Wu, Y.; Wan, T.; Zhou, X.; Wang, B.; Yang, F.; Li, N.; Chen, G.; Dai, S.; Liu, S.; Zhang, M.; et al. Hsp70-like Protein 1 fusion protein enhances induction of carcinoembryonic antigen–specific CD8+ CTL response by dendritic cell vaccine. *Cancer Res.* **2005**, *65*, 4947–4954.
29. Colaco, C.A.; Bailey, C.R.; Walker, K.B.; Keeble, J. Heat shock proteins: Stimulators of innate and acquired immunity. *BioMed. Res. Int.* **2013,** *2013*, doi:10.1155/2013/461230.
30. Ahmad, R.; Rasheed, Z.; Ahsan, H. Biochemical and cellular toxicology of peroxynitrite: Implications in cell death and autoimmune phenomenon. *Immunopharmacol. Immunotoxicol.* **2009**, *31*, 388–396.
31. Wong, R.S. Apoptosis in cancer: From pathogenesis to treatment. *J. Exp. Clin. Cancer Res.* **2011**, *30*, 87.
32. Elmore, S. Apoptosis: A review of programmed cell death. *Toxicol. Pathol.* **2007**, *35*, 495–516.
33. Lowe, S.W.; Lin, A.W. Apoptosis in cancer. *Carcinogenesis* **2000**, *21*, 485–495.
34. Beere, H.M. The stress of dying': The role of heat shock proteins in the regulation of apoptosis. *J. Cell Sci.* **2004**, *117*, 2641–2651.
35. Wolf, B.B.; Green, D.R. Suicidal tendencies: Apoptotic cell death by caspase family proteinases. *J. Biol. Chem.* **1999**, *274*, 20049–20052.
36. McIlwain, D.R.; Berger, T.; Mak, T.W. Caspase functions in cell death and disease. *Cold Spring Harb. Perspect. Biol.* **2013**, *5*, a008656.
37. Novara, G.; Galfano, A.; Berto, R.B.; Ficarra, V.; Navarrete, R.V.; Artibani, W. Inflammation, apoptosis, and BPH: What is the evidence? *Eur. Urol. Suppl.* **2006**, *5*, 401–409.
38. Takayama, S.; Reed, J.C.; Homma, S. Heat-shock proteins as regulators of apoptosis. *Oncogene* **2003**, *22*, 9041–9047.
39. Jolly, C.; Morimoto, R.I. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J. Natl. Cancer Inst.* **2000**, *92*, 1564–1572.

40. Leung, A.M.; Redlak, M.J.; Miller, T.A. Role of heat shock proteins in oxygen radical–induced gastric apoptosis. *J. Surg. Res.* **2015**, *193*, 135–144.
41. Bukau, B.; Weissman, J.; Horwich, A. Molecular chaperones and protein quality control. *Cell* **2006**, *125*, 443–451.
42. Garrido, C.; Bruey, J.M.; Fromentin, A.; Hammann, A.; Arrigo, A.P.; Solary, E. HSP27 inhibits cytochrome c-dependent activation of procaspase-9. *FASEB J.* **1999**, *13*, 2061–2070.
43. Rane, M.J.; Pan, Y.; Singh, S.; Powell, D.W.; Wu, R.; Cummins, T.; Chen, Q.; McLeish, K.R.; Klein, J.B. Heat shock protein 27 controls apoptosis by regulating Akt activation. *J. Biol. Chem.* **2003**, *278*, 27828–27835.
44. Ravagnan, L.; Gurbuxani, S.; Susin, S.A.; Maisse, C.; Daugas, E.; Zamzami, N.; Mak, T.; Jäättelä, M.; Penninger, J.M.; Garrido, C.; et al. Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat. Cell Biol.* **2001,** *3*, 839–843.
45. Goto, H.; Yano, S.; Matsumori, Y.; Ogawa, H.; Blakey, D.C.; Sone, S. Sensitization of tumor-associated endothelial cell apoptosis by the novel vascular-targeting agent ZD6126 in combination with cisplatin. *Clin. Cancer Res.* **2004**, *10*, 7671–7676.
46. Samali, A.; Cai, J.; Zhivotovsky, B.; Jones, D.P.; Orrenius, S. Presence of a pre-apoptotic complex of pro-caspase-3, HSP60 and Hsp10 in the mitochondrial fraction of Jurkat cells. *EMBO J.* **1999**, *18*, 2040–2048.
47. Gupta, S.; Knowlton, A.A. Cytosolic heat shock protein 60, hypoxia, and apoptosis. *Circulation* **2002**, *106*, 2727–2733.
48. Lobo, V.; Patil, A.; Phatak, A.; Chandra, N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn. Rev.* **2010**, *4*, 118.
49. Ji, L. Oxidative stress during exercise: Implication of antioxidant nutrients. *Free Radic. Biol. Med.* **1995,** *18*, 1079–1086.
50. Fubini, B.; Hubbard, A. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. *Free Radic. Biol. Med.* **2003**, *34*, 1507–1516.
51. Uttara, B.; Singh, A.V.; Zamboni, P.; Mahajan, R.T. Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. *Curr. Neuropharmacol.* **2009**, *7*, 65–74.
52. Alfadda, A.A.; Sallam, R.M. Reactive oxygen species in health and disease. *BioMed. Res. Int.* **2012**, doi:10.1155/2012/936486.
53. Oyinloye, B.E.; Adenowo, A.F.; Kappo, A.P. Reactive oxygen species, apoptosis, antimicrobial peptides and human inflammatory diseases. *Pharmaceuticals* **2015,** *8*, 151–175.
54. Lushchak, V.I. Free radicals, reactive oxygen species, oxidative stress and its classification. *Chem.-Biol. Int.* **2014**, *224*, 164–175.
55. Finkel, T. Signal transduction by reactive oxygen species. *J. Cell. Biol.* **2011**, *194*, 7–15.
56. Gonzalez, C.; Sanz-Alfayate, G.; Agapito, M.T.; Gomez-Nino, A.; Rocher, A.; Obeso, A. Significance of ROS in oxygen sensing in cell systems with sensitivity to physiological hypoxia. *Respir. Physiol. Neurobiol.* **2002,** *132*, 17–41.
57. Scandalios, J.G. Oxidative stress responses-what have genome-scale studies taught us? *Genome Biol.* **2002,** *3*, 1019–1021.
58. Mittler, R.; Vanderauwera, S.; Suzuki, N.; Miller, G.; Tognetti, V.B.; Vandepoele, K.; Gollery, M.; Shulaev, V.; Van Breusegem, F. ROS signaling: The new wave? *Trends Plant Sci.* **2011,** *16*, 300–309.
59. Coyle, J.T.; Puttfarcken, P. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **1993**, *262*, 689–689.
60. Schneeberger, K.; Czirják, G.Á.; Voigt, C.C. Inflammatory challenge increases measures of oxidative stress in a free-ranging, long-lived mammal. *J. Exp. Biol.* **2013**, *216*, 4514–4519.
61. Lee, J.; Koo, N.; Min, D.B. Reactive oxygen species, aging, and antioxidative nutraceuticals. *Compr. Rev. Food Sci. Food Saf.* **2004**, *3*, 21–33.
62. Hsieh, H.L.; Yang, C.M. Role of redox signaling in neuroinflammation and neurodegenerative diseases. *BioMed. Res. Int.* **2013**, *2013*, doi:10.1155/2013/484613.
63. Mayer, M.P.; Bukau, B. Hsp70 chaperones: Cellular functions and molecular mechanism. *Cell. Mol. Life Sci.* **2005**, *62*, 670.

64. Jacquier-Sarlin, M.R.; Fuller, K.; Dinh-Xuan, A.T.; Richard, M.J.; Polla, B.S. Protective effects of hsp70 in inflammation. *Cell. Mol. Life Sci.* **1994**, *50*, 1031–1038.
65. Trott, A.; West, J.D.; Klaić, L.; Westerheide, S.D.; Silverman, R.B.; Morimoto, R.I.; Morano, K.A. Activation of heat shock and antioxidant responses by the natural product celastrol: Transcriptional signatures of a thiol-targeted molecule. *Mol. Biol. Cell.* **2008,** *19*, 1104–1112.
66. Wu, C-W.; Biggar, K.K.; Zhang, J.; Tessier, S.N.; Pifferi, F.; Perret, M.; Storey, K.B. Induction of antioxidant and heat shock protein responses during torpor in the gray mouse lemur, Microcebus murinus. *Genom. Proteom. Bioinform.* **2015**, *13*, 119–126.
67. Ashley, N.T.; Weil, Z.M.; Nelson, R.J. Inflammation: Mechanisms, costs, and natural variation. *Ann. Rev. Ecol. Evol. Syst.* **2012**, *43*, 85–406.
68. Ferrero-Miliani, L.; Nielsen, O.H.; Andersen, P.S.; Girardin, S.E. Chronic inflammation: Importance of NOD2 and NALP3 in interleukin-1β generation. *Clin. Exp. Immunol.* **2007**, *147*, 227–235.
69. Villar, J.; Blanco, J.; Añón, J.M.; Santos-Bouza, A.; Blanch, L.; Ambrós, A.; Gandía, F.; Carriedo, D.; Mosteiro, F.; Basaldúa, S.; et al. The ALIEN study: Incidence and outcome of acute respiratory distress syndrome in the era of lung protective ventilation. *Intensive Care Med.* **2011,** *37*, 1932–1941.
70. Ware, L.B.; Matthay, M.A. The acute respiratory distress syndrome. *N. Engl. J. Med.* **2000**, *342*, 1334–1349.
71. Rubenfeld, G.D.; Caldwell, E.; Peabody, E.; Weaver, J.; Martin, D.P.; Neff, M.; Stern, E.J.; Hudson, L.D. Incidence and outcomes of acute lung injury. *N. Engl. J. Med.* **2005**, *353*, 1685–1693.
72. Slutsky, A.S. Hot new therapy for sepsis and the acute respiratory distress syndrome. *J. Clin. Invest.* **2002**, *110*, 737.
73. González-Reimers, E.; Santolaria-Fernández, F.; Martín-González, M.C.; Fernández-Rodríguez, C.M.; Quintero-Platt, G. Alcoholism: A systemic proinflammatory condition. *World J. Gastroenterol.* **2014**, *20*, 14660.
74. Hirsh, M.I.; Junger, W.G. Roles of heat shock proteins and γδT cells in inflammation. *Am. J. Respir. Cell Mol. Biol.* **2008**, *39*, 509–513.
75. Weiss, Y.G.; Bromberg, Z.; Raj, N.; Raphael, J.; Goloubinoff, P.; Ben-Neriah, Y.; Deutschman, C.S. Enhanced heat shock protein 70 expression alters proteasomal degradation of IκB kinase in experimental acute respiratory distress syndrome. *Crit. Care Med.* **2007**, *35*, 2128–2138.
76. Chu, E.K.; Ribeiro, S.P.; Slutsky, A.S. Heat stress increases survival rates in lipopolysaccharide-stimulated rats. *Crit. Care Med.* **1997**, *25*, 1727–1732.
77. Bromberg, Z.; Raj, N.; Goloubinoff, P.; Deutschman, C.S.; Weiss, Y.G. Enhanced expression of 70-kilodalton heat shock protein limits cell division in a sepsis-induced model of acute respiratory distress syndrome. *Crit. Care Med.* **2008**, *36*, 246.
78. Smolen, J.S.; Breedveld, F.C.; Burmester, G.R.; Bykerk, V.; Dougados, M.; Emery, P.; Kvien, T.K.; Navarro-Compán, M.V.; Oliver, S.; Schoels, M.; et al. Treating rheumatoid arthritis to target: 2014 update of the recommendations of an international task force. *Ann. Rheum. Dis.* **2016,** *75*, 3–15.
79. Aletaha, D.; Neogi, T.; Silman, A.J.; Funovits, J.; Felson, D.T.; Bingham, C.O.; Birnbaum, N.S.; Burmester, G.R.; Bykerk, V.P.; Cohen, M.D.; et al. 2010 rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum.* **2010**, *62*, 2569–2581.
80. Klareskog, L.; Malmström, V.; Lundberg, K.; Padyukov, L.; Alfredsson, L. Smoking, citrullination and genetic variability in the immunopathogenesis of rheumatoid arthritis. In *Seminars in Immunol*; Academic Press: 2011; Volume 23, pp. 92–98.
81. Millar, K.; Lloyd, S.M.; McLean, J.S.; Batty, G.D.; Burns, H.; Cavanagh, J.; Deans, K.A.; Ford, I.; McConnachie, A.; McGinty, A.; et al. Personality, socio-economic status and inflammation: Cross-sectional, population-based study. *PLoS ONE* **2013**, *8*, 58256.
82. Callahan, L.F.; Pincus, T. Education, self-care, and outcomes of rheumatic diseases: Further challenges to the "biomedical model" paradigm. *Arthritis Rheum.* **1997**, *10*, 283–288.
83. Schett, G.; Redlich, K.; Xu, Q.; Bizan, P.; Gröger, M.; Tohidast-Akrad, M.; Kiener, H.; Smolen, J.; Steiner, G. Enhanced expression of heat shock protein 70 (HSP70) and heat shock factor 1 (HSF1) activation in rheumatoid arthritis synovial tissue. Differential regulation of HSP70 expression and hsf1 activation in

synovial fibroblasts by proinflammatory cytokines, shear stress, and antiinflammatory drugs. *J. Clin. Invest.* **1998**, *102*, 302–311.
84. Kang, E.H.; Kim, D.J.; Lee, E.Y.; Lee, Y.J.; Lee, E.B.; Song, Y.W. Downregulation of heat shock protein 70 protects rheumatoid arthritis fibroblast-like synoviocytes from nitric oxide-induced apoptosis. *Arthritis Res. Ther.* **2009**, *11*, 130.
85. Van Roon, J.A.; van Eden, W.; van Roy, J.L.; Lafeber, F.J.; Bijlsma, J.W. Stimulation of suppressive T cell responses by human but not bacterial 60-kD heat-shock protein in synovial fluid of patients with rheumatoid arthritis. *J. Clin. Invest.* **1997**, *100*, 459–463.
86. Pockley, A.G. Heat shock proteins as regulators of the immune response. *Lancet* **2003**, *362*, 469–476.
87. Anderton, S.M.; Van Der Zee, R.; Prakken, B.; Noordzij, A.; Van Eden, W. Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *J. Exp. Med.* **1995**, *181*, 943–952.
88. Kaul, G.; Thippeswamy, H. Role of heat shock proteins in diseases and their therapeutic potential. *Indian J. Microbiol.* **2011**, *51*, 124–131.
89. Salinthone, S.; Ba, M.; Hanson, L.; Martin, J.L.; Halayko, A.J.; Gerthoffer, W.T. Overexpression of human Hsp27 inhibits serum-induced proliferation in airway smooth muscle myocytes and confers resistance to hydrogen peroxide cytotoxicity. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2007**, *293*, 1194–1207.
90. Lazaar, A.L.; Panettieri, R.A. Airway smooth muscle as a regulator of immune responses and bronchomotor tone. *Clin. Chest Med.* **2006**, *27*, 53–69.
91. Chiappara, G.; Gagliardo, R.; Siena, A.; Bonsignore, M.R.; Bousquet, J.; Bonsignore, G.; Vignola, A.M. Airway remodelling in the pathogenesis of asthma. *Curr. Opin. Allergy Clin. Immunol.* **2001**, *1*, 85–93.
92. Feigin, V. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: A systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **2016**, *388*, 1459–1544.
93. Vos, T.; Allen, C.; Arora, M.; Barber, R.M.; Bhutta, Z.A.; Brown, A.; Carter, A.; Casey, D.C.; Charlson, F.J.; Chen, A.Z.; et al. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015: A systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **2016**, *388*, 1545–1602.
94. Wolff, P.T.; Arison, L.; Rahajamiakatra, A.; Raserijaona, F.; Niggemann, B. High asthma prevalence and associated factors in urban malagasy schoolchildren. *J. Asthma* **2012**, *49*, 575–580.
95. Adeloye, D.; Chan, K.Y.; Rudan, I.; Campbell, H. An estimate of asthma prevalence in Africa: A systematic analysis. *Croatian Med. J.* **2013**, *54*, 519–531.
96. Bertorelli, G.; Bocchino, V.; Zhuo, X.; Chetta, A.; Del Donno, M.; Foresi, A.; Testi, R.; Olivieri, D. Heat shock protein 70 upregulation is related to HLA-DR expression in bronchial asthma. Effects of inhaled glucocorticoids. *Clin. Exp. Allergy* **1998**, *28*, 551–560.
97. Changchun, H.; Haijin, Z.; Wenjun, L.; Zhenyu, L.; Dan, Z.; Laiyu, L.; Wancheng, T.; Shao-Xi, C.; Fei, Z. Increased heat shock protein 70 levels in induced sputum and plasma correlate with severity of asthma patients. *Cell Stress Chaperones* **2011**, *16*, 663–671.
98. Vignola, A.M.; Chanez, P.; Polla, B.S.; Vic, P.; Godard, P.; Bousquet, J. Increased expression of heat shock protein 70 on airway cells in asthma and chronic bronchitis. *Am. J. Respir. Cell. Mol. Biol.* **1995**, *13*, 683–691.
99. Polla, B.S.; Bachelet, M.; Dall'Ava, J.; Vignola, A.M. Heat shock proteins in inflammation and asthma: Dr Jekyll or Mr Hyde? *Clin. Exp. Allergy* **1998**, *28*, 527–529.
100. Aron, Y.; Busson, M.; Polla, B.S.; Dusser, D.; Lockhart, A.; Swierczewski, E.; Favatier, F. Analysis of HSP70 gene polymorphism in allergic asthma. *Allergy* **1999**, *54*, 165–170.
101. Wong, H.R.; Wispe, J.R. The stress response and the lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **1997**, *273*, L1–L9.
102. Shingai, R.; Maeda, T.; Onishi, S.; Yamamoto, Y. Autonatibody against 70 kD heat shock protein in patients with autoimmune liver diseases. *J. Hepatol.* **1995**, *23*, 382–390.
103. Xu, Q.; Kiechl, S.; Mayr, M.; Metzler, B.; Egger, G.; Oberhollenzer, F.; Willeit, J.; Wick, G. Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis. *Circulation* **1999**, *100*, 1169–1174.
104. Salman, A.N. Serum Levels Evaluation of Heat Shock Protein70 during Gestation and Fetal Birth Weight in Asthmatic Women of Thi-QAR Province, Iraq. *J Al-Nahrain Univ.* **2015,** *18*, 117–122.

105. Schatz, M. Is maternal asthma a life or death issue for the baby? *Thorax* **2009**, *64*, 93–95.
106. Yang, M.; Wu, T.; Cheng, L.; Wang, F.; Wei, Q.; Tanguay, R.M. Plasma antibodies against heat shock protein 70 correlate with the incidence and severity of asthma in a Chinese population. *Respir. Res.* **2005**, *6*, doi:10.1186/1465-9921-6-18.
107. PeRIšIć, T.; Srećković, M.; Matić, G. Changes of antioxidant enzyme activity and heat shock protein content in lymphocytes of children with asthma. *Arch. Biol. Sci.* **2007**, *59*, 257–266.
108. Tong, W.; Luo, W. Heat shock proteins' mRNA expression in asthma. *Respirology* **2000**, *5*, 227–230.
109. Karin, M.; Greten, F.R. NF-[kappa] B: Linking inflammation and immunity to cancer development and progression. *Nat. Rev. Immunol.* **2005**, *5*, 749–759.
110. Torre, L.A.; Bray, F.; Siegel, R.L.; Ferlay, J.; Lortet-Tieulent, J.; Jermal, A. Global cancer statistics 2012. *CA Cancer J. Clin.* **2015**, *65*, 87–108.
111. Hanahan, D.; Weinberg, R.A. The hallmarks of cancer. *Cell* **2000**, *100*, 57–70.
112. Calderwood, S.K.; Stevenson, M.A.; Murshid, A. Heat shock proteins, autoimmunity, and cancer treatment. *Autoimm. Dis.* **2012**, *2012*, 486069.
113. Kapoor, C.; Vaidya, S. Heat shock protein (HSP) and cancer: An overview. *Am. J. Med. Dent. Sci.* **2013**, *1*, 31–34.
114. Beere, H.M.; Green, D.R. Stress management–heat shock protein-70 and the regulation of apoptosis. *Trends Cell Biol.* **2001**, *11*, 6–10.
115. Wu, J.; Liu, T.; Rios, Z.; Mei, Q.; Lin, X.; Cao, S. Heat shock proteins and cancer. *Trends Pharmacol. Sci.* **2017**, *38*, 226–256.
116. Vergara, D.; Simeone, P.; del Boccio, P.; Toto, C.; Pieragostino, D.; Tinelli, A.; Acierno, R.; Alberti, S.; Salzet, M.; Giannelli, G.; et al. Comparative proteome profiling of breast tumor cell lines by gel electrophoresis and mass spectrometry reveals an epithelial mesenchymal transition associated protein signature. *Mol. BioSyst.* **2013**, *9*, 1127–1138.
117. Shiota, M.; Bishop, J.L.; Nip, K.M.; Zardan, A.; Takeuchi, A.; Cordonnier, T.; Beraldi, E.; Bazov, J.; Fazli, L.; Chi, K.; et al. Hsp27 regulates epithelial mesenchymal transition, metastasis, and circulating tumor cells in prostate cancer. *Cancer Res.* **2013**, *73*, 3109–3119.
118. Lianos, G.D.; Alexiou, G.A.; Mangano, A.; Mangano, A.; Rausei, S.; Boni, L.; Dionigi, G.; Roukos, D.H. The role of heat shock proteins in cancer. *Cancer Lett.* **2015**, *360*, 114–118.
119. Jego, G.; Hazoumé, A.; Seigneuric, R.; Garrido, C. Targeting heat shock proteins in cancer. *Cancer Lett.* **2013**, *332*, 275–285.
120. Chaiwatanasirikul, K.A.; Sala, A. The tumour-suppressive function of CLU is explained by its localisation and interaction with HSP60. *Cell Death Dis.* **2011,** *2*, e219.
121. Ghosh, J.C.; Siegelin, M.D.; Dohi, T.; Altieri, D.C. Heat shock protein 60 regulation of the mitochondrial permeability transition pore in tumor cells. *Cancer Res.* **2010**, *70*, 8988–8993.
122. Chalmin, F.; Ladoire, S.; Mignot, G.; Vincent, J.; Bruchard, M.; Remy-Martin, J.P.; Boireau, W.; Rouleau, A.; Simon, B.; Lanneau, D.; et al. Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *J. Clin. Invest.* **2010**, *120*, 457–471.
123. Trinh, D.L.; Elwi, A.N.; Kim, S.W. Direct interaction between p53 and Tid1 proteins affects p53 mitochondrial localization and apoptosis. *Oncotarget* **2010**, *1*, 396–404.
124. Spooner, R.; Yilmaz, Ö. The role of reactive-oxygen-species in microbial persistence and inflammation. *Int. J. Mol. Sci.* **2011**, *12*, 334–352.
125. Simon, H.U.; Haj-Yehia, A.; Levi-Schaffer, F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* **2000**, *5*, 415–418.
126. Fischer, R.; Maier, O. Interrelation of oxidative stress and inflammation in neurodegenerative disease: Role of TNF. *Oxid. Med. Cell. Longev.* **2015**, *2015*, 610813.
127. Nowsheen, S.; Yang, E.S. The intersection between DNA damage response and cell death pathways. *Exp. Oncol.* **2012**, *34*, 243–254.

128. Nita, M.; Grzybowski, A. The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 3164734.
129. Barnes, P.J. Reactive oxygen species and airway inflammation. *Free Radic. Biol. Med.* **1990**, *9*, 235–243.
130. Henricks, P.A.; Nijkamp, F.P. Reactive oxygen species as mediators in asthma. *Pulm. Pharmacol. Ther.* **2001**, *14*, 409–421.
131. Winrow, V.R.; McLean, L.; Morris, C.J.; Blake, D.R. The heat shock protein response and its role in inflammatory disease. *Ann. Rheum. Dis.* **1990**, *49*, 128–132.
132. Chen, Y.; Voegeli, T.S.; Liu, P.P.; Noble, E.G.; Currie, R.W. Heat shock paradox and a new role of heat shock proteins and their receptors as anti-inflammation targets. *Inflamm. Allergy Drug Targets* **2007**, *6*, 91–100.
133. Hauet-Broere, F.; Wieten, L.; Guichelaar, T.; Berlo, S.; Van der Zee, R.; Van Eden, W. Heat shock proteins induce T cell regulation of chronic inflammation. *Ann. Rheum. Dis.* **2006**, *65*, 65–68.
134. Tóth, M.E.; Gombos, I.; Sántha, M. Heat shock proteins and their role in human diseases. *Acta Biol. Szeged.* **2015**, *59*, 121–141.
135. Arnal, M.E.; Lallès, J.P. Gut epithelial inducible heat-shock proteins and their modulation by diet and the microbiota. *Nutr. Rev.* **2016**, *74*, 181–197.
136. Goldstein, M.G.; Li, Z. Heat-shock proteins in infection-mediated inflammation-induced tumorigenesis. *J. Hematol. Oncol.* **2009**, *2*, doi:10.1186/1756-8722-2-5.
137. Hatfield, P.D.; Lovas, S. Role of Hsp70 in cancer growth and survival. *Protein Pept. Lett.* **2012**, *19*, 616–624.
138. Chauhan, D.; Li, G.; Hideshima, T.; Podar, K.; Mitsiades, C.; Mitsiades, N.; Catley, L.; Tai, Y.T.; Hayashi, T.; Shringarpure, R.; et al. Hsp27 inhibits release of mitochondrial protein Smac in multiple myeloma cells and confers dexamethasone resistance. *Blood* **2003**, *102*, 3379–3386.
139. Nylandsted, J.; Rohde, M.; Brand, K.; Bastholm, L.; Elling, F.; Jäättelä, M. Selective depletion of heat shock protein 70 (HSP70) activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 7871–7876.
140. Zininga, T.; Shonhai, A. Are heat shock proteins druggable candidates? *Am. J. Biochem. Biotech.* **2014,** *8*, 427–432.
141. Henstridge, D.C.; Febbraio, M.A.; Hargreaves, M. Heat shock proteins and exercise adaptations. Our knowledge thus far and the road still ahead. *J. Appl. Physiol.* **2016**, *120*, 683–691.s
142. Noble, E.G.; Shen, G.X. Impact of exercise and metabolic disorders on heat shock proteins and vascular inflammation. *Autoimmune Dis.* **2012**, *2012*, 836519.
143. Naughton, L.; Lovell, R.; Madden, L. Heat shock proteins in exercise: A review. *J. Exerc. Sci. Physiother.* **2006**, *2*, 13–26.

CHAPTER THREE

Interplay between Heat Shock Proteins, Inflammation and Cancer: A Potential Cancer Therapeutic Target

Accepted Manuscript: American Journal of Cancer Research 2019; 9(1): XXX-XXX

www.ajcr.us /ISSN:2156-6976/ajcr0090226

Review Article

Interplay between heat shock proteins, inflammation and cancer: a potential cancer therapeutic target

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Received December 21, 2018; Accepted December 28, 2018; Epub XXX, 2019; Published XXX, 2019

Abstract: The historical relationship between cancer and inflammation has long been evaluated, and dates back to the early work of Virchow (1863), where he hypothesised that chronic inflammation as a direct cause of tissue injury and infection, could actually promote tissue proliferation. At that period in time however, the exact mechanisms that mediated this relationship were little understood. Subsequent studies have since then demonstrated that chronic inflammation plays significant roles in microenvironments, mostly in the progression of tumours, probably, through over-secretion of proinflammatory cytokines and other immune-killing apparatus such as reactive oxygen species (ROS) which cause damage to normal cells leading to DNA damage and increased cellular mutation rates. Recently, the identification of DNA lesion 5-chlorocytosine (5-CIC) created by hypochlorous acid (HOCl) secreted to nullify or kill infectious agents and toll-like receptor 4 (TLR4)-mediated chronic inflammation in the human gut, has become the latest evidence linking inflammation directly to cancer. The key to cellular survival and adaptation under unfavour- able or pathological conditions is the expression of heat shock proteins (HSPs) also called molecular chaperones. These proteins play essential roles in DNA repair processes by maintaining membrane integrity, orderliness and sta- bility of client proteins that play prominent roles in DNA repair mechanisms. More so, HSPs have also been shown to modulate the effects of pro-inflammatory/apoptotic cytokines through the inhibition of cascades leading to the gen- eration of ROS-mediated DNA damage, while promoting the DNA repair mechanism, thus playing prominent roles in various stages of DNA repair and cancer progression. Hence, studies targeting HSPs and their inhibitors in inflam-mation, DNA damage, and repair, could improve current cancer therapeutic efficiency. Here the focus will be on the relationship between HSPs, inflammation and cancer, as well as roles of HSPs in DNA damage response (DDR).

Keywords: Heat shock proteins, inflammation, cancer, DNA damage response, 5-chlorocytosine, hypochlorous acid

Heat Shock Proteins

Due to continuous exposure to cellular stressors (e.g. heat, heavy metal stress, viral and bacterial infections, nutrient deficiency, exercise, oxidative stress, cold, acute or chronic inflammatory diseases, ischemia injury, drought, UV-light), cells frequently produce a family of evolutionary conserved proteins termed heat shock proteins (HSPs) or molecular chaperones [1, 2]. These proteins enable cell survival under pathological conditions, metabolic changes and environmental adaptation, which may lead to protein aggregation and denaturation thus resulting in several neurodenerative disorders such as motor neuron, frontal temporal lobar degeneration, Alzheimer's, Huntington's and Parkinson's diseases, as well as aging and cancer [3-5]. Although most of these polypeptides are generally expressed upon thermal induction and stress, some that are involved in the folding of nascent proteins, signal transduction and translocation of organelles across cellular membranes,

are constitutively expressed under physiological states and are termed heat shock cognate (Hsc) proteins [6-8]. These constitutively expressed HSPs perform important 'housekeeping' activities, which are critical in maintaining cellular homeostasis [9].

HSPs were first identified in the salivary glands of *Drosophila* larvae by Ritossa in the early 1960s and are broadly categorized into two main groups: high molecular weight and small heat shock proteins. High molecular weight HSPs (hHSPs) range from 40 to 110 kDa, (including HSPs 110, 100, 90, 70 and 60), and are ATP-dependent. They bind nascent and non-native proteins, and actively refold them into their 3-D structure. They also direct irreparable proteins to proteasomal degradation [9]. Small molecular weight HSPs (sHSPs) on the other hand, range from 8 to 30 kDa (including HSPs 40, 27, 15, 10 and ubiquitin), and are ATP-independent. These proteins, especially those which are encoded by *HSPB* genes, are structurally classified by flanking C-and N-terminal residues characterized by the existence of well-preserved α-crystallin and β-sandwich domains [10]. They are involved in the refolding of misfolded peptides, degradation of irreversible proteins and prevention of stress labile polypeptide aggregations [11]. More so, these proteins have been shown to be involved in complex immune responses to diseases such as cancer, as well as participate in the modulation of several inflammatory cascades, especially in the microenvironment [10].

Inflammation

Inflammation, forms part of the non-specific immune system and is the first line of defense in response to all forms of cellular injuries (e.g. viral or bacterial infections, toxins and chemicals) and is identified normally by heat, pain, redness, and swelling in the inflamed area [12]. Inflammatory response clears cellular damage caused by cellular injuries and initiates cellular repair and healing. It can be broadly classified into two: acute and chronic inflammation. Acute inflammation is a short-term and a self-limiting response that occurs under physiological conditions. It is thought to be beneficial to the cellular system when short-lived, but if long-termed and uncontrolled, it could degenerate into chronic inflammation characterized by increased inflammatory mediators, acute-phase reactants, inflammatory signalling pathway activation and increased production of abnormal cytokines [13]. Chronic inflammation leads to the destruction of several healthy cells and tissues, and in most cases, degenerates into organ failure and mortality, as observed in several human inflammatory diseases (HIDs) and cancer [14]. The major causes of chronic inflammation are currently still a topic of debate. Recent studies, however, have shown that inflammatory instigators such as microbial infections or bodily injuries are not the only causes. Hormonal imbalance and malfunctioned tissues caused by complications from other human diseases such as aging, HIDs and cancer also play a major role [15, 16].

Inflammatory response is normally mediated by proinflammatory signalling molecules as well as the secretion of proteins called cytokines. Cytokines are a large group of molecular proteins that are secreted normally by the Helper T-cells and macrophages. These molecules mediate specific interactions and communications between immune cells thus, stimulating inflammatory reactions and immune responses [17]. Generally, cytokines are named according to their mode of action and/or site of production; examples include interleukins produced by leukocytes, lymphokines produced by lymphocytes, chemokines produced to play chemotactic roles, monokines produced by monocytes and interferons [17, 18]. Interleukins (ILs) are the minute component of cytokines

immunomodulatory proteins secreted by one leukocyte to act on neighbouring leukocytes, therefore acting as "second molecular messengers" which activate these cells during inflammation and immune response [19]. Unlike other amino acids and steroidal-derived hormones which act on the endocrine system, interleukins act in an autocrine or paracrine manner due to their effects on neighbouring cells. ILs can also act as both pro- and anti-inflammatory stimulators and have been reported to modulate immune cell activation, growth, and differentiation during inflammatory reactions, infection and regulation of neuronal proteins, cell injury and invasion [20]. Those ILs which can stimulate inflammation and immune response under both physiological and pathological conditions are grouped into classes (IL1-IL17) according to their common structural elements and peptide sequence conservation. This makes them ideal therapeutic targets in several human diseases [18].

Cancer

Cancer is a group of diseases caused by uncontrolled cell growth, proliferation and invasion, and has the ability to metastasize to other parts of the body through the bloodstream and lymphatic nervous system [21]. Despite improvements in treatment modalities, this disease persists as one of the leading global health threats in both developed and developing countries. In the United States alone, cancer accounts for higher mortality rate more than heart diseases in individuals below the age of 85 years [22]. Furthermore, statistical and epidemiological studies compiled by the National Centre for Health Statistics (NCHS), National Cancer Institute (NCI), North American Association of Central Cancer Registries (NAACCR) and Centres for Disease Control and Prevention (CDC) have shown that 1 in every 4 deaths was as a result of cancer and 1,529,560 new cancer cases and 569,490 deaths were predicted to occur in 2010 [22].

Cancer can be broadly classified according to the tumour cells' originality; these include carcinoma, leukaemia, sarcoma, blastoma and germ cell tumour [23]. Carcinomas originate from epithelial cells such as colon, lung, breast, pancreas and prostate, and are most common in adults [24]**.** Leukaemia develops from the cell lining of the blood vessels, and they account for almost 30% of cancer cases in young children [25]. Sarcoma develops from connecting tissues outside the bone such as cartilage, nerve, fat and bone. Blastoma originates from immature cells or embryos and is more common in children than in adults [26]. Germ cell tumours originate from the gonads, testes and the ovaries [27].

Cancer and Inflammation

Genetic mutations, environmental factors (e.g. ultraviolet radiation, air pollution, ionizing radiation, cancer-causing chemicals and occupational exposures), lifestyle (e.g. smoking, lack of exercise, imbalanced diet and excess alcohol consumption) and hormonal imbalance, have emerged as the major sources of cancer morbidity and mortality worldwide [28, 29]. Although bacterial and viral infections such as human papillomavirus (HPV) are now gaining momentum, persistence chronic inflammation resulting from long-term invasion of infectious (e.g. *Helicobacter pylori*) and non-infectious (e.g. chronic pancreatitis, esophagitis, Barrett's and metaplasia) agents contribute immensely towards the development of several cancer types [30]. In fact, it is estimated that nearly 25% of all cancer cases are connected to chronic inflammation due to infectious or physico-chemical agents, with persistence *H. pylori* and hepatitis A and C promoting the risk of stomach and hepatocellular

carcinoma, respectively [31]. This could be due to improper stimulation and regulation of inflammatory cytokines, as well as immune cells leading to DNA damage and mutation of oncogenes, which in turn leads to tumour development. An adequate stimulation of inflammatory response can lead to quick clearance of the immune cell apparatus and inflammatory cytokines (e.g. chemokines, interleukins) via phagocytosis and apoptosis, immediately after acute inflammation [32]. The phagocytosis of apoptotic factors by phagocytes further supports anti-apoptotic action by promoting the production of anti-apoptotic transforming growth factor-β (TGF-β). Transforming growth factor-β in complex with a serine/threonine kinase binds to TGF-β receptors to trigger signalling cascades that regulate chemotaxis, cell differentiation, proliferation and several immune cell activators resulting in the inhibition of cancer progression [33, 34]. However, inadequate regulation of inflammatory response results in chronic inflammation characterized by massive cellular and tissue destruction and neoplastic transformation, which may lead to cancer progression and other inflammatory diseases [35, 36].

Link between HSPs, Inflammation and Cancer: A Potential Cancer Therapeutic Target

The link between chronic inflammation and cancer has long been an area of debate. Nevertheless, the precise mechanisms by which persistence inflammation leads to cancer are now beginning to unfold. A DNA lesion or site of structural damage in the DNA called 5-chlorocytosine (5-CIC) created by inflammatory cells during the inflammatory response, has been identified as a major DNA mutant that links chronic inflammation to cancer. In response to infectious agents, biological systems stimulate the synthesis of the immune apparatus referred to as ”battery of chemical warfare” with the aim of killing infectious agents and initiating cellular repair. These immune apparatus includes T-and B-lymphocytes, leukocytes, interleukins, cytokines, chemokines, reactive nitrogen species (RNS) (e.g. hydrogen peroxide, hypochlorous acid, and nitric oxide) and ROS. The reactive oxygen molecules designed to nullify the effects of invading pathogens can also in the process cause collateral damage to normal healthy cells resulting in irreparable DNA damage and mutation [37].

For instance, hypochlorous acid (HOCl) generated from the oxidative reaction between H_2O_2 and chloride ions (Cl^-) catalysed by the heme enzyme myeloperoxidase, plays major roles in the killing of foreign pathogens [38]. However, when in excess and under acidic conditions, this molecule is capable of chlorinating cytosine on the DNA strand to form 5-chlorocytosine, resulting in a lesion seen at the inflammatory site and present during inflammatory-related diseases such as atherosclerosis [39, 40]. Furthermore, because 5-chlorocytosine is relatively unstable, its conversion to 5-chlorouracil either spontaneously or through enzymatic deamination, causes irreparable damages and mutations in DNA which can lead to cancer [41, 42].

The other mechanism directly linking inflammation to cancer is the incidence of toll-like receptor 4 (TLR4) expressions in colon cancer [43]. TLR4 plays a significant role in activating the immune system, inflammatory response, as well as clearance of invading pathogens; however, this receptor can also sustain chronic inflammation, for example, the TLR4 response to bacteria found in the human gut, due to frequent food intake. These bacteria constantly gets replenished, therefore, cannot be completely cleared by the immune response. In this way, TLR4 can sustain persistent chronic inflammation, which is not only involved in cancer formation but also promotes different phases of cancer progression [44].

Moreover, one of the key factors of cellular survival under stressful conditions is the expression of highly regulated and conserved proteins termed HSPs. These proteins are upregulated in response to stress signals such as inflammation and the onset of tumours. Therefore, it is not surprising that these proteins are elevated in every facet of cancer development and inflammatory reactions. According to recent studies, HSPs form part of an immune-inflammatory complex that responds to infectious agents, cellular damage as well as DNA breaks [16]. The DNA is frequently subjected to lesion-inducing agents, it exogenous (genotoxic chemicals, UV or ionizing radiation) or endogenous (reactive oxidants), which subsequently leads to the generation of several DNA lesions including 5-CIC, double-stranded breaks, single-stranded breaks, and single base modifications. In the case where these errors are not properly repaired, they may lead to genome instability which increases the risk of oncogenesis, as a result, multi-dangerous gene mutations [45]. The evidence of heat shock proteins in reducing mutation frequency in *Saccharomyces cerevisiae* responding to elevated temperature, as well as the elevation of HSP70 in glioblastoma cell lines induced by UV or γ-rays interaction with p53, may however suggest their functions in apoptosis, cell cycle control and DNA repair processes [46, 47]. Furthermore, HSP70 and HSP27 in particularly have demonstrated association with base excision repair (BER) enzymes such as human AP endonuclease (APE1) and uracil DNA glycosylase (UDG). This observation suggests the significant roles of HSPs in DNA repair mechanisms, as well as presenting HSPs as modulators of certain DNA repair systems; and since most therapies targeting cancer exert their action by disrupting the normal integrity, structure, and functions of DNA, proper DNA repair mechanisms mediated by HSPs are therefore crucial in maintaining genome stability, integrity and orderliness, which may increase cancer therapeutics efficiency [48, 49].

Interestingly, scientific evidence exist showing that HSPs interact with and stabilize key client proteins that play crucial roles in DNA damage response (DDR) mechanisms and cancer progression. DDR mechanisms refer to pathways that are involved in cell-cycle checkpoints, activation of signalling networks, detection of DNA damage, and induction of cell death or DNA repair [50]. The mechanisms that are involved in the DNA repair processes include base excision repair (BER), direct DNA damage reversal and nuclear excision repair (NER). The ability of DDR to respond to DNA damage is controlled by the balancing act between client protein synthesis and their degradation mediated by HSPs. HSP90 in particular binds to DDR proteins and prevent them from E3 ubiquitin-CHIP (chaperoning) mediated ubiquitination and degradation [49]. These proteins include checkpoint kinase 1 (CHKI), O^6-meG-DNA methyltransferase (MGMT), MLH1 and MLH2, and APE1, among others.

Emerging evidence has shown that cells inability to repair DNA damage caused by stress has been associated with the loss of genomic integrity, organisation, structure, and function, which may increase the rate of tumour development [51]. These findings reveal the significant roles of HSPs in genetic stability, DDR and tumour progression [52]. Moreover, the roles of HSPs in inflammation cascades have been well elucidated. According to Ikwegbue and co-workers, HSPs exert cytoprotection against inflammatory reactions through the modulation of inflammation cascades that lead to the activation of pro-inflammatory cytokines such as TNF-α, thereby attenuating chronic inflammation [16]. So far there is no study linking HSPs directly to 5-CIC lesion and DDRs, but it can be hypothesised that a similar mechanism observed in other DNA damages and DDRs is followed. Based on these findings, it is tempting to propose that any study that is targeting HSPs (especially HSP90) and

their inhibitors in membrane and genetic stability, as well as inflammation, could give new mechanistic insight and may serve as a promising target to improve cancer therapeutics efficacy as proposed in the model (Figure 1).

Conclusion

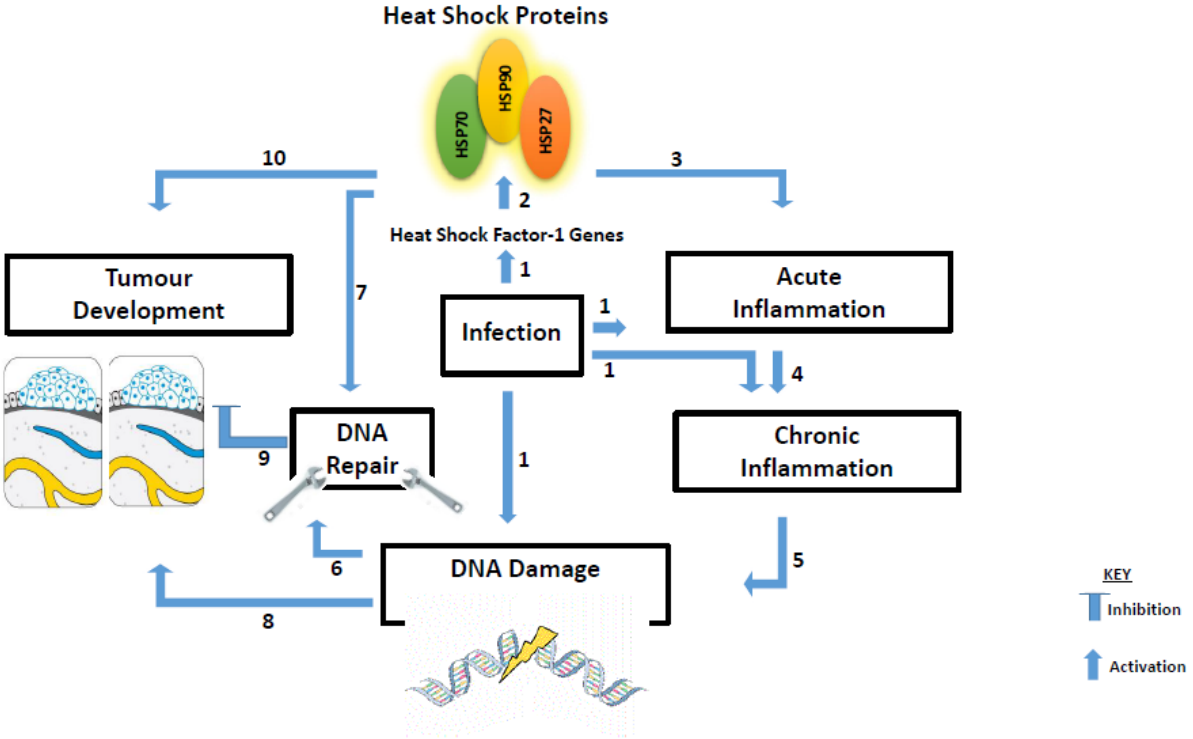
Tremendous efforts have been made over the years in devising strategies to prevent, manage, cure and even vaccinate against cancer, yet with several unsatisfactory results. More so, recent studies have focussed on testing therapeutic efficacy of HSPs and HSP inhibitors in cells and animal models, while other efforts are focussed on conducting clinical trials using HSPs in different stages of cancer development. However, any study aimed at understanding the functions of excess HOCl during inflammatory response, and the roles 5-CIC lesion (created by HOCl) which links chronic inflammation to DNA damage, and crucial roles of HSPs in maintaining the integrity and stability of the proteins that are involved in DNA repair processes, could be promising targets in improving current cancer therapies. Based on this promising aspect of HSPs and its inhibitors in inflammation, DNA repair, and cancer, one can therefore be optimistic that improved therapies and a cure for cancer is to be expected soon.

Acknowledgments: Abidemi Paul Kappo is sincerely grateful to the National Research Foundation (NRF), South Africa for a Thuthuka Research Grant and to the University of Zululand Research Committee for funding (Grant No: C387). Paul Ikwegbue and Priscilla Masamba are equally thankful to the National Research Foundation (NRF), South Africa for postgraduate bursaries towards their studies. Londiwe Mbatha acknowledges the University of Zululand Research Committee for a Postdoctoral Research Fellowship, while Babatunji Oyinloye is grateful to Afe Babalola University for an educational advancement scholarship.

Conflicts of Interest: None declared.

Figure Legend

Figure 3.1: Proposed model showing biological cooperativity between heat shock proteins and inflammation as a promising target towards improved cancer therapeutics. (1) The presence of infection causes the transcription of the heat factor -1 (HSF) gene, which enhances the synthesis of heat shock proteins (HSPs). Infection could also activate acute inflammation, and sometimes initiate chronic inflammatory response and DNA damage directly; (2) Synthesis and activation of HSPs (70, 90, and 27); (3) HSPs together with several immune cells including macrophages, neutrophils, interleukins, and several cytokines as well as ROS (HOCl), form part of acute inflammation aimed at eliminating infectious agents and initiating cellular repair; (4) Nevertheless, if the immune cell apparatus, especially ROS (HOCl in particular) are not properly stimulated, inflammation could persist and degenerates to chronic inflammation. (5) Chronic inflammation is characterized by massive cells and tissue destruction, resulting in DNA damage; (6) DNA damage activates several DDR pathways which can repair DNA damage and prevent accumulation of damaged proteins and tumour development; (7) Central to DNA repair mechanism is the synthesis of heat shock proteins, which maintain and stabilize the DDR proteins and prevent peptides aggregation and cancer; (8) DNA damage and lesions created by chronic inflammation can directly lead to tumour development; (9) If the DDR mechanisms fail due to the presence of HSPs inhibitors, the result could be malignant cells progression; (10) Improper stimulation of HSPs during tumour formation can lead to tumour invasion, progression, and metastasis.



References

[1] Laplante AF, Moulin V, Auger FA, Landry J, Li H, Morrow G, Tanguay RM and Germain L. Expression of heat shock proteins in mouse skin during wound healing. J Histochem Cytoch 1998; 46: 1291-1301.

[2] Al-Whaibi MH. Plant heat-shock proteins: a mini review. J KSUS 2011; 23: 139-150.

[3] Ou J-R, Tan M-S, Xie A-M, Yu J-T and Tan L. Heat shock protein 90 in Alzheimer's disease. BioMed Res Int 2014; 2014: 1-7.

[4] Zhang X, Shi J, Tian J, Robinson AC, Davidson YS and Mann DM. Expression of one important chaperone protein, heat shock protein 27, in neurodegenerative diseases. Alzheimer's Res Ther 2014; 6: 78.

[5] Leak RK. Heat shock proteins in neurodegenerative disorders and aging. J Cell Commun Signal 2014; 8: 293-310.

[6] D'Souza SM and Brown IR. Constitutive expression of heat shock proteins Hsp90, Hsc70, Hsp70 and Hsp60 in neural and non-neural tissues of the rat during postnatal development. Cell Stress Chaperon 1998; 3: 188.

[7] Somji S, Todd JH, Sens MA, Garrett SH and Sens DA. Expression of the constitutive and inducible forms of heat shock protein 70 in human proximal tubule cells exposed to heat, sodium arsenite, and CdCl (2). Environ Health Perspect 1999; 107: 887.

[8] Place SP and Hofmann GE. Constitutive expression of a stress-inducible heat shock protein gene, hsp70, in phylogenetically distant Antarctic fish. Pol Biol 2005; 28: 261-267.

[9] Edkins AL and Boshoff A. General structural and functional features of molecular chaperones. Edited by Heat Shock Proteins of Malaria. Springer, 2014. Pp. 5-45.

[10] Chatterjee S and Burns TF. Targeting heat shock proteins in cancer: a promising therapeutic approach. Int J Mol Sci 2017; 18: 1978.

[11] Finka A and Goloubinoff P. Proteomic data from human cell cultures refine mechanisms of chaperone-mediated protein homeostasis. Cell Stress Chaperon 2013; 18: 591-605.

[12] Ferrero-Miliani L, Nielsen O, Andersen P and Girardin S. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1β generation. Clin Exp Immunol 2007; 147: 227-235.

[13] Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006; 444: 860.

[14] Oyinloye BE, Adenowo AF and Kappo AP. Reactive oxygen species, apoptosis, antimicrobial peptides and human inflammatory diseases. Pharm 2015; 8: 151-175.

[15] Medzhitov R. Origin and physiological roles of inflammation. Nature 2008; 454: 428.

[16] Ikwegbue PC, Masamba P, Oyinloye BE and Kappo AP. Roles of heat shock proteins in apoptosis, oxidative stress, human inflammatory diseases, and cancer. Pharm 2017; 11: 2.

[17] Zhang J-M and An J. Cytokines, inflammation and pain. Int Anesthesiol Clin 2007; 45: 27.

[18] Feghali CA and Wright TM. Cytokines in acute and chronic inflammation. Front Biosci 1997; 2: d12-d26.

[19] Brocker C, Thompson D, Matsumoto A, Nebert DW and Vasiliou V. Evolutionary divergence and functions of the human interleukin (IL) gene family. Hum Genomics 2010; 5: 30.

[20] Commins SP, Borish L and Steinke JW. Immunologic messenger molecules: cytokines, interferons, and chemokines. J Allergy Clin Immunol 2010; 125: S53-S72.

[21] Adekiya TA, Aruleba RT, Khanyile S, Masamba P, Oyinloye BE and Kappo AP. Structural Analysis and Epitope Prediction of MHC Class-1-Chain Related Protein-A for Cancer Vaccine Development. Vaccines 2017; 6: 1.

[22] Jemal A, Siegel R, Xu J and Ward E. Cancer statistics, 2010. CA: Cancer J Clin 2010; 60: 277-300.

[23] Young Jr JL and Miller RW. Incidence of malignant tumors in US children. J Pediatr 1975; 86: 254-258.

[24] Morris J. Information: theoretical and applied. Prog Pathol 2001; 5: 165-183.

[25] Varricchio CG. A cancer source book for nurses. Edited by Jones and Bartlett Publishers, United Kingdom, 2004, pp 13-30.

[26] Scotting PJ, Walker DA and Perilongo G. Childhood solid tumours: a developmental disorder. Nat Rev Cancer 2005; 5: 481.

[27] Oosterhuis JW and Looijenga LH. Testicular germ-cell tumours in a broader perspective. Nat Rev Cancer 2005; 5: 210.
[28] Czene K, Lichtenstein P and Hemminki K. Environmental and heritable causes of cancer among 9.6 million individuals in the Swedish family-cancer database. Int J Cancer 2002; 99: 260-266.
[29] Danaei G, Vander Hoorn S, Lopez AD, Murray CJ, Ezzati M and group CRAc. Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. Lancet 2005; 366: 1784-1793.
[30] Shacter E and Weitzman SA. Chronic inflammation and cancer. Oncol 2002; 16: 217-229.
[31] Korniluk A, Koper O, Kemona H and Dymicka-Piekarska V. From inflammation to cancer. Ir J Med Sci (1971-) 2017; 186: 57-62.
[32] Vallespí MG and García I. Heat-shock proteins in inflammation and cancer. Biotecnol Apl 2008; 25: 208-215.
[33] Nakao A, Afrakhte M, Morn A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin N-E and Heldin C-H. Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling. Nature 1997; 389: 631.
[34] Massagué J. TGFβ signalling in context. Nature reviews Mol Cell Bio 2012; 13: 616.
[35] Lu H, Ouyang W and Huang C. Inflammation, a key event in cancer development. Mol Cancer Res 2006; 4: 221-233.
[36] Salem ML, Attia ZI and Galal SM. Acute inflammation induces immunomodulatory effects on myeloid cells associated with anti-tumor responses in a tumor mouse model. J Adv Res 2016; 7: 243-253.
[37] Fedeles BI, Freudenthal BD, Yau E, Singh V, Chang S-c, Li D, Delaney JC, Wilson SH and Essigmann JM. Intrinsic mutagenic properties of 5-chlorocytosine: A mechanistic connection between chronic inflammation and cancer. Proc Natl Acad Sci 2015; 112: E4571-E4580.
[38] Khor HK, Fisher M and Schoneich C. Potential role of methionine sulfoxide in the inactivation of the chaperone GroEL by hypochlorous acid (HOCl) and peroxynitrite (ONOO-). J Biol Chem 2004;
[39] Hazen SL and Heinecke JW. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. J Clin Invest 1997; 99: 2075-2081.
[40] Jiang Q, Blount B and Ames BN. 5-chlorouracil, a marker of DNA damage from hypochlorous acid during inflmmation: A GC-MS assay. J Biol Chem 2003;
[41] Chen H-JC, Row S-W and Hong C-L. Detection and quantification of 5-chlorocytosine in DNA by stable isotope dilution and gas chromatography/negative ion chemical ionization/mass spectrometry. Chem Res Toxicol 2002; 15: 262-268.
[42] Hale JT, Bigelow JC, Mathews LA and McCormack JJ. Analytical and pharmacokinetic studies with 5-chloro-2′-deoxycytidine. Biochem Pharmacol 2002; 64: 1493-1502.
[43] Fukata M, Chen A, Vamadevan AS, Cohen J, Breglio K, Krishnareddy S, Hsu D, Xu R, Harpaz N and Dannenberg AJ. Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. Gastroenterol 2007; 133: 1869-1869. e1814.
[44] Hunter P. The inflammation theory of disease: The growing realization that chronic inflammation is crucial in many diseases opens new avenues for treatment. EMBO Rep 2012; 13: 968-970.
[45] Pennisi R, Ascenzi Panddi Masi A. Hsp90: a new player in DNA repair? Biomolecules 2015; 5: 2589-2618.
[46] Nunes E, Candreva EC, Keszenman D and Salvo VA. The mutagenic effect of elevated temperatures in yeast is blocked by a previous heat shock. Mutat Res Fund Mol Mech Mut 1993; 289: 165-170.
[47] Matsumoto H, Wang X and Ohnishi T. Binding between wild-type p53 and hsp72 accumulated after UV and γ-ray irradiation. Cancer Lett 1995; 92: 127-133.
[48] Mendez F, Sandigursky M, Franklin WA, Kenny MK, Kureekattil R and Bases R. Heat-shock proteins associated with base excision repair enzymes in HeLa cells. Radiat Res 2000; 153: 186-195.
[49] Sottile ML and Nadin SB. Heat shock proteins and DNA repair mechanisms: an updated overview. Cell Stress Chaperon 2018; 23: 303-315.
[50] Ciccia A and Elledge SJ. The DNA damage response: making it safe to play with knives. Mol. Cell 2010; 40: 179-204.

[51] Bridge G, Rashid S and Martin SA. DNA mismatch repair and oxidative DNA damage: implications for cancer biology and treatment. Cancers 2014; 6: 1597-1614.
[52] Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. Nature 2001; 411: 366.

CHAPTER FOUR

MATERIALS AND METHODS

Despite all efforts towards improvement in cancer management and treatment, cancer remains one of the major global health threats of this century according to the World Health Organization. Central to the cellular response to infectious diseases and other stress signals including the onset of malignant tumours, is the synthesis of the highly conserved proteins termed heat shock proteins. These proteins play crucial roles in cell survival and adaptation under unfavourable conditions through folding of newly synthesis polypeptides, refolding of aberrant proteins, and translocation of organelles across membrane, thus enhancing the cellular survival rate (Ikwegbue et al., 2017). Among other classes of HSPs family that play crucial roles in the pathology of many diseases, HSP70-like protein (called HSP70.14) is now gaining momentum due its ability to interact with APCs and initiate its maturation, as well as activating Th1 and CTL responses, which play prominent roles in the killing of tumour cells. More so, using yeast-2-hybrid system has shown that HSP70.14 interact with human RING finger domain of RBBP6, this interaction suggests their function in protein quality control system (Kappo *et al.,* 2012).

Dysfunction of tumour suppressor protein p53 has been associated with almost 50% of cancer initiation, progression and invasion. The major negative regulator of p53 is MDM2, and its interaction with RING finger domain of RBBP6 has been reported to promote p53 degradation through ubiquitination mediated pathway (Li *et al.,* 2007). This observation suggests their roles in the ubiquitination of p53 where it acts as an E3 ligase enzyme, and mostly importantly, in the suppression of p53 and development of tumours. Hence, this project focused on expression, purification and characterization of novel HSP70.14 which has

been suggested as emerging candidate for the development of cancer vaccine, and also ascertained its interaction with RBBP6-RING finger domain as they have been postulated to play a role protein quality control system (anti-protein aggregation). The upreguation of these two proteins have been reported during cancer development, therefore their interaction could give a lead towards understanding the cascades of events that occur during cancer initiation, progression and invasion.

4.1 Preparation of competent cells *E. coli* BL21 (DE3) pLysS cells

The whole idea of making cells 'competent' is to manipulate and enable bacterial cells to take up foreign DNA. Firstly, all glassware used was washed, rinsed with distilled water and subjected to an autoclave to heat sterilize them. The bacterial strains XL1Blue and BL21 were streaked on Luria agar plates and incubated overnight at 37°C. Colonies were picked from the plates using a sterile yellow tip and was inserted into a test tube containing 5 ml YT broth. This was further incubated overnight at 37°C with shaking. The overnight culture was then poured into 50 ml YT broth the next day and was further incubated for 2hrs at 37°C with shaking, until the optical density (O.D.) was between 0.4-0.6. Thereafter, the absorbance was read at 600 nm wavelength using a spectrophotometer. The mixture was transferred into a sterile Eppendorf tube and spun at 5000 rpm for 5 mins. The supernatant was discarded and 10ml of ice cold and sterile pre-prepared $MgCl_2$ solution was added to the pellet, mixed gently and then incubated on ice for 20 mins. The mixture was then spun down for 10 minutes at 5000 rpm and the supernatant discarded. A 10 ml sterile and ice-cold $CaCl_2$ solution was then added to the pellet, mixed gently and incubated on ice for 4hrs. Thereafter, the mixture was spun down and the supernatant was again discarded. Finally, 3 ml $CaCl_2$ and 3 ml 30% sterile glycerol stock were added to the pellet and mixed gently. Thereafter, the mixture was aliquoted into 200 µl micro centrifuge tubes and stored at -80^0C until later use.

4.2 Bacterial transformation of *E. coli* BL21 pLysS competent cells with pGEX-6P-2-Hsp70.14 and RBBP6 RING plasmid

The already prepared competent cells were allowed to thaw on ice and 2 µl of plasmid DNA and 100 µl of the competent cells was mixed together in a sterile Eppendorf tube. This was then incubated on ice for 20 mins. The mixture was subjected to heat shock for 5mins at 37°C and was further incubated on ice for 5 mins. A 900 µl sample of pre-warmed YT broth without ampicillin was added to the mixture and mixed gently by inverting the tube a few times. Thereafter, the mixture was incubated with shaking at 37°C for 2hrs. Afterwards, 100 µl was taken from the incubated culture and spread on ampicillin-agar plates, which was then incubated at 37°C overnight. The remaining 800 µl was spun down at 15 000Xg and 700 µl of the supernatant discarded. This was mixed with the pellet, spread on ampicillin-agar plates and also incubated at 37°C overnight.

4.3 Small scale protein expression

Four colonies were picked randomly from overnight transformed plates and inoculated in 5 ml broth with ampicillin. These mixtures were incubated at 37°C for 4hrs, after which two 1 ml samples were taken out from the mixture and transferred into sterile micro-centrifuge tubes. One was induced with 0.5 mM IPTG whereas the other was left as the un-induced sample with no added IPTG. Both mixtures were incubated further with shaking at 37°C for 2hrs. The remaining 3 ml samples were also left for incubation until screening of the colonies was over. Cells from the cultures were harvested by centrifugation for 5mins at the highest speed. The supernatant was discarded and 50 µl of 2X sample buffer added to the pellets. The mixtures were vortexed and boiled at 90°C for 5 minutes. A 20 µl sample was taken from each culture and loaded onto an SDS PAGE gel (as described below in 2.5). A 100µl sample of the best expressing clone was used to induce an overnight culture for large scale

expression from the remaining 3 ml culture. The rest was made into glycerol stock in case of further use.

4.4 Large scale protein expression and extraction

A 100µl of the best expressing culture (taken from the remaining 3ml mixture) was used to inoculate 100ml of LB broth with ampicillin and was incubated at 37°C with shaking overnight. The next day, the culture was scaled up to 2L and incubated further at 37°C with shaking until the OD_{600} was between 0.4 – 0.6. The culture was then induced with 0.5mM IPTG and was left for further incubation at 25°C overnight. The next day, the cells were harvested by centrifugation at 5000rpm for 10mins. The supernatant was discarded and lysis buffer was added to the pellets kept at -80^0C. The cells were extracted by allowing the frozen cell pellets to thaw on ice which was followed by three cycles of freeze-thawing where the total bacterial cell lysate was placed in -80°C for 10 minutes, which was followed by incubation at room temperature for 10 minutes; this was done 3 times at 30 minutes interval. Thereafter, the mixture was sonicated for 5minutes and further incubated overnight at -80°C. The clear lysate (supernatant) containing the protein of interest was then obtained by centrifuging the cells at top speed (14000 Xg) for 30 minutes.

4.5 Affinity purification

Glutathione (GST)-Agarose beads were first prepared by weighing out the required amount and allowing the beads to swell overnight by suspending them in deionized water at 4°C. The next morning, the mixture in semi-liquid form was poured inside a 5 column volume affinity chromatograph column and was left to settle for a few hours. GSH cleansing buffer 1 (0.1 M borate buffer containing 0.5 M NaCl was prepared adjusted to pH 8.5 using NaOH) was prepared together with GSH cleansing buffer 2 (0.1 M acetate containing 0.5 M NaCl,

prepared using sodium acetate and adjusted accordingly to pH 4.5 with acetic acid). 250 ml of each cleansing buffer was passed through GST-agarose column to remove impurities. In between these runs, 0.02% NaN_3 was passed onto the column. Thereafter, equilibration buffer [(1% Triton-X 100, 100 mM lysozyme, 1 mM DTT, 50 mM $ZnS0_4$) prepared in PBS containing 137 mM NaCl, 27 mM KCl, 43 mM Na_2HP0_4)] was used to equilibrate 11CV for 20 times. The clear lysate was poured onto the column and the flow-through collected. This was followed directly by washing the column with 10CV equilibration buffer. GST-RING was then eluted with 10 mM glycine at pH 10 and the protein was collected in the fraction of 5 ml per 15 ml test tubes. The column was washed again with 0.1 M borate and 0.5 M acetate buffer and store at 4 ℃.

For 6XHis-tag (Hsp70.14), a Nikel-NTA column recharged with cobalt was used. The column was first connected to AKTA prime plus purification machine stripped and washed high concentration of salt to remove contaminants. Thereafter, the column was recharged with 0.1 M cobalt (which turns pink color). This was followed with equilibrating the column using equilibration buffer (50 mM Tris, 400 mM NaCl, 20 mM imidazole and 0.02% NaN_3 at pH 7.4) until the line become straight. The clear lysate was poured onto the column and the flow-through collected, and column was further incubated until the blue line wavelength become straight again. The 6XHis-Hsp70.14 was purified using (50 mM Tris, 400 mM NaCl, 500 mM imidazole and 0.02% NaN_3 at pH 7.4) elution buffer and rapid increase in blue line peak (Spec monitor) was observed and protein was collected. The column was then equilibrated for some time and washed with high concentration of salt to remove unbounded proteins. All the eluted protein was subjected to SDS-PAGE analysis using the reagents as listed in Table 2.1. The purified pure proteins were then subjected to other Biochemical and biophysical characteristics.

Table 4.1: Composition of a 16 % SDS PAGE gel

	Separating gel	**Stacking gel**
Deionised water	3.20 ml	3.17 ml
Separating buffer	2.63 ml	_
4X Stacking buffer	_	1.25 ml
40 % Acrylamide 37.5:1	4.00 ml	0.50 ml
10 % SDS	150.00 µl	50.00 µl
TEMED 7.50 µl 5.00 c	16µl	5 µl

4.6 Quantification and Qualitative analysis

Extinction coefficient is a measure of light absorption in a medium, which is used to estimate the concentration of purified proteins or to identify protein fractions. Aromatic amino acids (tryptophan, tyrosine and phenylalanine) are known to exhibit high UV-light absorption. The protein's UV-light absorption is directly proportional to their concentration and aromatic acid content. However, using UV-light absorption is not always accurate because other molecules like nucleic acids and some impurities that absorb UV-light at 280nm wavelength may be present, which will not give accurate protein concentrations. Light absorption of Hsp70.14 and that of RBBP6 RING finger domain were determined in a 100-300 nm wavelength range. A small amount of each sample was poured in a quartz cuvette of 1cm in length and light

absorbance of the protein was determined at 280nm wavelength. The relationship between concentration, absorbance, molar extinction coefficient and path length was used as describe by Beer-lambert law shown below: $\mathbf{A} = \varepsilon \boldsymbol{\ell}\, \mathbf{C}$

Where:

A is absorbance (no unit)

$\boldsymbol{\mathcal{E}}$ is molar extinction coefficient (M^{-1} CM^{-1})

C is concentration (M)

$\boldsymbol{\ell}$ is path length (CM)

The quantification and analysis of purified recombinant proteins (pQE-30-Hsp70.14 and pGEX-6P-2- RBBP6 RING) were done in serial dilution of 1:100 using Jasco V-630 spectrophotometer which monitored and measured absorbances at A_{280} and A_{340} wavelength respectively. The corrected calibration curve plotted against dilution factor was used to determine the gradients, which were employed in the equation below to estimate the concentration of both proteins:

$$\text{Conc (mg/ml)} = \frac{slope \times molecular\ mass}{\varepsilon}$$

The absorbance was measured at A_{280} and A_{340} respectively since most proteins absorb light at A_{280}, and absorbance at A_{340} wavelength was measure to monitor the protein aggregation.

4.7 Structural characterization

4.7.1 Secondary structure determination

Circular dichroism (CD) is an excellent technique that is used extensively in the field of structural genomics and proteomic studies to ascertain the secondary structure, binding characteristics and folding state of proteins (Greenfield, 2006). This technique works by passing a beam of light through a polarizer, which will polarize the light and pass it to a prism called a photoelectric modulator (PEM). The polarized light will oscillate in a sinusoidal wave-like manner, which when viewed cross-sectionally, will show a trace of circular wave motion. The PEM detector will then interpret light into the left-handed circular polarized light which rotates in an anti-clockwise direction and right-handed circular polarized light which rotates in a clockwise direction (Greenfield, 2006). The two circularly polarized light waves was passed through the optical active sample, which shows "differential absorption rates" and refraction between the two polarized waves since each one rotates in the opposite direction. The light produced as result of the two circular polarized waves is called "elliptically polarized light" (Greenfield, 2006). In this study, CD was used to determine the secondary structures of Hsp70.14 and RING finger domain, possible interaction, as well the correct folding state between them. Any changes due to ligand binding or conditions that change the basic structure of the β-sheets, α-helix or the turns (coils) in a protein, with the respect to the control, will give information as how or why the changes in the secondary structure occurred if the spectra are compared. In this way, CD gives useful information in ascertaining the folding or unfolding state of the protein, as well as secondary structure of the protein (Greenfield, 2006).

Hsp70.14 in an elution buffer of 50 mM Tris, 40 mM NaCl, 500 mM Imidazole, and 0.02% NaN_3 (W/V) at 7.4, was diluted with mili-Q water to reduce the effects of imidazole and salt signal noise which could interfere with light absorption and CD structural determination of the protein in general. In the same vain, RING finger in PBS (137 mM NaCl, 27 mM KCl, 43 mM Na_2PH0_4 and 14m M KH_2PO_4) was diluted with miliQ water to reduce the salt signal noise effects. 3 µl of Hsp70.14 and 100 µl of RING were measured and analyzed in the absence presence of denaturing urea (10 M urea). The far-UV CD spectra was measured using 2 mm pathlength quartz cuvette at scanning speed of 100 nm/min, bandwidth of 5nm, data pitch 0.5nm and every 1 second response time that start from 250nm and end 185nm with average number of 3 accumulations. Furthermore, the mean residue ellipticity calculation was employed to standardize the recorded spectra using the equation below:

$$\boldsymbol{\theta} = \frac{100\ x\ \theta}{Cnl}$$

Where,

$\boldsymbol{\theta}$ is molar mean ellipticity residue (degree.CM^2.$dmol^{-1}$)

C is molar concentration (mM)

n is total number of residue in the protein

l is pathlength (CM)

4.8 Tertiary structural characterization

4.8.1 Intrinsic tryptophan fluorescence spectroscopy

The continuous invention and improvement of sophisticated optics and electronics has led to useful application of fluorophores in various biochemical analyses which offers more advantage over difficult to handle and expensive radio tracers (Ladokh *et al.*, 2000). Fluorescence spectroscopy is a technique that is widely employed today to measure the radiation emitted energy, which absorbs light at a specific wavelength (λ). When an electromagnetic radiation is absorbed, it transfers radiation energy to the molecules (proteins, lipids, nucleic acids) leading higher energy state (excitation state). A portion of this energy will be re-emitted as light which usually has longer wavelength and lower energy compared to the excitation state. Stoke's shift which can be recorded as differences in excitation and emission, is loss of energy as a result of vibration relaxation (Mehta, 2013). This technique is concentration dependent and function better at lower concentration because fluorescence intensity is directly proportional to the concentration of the molecule. There are different kinds of fluorescence available commercially and the choice of fluorophore depends mostly on quantum yield and stokes shift, emission wavelength and absorption, ease and selectivity. One of the main challenges of fluorescence molecules is their photolability which may lead to irreversible photobleaching or degradation (Ghisaidoobe and Chung, 2014). The excitation wavelength of indole group found in tryptophan is highly sensitive to aqueous environment normally at 295nm and between 300nm to 350nm emission. When stoke shifts occur in Tryptophan fluorescence, it means that emission wavelength is totally dependent on tryptophan environment, especially in hydrophilic environment. Due to its hydrophobicity, tryptophan residues are buried in hydrophobic environment and exhibit high quantum jump and fluorescence intensity.

To obtain the fluorescence spectra of these proteins (RING finger and Hsp70.14), a Jasco FP-6300 with a spectra software manager (v1.5.00), and 10 mm path length quartz cuvette was used. An 11 µl RING and 12 µl were added to 189 µl and 188µl of size exclusion buffer (50 mM Tris and 0.5 M NaCl) respectively. Once more, these were done in the presence (denatured) and absence of 10M urea. Emission spectra were obtained at scan speed of 200 nm/min, 2.5nm (emission), 5nm slit widths and excited at 295 nm wavelength. Fluorescence spectra were recorded between 280 nm and 400 nm wavelength, and all graphs were plotted by subtracting the blank from each sample.

4.9 Extrinsic ANS-Fluorescence spectroscopy and ANS binding studies

8-Aminonaphthalen-1-sulfonic (ANS), alternatively called 1-anilino-8-naphthalensulfonate is an extrinsic fluorescence spectroscopy used to probe hydrophobic binding pocket of the partially folded proteins due to its strong affinity toward denatured proteins (Ali *et al.*, 1999). The hydrophobic interaction of ANS (hydrophobic fluorescent dye) with protein has been widely applied in different aspect of protein analysis to characterize folding state, detect protein aggregates as well as measure surface hydrophobicity of the proteins (Latypov *et al.*, 2008). In most cases, ANS is considered a non-fluorescence probe because of its low fluorescence intensity when bound to the proteins exposed binding sites in a polar environment; though its properties of increasing fluorescence lifetime, intensity and shifting of fluorescence emission maxima, suggests hydrophobicity functions suitable for florescence probe (Gasymov and Glasgow, 2007).

To determine ANS concentration, powdered form of ANS was dissolved in dH_2O and serial dilution of 1:100 was carried out to obtain a concentration of 260 mM stock solution which was stored in a dark place, covered with foil to prevent photodecomposition since ANS is

very sensitive to light. Furthermore, because of the high concentration of ANS, serial dilution (1:10) was done to bring the stock solution (260 mM) down to working concentration of 26 mM. Thereafter, RING sample (11µl) was incubated with 15.4 µl in a 173.6 µl size exclusion buffer (50 mM Tris and 0.5 M NaCl) and was allowed to stand for few hours. A 12 µl of Hsp70.14 was also incubated with 15.4 µl in a 172.6 µl in the same size exclusion buffer for few hours. More so, a mixture of 11 µl RING, 12 µl Hsp70.14 and 15.4 µl ANS was also incubated in the same of size exclusion buffer (161.6 µl) and was allowed to stand for few hours. All these samples (mixtures) were prepared in both presence (denature) and absence (native) of denaturing agent (urea). Blank samples were also prepared and analysed using Jasco FP-6300 fluorimeter, scan at speed of 200 $nm.mn^{-1}$, 5nm slit widths, excitation at 390nm and emission was recorded between the wavelength of 400-600 nm. The spectra of blank samples were subtracted from each sample (i.e native blank-/+native sample and denatured blank-/+ denatured sample) in both native and denatured samples, and this was done to all the samples.

4.10 Size exclusion high pressure liquid chromatography (SE-HPLC)

Size exclusion-high pressure liquid chromatography (SE-HPLC), preferably called size exclusion or gel filtration because its rapid molecule separation and high resolution, is high-throughput analytical technique which separate proteins based on diffusion coefficient, hydrodynamic size or volume and surface properties (Schrag *et al.,* 2014). Initially, it was thought this chromatography to be more suitable for separation of protein molecules than peptides, however recent application suggests that this techniques can operate in the fractionation range of less than 10000 Daltons (10KDa) (Irvine, 1997). The fractionation range is determined by the diameter pores in the stationary phase. When the Sephadex material is used, it is controlled by the degree of cross-linking which is inversely proportional

to the bead pores. The LC phenomenex HPLC column which has a length of 300 mm and a diameter of 7.8 mm is filled with silica beads and arranged in 3-dimensional shape to restrict the movement and migration of small molecules whereas allowing the free movement of larger molecules. In this way, small molecules penetrate the bead pores and become trapped, and this restrict their movement whereas larger molecules move freely and come out first (Alpert, 1999).

To ascertain the size of the proteins, Size exclusion silica column (Yarra 3u SEC-2000 coupled to Phenomenex Security Guard ULTRAguard column) was used. The column was equilibrated with size exclusion (50 mM Tris, 0.5 M NaCl and 0.02% NaN_3 at pH 7.4) and connected to Shimadzu Prominence HPLC system (SPD20A) prior to equilibration and ran at 0.25 $mL.mn^{-1}$ flow rate. Column calibration was done using bovine serum albumin (66.6 kDa), CLIC-1 (30 kDa) as standard. After few hours of equilibration, this was followed by injection of 20 µl of protein samples for a period of 30 minutes each. The mixture of the two proteins (RING and Hsp70.14) was incubated for 1 h, and a 20 µl of the sample was injected with a syringe into the column. The quaternary structure of the proteins and complex of the interaction were obtained using calibration curve of size exclusion standard.

4.11 PreScission protease Cleavage of GST and recovering of free RING

GST-RING were pooled together and poured into a freshly prepared 3C-PreScission™ Protease cleavage buffer (250 mM Tris, 750 mM NaCl, 5 mM EDTA and 5 mM DTT at pH 6.3). The high concentration of salt and lower pH was used to bring down the pH of elution buffer (10 mM glycine at pH 10) to pH 7.0, since glycine is amino acid with weaker ionic strength therefore there was no need to dialyze glycine out of the solution. Furthermore, some cleavage trials were carried out to ascertain when GST tag would be totally cleaved off the

protein. Firstly, 1 ml of the protein, 0.25 ml of cleavage buffer and 75 µl of PreScission protease were incubated over a period of time and 50 µl sample been taken out at 15 minutes interval. These samples were then analyzed on SDS-PAGE in order to ascertain the time interval when protease will completely cleave off GST; this was noted and used to incubate a larger volume of the mixture. A 4 ml of the protein (GST-RING), 1 ml of cleavage buffer and 250 µl of PreScission protease were incubated overnight at 4 ℃. The next morning, the sample was analyzed on the SDS-PAGE.

Following the cleavage, the mixture was then returned to the GST-agarose column connected to benzamidine column, which traps protease enzyme and RING finger was collected as unbounded protein during wash with equilibration buffer, and GST was eluted with 10 mM glycine.

4.12 Molecular docking studies

Docking is a well-known computational tool that is commonly used to predict the protein-protein or protein-ligands association complexes. Molecular docking actually helps to ascertain the interactions between two or more biomolecules and finding the best possible orientation that would minimize the total energy expenditure of the interactions, especially in the case of protein-ligands interactions (Dar *et al.,* 2016). The knowledge obtained from protein-ligand orientation could actually be used to predict the energy of the binding, free energy, binding affinity as well as the stability of the complex.

Molecular docking has been applied extensively in environmental clean-up (bioremediation) and most importantly in drug discovery and drug designing, making it the interesting aspect of research for pharmaceutical industries (Suresh *et al.,* 2008). Since the first application in the early 1980s, this technique has attracted wide array of interesting research in both

medicinal and pharmaceutical companies because it allows for protein-small molecule interaction at atomic level, as well as characterizing their behaviours and other fundamental biochemical processes (Meng *et al.*, 2011; McConkey *et al.*, 2002).

In the stimulation approach technique of docking, ligand is allowed to bind into the groove of the protein, causing "conformational moves" that limit the total energy released while allowing ligand flexibility. This theory suggests that ligand fits into the grove of the target protein in induced-fit manner (Meng *et al.*, 2011). In shape complementary approach, the ligand and protein have structural related features which enables their interaction with each other (Dar *et al.*, 2016), this can be linked to the enzyme lock-and-key theory as proposed by Fisher in enzyme model (Fisher, 1894).

In silico molecular docking of the proteins (Hsp70.14 and RING) was carried out using PatchDock to ascertain the putative interaction between the proteins. PatchDock is a type of rigid online server which is based on size and shape complementarity to predict interactions between two proteins in lock-and-key manner. In short, the PDB files 3-D structure of Hsp70.14 substrate binding domain (receptor) and RING finger domain of RBBP6 (ligand) were uploaded (https://bioinfo3d.cs.tau.ac.il/PatchDock) and RMSD default setting (cluster 4Å), and complex type set at protein-protein was used prior to submission. The result of interaction between the proteins was observed, examined and visualized using PyMol Molecular Graphics System (2003). DeLano Scientific, LLC, USA. http://www.pymol.org.

REFERENCES

Li L, Deng B, Xing G, Teng Y, Tian C, Cheng X, Yin X, Yang J, Gao X, Zhu Y and Sun Q (2007). PACT is a negative regulator of p53 and essential for cell growth and embryonic development. *Proceedings of the National Academy of Sciences USA*, **104**(19): 7951-7956.

Ali V, Prakash K, Kulkarni S, Ahmad A, Madhusudan KP and Bhakuni V (1999). 8-anilino-1-naphthalene sulfonic acid (ANS) induces folding of acid unfolded cytochrome c to molten globule state as a result of electrostatic interactions. *Biochemistry* **38**(41): 13635-13642.

Alpert AJ. (1999). Size exclusion high-performance liquid chromatography of small solutes. In *Column handbook for size exclusion chromatography* (249-266). Academic Press.

Dar AM, Khan MA, Mir S and Gatoo MA (2016). DNA binding, cleavage activity, molecular docking, cytotoxicity and genotoxicity studies of newly synthesized copper based metal complexes. *Pharmaceutica Analytica Acta* **7:** 1636-1616.

Fischer E (1894). Einfluss der Configuration auf die Wirkung der Enzyme. *European Journal of Inorganic Chemistry* **27**(3): 2985-2993.

Gasymov OK and Glasgow BJ. (2007). ANS fluorescence: potential to augment the identification of the external binding sites of proteins. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **1774**(3): 403-411.

Ghisaidoobe A and Chung S (2014). Intrinsic tryptophan fluorescence in the detection and analysis of proteins: a focus on Förster resonance energy transfer techniques. *International Journal of Molecular Sciences* **15**(12): 22518-22538.

Greenfield NJ (2006). Using circular dichroism spectra to estimate protein secondary structure. *Nature Protocols* **1**(6): 2876-2890.

Irvine GB (1997). Size-exclusion high-performance liquid chromatography of peptides: a review. *Analytica Chimica Acta* **352**: 387-397.

Kappo MA, Eiso AB, Hassem F, Atkinson RA, Faro A, Muleya V, Mulaudzi T, Poole JO, McKenzie JM, Chibi M and Moolman-Smook JC (2012). Solution structure of RING finger-like domain of retinoblastoma-binding protein-6 (RBBP6) suggests it functions as a U-box. *Journal of Biological Chemistry* **287**: 7146-7158.

Ladokhin AS, Jayasinghe S and White SH. (2000). How to measure and analyze tryptophan fluorescence in membranes properly, and why bother? *Analytical biochemistry* **285**(2): 235-245.

Latypov RF, Liu D, Gunasekaran K, Harvey TS, Razinkov VI and Raibekas AA (2008). Structural and thermodynamic effects of ANS binding to human interleukin-1 receptor antagonist. *Protein Science* **17**(4): 652-663.

Mehta A (2013). Animation for the Principle of Fluorescence and UV-Visible Absorbance. Available at the following url: https://pharmaxchange.info/2013/03/animation-for-the-principle-of-fluorescence-and-uv-visible-absorbance/ [Accessed 12 December 2018].

Meng XY, Zhang HX, Mezei M and Cui M (2011). Molecular docking: a powerful approach for structure-based drug discovery. *Current Computer-aided Drug Design* **7**(2): 146-157.

Schrag D, Corbier M and Raimondi S (2014). Size exclusion-high-performance liquid chromatography (SEC-HPLC). In *Monoclonal Antibodies* 507-512. Humana Press, Totowa, NJ.

Suresh PS, Kumar A, Kumar, R and Singh VP (2008). An *in silico* approach to bioremediation: Laccase as a case study. *Journal of Molecular Graphics and Modelling* **26**(5): 845-849.

Ikwegbue P, Masamba P, Oyinloye B, and Kappo A (2018). Roles of heat shock proteins in apoptosis, oxidative stress, human inflammatory diseases, and cancer. *Pharmaceuticals* **11**(1): 2.

CHAPTER FIVE

RESULTS

5.1 Bioinformatics Analysis

Prediction of protein structure, functions and possible interacting partners prior to wet-lab experiments has made a huge stride in the field of biomedical research. Bioinformatics, as the name applies, is the application of informatics techniques to study biological processes. Advances in bioinformatics studies in the last two decades have helped to gain more insight in predicting the structure and functionality of biological important molecules such as proteins, ligands and enzymes. For this reason, bioinformatics has attracted attention in recent years because it broadened our understanding of biological molecules, and most importantly, it helps to study and understand pathology of several diseases at molecular level (Chen *et al.,* 2014). Basically, the traditional ways of studying protein structure, function and interacting partners needed for diseases interventions, is through *in vitro*/*in vivo* experiments which is sometimes time consuming and difficult to understand the functions and structure of newly sequenced proteins. Thus, this could limit the utility of newly sequenced proteins. Hence, *in-silico* approaches for the interactions that occur between proteins and other important biological interacting partners as well as pathways within the living cell (Chang *et al.,* 2010), could help to overcome these challenges faced by *in vitro*/*in vivo* studies.

5.1.1 Construction of homology modelling of the proteins

Advancement of computational tools application to study and predict protein structures has increased in the last three decades. Most of the tools merely involves the use of protein of interest and compare it to other characterized (known) proteins in database, in order to gain and analyze statistical or evolutionary profile of the target protein. Determination or

prediction of 3-D structure of a protein is very important in different aspect of biological experiments such as in the discovery of the structural based specific inhibitors or side directed mutagenesis. It is estimated that the number of proteins that are structurally characterized in Protein Data Bank (over 33 000 proteins) are far less than the number of known sequences in UniProt protein database (~2.3 million sequences), hence, several computational modelling methods has been developed to overcome these challenges (Arnold *et al.,* 2006). Since the 3-D structure of the proteins are more conserved compared to their respective sequences, it is therefore possible to identify protein homologous that is structurally characterized for a given protein sequence. Homology modelling, which is routinely used in virtual screening or sequence variation has proven to be the best and most reliable method in generating 3-D model of a protein from its constituent amino acids. This modelling method is mostly employed to generate 3-D models when the experimental characterized protein structures are not available in PDB (Biasini *et al.,* 2014).

Among other homology modelling methods (e.g. Homer, Modweb, $(PS)^2$, $(PS)^2$-V2, CPHmodels and Phyre2), SWISS-MODEL has proven to be one of the best methods of choice for generating 3-D model of proteins without a need of downloading a large software or a complex software package. SWISS-MODEL is an automated web server used to build quality 3-D models in different functional states (Biaini *et al.,* 2014). For this reason, SWISS-MODEL was employed in this study to predict the 3-D models of SBD of Hsp70.14 and RING finger domain of RBBP6, and since the quality built 3-D models are the pre-requisite for docking (PachDock) experiment. Figure 5.1 (A) shows 3-D model of SBD of Hsp70.14, which predicted 4 β-sheets, 3 α-helices, and many random coils joining them together; whereas (B) depicts 3-D models viewed with Pymol, which revealed 2 β-sheets and 2 α-helices, joined by random coils.

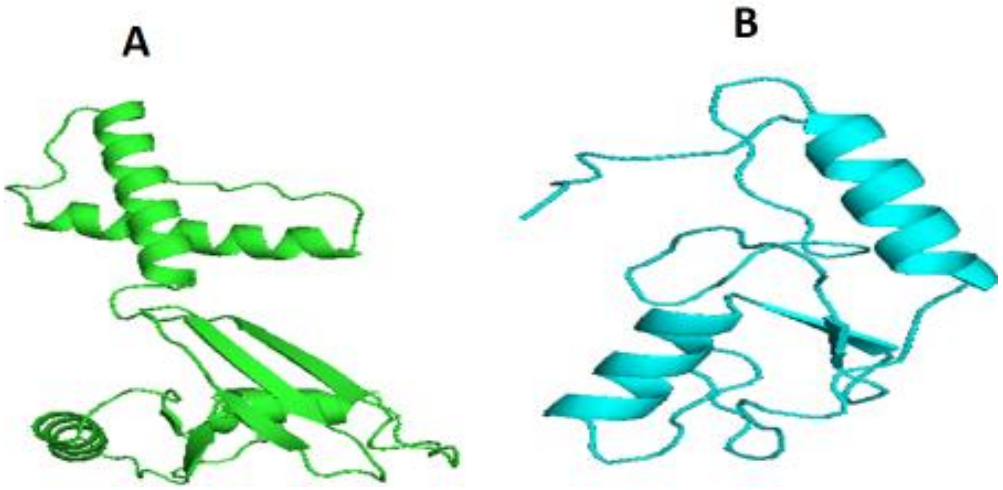


Figure 5.1: Homology modelling structure of the proteins. Diagram (A) represent Hsp70.14 substrate binding domain, whereas diagram (B) represent RING finger domain.

To validate the overall quality of the built models, Ramachandran plot was generated by employing the PROCHECK principles. PROCHECK is used to validate the quality or the accuracy of the built model, and also to generate residues of Proline and Glycine plots respectively. Ramachandran principle is on the basis of torsion angles φ, ψ and ω, which are among the key local parameters that determine rotation and control the folding states of a protein (Best *et al.*, 2012). In essence, if the torsion angles of a particular protein are predicted, it can actually predict the 3-D structure of the protein. Ramachandran plot also provides information on allowed and disallowed regions of torsion angles and their values. Therefore, it is seen as essential factor for the assessment of local proteins quality and 3-D folding states. PROCHECK validation server queries the final protein models against standard stereochemical parameters and experimental data (where they were generated), as well as give a broad view of the protein quality structures such as torsion angles, planarity,

covalent geometry, distribution of restrained violations, accessibility and precision (Laskowski *et al.,* 1996).

Ramachandran plot analysis showed that SWISS model has 94.0 % of residues of Hsp70.14 in most favourable region, 6.0 % in additional allowed region, and 0.0 % in both generously allowed region and disallowed region as shown in figure 4.2 (A). As inset in figure 5.2 (B), which shows that RING have 81.5 % residues are found in most favourable region, 16 % in additional allowed region, and 1.2 % in both generously allowed and disallowed regions. These results imply that the models built are of good quality and stable, therefore good enough to carry out PatchDock experiment.

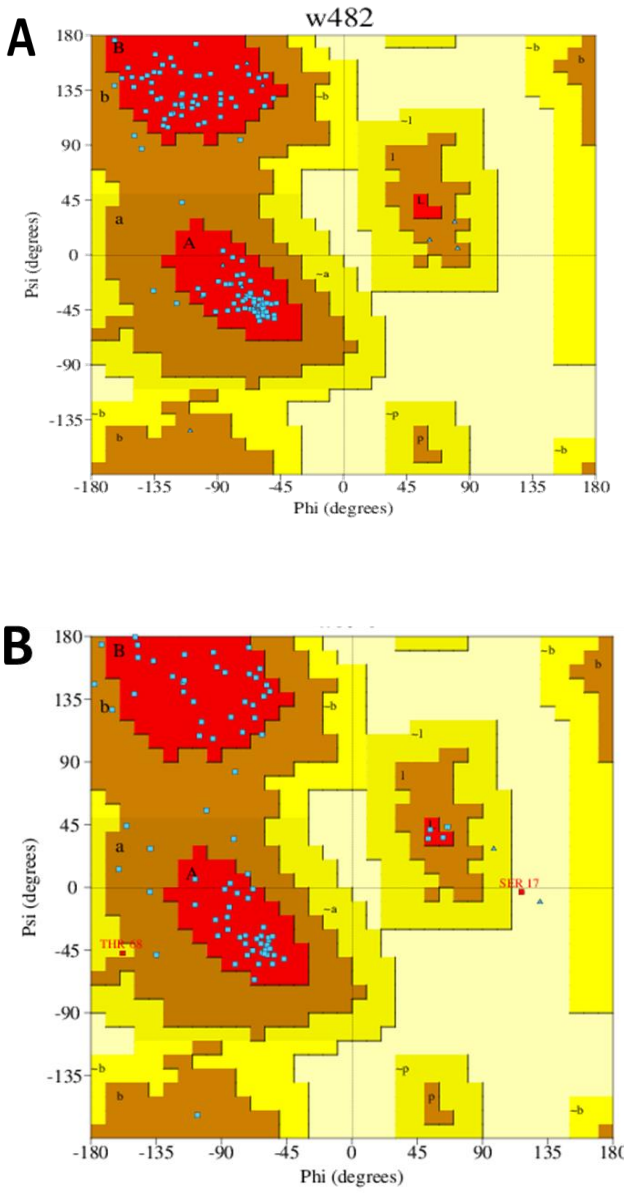


Figure 5.2: Ramachandran validation of the model built using PROCHECK. (A) Represents Ramachandran plot of Hsp70.14**.** It shows 94.0 % of residues in most favourable region, 6.0 % in additional allowed region, and 0.0 % in both generously allowed and disallowed region. **(B)** Presents Ramachandran plot of RING finger domain. 81.5 % are found in most favourable region, 16 % in additional allowed region, and 1.2 % in both generously allowed and disallowed region.

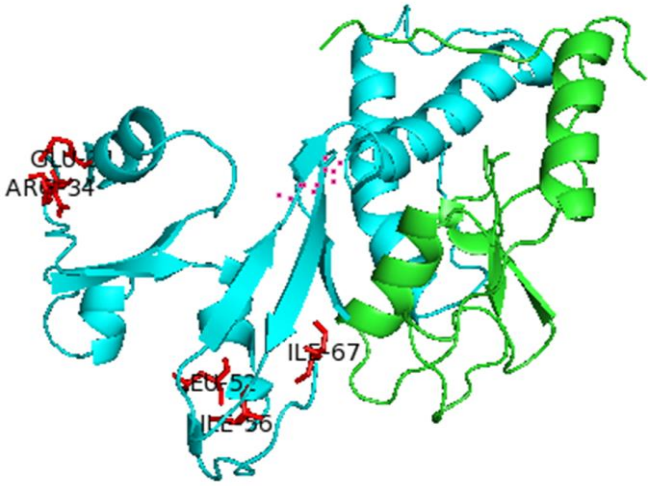


Figure 5. 3: Docking prediction of putative interaction between Hsp70.14 and RING finger domain. The diagram was viewed with PyMol.

PatchDock is an effective algorithm used for protein-protein and small ligand-protein interaction, which is based on the principle of size and shape complementarity. This aimed at finding the best transformations of which when applied, produces both little amounts of steric clashes and wide interface area (Schneidman-Duhovny *et al.,* 2005). PatchDock algorithm predicted the strong interaction between the proteins (Hsp70.14 and RING). The residues that are involved in the interaction were mapped out using PyMol. These interacting residues include Glu21, Arg34, Ile56, Ile67 and Leu52. PatchDock also predicted significant important values such as the binding score (16038), area of the binding/interaction (2603.70) and contact atomic energy (-125.15).

5.2 *In vitro* experiments

Expression and purification of proteins are very integral part of Molecular Biology and Biochemistry in general. Among other microorganisms used in protein expression, *Escherichia coli* (*E. coli*) is the most preferable and widely used organism for recombinant expression owing to the facts that it is readily available, cost effective, fast to grow and

genetically easy to manipulate for the construction of recombinant proteins. Although recombinant proteins can be produced in this way, there has been a challenge of inclusion bodies formation due to the presences of consecutive disulfide bonds, thus, resulting in the production of insoluble proteins aggregates (Rosano and Ceccarelli, 2014). Recently, various fusion tags have been employed to overcome this challenge and to improve the solubility and expression of recombinant peptides (Esposito and Chatterjee, 2006). Hence, enhancing the purity and quality of purified proteins is needed for structural and functional studies. Purely purified proteins are very essential for characterization and interaction studies used in different facets of drug discovery and vaccine interventions for several diseases including cancer (Kosano *et al.*, 1998). For this purpose, this section lays emphasis on protein expression, purification, characterization and interaction between RING finger domain of RBBP6 and Hsp70.14.

5.2.1 Transformation of E. coli BL21 competent cells with substrate binding domain (SBD) of Hsp70.14 and RING finger domain constructs

Prior to transformation procedure, BL21 competent cells were first prepared. This is a process that makes use of high concentration of calcium chloride to alter and neutralize the negative charge on the bacterial cell membrane for easy uptake of foreign DNA. Based on the coding sequences of SBD of Hsp70.14, fused to 6xHis was constructed and bought from Genescript, while RING finger domain fused to Glutathione-S-transferase (GST) was mini-prepped from the previous purchased constructs. ExPASy ProtParam web-sever https://web.expasy.org/cgi-bin/protparam/protparam was visited in order to calculate the molecular weight of the SBD-Hsp70.14 using amino acid sequences, as well as to obtain isoelectric point of the protein. Approximately 14.6 kDa molecular weight and 6.4 isoelectric point was obtained. Given that the molecular weight of 6xHis tag is less than 1 kDa, hence

the expected weight of 6xHis-SBD-Hsp70.14 fusion protein is around 16.0 kDa. Transformation is a molecular biology process by which bacterial cell walls are genetically altered for the purpose of up-taking foreign DNA from their immediate environment. BL21 cells were competent cells of choice due to their availability and their ability to fold proteins to their 3-D as well as enhancing their solubility. The cells were transformed using both SBD-Hsp70.14 and RING finger constructs in order to determine their competency. No growth was observed in the control plate, whereas the test plate (supernatant and pellet) shows moderate and high growths respectively. This is simple indicator of positive transformation and the cells were competent since there was no contamination in the control plate.

5.2.2 Expression screening of the positive transformants

Prior to large scale expression, four colonies were randomly picked from the positive transformed plate in order to check for the presence of protein of interest. This was followed by the analysis of total bacterial lysate using 16% SDS-PAGE. Figures 5.4 and 5.5 show the proteins to be expressing at the correct weights which are approximately 15 kDa for Hsp70.14 and 37 kDa for RING, respectively. Lane 1,3,5,7 represents un-induced samples whereas lanes 2,4,6,8 represent induced samples. Ideally, more expression (dark bands) are expected from induced lane due the presence of 0.5 mM IPTG used to induce protein expression, however, this was not the case in this study. Figure 5.6 shows good expression (dark bands) in both induce and un-induced lanes, this could be due to leaky expression where protein expression takes place without induction of IPTG (Weickert *et al.,* 1996). The results obtained from SDS-PAGE analysis in this study indicate that these proteins were successfully expressed BL21 and T7 cells.

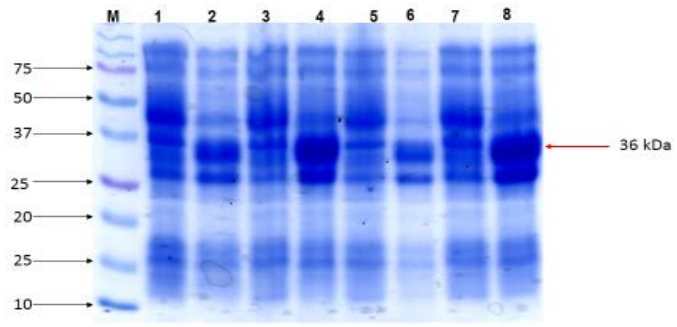


Figure 5.4: Expression screening result of GST-RING finger domain transformed into BL21 cell. Lane M represents molecular marker in kDa. Lanes 1, 3, 5 and 7 represents un-induced samples of total bacterial lysate whereas lanes 2, 4, 6 and 8 represents total bacterial lysates induced with 0.5 mM IPTG.

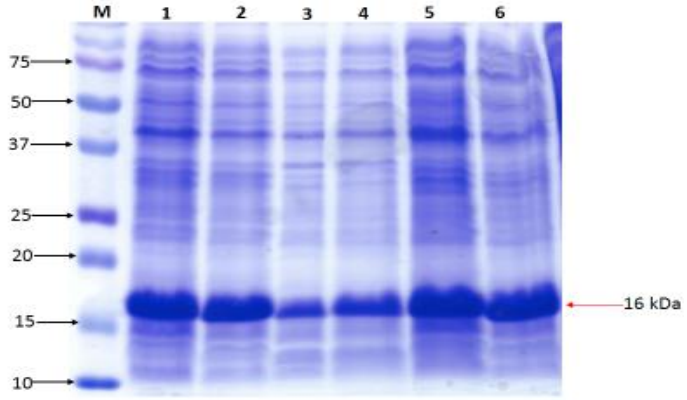


Figure 5.5: **Expression screening result of 6xHis-SBD of Hsp70.14 transformed into BL21 cells.** Lane M represents molecular marker in kDa. Lanes 1, 3, 5 and 7 represents un-induced samples of total bacterial lysate whereas lanes 2, 4, 6 and 8 represents total bacterial lysates induced with 0.5 mM IPTG.

The advantages of expression screening are: it is less time and quicker way of determining the presence and expression of protein of interest, also it could help to overcome the challenges of large scale expression which are inclusion body formation and insoluble protein aggregates. Using the SDS-PAGE gels as referencing points, the best expressing lanes (dark bands) were used to scale up for large expression.

5.2.3 Large scale expression

Well folded and soluble proteins are required for structural and functional characterization in the various fields of Biotechnology, Biochemistry and Molecular Biology. Hence the use of recombinant protein expression in pharmaceutical industries for the production of recombinant vaccines and drugs, is now gaining momentum. Subsequent studies on recombinant protein expression (using *E. coli*) over the years has increased the knowledge of its application in various arrays of *in vitro* studies such as protein, ligands or enzyme interactions (Papaneophytou and Kontopidis, 2014). Sufficient and soluble proteins are need for ion exchange, gel filtration and affinity purification; it is for this purpose that large scale expression was done to obtain the large quantity and soluble proteins needed for interaction and characterization studies.

The best expressing lanes from SDS-PAGE analysis were used to inoculate 100 µm (both proteins) overnight at 37 ℃ and next morning, the cultures were scaled up to 2 litres and induced with 0.5 mM IPTG at 25 ℃. The cells were harvested and extracted by centrifugation, and SDS-PAGE gels were used to evaluate the expression of recombinant proteins. The figures 5.6 and 5.7 shows the proteins were expressing and progression of expression at (5µl, 10µl, 15µl and 20µl of the loaded samples), and that 0.5 mM IPTG was able to induce sufficient recombinant expression of the two proteins.

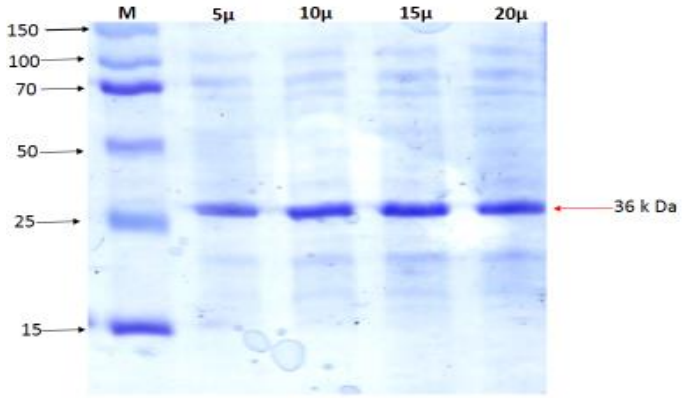


Figure 5.6: Large scale result of GST-RING finger domain transformed into BL21 cells. Lane M represents molecular marker in kDa. Lane 5µl, 10µl, 15µl and 20µl represents the samples obtained after overnight incubation of post 0.5 mM IPTG induction.

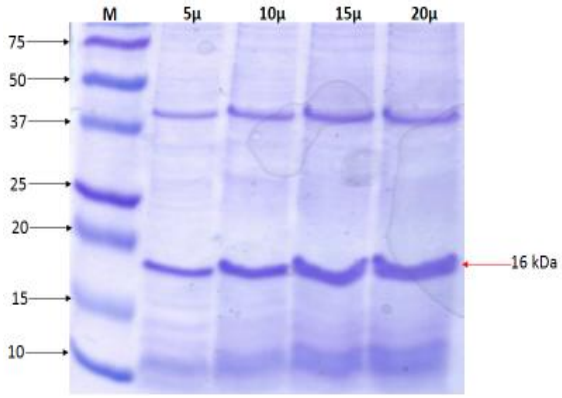


Figure 5.7: Large scale result of 6xHis-SBD of Hsp70.14 transformed into BL21 cells. Lane M represents molecular marker in kDa. Lane 5µl, 10µl, 15µl and 20µl represents the samples obtained after overnight incubation of post 0.5 mM IPTG induction.

5.2.4 Affinity purification of the proteins

The soluble recombinant 6XHis-Hsp70.14 and GST-RING were purified using Nikel-NTA column (recharged with cobalt) and GST-agarose affinity column respectively. These columns allow for non-denaturing and fast purification of tag proteins. These proteins bind to their respective columns which allowed impurities and non-specific proteins that do not have affinity for either GSH or Nikel column to flow out. A single step elution was carried out; where GST-RING was eluted with 10 mM glycine at pH 10, whereas 6XHis-Hsp70.14 was eluted with 50 mM Tris, 400 mM NaCl, 500 mM imidazole and 0.02% NaN_3 at pH 7.4. The collected fraction of proteins was subjected to SDS-PAGE analysis to determine how pure the proteins are, as shown in figure 5.8 (A and B).

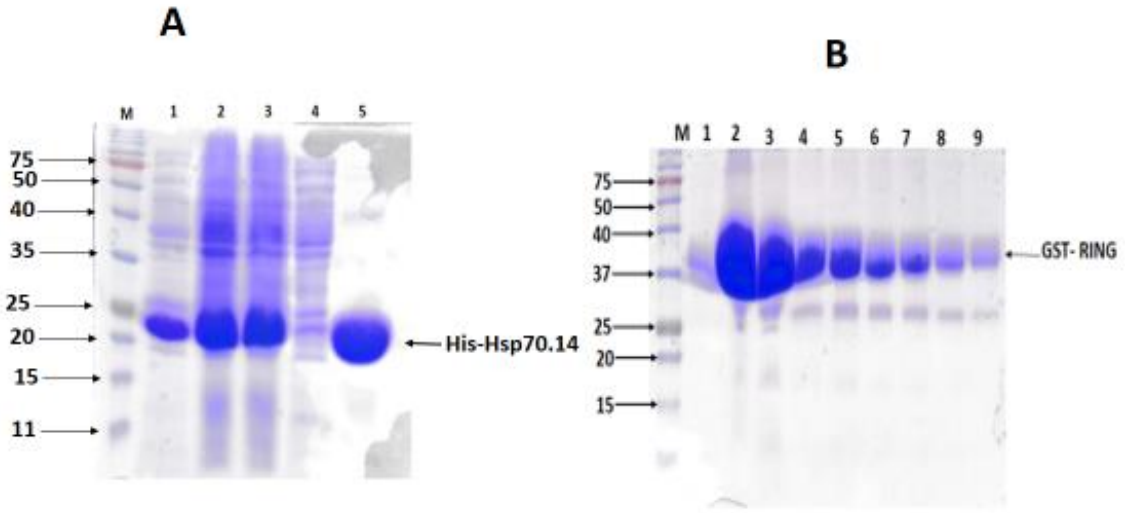


Figure 5.8: Purification gels. Diagram (A) represent Hsp70.14; M stand for molecular weight marker, lane 1 is uninduced sample, lane induced sample, lane is total bacterial lysate, lane is flow through and lane 5 is elution. Diagram (B) represent RING domain; lane (1-9) represent purified RING elution collected at different fraction of 5 ml.

The SDS-PAGE analysis showed that the proteins are pure enough for other biophysical and biochemical characterization owing to the clear-single band that is observed in lane 5 figure 5.8 (A) and lane (1-9) figure 5.8 (B), respectively. No single band corresponding to the size proteins on the flow through which indicates that all the proteins of interest bond to the respective column, and simple indication of successful purification. The good expression observed in un-induced sample (lane 1 in figure 5.8. A) is an indication of leaky expression and uncontrolled lac promoter, which means that the protein expresses in the presence and absence of IPTG. The slight band that appeared at approximately 26 kDa [figure 5.8 (B)] corresponds to GST cleaving itself off, this could due to the absence of PMF which inhibit its protease activities.

5.2.5 GST cleavage trials

Due to the big size of GST (26 kDa), it could interfere with protein (RING) absorption at 280 nm wavelengths and most especially, its interaction with other peptides (especially Hsp70.14) thus there is a need to separate GST from RING before it could be subjected to characterization and interaction studies. Following the incubation of GST-RING with 3c-protease, a 50 µl sample was taken out at every 15 minutes interval. As shown in the figure 5.9 (B), after T240 and O/N incubation which was where the protein was completely separated from GST tag. Additionally, figure 5.9 (A) showed cleavage trial in the presence and absence of chelating agent (EDTA). According to studies (Biegel and Makriyannis, 2003), the 3C-protease activity is inhibited by metal ion (such as Zn^{+2}) and figure 5.9 (A) shows there is a little different between lane 1 and 2 in terms of protease activities thus, a buffer containing EDTA was used. This was done to determine the activity of 3C protease in the presence and absence of its inhibitor; the result shows it has little or no effect.

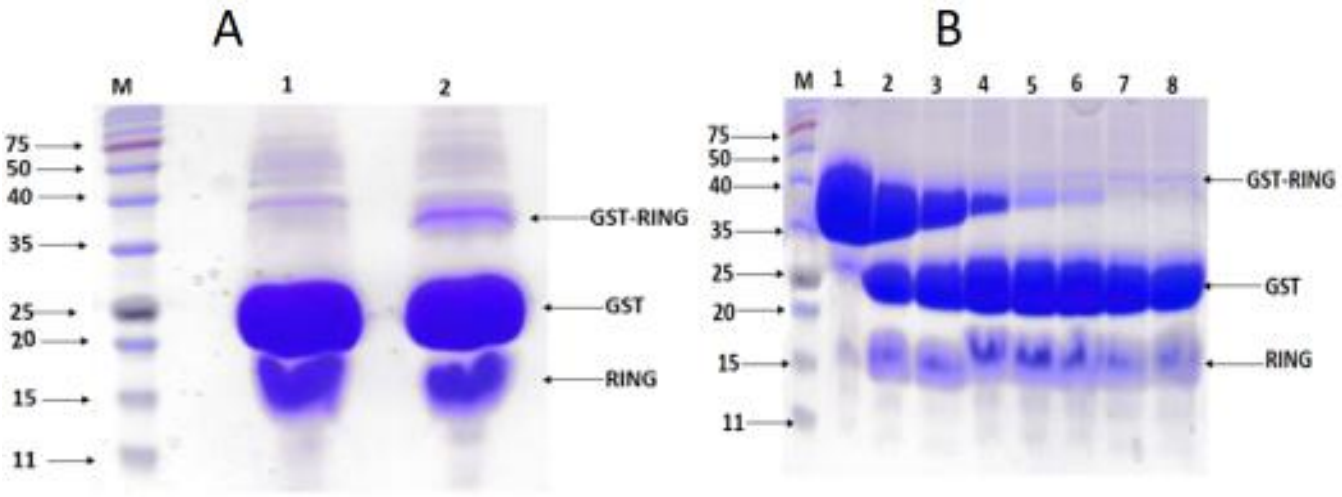


Figure 5.9: Cleavage trials. Diagram (A) present cleavage trial in the presence and absence of EDTA; lane 1 shows the activity of protease in the present of EDTA whereas lane presents action of protease in the absence EDTA. (B) represents cleavage trials over a period of time with sample taken at every 15 and 30 mins interval; lane is T_0, lane 2 is T_{15}, lane 3 is T_{30}, lane 4 is T_{60}, lane 5 is T_{90}, lane 6 is T_{120}, lane 7 is T_{240}, lane 8 is O/N.

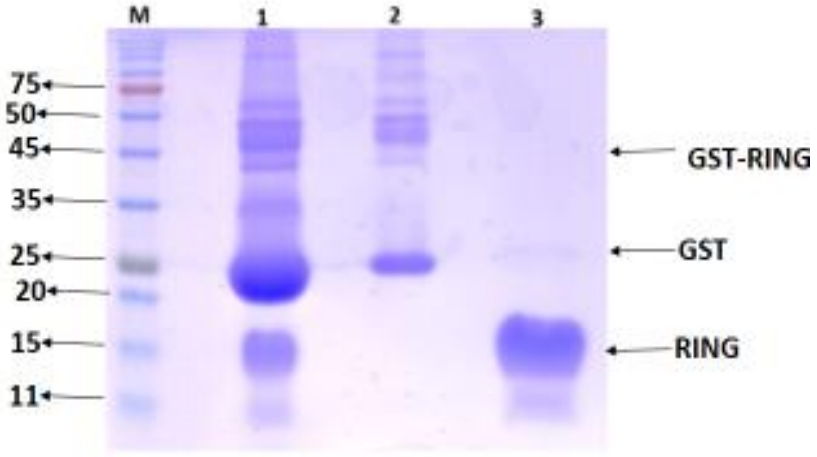


Figure 5.10: Removal of GST tag and recovery of RING. Lane M represent molecular marker, lane 1 is cleavage (GST+RING), lane 2 is cleaved GST and lane 3 is RING finger protein.

5.3 Determination of the proteins concentration

Ultraviolet (UV) spectroscopy was used to analyse the quality and concentrations of the purified proteins. UV spec aids to detect nucleic acid impurities or aggregates between 240-340 nm wavelengths. A serial dilution (1:100) was used to ascertain the proteins concentration, and absorbance was monitored in the range of 260 nm-340 nm wavelengths. The absorbance was plotted against the dilution. The equation below was derived from linear plotted graph.

y=15.05x-0.312

$$Conc.\ (mg/ml) = \frac{Molecular\ weight\ x\ slope}{\varepsilon} \qquad \ldots. (1)$$

$$= \frac{16700\ X15{,}05}{4470}$$

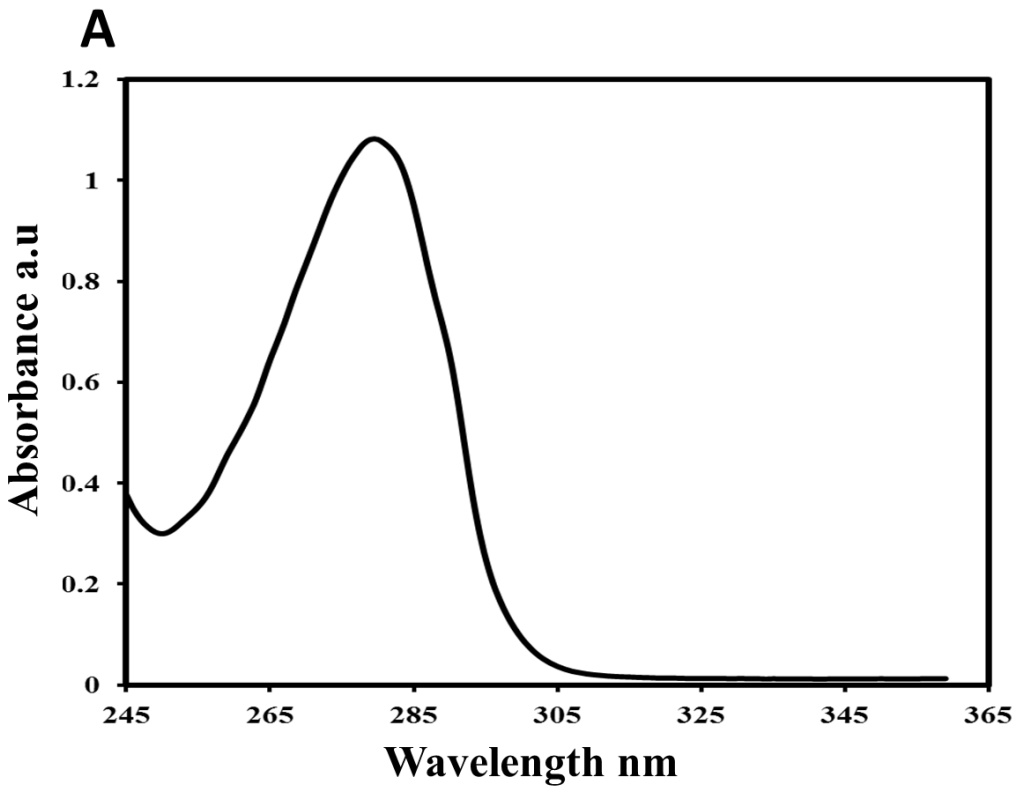
$$= 56.2\ mg/ml$$

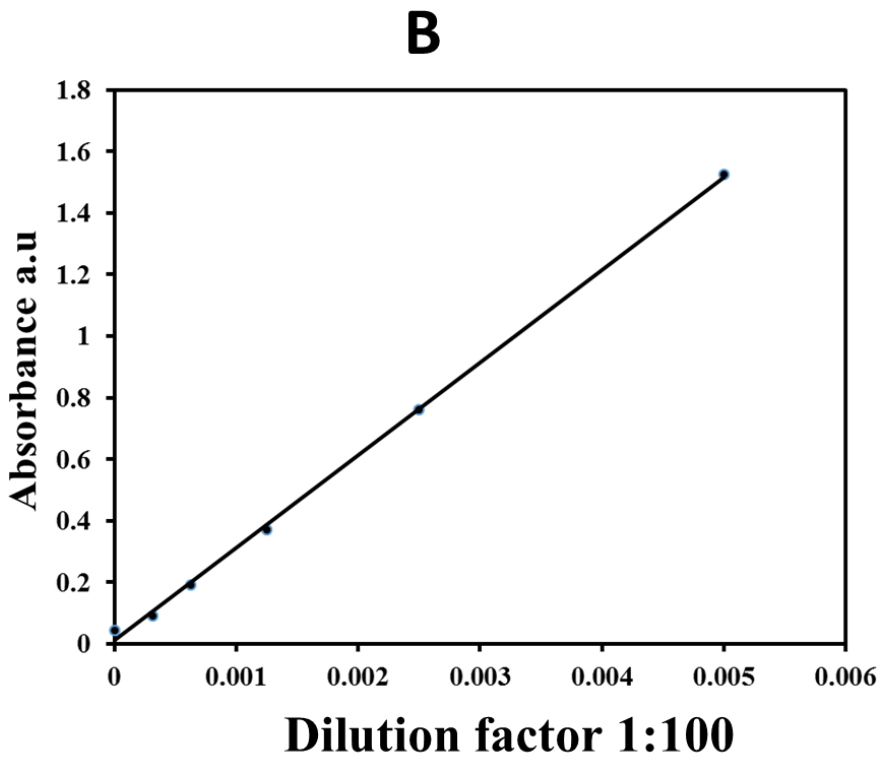
$$Conc.\ (mol/L) = \frac{reacting\ mass}{molar\ mass}$$

$$= \frac{56.2}{16700} = 0.0034\ mol/L \sim 3400\ \mu M$$

Therefore, the calculated protein concentration is 3400 µM

No peak was observed at 260 and 340 nm wavelength and a single peak seen at 280 nm wavelength, is an indication of pure protein with no impurities such as nucleic and non-nucleic acids contaminant.


A
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance a.u
245
265
285
305
325
345
365
Wavelength nm


B
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance a.u
0
0.001
0.002
0.003
0.004
0.005
0.006
Dilution factor 1:100

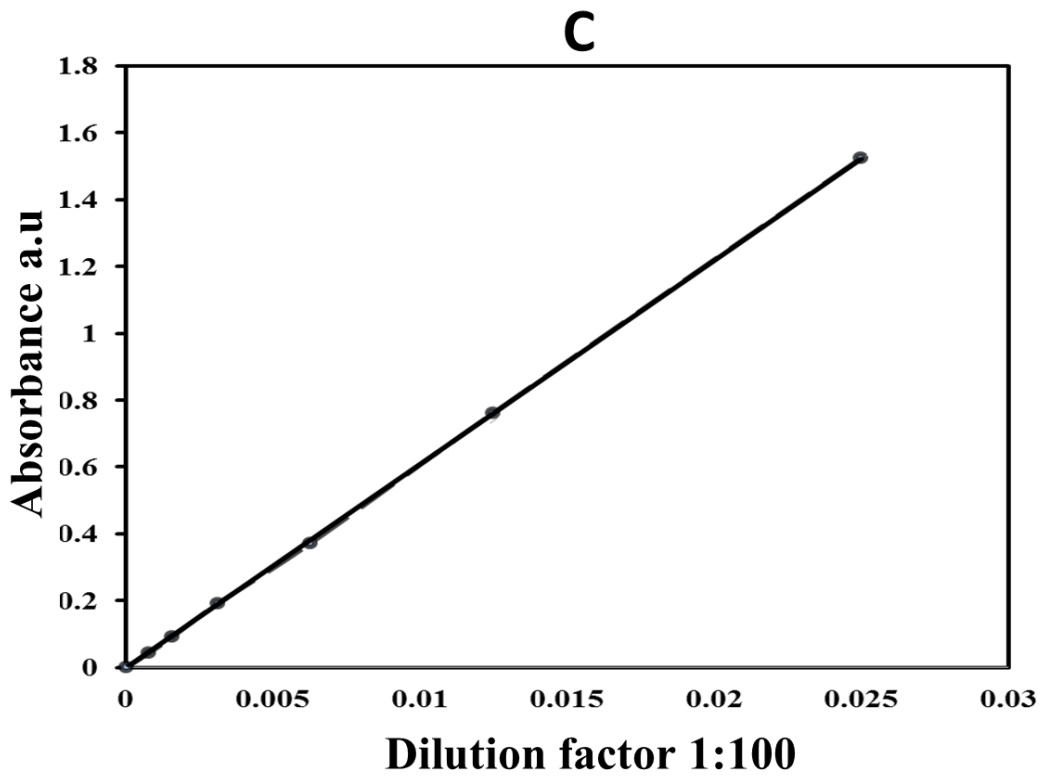
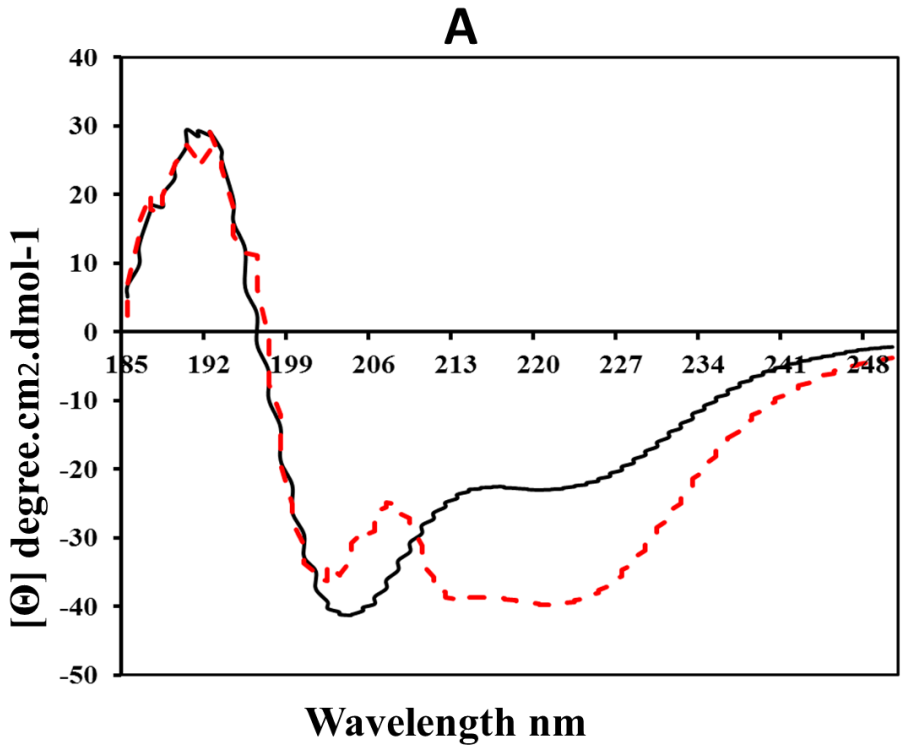


Figure 5.11: Quantitive and qualitative assessment of Hsp70.14. (A) UV absorption analysis and quality of the purified Hsp70.14, monitored between 245 and 365 nm. The absence of peaks at 260 nm and 340 nm indicates absence of protein aggregates and DNA impurities. (B) Dilution series (1:100) was used to ascertain the concentration of pure Hsp70.14, and corrected absorbance was plotted against the dilution factor (R^2=0.999) which was obtained. The resulting slope 15.05 was used to determine the concentration of the protein (mg/ml). (C) Dilution series (1:100) was used to ascertain the concentration of purified RING, and corrected absorbance was plotted against the dilution factor (R^2=0.999) which was obtained. The resulting slope 12.95 was used to determine the concentration of the protein (mg/ml), by substituting the value in equation (1).

5.4 Determination of the protein secondary structures

In the far-ultraviolet, the circular dichroism spectra for protein in a particular region have peculiar characteristics based on the proteins secondary structure adaptation (Hamilton and Sion, 2008). Therefore, far-UV was used to determine the secondary structural nature of the proteins (Hsp70.14 and RING). RING is predominately α-helix which have the highest and most characteristic spectra with distinct peak between 185-195 nm, with minima or trough at 200 nm wavelength (figure 5.12A). Like RING, Hsp70.14 composed mainly α-helix with highest peak at 192 nm and distinct trough between 206-277 nm wavelengths (figure 5.12B).

This is in contrast with bioinformatics results using I-TASSER (homology modelling) which predicted predominate β-sheets (4:2) for Hsp70.14 and evenly composition of α-helices and β-sheets (2:2) for RING, respectively.



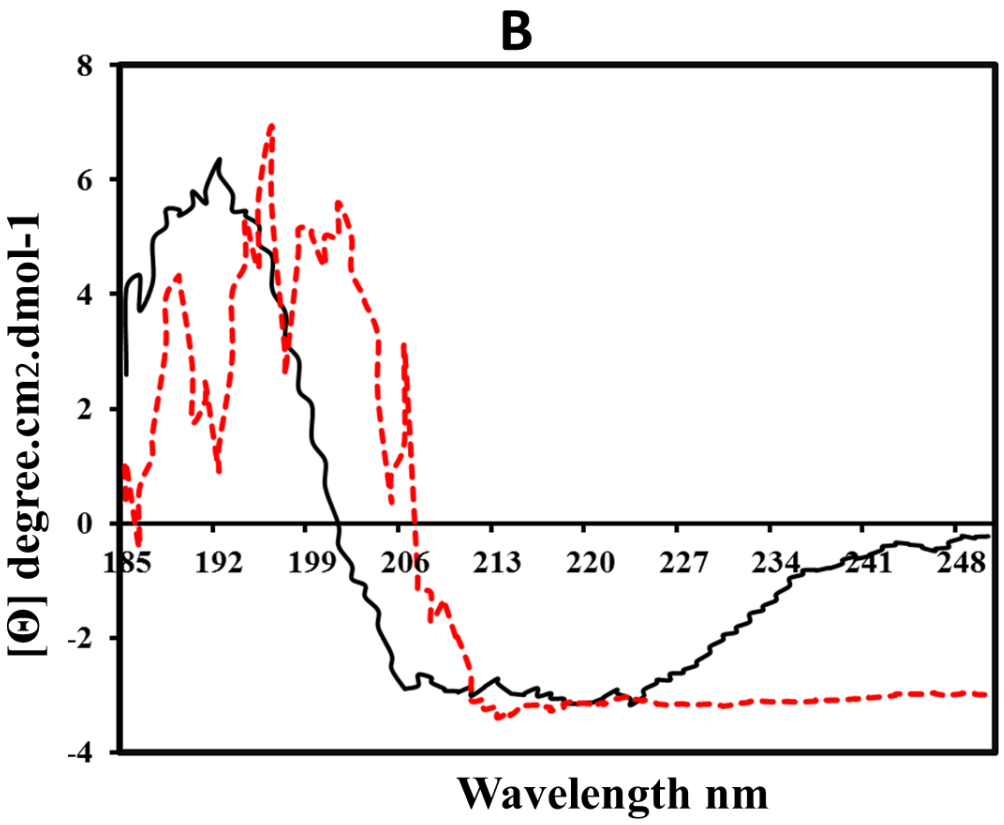
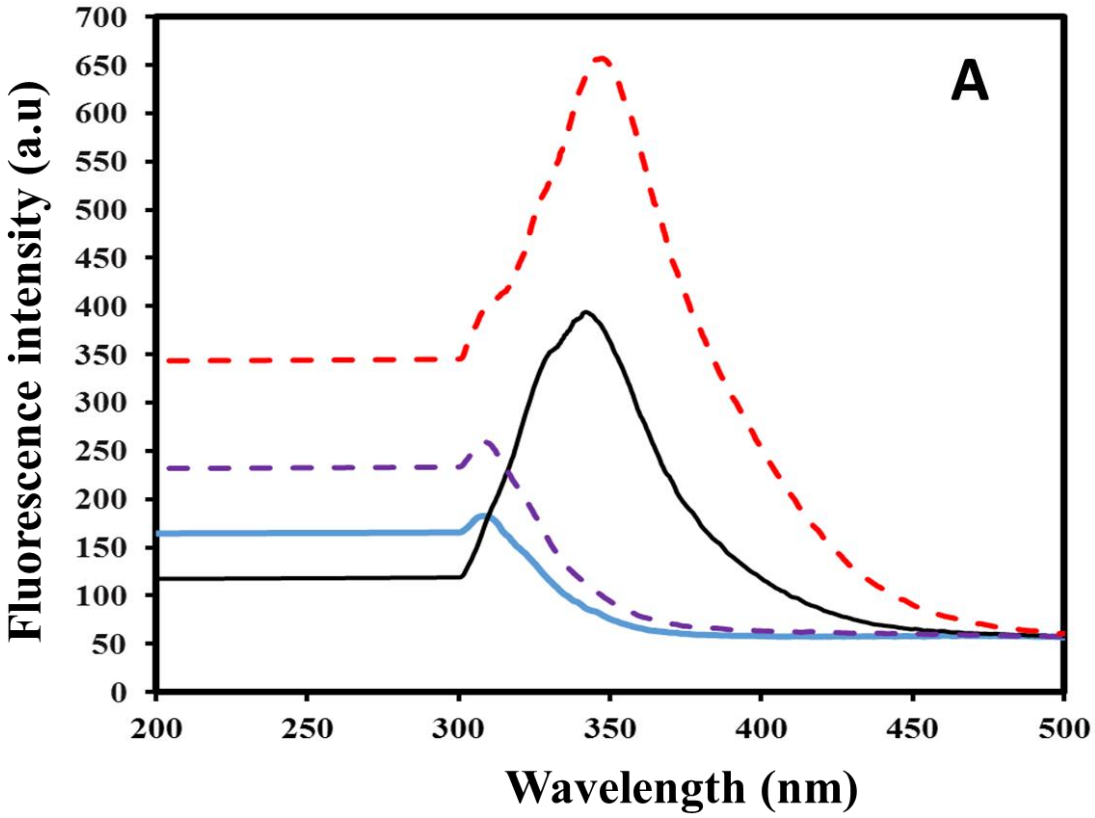


Figure 5.12: Circular dichroism spectra of the proteins using Far-UV spec. (A) represent the far-CV of RING in the absence (native) and presence (denatured) of 8 M Urea. The red dotted line represent denature whereas black solid line represent native sample which predominately α-helices. (B) Represent the secondary of Hsp70.14 in the presence and absence of 8 M urea. The red dotted line represent denature sample whereas black solid line represent native sample which predominately α-helices with a few β-sheet. All the experiment was carried using JASCO J810 far-UV spec at 20℃.

5.5 Determination of tertiary structure of the proteins

Tryptophan, phenylalanine and tyrosine are aromatic and dominantly fluorescent amino acids found in proteins due to their ability to absorb light frequency at 280 nm wavelengths. Any protein which lacks these amino acids especially tryptophan is likely to have little light absorbance capacity. Among the three, tryptophan has the highest light absorbing capacity and can absorb light at wavelengths longer than 295 nm owing to its indole group which absorbs and emit light at different wavelength, even though it is solvent dependent (Kerwin and Remmele, 2007). Intrinsic fluorescence spectroscopy was employed to characterise the

tertiary structures of the proteins in the presence and absence of denaturing agent (urea) in relation with their local environment. RING has total of 3 Phe, 2 Tyr and 0 Trp residues; whereas Hsp70.14 has total of 8 Phe, 0 Trp and 3 Tyr residues. The spectra were selectively excited at 295 nm and emission of light and quantum yield was monitored at 300 -500 nm wavelengths. The maximum emission wavelength was observed between 300 nm and 400 nm (280 nm) and 350 nm and 400nm (295 nm) for the proteins (Hsp70.14, RING and RING + Hsp70.14), in presence and absence of urea as shown in figure 5.13 (A and B).



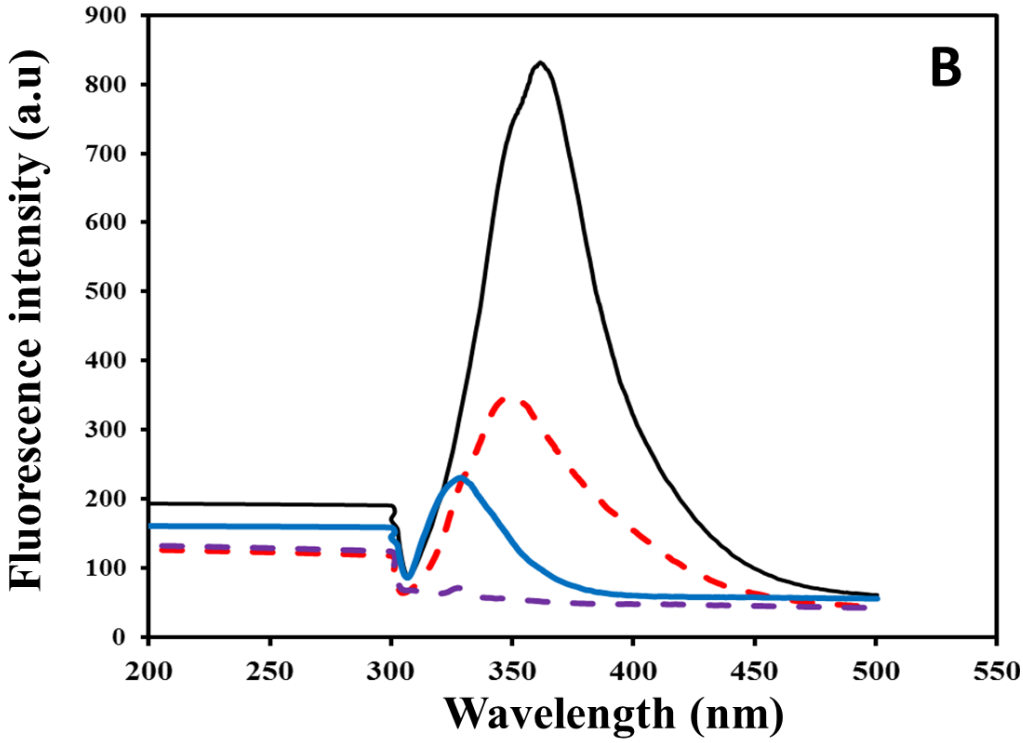
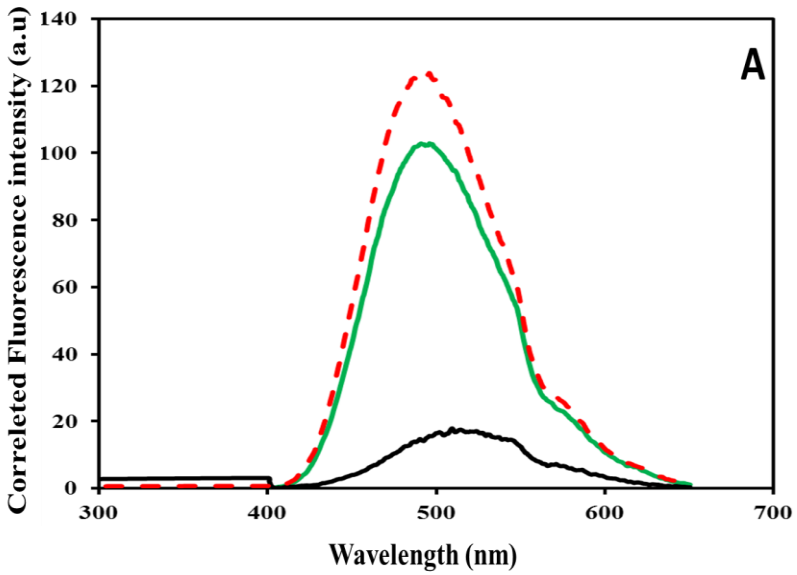


Figure 5.13: Intrinsic fluorescence emission spectra of the proteins. (A) Shows the absorption spectra of the proteins at 280 nm in the presence (denatured) and absence (native) of 8 M urea. The red dotted line represents denatured Hsp70.14, and solid black line represent native its native sample. In addition, the dotted pink line represent denature RING sample whereas solid blue line represent its native sample. (**B)** Shows the absorption spectra of the proteins at 295 nm in the presence (denatured) and absence (native) of 8 M urea. Once again, the red dotted line represents denatured Hsp70.14, and solid black line represent native its native sample. Whereas the pink dotted and blue line represent denatured and native RING samples, respectively. Each spectrum was excited at 295 nm and collected in triplicate at average of three accumulations. The emission was monitored between 300 nm and 500 nm wavelength at 20℃.

5.6 Extrinsic ANS binding studies

8-Aminonaphthalen-1-sulfonic (ANS) is a probe that is used to ascertain the existence of hydrophobic pockets in the proteins. ANS binding assay was used to investigate the presence of hydrophobic and ANS binding patches in Hsp70.14 substrate binding domain, RING and the effects of ANS on the Hsp70.14-RING complex. All the aforementioned binding studies were done in the presence and absence of denaturing agent (8 µM urea).

Following the excitation at 395 nm, a major rise in the level of quantum yield associated with blue shift in emission maxima was witnessed when far-UV monochromatic light was passed through native Hsp70.14 compared to denatured sample. This could signify binding between Hsp70.14 and ANS. RING finger samples (denatured and native) showed no significant increase in the emission spectra, and it is likely it has little or no hydrophobic pockets therefore, leading to decrease of the quantum yield. In addition, native Hsp70.14-RING complex showed notable increase in quantum yield, shift in emission maxima and fluorescence intensity compared to the denatured one which decreased in fluorescence intensity (figure 5.14. A and B).



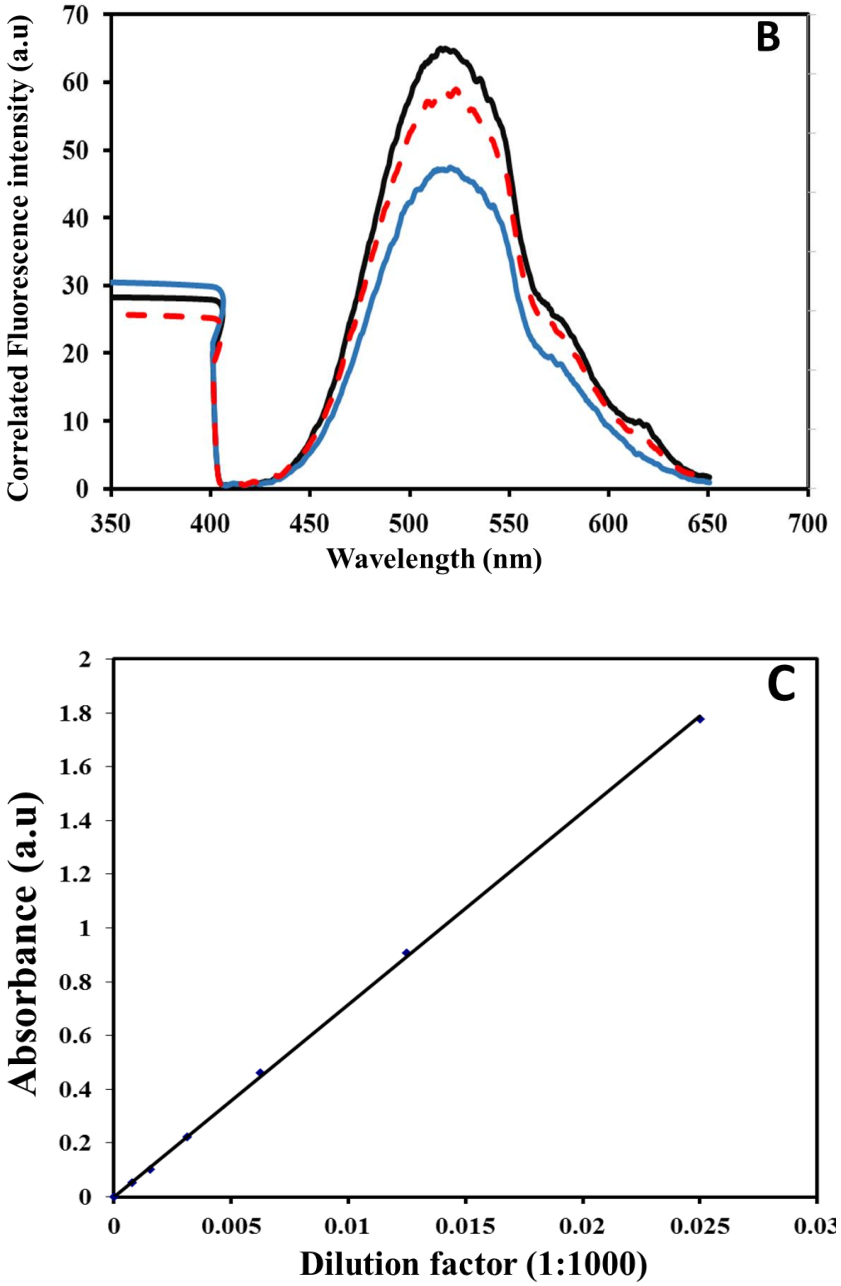


Figure 5.14: ANS binding fluorescence emission analysis. (A) Extrinsic ANS fluorescence spectra of native samples. Lime green line represent native Hsp70.14, solid black line shows RING and red dotted line shows Hsp70.14 + RING. The emission spectra was monitored and collected between 300 nm to 500 nm. (B) Extrinsic ANS binding spectra of denatured samples. Black line represent ANS binding to denatured Hsp70.14, blue line shows ANS binding to RING and red dotted line shows ANS binding to Hsp70.14-RING complex. A 15.4 µl of 200 µM of ANS concentration was, and it allowed to bind to 11 µl RING and 12 µl Hsp70.14. ANS was excited at 395 nm, and spectrum emission collected between 300-500 nm wavelengths. (C) Represent the serial dilution ANS binding essay used to determine the concentration of ANS (200 µM).

5.7 Size exclusion high pressure liquid chromatography (SE-HPLC)

SE-HPLC is often employed to separate protein molecules based on diffusion coefficient, hydrodynamic size or volume and surface properties. SE-HPLC was used to characterize the quaternary structure of proteins (Hsp70.14 and RING), and also to ascertain the possible binding between the proteins by monitoring their retention. Ideally, the bigger proteins are supposed to be eluted first but sometimes proteins behave differently on SE-HPLC column because of their hydrodynamic volume which increases their retention time. To determine the size and retention time of the proteins, a column calibration was done using bovine serum albumin (66.6 kDa), CLIC-1 (30 kDa) as standard. This standard of known size was used to estimate the molecular size of Hsp70.14 as shown in (figure 5.15.A). In the light of this, the possible interaction between Hsp70.14 and RING was ascertained considering the graphical shift, the retention times of the proteins and the complex (figure 5.15.B).

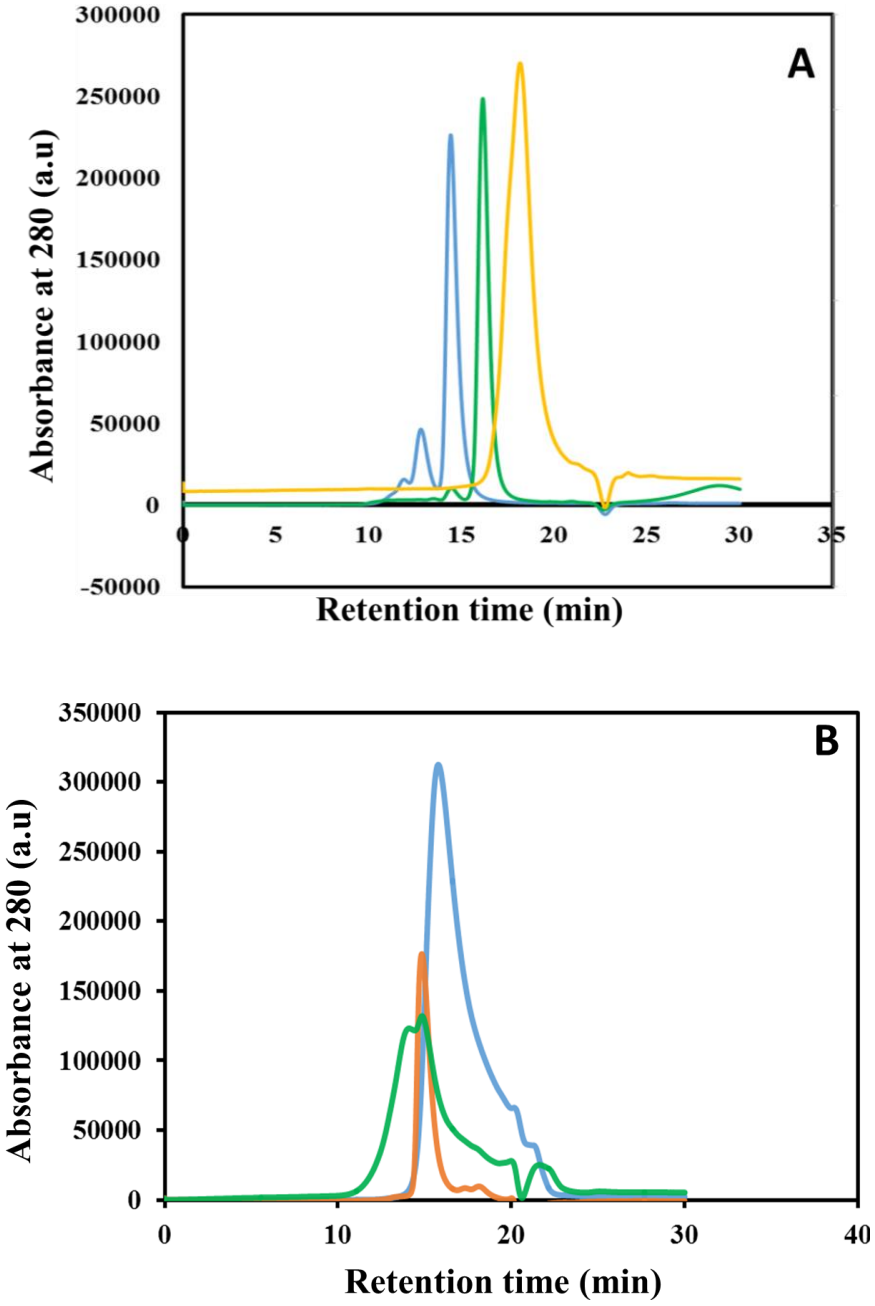


Figure 5.15: SE-HPLC chromatogram of the proteins. The elution profile of the proteins was resolved using SE-HPLC. **(A)** Represent the elution and retention of the proteins [BSA-65 kDa (blue line), CLIC-31 kDa (green line) and Hsp70.1 (yellow line)] which was used to estimate the size of Hsp70.14 based on their retention time using gel filtration standard. **(B)** Shows the possible interaction between the two proteins using SE-HPLC. Blue solid line-Hsp70.14, orange line-RING and green line-Hsp70.14-RING complex. Gel filtration standard was used to estimate their molecular mass based on their hydrodynamic volume.

References

Chang DTH, Syu YT and Lin PC (2010). Predicting the protein-protein interactions using primary structures with predicted protein surface. *BMC Bioinformatics* **11**(1): 3.

Chen W, Hong YQ and Meng ZL (2014). Bioinformatics analysis of molecular mechanisms of chronic obstructive pulmonary disease. *European Review for Medical and Pharmacological Science* **18**(23): 3557-3563.

Arnold K, Bordoli L, Kopp J and Schwede T (2006). The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22**(2): 195-201.

Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L and Schwede T (2014). SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Research* **42:** 252-258.

Best RB, Zhu X, Shim J, Lopes PE, Mittal J, Feig M and MacKerell Jr AD (2012). Optimization of the additive CHARMM all-atom protein force field targeting improved sampling of the backbone ϕ, ψ and side-chain $\chi 1$ and $\chi 2$ dihedral angles. *Journal of Chemical Theory and Computation* **8**(9): 3257-3273.

Schneidman-Duhovny D, Inbar Y, Nussinov R and Wolfson HJ (2005). PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucleic Acids Research* **33**: 363-367.

Laskowski RA, Rullmann JAC, MacArthur MW, Kaptein R and Thornton JM (1996). AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *Journal of Biomolecular NMR* **8**(4): 477-486.

Backert L and Kohlbacher O (2015). Immunoinformatics and epitope prediction in the age of genomic medicine. *Genome Medicine* **7**(1): 119.

Tomar N and De RK (2010). Immunoinformatics: an integrated scenario. *Immunology* **131**(2): 53-168.

Flower DR. (2007). Immunoinformatics and the *in Silico* Prediction of Immunogenicity. In *Immunoinformatics* 1-15. Humana Press.

Rosano GL and Ceccarelli EA (2014). Recombinant protein expression in Escherichia coli: advances and challenges. *Frontiers in Microbiology* **5:** 172.

Esposito D and Chatterjee DK (2006). Enhancement of soluble protein expression through the use of fusion tags. *Current Opinion in Biotechnology* **17**(4): 353-358.

Kosano H, Stensgard B, Charlesworth MC, McMahon N. and Toft D (1998). The assembly of progesterone receptor-hsp90 complexes using purified proteins. *Journal of Biological Chemistry* **273**(49): 32973-32979.

Weickert MJ, Doherty DH, Best EA and Olins PO (1996). Optimization of heterologous protein production in Escherichia coli. *Current Opinion in Biotechnology* **7**(5): 494-499.

Papaneophytou CP and Kontopidis G (2014). Statistical approaches to maximize recombinant protein expression in Escherichia coli: a general review. *Protein Expression and Purification* **94:** 22-32.

Hamilton RT and Sion EM (2008). Far-ultraviolet spectral analysis of the prototype nova-like variable VY sculptoris from the high state to the low state. *Publications of the Astronomical Society of the Pacific* **120**(864): 165.

Kerwin BA and Remmele Jr RL (2007). Protect from light: photodegradation and protein biologics. *Journal of Pharmaceutical Sciences* **96**(6): 1468-1479.

CHAPTER SIX

GENERAL DISCUSSION AND FUTURE RECOMMENDATIONS

6.1 General discussion

Cancer is a group of diseases characterized by abnormal cell growth and invasion resulting from mutated tumour suppressor genes or uncontrolled cell divisions. Despite improvement in cancer treatment modalities, cancer remains one of the leading causes of death worldwide, in both developed and developing countries (Ikwegbue *et al.,* 2017). Cancer has a huge impact on society well-being across the globe. In United State alone, ~609,640 mortalities resulting from cancer were expected to occur in 2018, which are nearly 1,700 deaths every day. With lung cancer been the most prevalence sources of death, breast, prostate and colorectum cancers accounts for nearly 45 % of all the cancer mortalities (Torre *et al.,* 2018). Though cancer statistics in Africa setting are poorly documented due to the lack of nationwide surveillance in Sub-Sahara Africa, studies have reported prostate cancer as the most common among men and boys and breast cancer among women and girls (Singh *et al.,* 2015).

Over the years, the several unsuccessful attempts to completely eradicate cancer diseases from human life has led to variety of application therapies (such as immunotherapy, targeted therapy, antibody based therapy, chemotherapy, surgery) aimed at finding treatment and radical cure for these diseases (Padayachee *et al.,* 2019). Although there has been a major improvements in the application of these therapies over a decade now (Siegel *et al.,* 2017), yet, they are still faced with challenges of non-specificity and several side effects, resulting in the killing of normal cells by chemotherapeutic agents. This has led to continuous look out for alternative therapies aimed at finding alternative drugs or to improve the current available treatment modalities. In the same vain, many researchers have exploited the application of

heat shock proteins (Hsps) as a therapeutic intervention against cancer because of their ever-presence in the pathology of different facets of cancer development. Studies have shown that tumour cells requires Hsp chaperonage activities for survival and proliferation than normal cells, owning to the fact that most oncoprotein in invading cells are often unfolded therefore require chaperonage protection of Hsps for survival (Chatterjee and Buns, 2017). Hsp70.14, alternatively known as HSP70L1, is a variant of Hsp70 that lacks c-terminal but contains substrate binding domain and ATPase domain. The involvement of this protein in cancer development has been well documented. According the work by Kappo and colleagues (2012) which postulated the putative interaction between this protein (Hsp70.14) and RING finger domain of RBBP6, this interaction suggests their function in the protein quality control system (Kappo *et al.,* 2012). RING finger domain of RBBP6 is a cysteine rich domain that coordinate Zn^{+2} in a "cross braced" topology. Additionally, studies have implicated RING finger domain in the ubiquitination and proteolysis degradation of tumour suppressor protein, p53 (Pugh *et al.,* 2006). These findings suggest its function in the events that lead to cancer progression and proliferation.

In order to ascertain the interaction between these proteins, these proteins were recombinatly expressed in *E. coli* cells (BL21 and T7 cells), purified and characterized using Far-UV, CD spec, SE-HPLC. Prior to expression, several bioinformatics and computational tools were employed to analyze, predict, interpret and validate the physiochemical and structural characterization of the proteins. In the last decade, bioinformatics has been employed in the different areas of drug design and discovery to predict the structure of a protein or to get useful information about proteins or ligands or protein-protein interaction prior to wet-lab experiment (Gill *et al.,* 2016). As presented in chapter five [figure 5.1 (A)], the 3-D structural analysis of Hsp70.14 revealed 4 β-sheets, 3 α-helices, and many random coils joining them

together; whereas figure [5.1 (B)] depicts 3-D models viewed with Pymol, which revealed 2 β-sheets and 2 α-helices, joined by random coils.

Homology modelling revealed about 90 % residues of Hsp70.14 and 81 % residues RING are found in most favored region. Furthermore, Ramachandran plot analysis in RAMPAGE which employs PROCHECK was used to validate the overall stereeochemical parameters and quality of models built, which showed that overall structures are of good quality and stable (figure 5.2 A and B). Following the homology modelling, molecular docking was carried out using PatchDock which based on principle of size and shape complementarity (Schneidman-Duhovny *et al.,* 2005). The result (figure 5.3) revealed the interaction and binding behaviour of Hsp70.14 with RING finger domain, which when visualized with PyMol, gives information on the residues (GLU-21, ARG-34, ILE-56, ILE-67 and LEU-52) that are involved in the binding. PatchDock result also gave useful information on binding affinity (Kd)-16038, area of the binding/interaction (2603.70) and contact atomic energy (-125.15). Overall PatchDock studies revealed there is strong binding between the proteins judging by high binding affinity score (16038) of the interaction.

Recombinant protein expression and purification are essential and prerequisite for many structural, functional, characterization and interaction studies use in different facets of drug discovery and vaccine interventions for several diseases including cancer (Kosano *et al.,* 1998). Recombinant 6xHis-Hsp70.14 and GST-RING were successfully expressed (BL21 and T7 cells) and purified using Nikel NTA column (recharged with 0.1 M cobalt) and GST agarose column (agarose beads) respectively as shown (figure 5.4&5.5). The SDS-PAGE analysis of the recombinant proteins revealed ~37 kDa for GST-RING and ~17 kDa for 6xHis-Hsp70.14.

Prior to characterization and interaction studies, GST tag was cleaved off the RING finger domain to get a pure soluble RING protein for characterization and interaction studies as shown in figure 5.9. Different cleavage trials and at different concentration of PreScission protease were carried out in the presence and absence metal chelating agent (EDTA). The results revealed that in the absence of EDTA, the activity of 3C protease is slower compared to the presence of EDTA which chelate Zn^{+2} present in the RING finger elution buffer (fig 5.9.A). SDS-PAGE analysis revealed GST tag was completely cleaved off after overnight incubation with the protease. These results indicated that purified proteins were suitable for characterization and interaction studies.

Assessment and characterization of the purified proteins are essential step for application in this and further studies. For these reasons, UV spec, far-UV, SE-PLC, CD spec, among others was employed for the characterization of these proteins as well as obtaining their ANS binding affinities. A typical UV protein spectrum (figure 5.11.A) showed a peak at 280 nm which is wavelength where most proteins with exposed aromatic rings (tryptophan, tyrosine or phenylalanine) residues absorb light. The number of peak observed at 340 nm indicates the absence of protein aggregates and contamination by nucleic acid molecules. Serial dilutions (Figure 5.11 B&C) used to determine the concentration of proteins, with Hsp70.14 (3.4 mM) and RING 3.7 mM, respectively. Furthermore, SE-HPLC was used to determine the quaternary structure and molecular weight of Hsp70.14 using known molecular size BSA (65 kDa) and CLIC 31 kDa as gel filtration standard. According result (fig 5.15A), the molecular size of Hsp70.14 was estimated to be 9 kDa whereas the expression and purification for SDS-PAGE analysis showed 17 kDa. This could be due to gel filtration standard used (BSA). BSA is globular protein with ability to dimerize with other proteins (Sun *et al.*, 1985). Nevertheless, the fact the proteins were eluted according to their molecular weight (from

biggest to smallest) is evidence of successful size exclusion experiment and quaternary determination of Hsp70.14.

Secondary structures of the proteins were determined using CD spectrometer. The CD spectra of the proteins depicted in (figure 5.12A&B) revealed a predominantly α-helices for both proteins in contrast with bioinformatics homology modelling which predicted predominately β-sheets for Hsp70.14 and evenly balance (2α-helices and 2β-sheets) for RING (figure 5.1A&B). Secondary structure analysis revealed the polypeptide backbone of the proteins, however, there is no observable difference between the native and denatured RING samples, and this could spell no interaction between the protein and denaturing agent unlike Hsp70.14 which shows distinctive interaction with urea.

Intrinsic fluorescence spectroscopy was employed to characterize the tertiary structures of the proteins in the presence and absence of denaturing agent (urea) in relation with their local environment. RING has total of 3 Phe, 2 Tyr and 0 Trp 2 residues; whereas Hsp70.14 has total of 8 Phe, 0 Trp and 3 Tyr residues. It is observable that almost all the Trp residues in RING is buried in the hydrophobic core whereas few of Hsp70.14 Trp are exposed to hydrophilic environment. This is evidenced in figure 5.13A where absorption of light intensity increased in the presence of denaturing agent represented with dotted red and pink line, and this indicates that the proteins are in their 3-D structure (properly folded). The inability for RING to absorb more light owes to its less composition of aromatic amino acids. The ability of native Hsp70.14 to absorb light at 295 is further evidence of its abundance composition of aromatic amino acids and also the presence of some aromatic amino acids in hydrophilic environment.

Furthermore, the ANS fluorescence characteristics hydrophobic probe is very valuable in assessing conformational changes in protein structures as well as in the determination of hydrophobic binding pockets. Hsp70.14 shows interaction with ANS as a result of an increase in the quantum yield (green line) between 420-620 nm wavelengths compared RING (black line) which shows little ANS binding capability (5.14A). This is an indication of hydrophobic patches found in Hsp70.14 where ANS can bind. Interestingly, Hsp70.14-RING complex (dotted red line) shows an increase in quantum yield compared to RING alone and decrease compared to Hsp70.14. The shift in quantum yield in this complex could signal interaction between the two proteins and ANS. It could also be possible that Hsp70.14 bound to RING, and that decreases its ability to bind ANS hence maxima emission spectra reduces. Nevertheless, it is not surprising that there was no observable increase in quantum yield of denatured sample of the proteins and complex, as well as their interaction with ANS. This is clear evidence in (figure 5.14B), owning to the fact that proteins have been denatured with 8 M urea therefore could not interact properly (weak interaction) with themselves or with ANS in an unfolded form.

In support of ANS binding studies which showed a shift in the maxima emission quantum yield or increase in the fluorescence intensity as result of binding, and PatchDock molecular docking which predicted a strong binding between Hsp70.14 and RING finger domain. SE-HPLC which separates proteins based on their size and hydrodynamic volume was used to assess the possible binding between the proteins taking into consideration their retention time at 280 nm wavelength. Following the SE-HPLC analysis RING (smaller in size) was eluted first around 17 mins, whereas Hsp70.14 was eluted one minute later (18 mins) depicted in figure 5.15B. Ideally, the bigger proteins are supposed to be eluted first from the column but sometimes proteins behave differently on SE-HPLC column because of their hydrodynamic

volume which increases their retention time. Thus, RING may have bigger hydrodynamic volume than substrate binding domain of Hsp70.14. Also, for the fact that molecular weight of Hsp70.14 obtained from SE-HPLC (figure 5.15A) is 9 kDa, could explain its behaviour in size exclusion chromatography and why RING (11 kDa) was eluted first. Furthermore, the complex of Hsp70.14-RING showed different elution pattern (two peaks) in such a way that the peak of the complex was eluted first and as retention time decreased whereas the second peak corresponds to RING elution peak (figure 5.15B). The decrease in the elution profile of the complex (first peak) is further evidence suggesting the interaction between two proteins as well as formation of protein complex (Hsp70.14-RING). It is difficult to calculate the binding affinity and strength of interaction at this point, however, based on molecular docking result, ANS binding studies and SE-HPLC results, it can be postulated that there is interaction between the proteins.

6.2 Recommendation for future studies

The finding in this study shows that the proteins can recombinatly expressed, purified, solublized and stablized. Additional, ANS binding study, SE-HPLC and molecular docking have shown interaction between the two proteins. The future work will be to ascertain binding affinity (K_D) and stoichiometry of the binding using MST or NMR, as well as solve the structure of the complex using X-ray crystallography, in order to test their therapeutic applications in cancer drug.

References

Ikwegbue PC, Masamba P, Oyinloye BE and Kappo AP (2018). Roles of heat shock proteins in apoptosis, oxidative stress, human inflammatory diseases, and cancer. *Pharmaceuticals* **11**(1): 2.

Torre LA, Trabert B, DeSantis CE, Miller KD, Samimi G, Runowicz CD, Gaudet MM, Jemal A and Siegel RL (2018). Ovarian cancer statistics, 2018. *CA: a Cancer Journal for Clinicians* **68**(4): 284-296.

Padayachee ER, Adeola HA, Van Wyk JC, Nsole Biteghe FA, Chetty S, Khumalo NP and Barth S (2019). Applications of SNAP-tag technology in skin cancer therapy. *Health Science Reports* 103.

Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RG, Barzi A and Jemal A (2017). Colorectal cancer statistics, 2017. *CA: a Cancer Journal for Clinicians* **67**(3): 177-193.

Chatterjee S and Burns T (2017). Targeting heat shock proteins in cancer: a promising therapeutic approach. *International Journal of Molecular Sciences* **18**(9): 1978.

Kappo MA, Eiso AB, Hassem F, Atkinson RA, Faro A, Muleya V, Mulaudzi T, Poole JO, McKenzie JM, Chibi M and Moolman-Smook JC (2012). Solution structure of RING finger-like domain of retinoblastoma-binding protein-6 (RBBP6) suggests it functions as a U-box. *Journal of Biological Chemistry* **287**: 7146-7158.

Pugh DJ, AB E, Faro A, Lutya PT, Hoffmann E and Rees DJ (2006). DWNN, a novel ubiquitin-like domain, implicates RBBP6 in mRNA processing and ubiquitin-like pathways. *BMC Structural Biology* **6:** 1

Gill SK, Christopher AF, Gupta V and Bansal P (2016). Emerging role of bioinformatics tools and software in evolution of clinical research. *Perspectives in Clinical Research* **7**(3): 115.

Kosano H, Stensgard B, Charlesworth MC, McMahon N. and Toft D (1998). The assembly of progesterone receptor-hsp90 complexes using purified proteins. *Journal of Biological Chemistry* **273**(49): 32973-32979.

Sun SF, Del Terzo S and Nash RA (1985). Size-exclusion chromatographic behaviour of bovine serum albumin in uni-uni valent ions solutions. *Chromatographia* **20**(5): 303-307.