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Investigation of the effect of HSP90 inhibitor treatment on tumour cell biology and the bone microenvironment

by

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Submitted in total fulfillment of the requirements of the degree of Doctor of Philosophy

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TABLE OF CONTENTS

GENERAL DECLARATION

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Declaration for thesis based or partially based on conjointly published or unpublished work.

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 unpublished publications. The core theme of the thesis is **the investigation of the effect of HSP90 inhibitor treatment on tumour cell biology and bone microenvironment**. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the **Department of Biochemistry and Molecular Biology** under the supervision of **Dr. John Price**.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 2, 3 and 4, my contribution to the work involved the following:

Thesis chapter	Title	Publication status	Nature and extent of candidate's contribution
2	HDAC Family Members Contribute To Cancer Cell Acquired Resistance Towards Structurally Diverse HSP90 Inhibitors	Manuscript in preparation	Participated in the development of project hypothesis, designed and performed all experimental procedures, analysed data, prepared and wrote the manuscript.
3	Acquired Resistance Towards HSP90 Inhibitors Is Associated With Decreased Metastatic Potential But An Enhanced Osteolytic Phenotype	Manuscript in preparation	Participated in the development of project hypothesis, designed and performed all experimental procedures except for Figure 3.4, analysed data, prepared and wrote the manuscript.
4	Molecular Stress Inducing Compounds Increase Osteoclast Formation In A Heat Shock Factor 1 Dependent Manner	Manuscript published	Participated in the development of project hypothesis, designed and performed all experimental procedures except for Figure 4.5, analysed data, prepared and wrote the manuscript.

I have renumbered sections of yet-to-be submitted papers in order to generate a consistent presentation within the thesis.

Signed: ……  ……

Date: 22/06/2014 ……

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PUBLICATIONS

Lang B.J., Nguyen L., Nguyen C.H., Vieusseux J.L., **Chai R.C.C.**, Christophi C., Fifis T., Kouspou M.M., and Price J.T., *Heat stress induces epithelial plasticity and cell migration independent of heat shock factor 1.* Cell Stress Chaperon., 2012. **17**(6): p. 765-778.

Nguyen C.H., Lang B.J., **Chai R.C.C.**, Vieusseux J.L., Kouspou M.M., and Price J.T., *Heat-shock factor 1 both positively and negatively affects cellular clonogenic growth depending on p53 status.* Biochem. J., 2013. **452**(2): p. 321-329.

van der Kraan A.G., **Chai R.C.C.**, Singh P.P., Lang B.J., Xu J., Gillespie M.T., Price J.T., and Quinn J.M., *HSP90 Inhibitors Enhance Differentiation and Microphthalmia Transcription Factor (MITF) Activity in Osteoclast Progenitors.* Biochem. J., 2013. **451**(2): p. 235-244.

Chai R.C.C., Kouspou M.M., Lang B.J., Nguyen C.H., van der Kraan A.G., Vieusseux J.L., Lim R.C.C., Gillespie M.T., Benjamin I.J., Quinn J.M. and Price J.T., *Molecular Stress Inducing Compounds Increase Osteoclast Formation in a Heat Shock Factor 1 Dependent Manner.* J. Biol. Chem., 2014. **289**(19):13602-13614.

Chai R.C.C., Vieusseux J.L., Lang B.J., Nguyen C.H., Kouspou M.M. and Price J.T., *HDAC Family Members Contribute to Cancer Cell Acquired Resistance Towards Structurally Diverse HSP90 Inhibitors*. **Manuscript in preparation.**

Chai R.C.C., Vieusseux J.L., Nguyen C.H., Lang B.J., McGregor N.E., Sims N.A., Britt K., Kouspou M.M. and Price J.T., *Acquired Resistance towards HSP90 Inhibitors is Associated with Decreased Metastatic Potential but an Enhanced Osteolytic Phenotype.* **Manuscript in preparation.**

SUMMARY

HSP90 is a molecular chaperone that is ubiquitously expressed in cells, participating in the stabilization and activation of over 200 proteins, many of which are essential for cell signaling and adaptive stress responses. To accomplish this, HSP90 forms an ATP-dependent multi-protein dynamic complex termed the HSP90 chaperone machine. Cancer cells are dependent on this machinery to protect numerous mutated and overexpressed oncoproteins from misfolding and degradation. As such, pharmacological inhibitors of HSP90, such as 17-allylamino-17-demethoxy geldanamycin (17-AAG), act as potent anticancer agents in preclinical tumour models. However, their success within the clinical setting has been less pronounced. As with other anticancer agents, intrinsic and acquired drug resistance may significantly limit the utility and efficacy of HSP90 inhibitors. Therefore, the prediction and reversal of HSP90 inhibitor resistance may be a potential way of significantly improving the therapeutic efficacy of these anticancer agents. In addition to resistance, prolonged drug treatment of cancer cells can also result in phenotypic changes that promote their ability to metastasise, whether this occurs with respect to HSP90 inhibitors has yet to be determined. Therefore, one aspect of this study was to investigate the underlying molecular changes that contribute to the acquired resistance to HSP90 inhibitors and the associated phenotypical alterations that occurred. To achieve this we generated MDA-MB-435 and MDA-MB-231 human cancer cell lines that were resistant towards 17-AAG by gradual dose escalation. When cultured in the absence of drug pressure, cells maintained their respective levels of 17-AAG resistance (7–240x), and were cross-resistant towards other benzoquinone ansamycin that are structurally related to 17-AAG. Additionally, the resistant cell lines were also cross-resistant to structurally distinct HSP90 inhibitors, such as radicicol and other radicicol-based compounds. Altered expression of NQO1, histone deacetylase 6 (HDAC6) and histone deacetylase 1 (HDAC1) were identified in the resistant cell lines and were considered potential mechanisms of resistance. Consistent with increased HDAC expression, histone 3 acetylation was increased in the resistant cells. Moreover, HDAC inhibition with pharmacological inhibitors significantly re-sensitized resistant cells towards 17-AAG. Similarly, HDAC inhibition dramatically re-sensitized the resistant cells towards other structurally-distinct HSP90 inhibitors. In conclusion, prolonged 17-AAG treatment was found to result in cancer cell resistance towards a spectrum of HSP90 inhibitors to which HDAC upregulation makes a significant contribution.

To investigate the effects of chronic exposure to HSP90 inhibitors on cancer cell biology, we examined whether the MDA-MB-435 and MDA-MB-231 resistant cell lines displayed altered

growth, survival and metastatic properties. Despite both cell lines being significantly resistant to HSP90 inhibitors, each displayed reduced growth both *in vitro* and *in vivo*. Consistent with this, gene expression profiling revealed that genes involved in cell cycle progression were downregulated and markers of cancer stem cells (CSC) associated with slower growth were upregulated in the resistant cells. Despite the decrease in growth, the migratory capacity of the resistant cells was found to be significantly greater than that of the parental cells. This was supported by the increased expression of epithelial to mesenchymal transition (EMT) markers, commonly associated with an increased migratory phenotype. Despite this, the metastatic potential of the resistant cells was actually found to be markedly decreased *in vivo*, developing less metastatic tumour burden at both visceral and skeletal sites. Interestingly, within the bone, although the tumour burden was significantly lower in mice bearing the resistant cells, x-ray and bone morphometric analysis demonstrated that the extent of osteolytic lesions were similar in both the parental and resistant cell lines, demonstrating an enhanced pro-osteolytic phenotype in the resistant cells. Taken together, these findings show for the first time that acquired resistance towards HSP90 inhibitors results in phenotypical changes at the cellular level that negatively impact upon tumour growth and metastasis, yet paradoxically enhances a pro-osteolytic phenotype, potentially through multiple molecular changes as observed in the resistant cells by gene expression profiling.

In relation to bone, 17-AAG has been previously shown to increase bone loss in mouse models through the direct stimulation of osteoclast formation. Heat shock factor 1 (HSF1), the master transcriptional regulator of heat shock response (HSR) associates with HSP90 under normal conditions, maintaining HSF1 in an inactive monomeric state. However, upon 17-AAG binding to the N-terminal ATPase domain of HSP90, or upon cellular stress, HSF1 dissociates from the HSP90 complex and binds to heat shock element (HSE) sites within the promoters of target genes. Hence, the activation of HSF1 by 17-AAG may be involved in the activation of osteoclast formation that subsequently leads to bone loss. Therefore an additional aim of this study was to definitively determine the role of HSF1 in HSP90 inhibitor-induced osteoclast formation. It was determined that HSP90 inhibitors that induced a heat shock response also enhanced osteoclast formation while HSP90 inhibitors that did not, had no impact upon osteoclast formation. Pharmacological inhibition or shRNAmir knockdown of Hsf1 in RAW264.7 cells, as well as the use of *Hsf1* null bone marrow cells, demonstrated that enhanced osteoclast formation by 17-AAG was HSF1-dependent. Moreover, ectopic over-expression of Hsf1 enhanced the effect of 17-AAG upon osteoclast formation. Consistent with these findings, protein levels of the essential osteoclast transcription factor, microphthalmia-associated transcription factor (Mitf) were increased by 17-AAG in a HSF1-dependent manner. In addition to HSP90 inhibitors, we also identified

that other heat shock response inducing agents, such as alcohol, doxorubicin and methotrexate, can also directly increase osteoclast formation in a stress-dependent manner. These results indicate that cellular stress can enhance osteoclast differentiation via HSF1-dependent mechanisms and may significantly contribute to pathological and therapeutic related bone loss.

Therefore, this study demonstrates that acquired resistance to a broad spectrum of HSP90 inhibitors in cancer cells is partially mediated by a HDAC-dependent mechanism and is associated with decreased tumour growth and metastasis. In addition, the induction of osteolysis by HSP90 inhibitors and other stress-inducing agents is mediated by the action of HSF1, most likely via altering the steady-state levels of the osteoclastogenic gene MITF. Hence, this thesis provides a potential treatment strategy to prevent or reverse the development of resistance towards HSP90 inhibitors via HDAC inhibition as well as providing a molecular mechanism by which these agents cause osteolysis of the bone.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

17-AAG	17-allylamino-17-demethoxy geldanamycin
17-DMAG	17-(2- dimethylamino ethylamino)-17-desmethoxygeldanamycin
5-FU	5-fluorouracil
ABC	ATP-binding cassette
ADAMTS1	A disintegrin-like and metalloproteinase with thrombospondin motifs 1
ADP	Adenosine diphosphate
AHA1	Activator of heat shock 90kDa protein ATPase homolog 1
AhR	Aryl hydrocarbon receptor
AI	Aromatase inhibitor
ALA	Alpha-lipopic acid
ALK	Anaplastic lymphoma kinase
AP-1	Activator protein 1
AR	Androgen receptor
ATFs	Activating transcription factors
ATP	Adenosine triphosphate
BA	Benzoquinone ansamycin
BAD	Bcl-2-associated death promoter
BAG	Bcl-2-associated agonist of cell death
Bak	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BLI	Bioluminescence imaging
BMM	Bone marrow macrophage
BMPs	Bone morphometric proteins
BNIP	Bcl-2/adenovirus E1B 19 kDa interacting protein
BSA	Bovine serum albumin
BST2	Bone marrow stromal antigen 2
CASR	Calcium ions to calcium-sensing receptors
CDC37	Cell division cycle 37
CDC42	Cell division cycle 42
CDKs	Cyclin-dependent kinases
CHIP	C-terminus of HSP70-interacting protein
CSC	Cancer stem cell
CTD	Carboxy-terminal domain
CXCL	Chemokine (C-X-C motif) ligand

CXCR	CXC chemokine receptor
CYP40	Immunophilin-cyclophilin 40
DBD	DNA-binding domain
DC-STAMP	Dendritic cell-specific transmembrane protein
DMEM	Dulbecco's Modified Eagle's Medium
DTCs	Disseminated tumour cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF2a	Eukaryotic translation initiation factor 2A
EMT	Epithelial-to-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERSE	Endoplasmic reticulum stress element
FAK	Focal-adhesion kinase
FbCM	Fibroblast-conditioned media
FBS	Fetal bovine serum
FGFR	Fibroblast growth factor receptor
FKBP	FK506-binding proteins
GA	Geldanamycin
GHKL	Gyrase, HSP90, histidine kinase and MutL
GIST	Gastrointestinal stromal tumor
GnRH	Gonadotropin-releasing hormone
GR	Glucose receptor
GRB7	Growth factor receptor-bound protein 7
GRP94	94kDa glucose regulated protein
GSEA	Gene set enrichment analysis
GSH	Glutathione
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HER2	Human epidermal receptor 2
HIFs	Hypoxia-inducible factors
HIP	HSP70-interacting protein
HOP	HSP70/HSP90 organizing protein
HR	Hydrophobic repeats
HSC	Haematopoietic stem cell
HSC70	Heat shock cognate 70kDa protein
HSE	Heat shock element
HSFs	Heat shock factors

HSPs	Heat shock proteins
HSR	Heat shock response
hTERT	Human telomerase reverse transcriptase
IFN	Interferon
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor 1 receptor
IKK	Inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
ISP	Iron-sulphur protein
ITAM	Immunoreceptor signalling motif
JNK	c-Jun N-terminal kinases
KNK437	N-Formyl-3,4-methylenedioxy-benzylidene-gamma-butyrolaetam
LHRH	Luteinizing hormone-releasing hormone
LRR	Leucine rich repeat
M-CSF	Macrophage colony-stimulating factor
MAPK	Mitogen-activated protein kinases
MD	Middle domain
MDR	Multidrug resistance
MHC	Major histocompatibility complex
MITF	Microphthalmia-associated transcription factor
MMP	Matrix metalloproteinase
MR	Mineralcorticoid receptor
MRP	Multidrug resistance protein
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
MTX	Methotrexate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFATc1	Nuclear factor of activated T cells cytoplasmic 1
NIK	NF-κB-inducing kinase
NQO1	Quinone-metabolising enzyme NAD(P)H: quinone oxidoreductase
NSCLC	Non-small cell lung cancer
NTD	Amino-terminal domain
OPG	Osteoprotegrin
OPN	Osteopontin
OSCAR	Osteoclast-associated receptor
P-gp	P-glycprotein
PARP	Poly (ADP-ribose) polymerase
PBMCs	Peripheral blood mononuclear cells
PI3K	Phosphoinositide 3-kinase

PiC	Phosphate carrier
PPIase	Peptidyl-prolyl cis-trans isomerase
PPT1	Protein phosphatase 1
PR	Progesterone receptor
PSA	Prostate-specific antigen
PT	Peptide transporter
PTEN	Phosphatase and tensin homolog
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
PVDF	Polyvinylidene difluoride
RA	Rheumatoid arthritis
RANK	Receptor Activator of Nuclear Factor κ B
RANKL	Receptor activator of nuclear factor kappa-B ligand
Rb	Retinoblastoma-associated protein
RD	Radicicol
RelA	Reticuloendotheliosis viral oncogene homolog-A
RI	Resistance index
RISC	RNA-induced silencing complex
ROI	Region of interest
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RunX2	Runt-related transcription factor
SHR	Steroid hormone receptor
sHSPs	Small heat shock proteins
SRB	Sulforhodamine B
STR	Short-tandem repeat
TAD	Transactivation domain
TCA	Trichloroacetic acid
TFE	Transcription factor E
TGF-β	Transforming growth factor-β
TKI	Tyrosine kinase inhibitor
TNBC	Triple negative breast cancer
TNF	Tumour necrosis factor
TPR	Tetratricopeptide repeat
TRAF	TNF receptor-associated factors
TRAP	Tartrate resistant acid phosphatase
TRAP100	Thyroid hormone receptor (TR)-associated protein 100
TSA	Trichostatin A
UBC	Ubiquitin-conjugated enzyme

VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
XBP1	X-box binding protein 1

Chapter 1

Introduction

1.1 Breast cancer

Cancer is the second leading cause of death and remains a major public health problem in the western world. In Australia, breast cancer is the most common cancer amongst women (excluding basal and squamous skin cell carcinoma), accounting for 28.2% of all new cases diagnosed in 2008 [1]. It is estimated that the current age-standardised incidence rate of breast cancer is 115.4 per 100,000 women [1]. On average there are greater than 13,000 new cases of breast cancer diagnosed and close to 3000 deaths each year from the disease [1].

Breast cancer is a heterogeneous disease encompassing multiple sub-types with different morphologies, molecular signatures, prognoses and responses to therapies [2]. In breast cancer patients, the main cause of death is due to tumour metastasis, a multistage process in which cancer cells spread from the tumour of origin to colonise distant organs [3]. A substantial proportion of patients are diagnosed with metastatic disease at either presentation (5%) or during the course of treatment (30%) [4]. Improvements in treatment efficacy and earlier diagnosis in recent years have led to a 98.6% 5-year relative survival rate for patients with localised breast cancer [5]. In contrast, the 5-year survival rate of patients with advanced metastatic breast cancer is only 23.3% [5]. Metastatic breast cancer is typically incurable with current conventional therapies being employed for palliative care of patients [4]. In addition to that of metastatic disease, drug resistance is a major hurdle to successful treatment, accounting for 50% of treatment failures in patients that have metastatic disease when treated with drugs such as tamoxifen [6]. Therefore, the development of new diagnostic and efficacious treatments that will predict and circumvent drug resistance and are able to treat metastatic tumours are likely to improve disease outcomes in breast cancer patients.

1.1.1 Breast cancer sub-types

A number of clinical and molecular markers are routinely used to categorize breast cancers [7]. Currently, all breast cancers are tested for the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal receptor 2 (HER2). Based on these molecular markers, breast cancer can be classified into a number of different molecular subtypes referred to as luminal A, luminal B, HER2-overexpressing and triple negative (Figure 1.1). The different breast cancer sub-types have distinct histopathological markers, prognosis, response to therapy and metastatic propensity.

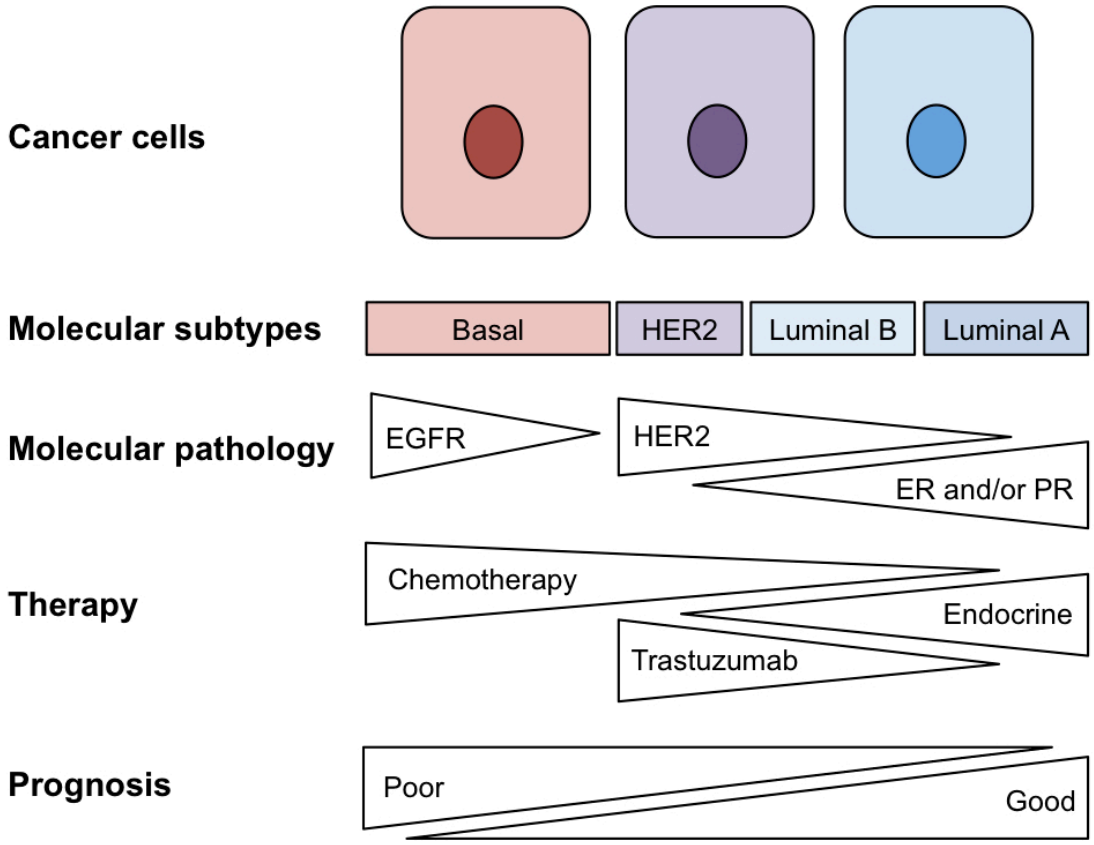


Figure 1.1 The relationship between different breast cancer subtypes with the expression of molecular markers, treatment strategies and prognosis. Adapted from [8].

1.1.1.1 Luminal sub-types

Estrogens and progesterones are crucial in the regulation of mammary gland proliferation and differentiation. These sex hormones exert their effects by binding to their corresponding receptors, ER and PR, respectively. The luminal subtypes consist of breast cancers that express ER and PR, which are further characterized by the expression pattern of molecular markers that equates to the epithelium lining of the luminal compartment of normal breast ducts [7, 9]. Tumours that are ER positive and/or PR positive, and HER2 negative are classified as luminal A, and those that are ER positive and/or PR positive and HER2 positive are luminal B [7]. Currently available endocrine strategies for the treatment of luminal breast cancers include targeting the ER itself with anti-estrogens such as tamoxifen, or suppressing the amount of available ligand (estrogen) for the receptor either with gonadal suppression in premenopausal women (ovariectomy or luteinizing hormone-releasing hormone agonists), or with aromatase inhibitors in postmenopausal women [10]. Luminal A tumours have the best overall prognosis among the breast cancer subtypes due to their favourable response to endocrine treatments. In contrast, luminal B tumours are more proliferative and can require chemotherapy in addition to endocrine treatment.

1.1.1.2 HER2 overexpression

The HER2 (also referred to as ERBB2) receptor tyrosine kinase is a member of the epidermal growth factor receptor (EGFR) family and is involved in the regulation of cell growth, survival and differentiation through cross-talk with other signaling pathways [11]. Amplification of the *HER2* gene occurs in about 20% of patients with early stage breast cancer. The HER2 subtype is also defined by tumours with high expression of genes from the *HER2* amplicon, including HER2, Growth factor receptor-bound protein 7 (GRB7) and TRAP100 [12]. HER2 subtype tumours have a poor prognosis due to a high proportion of *TP53* mutations and higher histopathological grade than luminal A tumours [13]. However, the clinical use of the anti-HER2 monoclonal antibody, trastuzumab, as an adjuvant therapy in addition to other chemotherapies has shown remarkable reductions in the relapse rates amongst HER2 positive breast cancers, and a subsequent increase in overall survival [14]. However, not all HER2 positive tumours respond to trastuzumab as resistance can occur as a result of the activation of downstream signaling pathways, e.g. phosphoinositide 3-kinase (PI3K) mutations or loss of function of phosphatase and tensin homolog (PTEN) [15, 16]. Clinical studies with the combination of mammalian target of rapamycin (mTOR) inhibitor, paclitaxel and trastuzumab in patients with HER2-overexpressing metastatic breast cancer has demonstrated encouraging results with an overall response rate of 44% [17]. This highlights the importance of identifying novel combination strategies with anti-HER2 therapies that can lead to improved clinical outcomes.

1.1.1.3 Triple negative or basal type breast cancer

Basal type breast cancers are invasive ductal carcinomas that feature high histological grade, solid architecture, absence of tubule formation and a high mitotic rate [7]. A large proportion of basal type breast cancers are found to be negative for ER, PR as well as lacking amplification or overexpression of HER2 [18]. Such tumours that lack these three markers are termed triple negative breast cancer (TNBC). About 70% of breast tumours that are triple negative also cluster with the gene expression signatures of basal-like breast cancers [12] and conversely about 80% of basal cell cancers are triple negative [18]. TNBC accounts for roughly 15% of all breast cancer cases but causes a disproportionately high number of deaths due to intrinsic aggressiveness and a lack of treatment options [18]. TNBC patients do not benefit from endocrine or anti-HER2 therapies, limiting their treatment to that of chemotherapy. However, TNBC patients have a worse outcome after chemotherapy than do patients with other subtypes of breast cancer [19]. In recent years, many molecular alterations have been discovered in TNBC, which led to investigations in the use of other newly developed targeted therapies. These potential targets and predictive markers, including EGFR, poly (ADP-ribose) polymerase (PARP), MET, SRC, NOTCH, insulin-like growth factor 1 receptor

(IGF-1R), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor (VEGF) and MEK are currently under pre-clinical and clinical evaluation [19].

1.1.2 Breast cancer metastasis

Metastasis is the spread of cancer cells from a primary site of tumor growth to the formation of new tumours at distant organs. A significant proportion of breast cancer patients are diagnosed with metastatic disease at initial presentation (5%) or during the course of treatment (30%). Metastases at distant sites are the leading cause of morbidity and mortality in breast cancer patients and treatment options remain limited to date. Furthermore, metastases can occur years or even decades after successful treatment of the primary tumour by surgery and adjuvant therapy [20].

The metastatic process consists of multiple steps (Fig. 1.2), all of which must be successfully completed for the development of a metastatic tumour [21]. Local invasion is the first step of metastasis requiring altered cell-to-cell and cell-to-extracellular matrix (ECM) adhesion [22]. The invasive phenotype involves loss of the epithelial cell markers, such as adherens and tight junction proteins that include E-cadherin and ZO-1 [23]. This is followed by the upregulation of the ECM component fibronectin and the cellular intermediate filament protein vimentin, which are both associated with mesenchymal cells [23]. Epithelial-to-mesenchymal transition (EMT), which is involved in embryonic development, can occur in cancer cells upon the activation of specific genes, e.g. Snail, Twist and Slug that contributes to an invasive phenotype and malignant progression [24]. Invasion is also facilitated by the proteolytic degradation of the ECM and basement membranes that underlie the epithelial and endothelial cell layers. This enables tumour cells to penetrate tissue boundaries and is mediated, amongst others, by matrix metalloproteinases (MMPs) and the urokinase plasminogen activator pathways [25, 26]. Adherence of tumour cells to the ECM is primarily mediated by the integrin family of cell surface receptors, which bind to ECM proteins and form signaling complexes with oncogenic receptor tyrosine kinases [27].

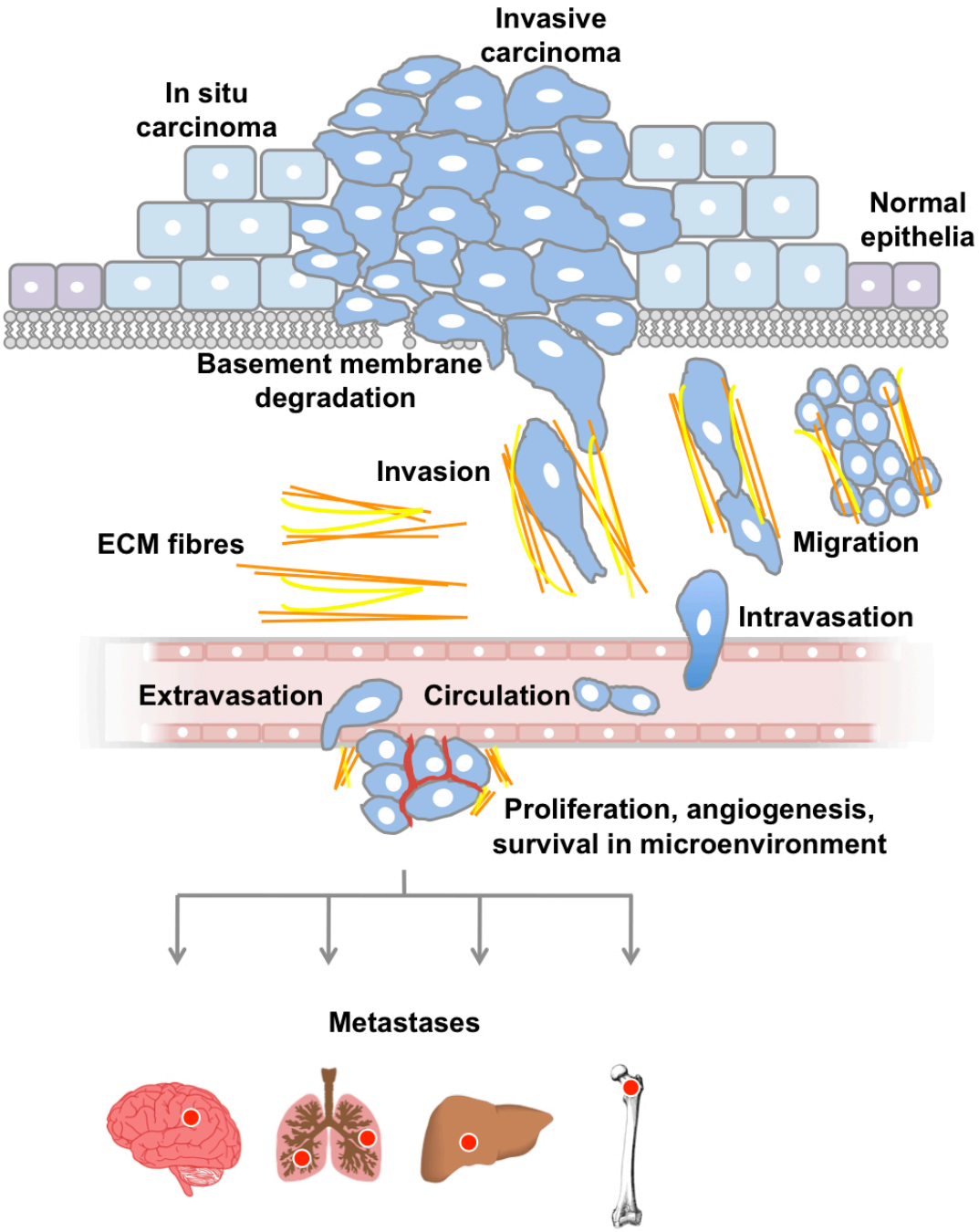


Figure 1.2 The metastatic cascade. Transformation of normal epithelia cells results in the formation of carcinoma in situ. Molecular changes such as loss of cell-to-cell junctions lead to the invasive carcinoma stage. After degrading the basement membrane, tumour cells invade the surrounding stroma, migrate and intravasate into blood or lymph vessels. The tumour cells are then transported in the circulation until they arrest in the capillaries of a distant organ. The disseminated cells subsequently have to extravasate, establish angiogenic growth, proliferate and adapt to the new microenvironment. Adapted from [28].

The regulation of tumour cell migration is crucial to the metastatic cascade as the process fundamentally involves the movement of cancer cells from one site to another [24]. Invasive tumour cells acquire a migratory phenotype, allowing them to respond to cues from the microenvironment that trigger tumour invasion. Migrating tumour cells often are observed moving linearly in association

with ECM fibres, such as collagen, laminin and fibronectin [29]. Different molecular pathways determine the distinct types of migration. The activation of RhoGTPases has an important role in single-cell migration, which involves their loose attachment to the ECM fibres and loss of cell polarity, resulting in rapid movement along the path of least resistance [4, 30]. By comparison, collective-cell migration requires the continued presence of intercellular junctions to allow cells to invade and disseminate as clusters, which are more efficient in embolizing and surviving within the circulation [4]. ECM fibres often converge onto blood vessels, enabling tumour cells to disseminate systemically through the blood circulation. The directional migration of cancer cells is mediated by chemoattractant gradients that diffusing from blood vessels or are produced by other cell types, such as fibroblasts and macrophages in the surrounding stroma [29]. EGFR and IGF-1R are associated with increased chemotactic migration in metastatic breast cancer cells [31, 32], hence their ligands, epidermal growth factor (EGF) and insulin-like growth factor (IGF) are thought to be important chemoattractants that drive breast cancer cell migration and invasion.

When tumour cells reach the blood or lymph vessels they penetrate endothelial cell and basement membrane barriers, in a process known as intravasation by extending invadopod-like protrusions [29]. A study of gene expression involved in lung metastases of a mouse mammary tumour cell line revealed that enhanced expression of TWIST, a transcription factor involved in the EMT, increases metastasis due to the augmentation of hematogenous intravasation rates [24, 33]. Once tumour cells have entered the bloodstream, they must be able to survive physical shear forces, immune cell-mediated killing and anoikis. The binding of tumour cells to platelets and coagulation factors, including fibrinogen, fibrin and thrombin, creates an embolus that is resistant to immune cell-mediated killing and to physical shear forces [34]. Having survived the circulation, metastatic tumour cells become arrested in capillary beds of distant organs. The cells escape the vasculature into target tissues through a process termed extravasation which involves the adhesion of circulating tumour cells to the vascular wall, the disruption of endothelial cell-junctions and the transendothelial migration of the cancer cells [35-37]. Once in the new site, tumour cells must initiate and maintain growth to form micrometastases, sustained by the development of new blood vessels, termed angiogenesis, in order to form macroscopic tumours [21].

It has long been recognized that dissemination of cancer cells from a primary tumour is not a random process, as exemplified by the frequent metastasis of breast cancer to the bone, liver, brain and lungs. Stephen Paget proposed that disseminated cancer cells, or “seeds”, would only colonise organ microenvironments, or “soils”, that are favourable to the growth of cancer cells [38]. Tumour cells can ‘home’ to specific sites through the expression of chemokine receptors that help direct the migrating cells [39]. Furthermore, chemokines secreted from distant sites can also dictate the dissemination and colonization of tumour cells. A “premetastatic-niche” theory has also been

proposed, which states that a permissive distant site for growth and colonization can be established by bone marrow-derived hematopoietic cells in response to hormonal factors emitted by primary tumours [40, 41]. After homing to a specific site, disseminated tumour cells must be able to productively interact with the new microenvironment in order to proliferate and survive. One of the best characterized examples of the cellular and molecular adaptation of cancer cells to the host stroma of a distant metastatic site is seen when breast cancer cells metastasize to bone. This phenomenon will be further discussed in section 1.5.2 of this chapter.

1.2 Heat shock response

Ritossa reported in 1962 that elevated temperature led to the development of chromosomal puffs in the salivary glands of *Drosophila busckii* [42]. Subsequent molecular analysis by Tissieres and Mitchell revealed that the induction of these chromosomal puffs coincided with the synthesis of a small number of proteins termed heat shock proteins (HSPs), which belong to the molecular chaperone family of proteins. The process of HSP induction by a variety of stimuli has been termed as the heat shock response (HSR), which is evolutionarily ancient and well conserved across species. The HSPs participate in the folding of proteins under resting conditions during cell growth and development [43], however, upon cell exposure to stress, an increase in unfolded and misfolded proteins challenges protein homeostasis. This leads to the activation of the transcription factor, heat shock factor 1 (HSF1) and the subsequent induction of HSPs to assist in refolding proteins to their native states or facilitate their degradation via the ubiquitin proteasomal pathway. HSPs can be induced by a diverse number of stress conditions that include environmental stresses such as elevated temperature, oxygen-free radicals, heavy metals and other chemicals. Similarly, pathophysiological conditions of disease states such as cancer, neuronal injury, infection and ischemia amongst others, often have microenvironmental alterations that include hypoxic, acidic and nutrient deprived conditions, which can also result in the induction of HSPs.

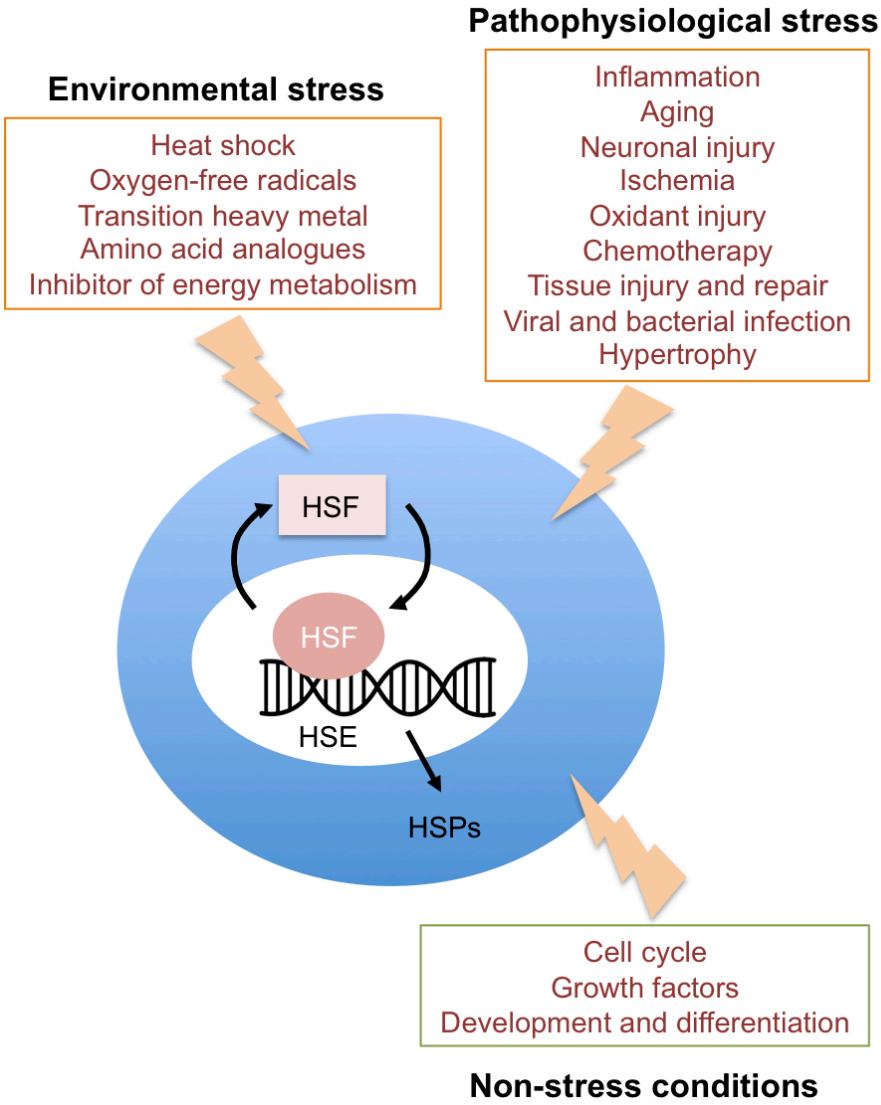


Figure 1.3 Conditions that induce the heat shock response. Cells respond to environmental stress, pathophysiological stress as well as during non-stress conditions by the activation of the transcription factors, heat shock factors (HSFs) that bind specifically to the heat shock elements (HSE), resulting in the elevated expression of the HSPs. Adapted from Morimoto et al [43].

1.2.1 Heat shock factors

Stress-induced transcription of HSP genes requires activation of heat shock factors (HSFs) [44, 45]. To date, there are four HSFs that have been characterized. Among vertebrates, HSFs 1, 2 and 4 are ubiquitous, of which HSF1 is the master regulator of heat shock response. HSF3 has been characterized in only avian species and more recently in mice [43, 46]. There are different functional domains in HSFs and these have been most thoroughly characterized for HSF1, as shown in Fig. 1.4A [43]. The DNA-binding domain (DBD) is the most preserved domain among the HSFs and belongs to the family of winged helix-turn-helix DBDs [47]. The DBD forms a globular structure with a loop

that generates a protein-protein interface between adjacent subunits of the HSF trimer that enhances high-affinity binding to DNA [48, 49]. Adjacent to the DBD are hydrophobic repeats (HR-A and HR-B) that form a coiled-coil structure to regulate HSF trimer formation [50]. Suppression of spontaneous HSF trimerization is mediated by another hydrophobic repeat, HR-C, which is absent in HSF4 [51]. In addition, the sequence between HR-A/B and HR-C, known as the regulatory domain (RD) negatively regulates DNA binding and transcriptional activation [52]. Positioned at the carboxyl terminus of HSFs is the transactivation domain (TAD), consists of hydrophobic and acidic residues that enables a rapid and prolonged stress response [53].

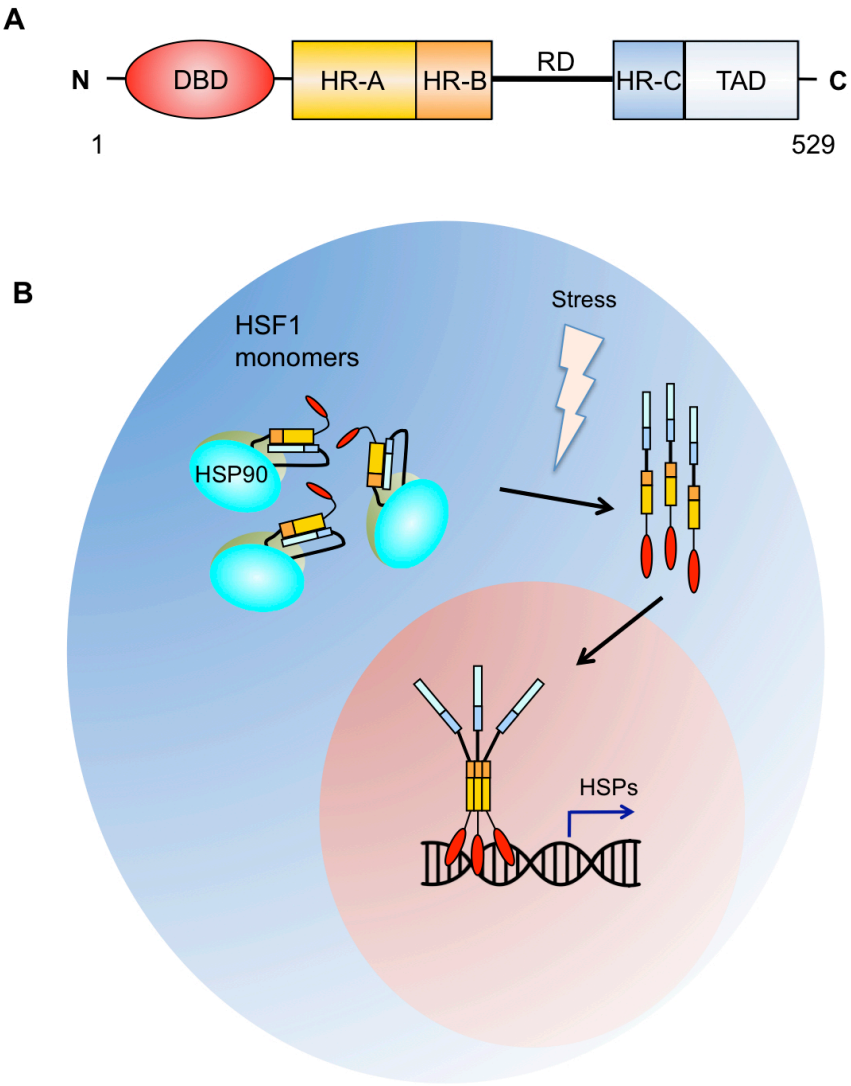


Figure 1.4. The structure and regulatory features of HSFs. (A) Schematic representation of HSF1 structural motifs corresponding to the DNA-binding domain (DBD), hydrophobic repeats (HR-A/B and HR-C), the regulatory domain (RD) and the transcriptional activation domain (TAD). (B) HSF1 monomers dissociate from HSP90 upon stress to form homotrimers with DNA-binding activity that results in the activation of target gene expression. Adapted from [43].

HSF1 exists as a monomer that forms a complex with heat shock protein 90 (HSP90). The latent HSF1 monomer lacks DNA binding and transcriptional activity under resting conditions (Fig. 1.4B). Upon stress, it dissociates from the complex to form active homotrimers that bind to heat shock promoter elements (HSEs) of target genes, characterized as multiple adjacent and inverse iterations of the pentanucleotide motif 5'–nGAAn-3' [54]. Although the inhibitory mechanism is largely unknown, the negative feedback from the end products of HSF1-dependent transcription provides an important regulating step in adjusting the duration and intensity of the heat shock response according to the levels of chaperones as well as the levels of unfolded or misfolded proteins [48].

Tumour cells are constantly exposed to stressors, including hypoxia, acidosis, nutrient deprivation and immune attacks from the host [55]. It is therefore imperative that tumour cells are able to adapt and survive the harsh tumour microenvironment. Indeed, Dai and colleagues showed that HSF1 deficiency in mice significantly decreased spontaneous tumour formation induced by p53 mutation [55]. In addition, HSF1 deficiency also rendered cultured cells refractory to oncogenic transformation by oncogenic, H-RAS [55]. This indicates that HSF1 enables tumour cells to accommodate drastic alterations, survive initial oncogenic stresses and adapt to the malignant state [55]. These effects are largely thought to be attributable to the induction and regulation of HSPs such as HSP90, which will be discussed in detail in sections 1.2.2 and 1.3. However, a recent study has also identified a subset of HSF1-regulated genes that are specific to cancer cells and distinct from the heat shock genes [56]. These HSF1-regulated non-heat shock genes regulate core cellular functions of cancer cells such as DNA repair, cell cycle, apoptosis, energy metabolism, transcription and translation. Hence, further studies on the far-reaching effect of HSF1 upon tumourigenesis and metastasis beyond the classical induction of HSPs are highly warranted.

1.2.2 Heat shock protein families and their functions

HSPs are classified broadly into conserved families that include HSP110 (HSPH), HSP90 (HSPC), HSP70 (HSPA), HSP40 (DNAJ) and small HSPs (HSPB) [57]. HSPs act as molecular chaperones that interact with a large range of unfolded proteins. This interaction is mediated by the recognition and binding of HSPs to exposed hydrophobic amino acids and other specific peptide sequences of non-native unfolded proteins [57]. Amongst all of the HSPs, HSP70, small HSPs and HSP90 are the most widely studied.

Human cells contain two major types of HSP70 family members and include two stress-inducible HSP70 isoforms (HSPA1A and HSPA1B) and the constitutively expressed HSC70 (HSPA8) [58]. Under normal conditions, HSP70s are involved in the folding of newly synthesized proteins, the assembly of multi-protein complexes and the transport of proteins across cellular membranes [59].

Under stress, HSP70 family members prevent the aggregation of unfolded proteins and are able to refold aggregated proteins [57, 60]. The binding of HSP70 proteins to other proteins is mediated by ATP hydrolysis at the amino-terminal ATPase site [59]. In addition, several co-chaperones bind to HSP70 to regulate chaperone function [59]. The J-domain co-chaperones, e.g. HSP40, stimulate the ATPase activity of HSP70 [61]. The nucleotide exchange factor co-chaperones, e.g. Bag-1, HSP110 (HSPH1), HSPBP1, catalyze the release of ADP during the ATPase cycle [62-64]. The TPR domain co-chaperones, e.g. HOP and CHIP, are essential for the assembly of HSP90/HSP70 complex and CHIP has been implicated in the ubiquitination of some bound proteins [65-67]. HSP70 proteins are expressed at high levels in many cancers [68-70]. HSP70 expression increases after chemotherapy and is functions to inhibit programmed cell death and senescence [71]. Resistance to cisplatin is associated with high HSP70 expression in human ovarian cancer cells and the downregulation of HSP70 reverses drug resistance in cancer cells due to its anti-apoptotic role [71]. The cytotoxic effect of HSP70 down-modulation is particularly strong in transformed cells but undetectable in normal cells lines [72]. This highlights the role of HSP70 in the survival of transformed cells due to internal stress such as the increase in mutated and misfolded proteins, and the inappropriate activity of deregulated signaling pathways [73].

The small HSPs (sHSPs) family of molecular chaperones was discovered as a set of proteins with small molecular weight (15-30 kDa) and is characterized by a conserved sequence of 80-100 amino acids called α-crystallin [74]. The human sHSP family contains ten members (HSPB1-HSPB10) [75]. sHSPs have high capacity to bind unfolded proteins and protect cells against protein aggregation. However, they must release aggregate-prone client proteins to other downstream chaperones for refolding [76]. HSP27, also known as HSPB1, is the most characterized member of the sHSP family. Clinically, an increased level of HSP27 is apparent in many cancers when compared to matched non-transformed tissue [59]. Like other HSPs, HSP27 has strong anti-apoptotic properties and is highly expressed in cancers that are resistant to treatments [77, 78]. HSP27 enhances cell survival through inactivation of caspases by sequestering cytochrome C [79] and inhibition of AKT-dependent Bax activation [80]. In addition to its role in cell survival, HSP27 enhances cell migration and invasion via FAK-dependent actin organization and STAT3-dependent MMP expression [81, 82].

1.3 HSP90

HSP90 is one of the most conserved HSPs, present in organisms from all kingdoms of life except archaea. The role of HSP90 extends beyond the stress response, as shown by the abundance of HSP90 even in unstressed cells and is typically induced by only a few-fold by stress [83]. HSP90 participates in stabilizing and activating more than 200 proteins, many of which are essential for constitutive cell

signaling and adaptive responses to stress [84]. To accomplish this, HSP90, HSP70 and other proteins termed the co-chaperones form an ATP-dependent dynamic complex known as the HSP90 chaperone machine. Cancer cells exploit the HSP90 chaperone machinery to protect an array of mutated and overexpressed oncoproteins from misfolding and degradation, hence facilitating the phenomenon known as oncogene addiction and promoting cancer cell survival and progression [84].

1.3.1 HSP90 family and classification

The vertebrate HSP90 (HSPC) family can be divided into three subtypes: mitochondrial TNFR-associated protein (TRAP/HSPC5), endoplasmic reticulum (ER) –localized HSP90B (HSPC4) and cytosolic HSP90A (HSPC1, HSPC2 and HSPC3) [85]. The different members of the vertebrate HSP90 protein family and their functions are summarized in Table 1.1.

TABLE 1.1 Members of the HSP90 (HSPC) family in vertebrates

Name	New name	Localisation	Functions
HSP90AA1 HSP90AA2 HSP90AB1	HSPC1 HSPC2 HSPC3	Cytoplasm	Mediate correct folding of proteins involved in signal transduction, protein trafficking, receptor maturation, and innate and adaptive immunity
HSP90B/GRP94	HSPC4	ER	Mediate responses to ER stress: disruption of calcium or redox homeostasis, inhibition of glycosylaton, protein misfolding and hypoxia
TRAP/HSP75	HSPC5	Mitochondria	Regulation of mitochondrial integrity and oxidative cell death; mitochondrial protein folding; transcriptional responses to stress

TRAP is compartmentalized within mitochondria and was initially discovered as a HSP90-like chaperone [86, 87]. It is most closely related to the bacterial HSP90, HtpG, and has evolved independently of other HSPs during early eukaryotic evolution (Fig. 1.5) [88]. TRAP expression is dependent on HSF1 and its activity is ATP-driven [89]. Unlike other HSPs, TRAP does not bind co-chaperones, e.g. p23 or HOP [89]. TRAP has critical roles in the pathways of mitochondrial integrity, oxidative cell death, organelle-compartmentalized protein folding and transcriptional responses to proteotoxic stress [90]. TRAP has been shown extensively to be cytoprotective in cells against anti-tumour agents by reducing reactive oxygen species (ROS) production and oxidative stress [91-93]. A study also identified TRAP as one of the target genes upregulated by the Myc oncogene, indicating a role for TRAP in oncogenic stress response and transformation [94]. Consistent with these data, TRAP levels are elevated in primary human tumour specimens, while present at very low levels in corresponding normal tissues [90].

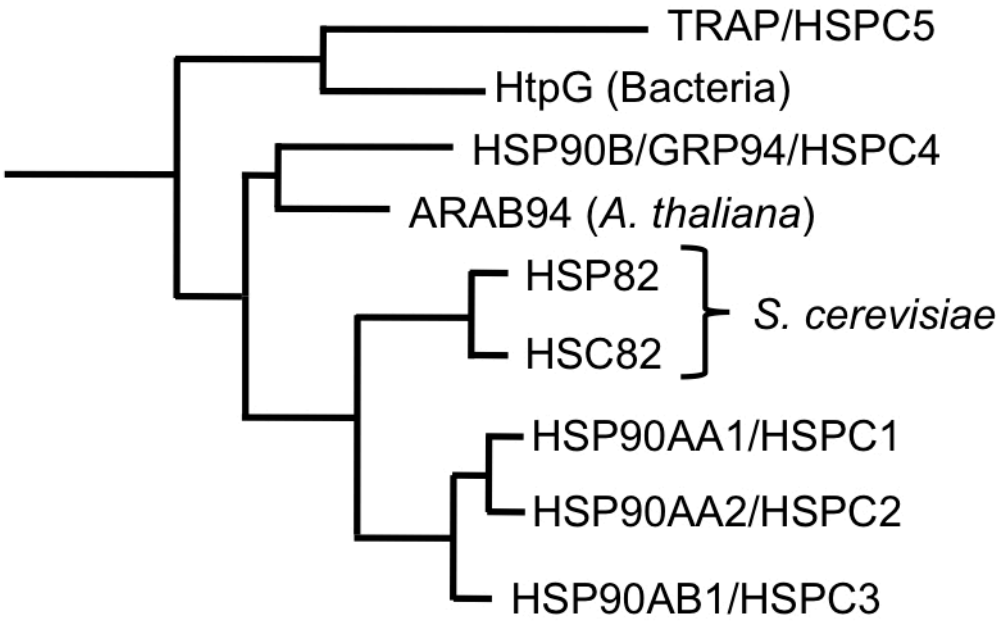


Figure. 1.5. Phylogenetic tree of the major HSP90 family members. The analysis is based on sequence comparisons of human cytosolic HSP90AA and HSP90AB, human HSP90B (GRP94), *Arabidobsis thaliana* GRP94 (ARAB94), TRAP, *E. coli* HtpG, as well as yeast HSP82 and HSC82. Adapted from [95].

The ER-localised HSP90B protein, also known as GRP94 or HSPC4, has evolved from a common ancestor as cytosolic HSP90A, distinct from TRAP and HtpG (Fig. 1.5) [88]. The promoter of HSP90B is characterized by the presence of ER stress elements (ERSE) which are cis-acting elements shown to mediate the responses to ER stress [96] induced by perturbations in ER homeostasis, such as disruption of calcium or redox homeostasis, glycosylation inhibition, nascent polypeptide misfolding and hypoxia [95]. Transcription of HSP90B is initiated by signaling pathways responsive to changes in the ER lumen and activate transcription factors that bind ERSE, such as xbob-binding protein 1 (XBP1), eukaryotic translation initiation factor 2A (eIF2a) and a (ATF6) [97]. Interestingly, HSP90B is not induced by high temperature or other stresses that are unique to the cytosol [95]. Increased levels of GRP94 have been associated with cellular transformation, tumorigenicity, as well as a decreased sensitivity towards radiation and chemotherapeutic treatments [98]. HSP90B has also been shown to be essential in the binding of tumour specific antigens in the ER and the initiation of immune responses against tumour cells [99].

The cytosolic HSP90A (HSPC) group is the largest and most widespread of all the HSP families. Evolutionarily recent duplication events have resulted in multiple genes encoding HSP90A in vertebrates: the stress-inducible HSP90AA, including HSP90AA1 (HSPC1) and HSP90AA2 (HSPC2), as well as the constitutively expressed HSP90AB (HSPC3) (Fig. 1.5) [85]. Transcription of inducible HSP90AA is regulated by the binding of HSF1 to the HSE promoter sequences [100].

Interestingly, although the constitutively expressed HSP90AB is not highly inducible, HSEs are present within its promoter and play a critical role as stress-dependent enhancers of transcription [101]. Notably, HSF1 is a bound client protein of HSP90 that is held in an inactive state under normal conditions. This interaction is interrupted under stress, releasing HSF1 for transcriptional activation. Thus HSP90 plays an important role in regulating its own transcription [85].

The cytosolic HSP90A family has been extensively studied and has appeared in the last decade of research to be a promising target for cancer therapy. The subsequent sections of this chapter will focus on cytosolic HSP90A proteins.

1.3.2 HSP90 conformation and the chaperone cycle

HSP90 is a member of the ATPase superfamily, GHKL (gyrase, HSP90, histidine kinase and MutL) [102]. The highly conserved structure of each monomer in the HSP90 dimer consists of three domains: amino-terminal domain (NTD) connected to a middle domain (MD), followed by a carboxy-terminal domain (CTD) (Fig. 1.6A) [85].

The structure of the NTD was first determined from studies of yeast and human HSP90 and found to contain a two-layer α/β sandwich structure that forms a pocket [103, 104]. The true function of the NTD and its pocket was revealed by the sequence homology identified between HSP90 and ATP-dependent DNA-manipulating proteins, as well as the determination of the co-crystal structure of HSP90 NTD in yeast [105, 106]. The binding of an antitumour agent geldanamycin at the ATP-binding pocket was shown to displace ATP and inhibit HSP90 function with high specificity. Efficient ATP hydrolysis was found to be highly dependent on the presence of a catalytic Arg residue from a loop in the MD domain to dock with the ATP-binding pocket [107]. In addition, an interaction between the N-termini of both monomers within the HSP90 dimer promotes the activity [108].

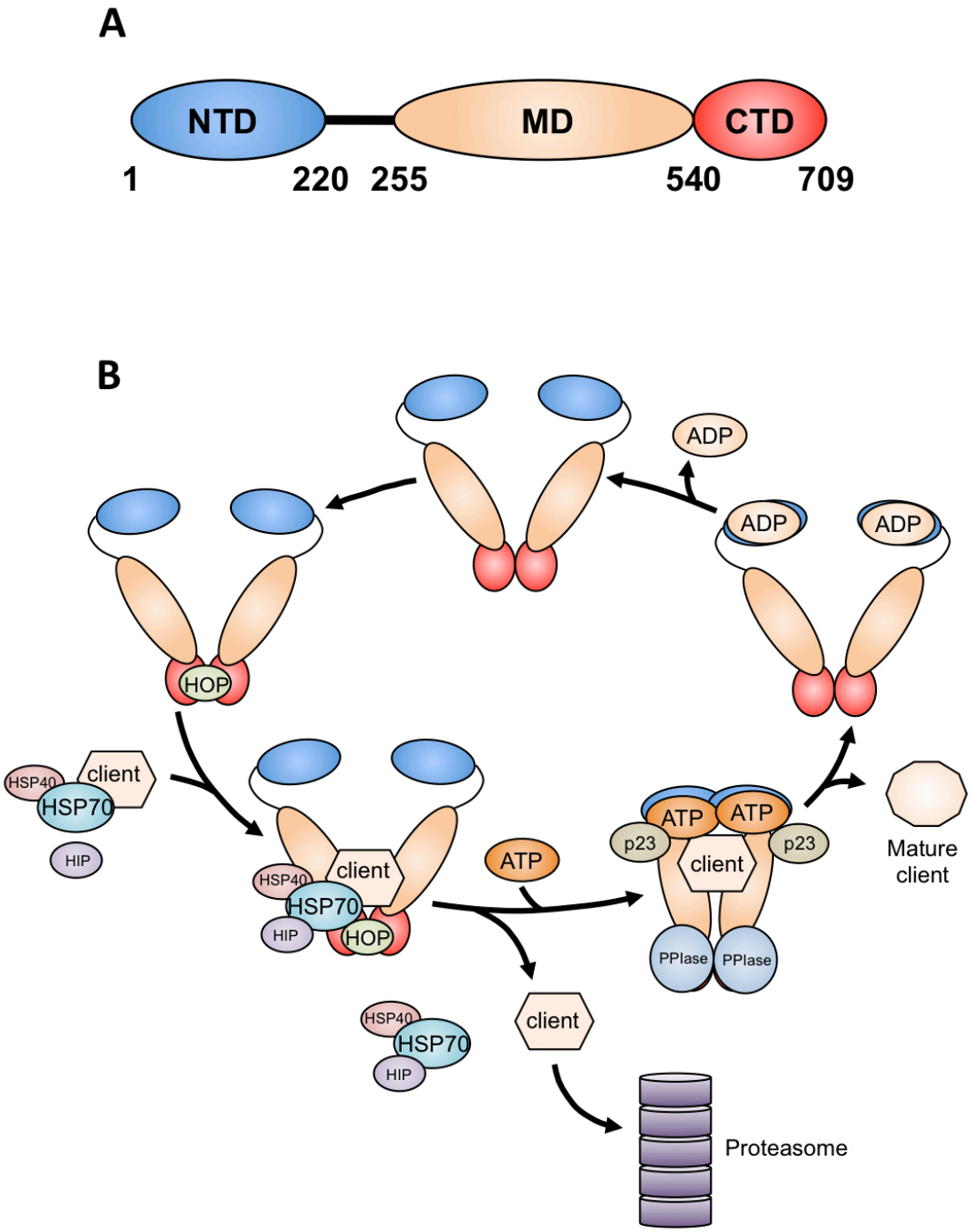


Figure 1.6. HSP90 structure and chaperone cycle. (A) The structure of HSP90, consisting of an amino-terminal domain (NTD), middle-domain (MD) and carboxy-terminal domain (CTD). **(B)** Client protein, e.g. steroid hormone receptor (SHR), binds to HSP70 and HSP40 before binding to the HSP90-HOP complex with HIP. ATP and co-chaperones (e.g. p23, CYP40, AHA1, CDC37) then bind the complex, promoting client maturation and complex dissociation. Terminally misfolded client proteins are targeted for degradation by the ubiquitin-proteasome pathway. Figure adapted from [85].

The MD of HSP90 is connected to the NTD by a charged linker region. Mutations in this region impair the binding of co-chaperones to HSP90 and the activation of client proteins [109]. The MD

consists of a large αβα domain at the N-terminus of the region connecting to a small αβα domain at the C-terminus by a series of α-helices [102]. The primary role of the MD is client protein recognition and interaction that is mediated by a hydrophobic patch and amphipathic protrusion [85].

The CTD of HSP90 is a dimer of small α/β monomers, linked by two α-helices from each monomer to form a four-helix bundle at the dimer interface [85, 102]. The 35-residue segment at the C-terminus of the CTD provides the MEEVD motif, which is implicated in the binding of co-chaperones that contain tetratricopeptide repeat (TPR) domains [102].

HSP90 undergoes many conformational changes to interact with a vast array of client proteins. During the maturation or refolding of client proteins, HSP90 depends on a large set of co-chaperones to drive the chaperone cycle [110]. The different assembly and make-up of the HSP90 complex, which depends on the types of client proteins, is a reflection of the dynamic nature of the HSP90 chaperone cycle. The HSP90 complex responsible for the maturation of steroid hormone receptors (SHR) is well studied and represents a typical example of a dynamic HSP90-client chaperone cycle [85, 110-113] (Fig. 1.6B).

The newly synthesized or misfolded client protein first associates with HSP70, HSP40 and the adapter HSP70-interacting protein (HIP) to form a complex. HSP70/HSP90 organizing protein (HOP) facilitates the association of this early complex with HSP90 by serving as an adaptor protein between HSP70 and HSP90 [114]. ATP then binds to the ATP-binding pocket at the NTD of HSP90, which subsequently exposes the client protein-binding region of the MD. Many co-chaperones that either inhibit or enhance HSP90's ATPase activity can bind to the complex at this stage. The co-chaperone p23 stabilises the closed conformation of HSP90 to facilitate client and ATP-binding. ATP hydrolysis is crucial for the release of the client protein, however, the binding of p23 at this stage partially inhibits ATP hydrolysis to ensure maturation of the client before it is released [115]. Another co-chaperone, cell division cycle 37 (CDC37), that can also inhibit the ATPase activity of HSP90 can also bind to the complex and can link kinase-type clients to HSP90 [116]. In contrast, activator of heat shock 90kDa protein ATPase homolog 1 (AHA1) is one of the only few co-chaperones that can enhance ATP hydrolysis of HSP90. AHA1 facilitates contact between the MD and the NTD ATP-binding site to stabilize the conformation and promotes efficient ATP hydrolysis [85, 117]. In general, the co-chaperones that inhibit ATPase activity are involved in client binding or formation of a mature HSP90 complex, whereas those that enhance the activity of ATPase are activators of the HSP90 conformational cycle [85]. The HSP90 complex at this late stage of the chaperone cycle also contains peptidyl-prolyl cis-trans isomerases (PPIases) e.g. the immunophilin cyclophilin 40 (CYP40) and FK506-binding proteins (FKBPs) [118, 119]. These co-chaperones contain TPR sequences that

interact with the CTD of HSP90 and are crucial for the maturation of client proteins e.g. SHRs but their actual function in the complex is still not well understood. After client maturation and ATP hydrolysis, the co-chaperones and folded client are released from the HSP90 complex. Finally, the dissociation of ADP returns HSP90 to its open conformation. HSP90 inhibition or terminal misfolding of the client protein can lead to the degradation of client proteins through the proteasomal pathway, which will be discussed in detail in section 1.4.1.

An additional layer of regulation of the chaperone cycle is the post-translational modification of HSP90 such as phosphorylation, acetylation and nitrosylation**.** Phosphorylation of HSP90 has been shown to affect the binding of a client protein, arylhydrocarbon receptor (AhR), to the middle domain (MD) [120]. Mutation of the phosphorylation sites in this domain increases HSP90 binding to AhR, suggesting that phosphorylation negatively regulates the formation of a mature complex [120]. Furthermore, deletion of the co-chaperone protein phosphatase 1 (PPT1) in yeast induces hyperphosphorylation of HSP90 and inhibits the proper folding of client proteins [121]. However, Grattan and colleagues demonstrated that c-SRC-dependent phosphorylation of HSP90 resulted in the increased association of HSP90 with endothelial nitric oxide synthase (eNOS) [122]. This highlights the complex nature of phosphorylation/dephosphorylation events that can affect chaperone function. HSP90 is also acetylated at many sites including Lys294 in the MD of HSP90, which impedes co-chaperone binding and client maturation [123]. Histone deacetylase 6 (HDAC6) has been reported to deacetylate this residue, and the inhibition of HDAC6 compromises the binding of client proteins and co-chaperones leading to their degradation [124]. The acetyltransferase p300 has also been found to be responsible for acetylating HSP90 at multiple sites but notably Lys294 was not affected [125]. HSP90 is also a target for nitrosylation, which negatively regulates the ATPase activity and inhibits the maturation of client activation through the disruption of inter-domain interactions within the HSP90 dimer [126-128].

1.3.3 HSP90 function and client proteins

HSP90 contributes to many key housekeeping functions in cells through the chaperoning of client proteins (Fig. 1.7). HSP90 is involved in *de novo* protein folding during nascent polypeptide synthesis as well as stress-induced denaturation. HSP70 initiates the process by binding the hydrophobic region of nascent or unfolded proteins and HSP90 subsequently recognizes the unfolded intermediates with defined secondary structure but that lack complete tertiary structure [129]. This leads to the correct folding of proteins and suppresses the formation of protein aggregation. HSP90 also assists in the intracellular trafficking of proteins across the ER, cytoplasm and nucleus. For example, the association of nascent HER2 with HSP90 is essential for the protein's maturation and trafficking through the ER and Golgi network [130]. Similarly, the trafficking of steroid hormone receptors

(SHRs) from a cytoplasmic localization to the nucleus in the event of receptor activation is HSP90-dependent [131]. This translocation occurs when SHR is in heterocomplex with HSP90, which results in the movement of the complex towards the nucleus along the cytoskeletal network [131]. HSP90 also facilitates protein activation through ligand binding and post-translational modification. The association of client proteins that are involved in signal transduction with HSP90 maintains the proteins in a metastable state to allow the access of ligands, such as steroids, ATP and heme, to their binding sites in the protein [131]. Client proteins are also conformationally set up to respond to protein modifications, e.g. phosphorylation, when bound to HSP90, which facilitate subsequent protein interactions [131]. HSP90 is also involved in the assembly of multi-protein complexes, such as RNA polymerase II [132], PIKK complexes [133], kinetochore [134, 135], the telomere complex [136, 137], RNA induced silencing complex (RISC) [138, 139], and 26S proteasome [140]. The role of HSP90 in coordinating the assembly of protein complexes is consistent with the function of HSP90 at the late stages of protein folding with clients in the near-native state, whereas other chaperones, such as HSP70, function at earlier stages and facilitate the folding of nascent proteins [141]. Terminally misfolded protein with non-native conformation or in the absence of appropriate stimuli can be targeted by HSP90 for degradation through the ubiquitin-proteasome pathway, thereby regulating its steady-state cellular level [142].

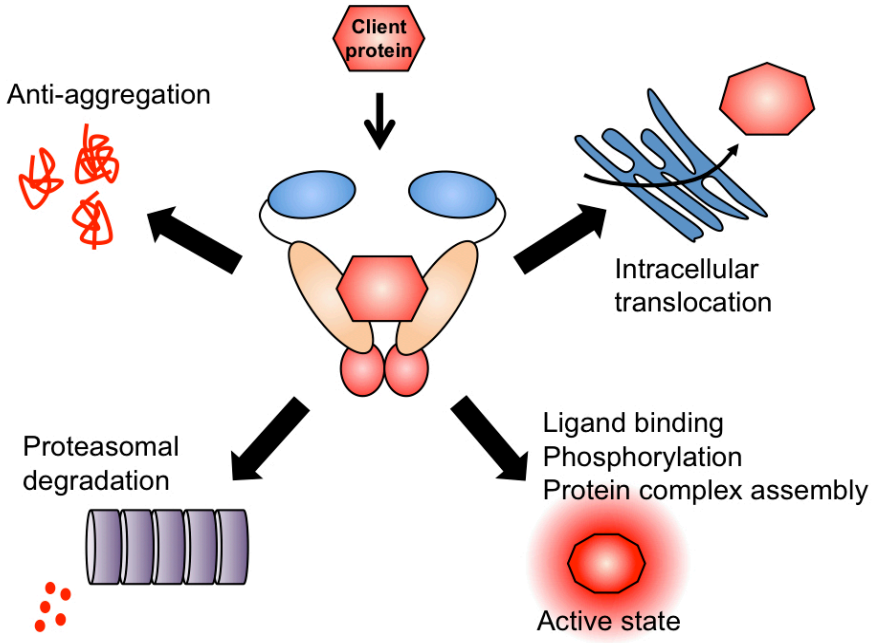


Figure. 1.7. The role of HSP90 in regulating different aspects of protein homeostasis. HSP90 completes the folding process of newly synthesized, conformationally labile client proteins in order to reach mature native conformation and prevent protein aggregation. Association of client proteins with HSP90 also facilitates the intracellular translocation of clients across ER, cytoplasm and nucleus. For client proteins involved in signal transduction, HSP90 maintains the proteins in a metastable state for the correct stimuli such as ligand binding, phosphorylation or assembly into protein complexes. Terminally misfolded proteins or in the absence of correct stimuli, HSP90 targets the client for degradation through the proteasome pathway. Figure adapted from [142].

To establish that a protein is a client of HSP90, it has to be shown to physically interact with the chaperone. Secondly, the inhibition of HSP90 must lead to a lower level of client protein amount or activity [85]. HSP90 has a wide range of client proteins that are involved in a host of cellular functions. Figure 1.8 summarises a sample of the large number of HSP90 client proteins and is organized according to their respective functions within the cell. The client proteins of HSP90 feature a high proportion of signal transduction proteins, in particular protein kinases and steroid hormone receptors (SHRs). Indeed, HSP90 was first discovered through the co-precipitation with the tyrosine kinase v-SRC [143]. Subsequently, other mutated kinases were found to be HSP90 clients, such as RAF and EGFR, which are part of the EGFR-RAS-RAF oncogenic pathway [144, 145]. Wild type kinases involved in pathways regulating proliferation, cell cycle and survival, such as HER2, CDK4 and AKT, are also HSP90-dependent [146-148]. Co-precipitation experiments also revealed the interaction of nuclear receptors such as SHRs with HSP90 [149-151]. SHRs have multiple roles in the body, including sexual development (ER, PR and AR), glucose metabolism (Glucose receptor, GR), and mineral uptake (Mineralcorticoid receptor, MR).

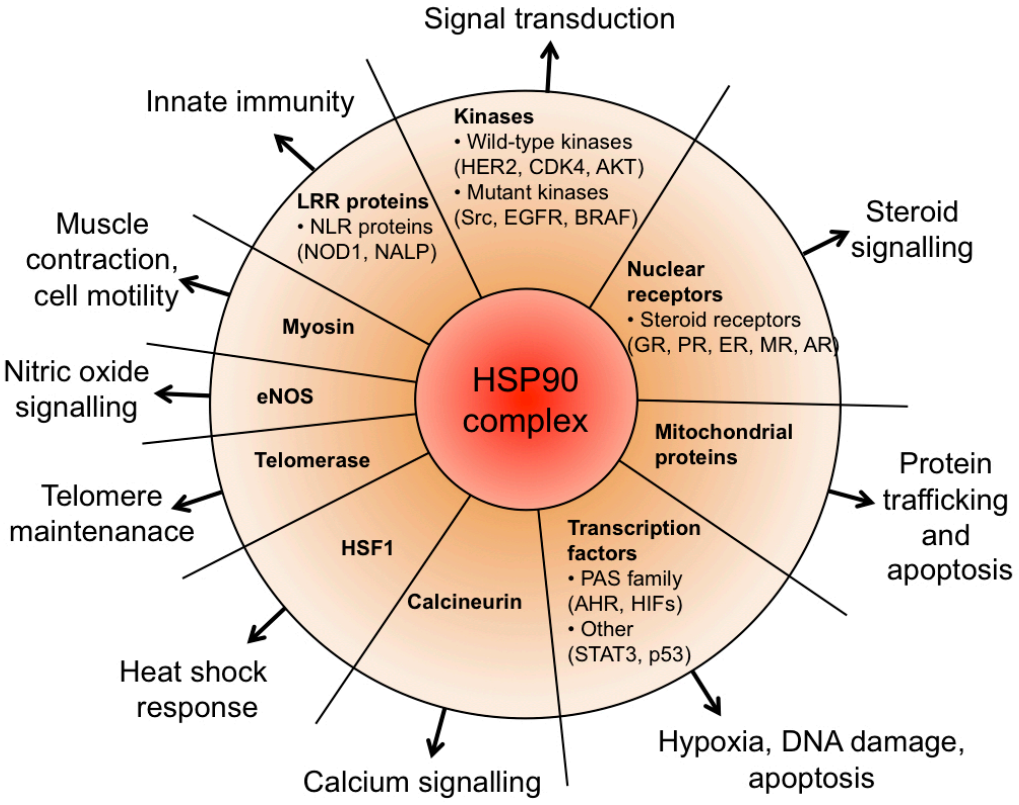


Figure 1.8. The central role of HSP90 in the regulation of multiple cellular functions through the interaction with client proteins. HSP90, in association with co-chaperones and adaptor proteins, facilitate the maturation and activity of a wide range of client proteins, thereby regulating many cellular processes, including: signal transduction by kinases and steroid hormone receptors (SHRs), mitochondrial integrity, transcriptional regulation of response to hypoxia, DNA damage and apoptosis, calcium signaling, heat shock response, telomere maintenance, nitric oxide signaling, muscle contraction and cell motility, and innate immunity. Figure adapted from [85].

Even though protein kinases and SHRs that are involved in many signaling pathways are the most well characterized clients of HSP90, many other clients have been discovered from co-precipitation studies and more recently, genomic and proteomic analyses of cells treated with HSP90 inhibitors. HSP90 has been shown to mediate the targeting of a subset of nuclear-encoded mitochondrial pre-proteins, such as phosphate carrier (PiC), peptide transporter (PT) and iron-sulfur protein (ISP), to membrane translocation [152]. HSP90 also regulates protein networks that are involved in transcriptional activities. These include transcription factors in the bHLH PER/aryl hydrocarbon receptor nuclear translocator (ARNT)/single minded (PAS) family, such as aryl hydrocarbon receptor (AhR), hypoxia inducible factors (HIFs) [153, 154], as well as other factors including STAT3 and p53 [155, 156] that are involved in the regulation of the response to hypoxia, DNA damage and apoptosis. Other clients include calcineurin, telomerase, eNOS, myosin and leucine rich repeat (LRR) proteins that regulate many core cellular functions critical for cell viability [157-161]. As discussed in section 1.2.1, HSF1 is tightly regulated by HSP90 and is activated upon stress stimuli to initiate the heat shock response. These examples are only a subset of the extensive network of client proteins dependent on HSP90, which highlight the role of HSP90 as a key player in many cellular regulatory hubs.

1.3.4 HSP90 function in cancer

The increased expression of HSP90 over the level observed in normal tissues is a common feature of many human cancers [162-164]. This phenomenon has been studied most extensively in breast cancer, in which overexpression of HSP90 is correlated with poor prognosis [165, 166]. As discussed in the previous section, HSP90 clients are involved in different signaling pathways and key cellular processes, many of which are especially important in cancer cells. The ability of HSP90 to maintain protein homeostasis is exploited by tumour cells to support the activated forms of oncoproteins that are mutated, translocated, amplified or overexpressed in malignancy [167]. In addition, HSP90 acts as a cytoprotective buffer against cellular stress induced by the hostile hypoxic, proteotoxic, acidotic and nutrient deprived microenvironment that is characteristic of tumours (Fig. 1.9) [142]. Hanahan and Weinberg have proposed the transformation of cancer cells from normal cells requires alterations in cell physiology that includes self sufficiency in growth signals, insensitivity to anti-growth signals, resistance to apoptosis, limitless replicative potential, sustained angiogenesis, invasion and metastasis, evasion of immune destruction and reprogramming of metabolism [168, 169]. Many HSP90 clients play significant roles in each of these alterations, hence highlighting the important role of HSP90 in the regulation of the hallmarks of cancer (Fig. 1.9).

In normal cells, the production and release of growth promoting signals are tightly regulated to ensure the homeostasis of cell number and tissue architecture. These signals are deregulated in cancer cells, conveyed via growth factors that bind transmembrane receptors, which often are tyrosine kinases. As discussed in section 1.3.3, kinases represent a large proportion of the client proteins of HSP90. Cancer cells can maintain a sustained proliferative signaling by elevating the levels of receptor proteins, resulting in a hyper-responsiveness to otherwise limiting amounts of growth factor ligand [169].

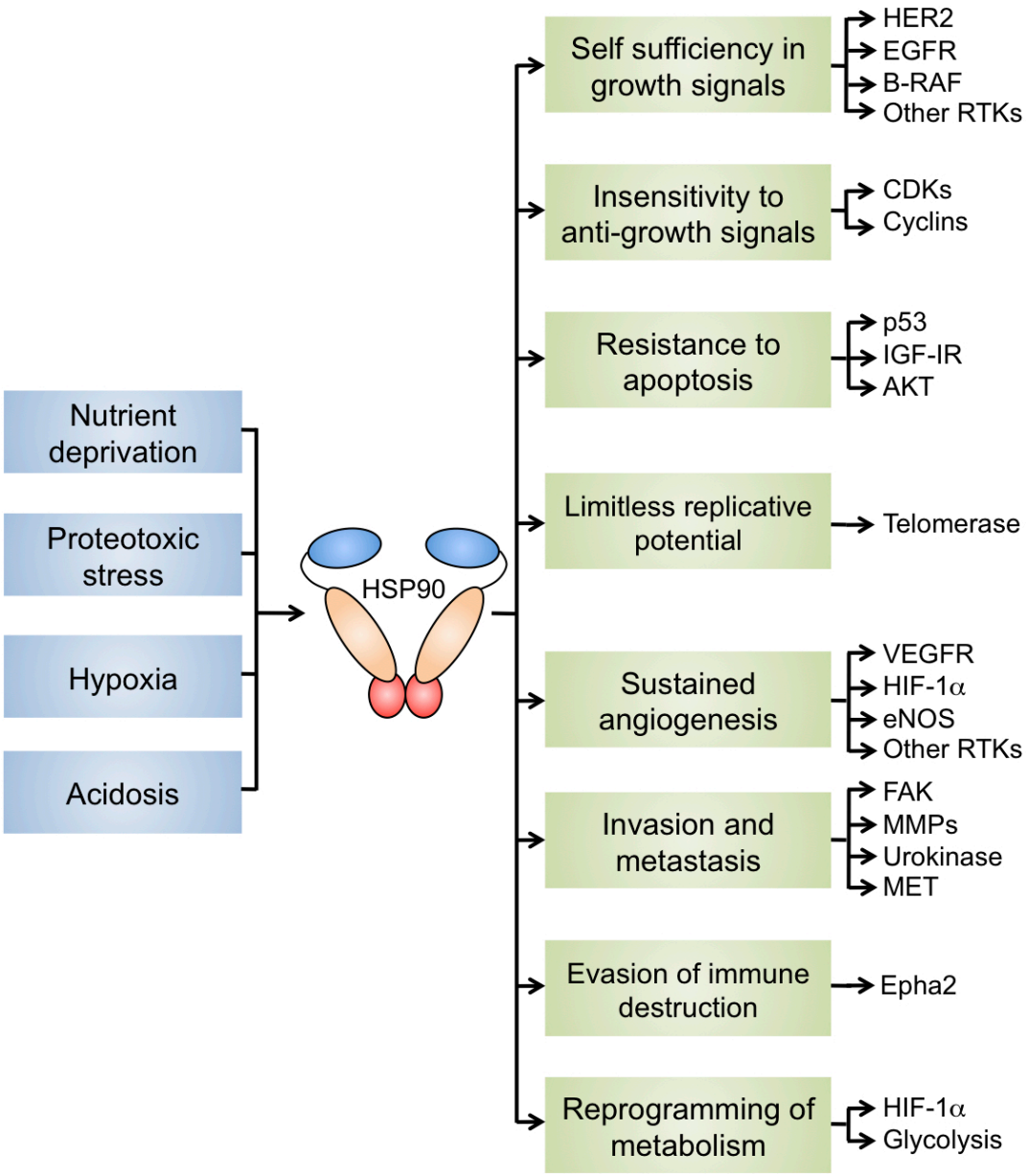


Figure 1.9. HSP90 is upregulated in response to multiple stresses in the tumour microenvironment. Multiple client proteins of HSP90 are involved in the acquisition and maintenance of the different properties necessary for the transformation, growth and progression of cancer cells. Adapted from [167].

The tyrosine kinase receptor HER2 is overexpressed in patients of breast and prostate cancers, and its elevated level is associated with poor prognosis [170]. HER2 is highly dependent on HSP90 for its maturation and stability. The inhibition of HSP90 leads to the endocytosis and receptor downregulation by proteasomal and lysosomal mechanisms [171, 172]. Proliferative signaling can also be maintained by activating mutations that lock kinases in an active conformation. Mutant EGFRs induce oncogenic effects through the activation of downstream signaling pathways that promote growth and survival [173]. EGFRs harbouring mutations have been found to be dependent on HSP90 for their stability and activity [174]. Consistent with this, HSP90 inhibition causes the degradation of mutant EGFR and suppression of its downstream effectors [174]. The protein kinase C-RAF is an important component of many signaling pathways including that of HER2 and EGFR. The C-RAF-related protein B-RAF is mutated in 70% of melanoma patients and many of its mutations result in enhanced kinase activity [175]. These mutant forms of B-RAF are found to be significantly more dependent on HSP90 than their wild-type counterpart, as demonstrated by their increased sensitivity to HSP90 inhibition [176]. Hence, The role of HSP90 in maintaining sustained growth signals is largely due to the increased dependence or addiction of cancer cells to increased levels and mutated forms of oncogenic protein kinases.

Cancer cells are also able to counter the effects of pathways that negatively regulate cell growth. Many signaling pathways are limited by the retinoblastoma-associated protein (Rb), which acts as a cell cycle gatekeeper by controlling cell transit through the G1 phase of cell division. Rb that has a low level of phosphorylation blocks cell proliferation by inhibiting the function of E2F transcription factors, which regulate the expression of genes critical for the progression of the cell cycle from G1 to the S phase[177]. The inhibition of E2F is attenuated when Rb is phosphorylated by cyclin-dependent kinases 2 and 4 (CDK2 and CDK4), which subsequently allows cell cycle progression [178]. The activity of CDKs are deregulated in cancer cells due to genetic or epigenetic changes in either the CDKs or their upstream pathways such as HER2, RAS or MYC oncogenes [179]. This enables cancer cells to proceed in the cell cycle by circumventing the anti-proliferative effects of Rb. CDK2 and CDK4 require HSP90 for their stability, and are degraded upon HSP90 inhibition[180, 181]. Moreover, cyclin E and cyclin D, which form activating complexes with CDK2 and CDK4 respectively, are rapidly downregulated upon HSP90 inhibition [182]. Hence, HSP90 function is critical in regulating the entering and progression of cells in the cell cycle.

Apoptosis is the default mechanism against cancer development, triggered by oncogene-induced signaling imbalance and DNA damage associated with increased proliferation [169]. Cancer cells are able to circumvent the apoptotic pathway through a number of strategies, of which the loss of p53 tumour suppressor function is the most common. This loss of function stems from mutations in the DNA-binding domain of p53, rendering it incapable of activating target genes that suppress

tumourigenesis [183]. In addition, mutant p53 exerts a dominant negative effect on wild type p53 through oligomerization, as well as acquiring oncogenic properties that promote growth and progression [183]. HSP90 binds to the DNA-binding domain of p53 and is essential for the stabilization of p53 at physiological temperatures as well as for the prevention of irreversible thermal inactivation of p53 [184, 185]. Interestingly, mutant p53 is highly dependent on HSP90 for its stabilization compared to its wild type counterpart, most probably due to the aberrant conformation of the mutant [186]. HSP90 inhibition has been shown to decrease the level of mutant p53 and increase the level of wild type p53 [187].

Apoptosis can also be inhibited in cancer cells through the activation of pro-survival signaling pathways such as the insulin-like growth factor (IGF-1) and its HSP90-dependent receptor (IGF-1R) [188, 189]. The activation of IGF-IR pathway induces the expression of pro-survival factors that include Bcl-2 and Bcl-xL, while simultaneously suppressing the expression of pro-apoptotic factors such as BIM [188, 190]. HSP90 inhibition has been shown to degrade IGF-IR and subsequently to promote apoptosis in cancer cells [189]. Another downstream effector of IGF-1R, Akt, is also a critical regulator of apoptosis. Activated Akt can phosphorylate BAD and caspase-9, leading to their inactivation and cell survival [191, 192]. The blockade of HSP90 inactivates Akt and increases cancer cell sensitivity towards apoptotic stimuli [148].

Continuous proliferation is required by cancer cells in order to generate macroscopic tumours. Such proliferative capability is limited in normal cells due to the inability of DNA polymerase to completely replicate the 3' ends of chromosomes known as telomeres. The loss of telomeric DNA after successive rounds of proliferation eventually leads to end-to-end chromosomal fusions that result in karyotype scrambling and cell death [193]. Telomerase is a specialized DNA polymerase that adds telomeric DNA to the ends of chromosomes. It is largely absent in normal cells but is expressed at functional levels in almost 90% of cancers [194]. Telomerase consists of the catalytic subunit, hTERT and the associated template RNA component, hTR. The active assembly of the telomerase complex and the enzymatic activity require association with the HSP90 complex [158] and the inhibition of HSP90 disrupts the interaction of hTERT with the chaperone, resulting in the proteolytic degradation of hTERT. A more recent study has shown that the interaction with HSP90 and p23 influences the telomerase binding to telomere sequences [184, 195]. Moreover, HSP90 is important *in vivo* for the maintenance of telomeric DNA length that decreases when HSP90 is inhibited [196].

In order to survive and grow in the tumour microenvironment, tumour cells require nutrients and oxygen, as well as the means of removal of metabolic waste and carbon dioxide. The establishment of a neovasculature within a tumour mass via the programmed assembly of endothelial cells to generate new blood vessels, termed angiogenesis, is critical to the growth of a tumour. During tumour

progression, angiogenesis is activated by the vascular endothelial growth factors (VEGFs) and their receptors (VEGFR). The induction of VEGF expression is switched on by hypoxia, which is typical of the stressful tumour microenvironment [197]. The HSP90 dependent hypoxia inducible factor-1α (HIF-1α) is the master regulator of the cellular response to hypoxia and has been shown to transcriptionally regulate VEGF [198]. Moreover, VEGFR as well as the downstream effector, eNOS, are dependent on HSP90 [199, 200]. The inhibition of HSP90 has been shown to result in the reduced secretion of VEGF by tumour cells and the impairment of VEGFR activity in tumour endothelial cells [201].

The ability to metastasise to distant organs is a major feature of malignant cancer cells. As discussed in section 1.1.3, metastasis is a multi-step process involving many signaling proteins that are clients of HSP90. The adhesion of tumour cells to the ECM is mediated by a complex consisting of focal-adhesion kinase (FAK) and integrins. HSP90 inhibition markedly reduces the level of newly synthesized FAK protein without changing its mRNA level, indicating a dependency on HSP90 at the post-transcriptional level [202]. The remodeling of the ECM by matrix-degrading enzymes, such as the matrix-metalloproteinases (MMPs) and plasmin, is a critical step in tissue invasion by cancer cells [203, 204]. HSP90 inhibition has been shown to impede the activation of the plasminogen activator, urokinase that leads to subsequent activation of MMP-2 and MMP-9 [205]. Increased cell motility is also another characteristic of metastatic cancer cells, in which receptor kinases such as MET plays a critical role [206]. HSP90 inhibition disrupts the association of MET with FAK and subsequently inhibits actin cytoskeleton rearrangement that is crucial for cell migration [207]. Many downstream effectors of receptor tyrosine kinases implicated in cell motility are also known clients of HSP90. The HSP90-dependent PI3K-AKT pathway directly downstream of many RTKs modulates signaling molecules that are crucial for cell motility, such as the rho-GTPases (RHO, RAC and CDC42) [208]. CDC42 forms a complex with activated CDC42-associated kinases 1 and 2 (ACK1 and ACK2) to stimulate the rearrangement of the actin cytoskeleton. The association of ACK1 and ACK2 with HSP90 is crucial for their kinase activity and the inhibition of HSP90 disrupts this interaction and subsequently impedes CDC42 signalling [209, 210]. Taken together, HSP90 plays a central role in conferring and maintaining of the metastatic phenotype in cancer cells.

Cells and tissues are under constant surveillance by the immune system to eradicate any spontaneously arising cancerous cells. Tumours that manage to appear thus possess mechanisms that are able to circumvent immune detection or are able to limit the effect of immunological destruction [169]. EphA2 is a member of the receptor tyrosine kinase (RTK) family that mediates intercellular interactions through binding of its ligand, ephrin-A1. EphA2 is overexpressed in many cancer types and correlates with poor prognosis [211, 212]. Presentation of EphA2 protein to MHC class I complexes is observed in tumour cells leading to the recognition and eradication of the tumour cells

by CD8+ T-cells [213]. In fact, EphA2 specific CD8+ T-cells have been detected in the blood of patients of different types of cancer [214, 215]. HSP90 has been shown to exert a dual role in regulating the stability of nascent EphA2 protein and maintaining the signaling activity of the mature receptor [216]. The treatment of EphA2 expressing tumour cells with HSP90 inhibitor results in the degradation of EphA2 and enhances the recognition and eradication of tumour cells by CD8+ T-cells via a mechanism that is dependent on the proteasome and the retro-translocation of EphA2 into the tumour cytoplasm [217].

Deregulated proliferation in cancer cells is coupled with adjustments of energy metabolism to support tumour cell growth. Normal cells metabolize glucose first to pyruvate via glycolysis in the cytosol and eventually to carbon dioxide in the oxygen-dependent mitochondria. Warburg and colleagues were the first to observe that cancer cells favoured glycolytic metabolism and largely bypass the mitochondrial pathway, partly due to the hypoxic conditions within the tumour [218]. The HIF-1α dependent hypoxia response system upregulates glucose transporters and many enzymes involved in the glycolytic pathway [219, 220]. The inhibition of HSP90 has shown synergistic effects with glycolysis inhibitors in many cancers [221]. The inhibition of HSP90 promotes rapid degradation of HIF-1α by inducing the binding of RACK1 in hypoxic conditions, indicating an essential role of HSP90 in mediating the reprogramming of metabolism in cancer cells [222].

Cancer is a disease that is known to involve crosstalk between multiple signaling pathways and molecular mediators to promote tumour growth and progression. From the data discussed above, HSP90 has emerged as a key gatekeeper of oncogenic pathways critical for the maintenance of cancer hallmarks through its regulation of a plethora of client proteins.

1.4 HSP90 inhibitors

In light of the role of HSP90 in regulating multiple pathways critical for cancer cells, targeting HSP90 may provide a broader and effective approach than single, oncogenically activated but dispensable signaling pathways that are the focus of most current drug discovery efforts [142]. To date, there is no approved treatment with HSP90 inhibitors but many compounds are under pre-clinical and clinical evaluation for the treatment of various cancer types.

1.4.1 Mechanism of HSP90 inhibitors

As discussed in section 1.3.2, the chaperoning activity of HSP90 is ATP-dependent. The N-terminal ATP-binding pocket of HSP90 is also a binding site for natural inhibitors of HSP90 such as

geldanamycin (GA) and radicicol. These compounds replace the nucleotide at the ATP-binding site with an affinity much higher than that of ATP (Fig. 1.10) [223]. This restrains HSP90 in a conformation equivalent to its ADP-bound form and prevents the subsequent binding of HSP90 to client proteins, resulting in the recruitment of E3 ligase such as C-terminus of HSP70-interacting protein (CHIP) and ubiquitin-conjugated enzyme (UBC) [224]. CHIP has a TPR domain that enables interaction with the C-terminal of HSP90 and HSP70, and the U-box domain binds to the UBC [224], which are crucial in the formation the complex to mediate the polyubiquitinylation of HSP90 client proteins and their degradation via the proteasome.

The role of HSP90 in maintaining protein homeostasis and its ubiquitous expression raised early concerns about the suitability of the chaperone to act as a drug target. However, many of the HSP90 inhibitors showed selective binding towards HSP90 within tumour cells [225-227]. Moreover, tumour cells are significantly more sensitive to HSP90 inhibition than nontransformed cells [228, 229]. The tumour-selectivity of HSP90 inhibitors is largely because of the addiction of tumour cells to HSP90 due to the increase in oncoproteins and the harsh tumour microenvironment, as described in previous section of this chapter. HSP90 exists as latent complexes that are still able to regulate most chaperoning processes in normal cells. In contrast, the high demand for chaperoning activity in tumour cells results in the presence of HSP90 in multi-chaperone complexes with an increased ATPase activity and binding affinity for ligands [225, 230]. Recent findings by Moulick *et al* confirmed and extended this view by showing that a majority of HSP90 in tumour cell retains chaperone functions similar to normal cells, whereas a functionally distinct pool of HSP90 is enriched and expanded in tumour cells that are required by oncoproteins to sustain the malignant phenotype [231]. The underlying mechanism for the formation of the tumour-specific HSP90 is not clear but may be dictated by post-translational modification of HSP90 that can alter chaperone activity, as discussed in section 1.3.2.

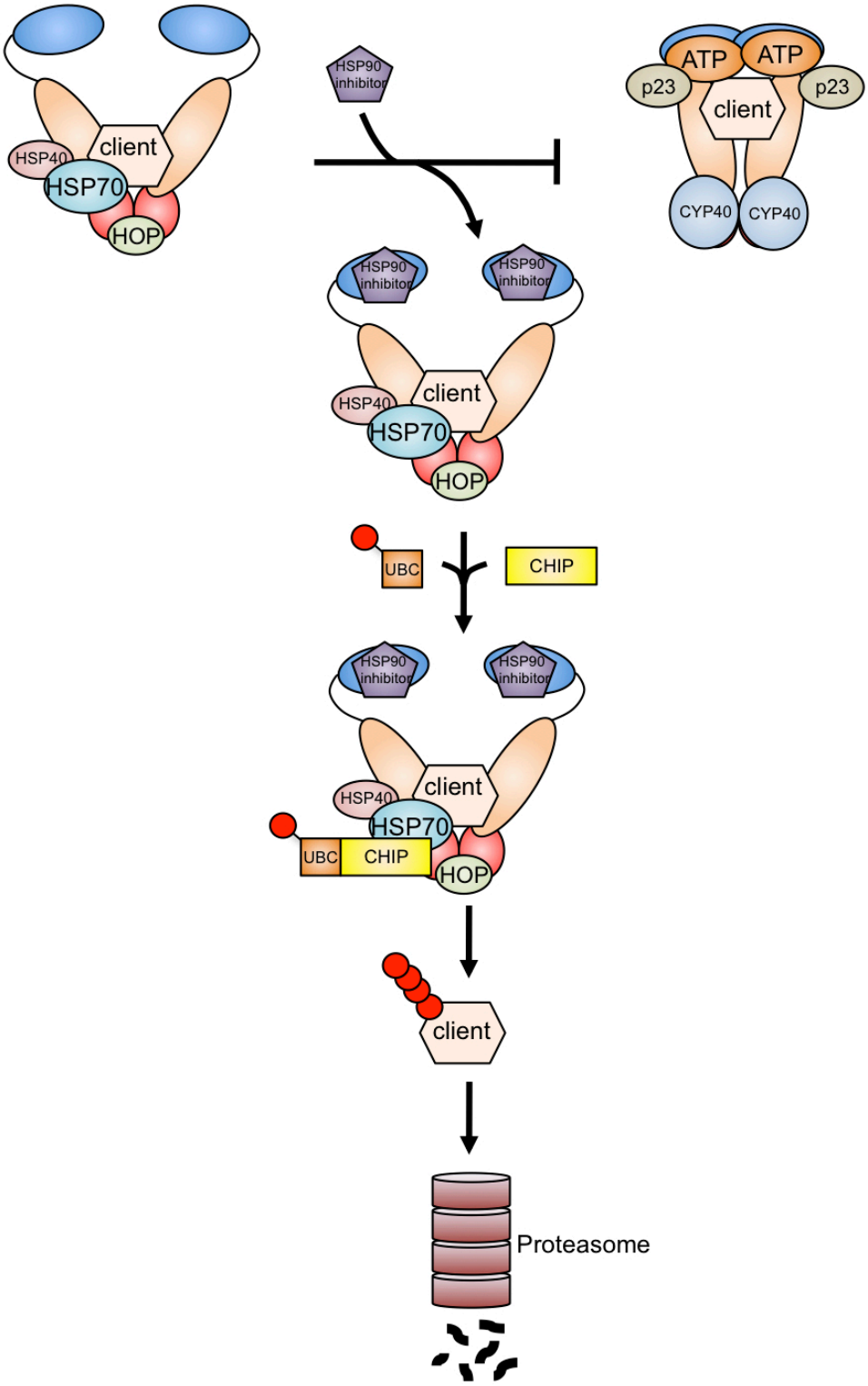


Figure 1.10. The mechanism of HSP90 blockade by specific inhibitors. HSP90 inhibitors replace the nucleotide at the N-terminal ATPase site and prevents the normal cycling of HSP90 and the formation of a mature complex. The conformation of the complex bound by HSP90 inhibitor facilitates the recruitment of C-terminus of HSP70-interacting protein (CHIP) and ubiquitin-conjugated enzyme (UBC) that ubiquitinate the client protein thus driving proteasomal degradation.

1.4.2 Classification and clinical efficacy

The discovery of HSP90 as a multigenic regulator of cancer and the availability of natural compounds that inhibited its function sparked the interest for the development of HSP90 inhibitors as a potential strategy for cancer therapy. HSP90 inhibitors are generally classified according to their structural similarities to the benzoquinone ansamycins (BAs), radicicol or purine, all of which will be discussed in the following sections. Table 1.2 summarises the pharmacological properties and clinical status of the different classes of HSP90 inhibitors.

TABLE 1.2 HSP90 inhibitors in clinical trials

Class	Inhibitor	Company	Route	Phase	Current status
Benzoquinone ansamycins	17-AAG (Tanespimycin)	Kosan	IV	II/III	Suspended
	17-DMAG (Alvespimycin)	Kosan	IV	I	Completed phase I trials - single agent and in combination with trastuzumab
	IPI-504 (Retaspimycin)	Infinity	IV	II/III	Phase I/II trials in prostate cancer, breast cancer and NSCLC. Phase III trial in GIST terminated due to toxicity. Phase II trials with docetaxel +/- IPI504 in NSCLC
Resorcinol derivatives	STA-9090 (Ganetespib)	Synta	IV	II/III	Ongoing phase II trials in prostate cancer, breast cancer, hematological malignancies, melanoma, SCLC, NSCLC, GIST. Ongoing phase II/III trial of docetaxel +/- STA-9090 in NSCLC
	NVP-AUY922	Novartis	IV	I/II	Phase II single agent trials in NSCLC, breast cancer. Combination studies with bortezomib, trastuzumab, erlotinib, cetuximab, capecitabine
	KW-2478	Kyowa Hakko Kirin	IV	I/II	Phase I in haematological malignancies completed. Phase I/II trial in combination with bortezomib in multiple myeloma ongoing.
	AT13387	Astex	Oral/IV	I/II	Phase I single agent trials in solid tumours. Phase II trials +/- imatinib in GIST.
Purine derivatives	CNF 2024 (BIIB021)	Conforma/ Biogen Idec	IV	I	Phase I trials in solid tumoursand hematological cancers completed. Phase I combination studies with trastuzumab in breast cancer. Compound is currently suspended
	MPC-3100	Myrexis	Oral	I	Phase I trials still awaiting results. No other trials listed.
	Debio 0932	Debiopharm	Oral	I	Phase I single agent trial still awaiting results.
	PU-H71	Memorial Sloan Kettering	IV	I	Phase I single agent trial in advanced solid tumours/lymphoma
Pyrazole derivative	SNX-5422	Serenex/Pfizer	Oral	I	Development halted due to ocular toxicity

1.4.2.1 Benzoquinone ansamycins

Geldanamycin (GA) is a benzoquinone ansamycin that was first isolated from the fermentation broth of *Streptomyces hygroscopicus* in the 1970s (Fig. 1.11) [232]. Its anti-cancer properties were discovered through a screening of compounds that were capable of reversing *v-src* transformation in cells [229]. Further studies revealed that GA inhibits HSP90 by competing with ATP and binding to the NT ATP-binding site with a dissociation constant (K_d) of 0.2μM [233-235]. GA was deemed unsuitable for clinical evaluation due to its poor solubility, limited *in vivo* stability and hepatotoxicity [236].

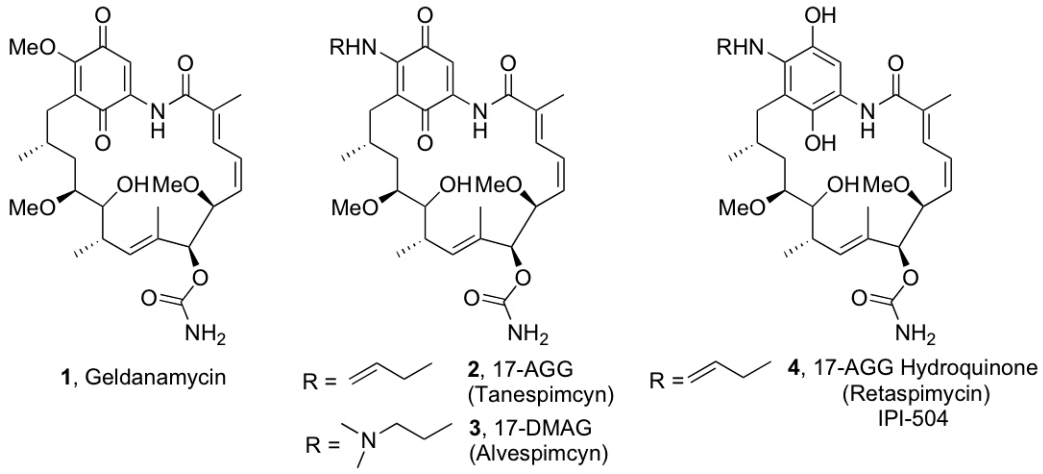


Figure 1.11 Chemical structures of benzoquinone ansamycins. Adapted from [237].

The non-essential C-17 methoxy group of GA was substituted with an allylamino group resulting in the formulation of 17-allylamino-17-demethoxy geldanamycin (17-AAG), a compound with similar biological activity to GA but less toxic (Fig. 1.11) [238]. 17-AAG has a moderate HSP90 binding affinity with a K_d of 0.6μM *in vitro* [235] and was the first HSP90 inhibitor to enter clinical trials to provide proof-of-concept studies for the feasibility of HSP90 inhibition in humans. Phase I pharmacokinetic studies of 17-AAG in patients with advanced malignancies revealed that at dose levels of 320 and 450 mg/m^2/week resulted in the inhibition of HSP90 client proteins such as c-RAF, CDK4 and HSP70 induction in tumour biopsies 24 hours post-treatment [239]. The major side effects reported were hepatotoxicity, diarrhea and fatigue, exacerbated by its vehicle consisting of dimethyl sulfoxide and egg phospholipid. Single agent phase I and phase II studies in patients with melanoma, breast cancer, prostate cancer and renal cell carcinoma showed no significant responses, however some patients did show stable disease [240-243]. The first significant clinical outcome of 17-AAG was reported in a phase I/II study of patients with HER2 positive metastatic breast cancer in combination with the HER2 inhibitor, trastuzumab. Patients that had HER2+ tumours that had been previously refractory to trastuzumab treatment alone showed an overall response rate of 22% and

median overall survival of 17 months after the combination treatment [244, 245]. The effective nature of these trials may be attributable to the degradation of HER2 by 17-AAG and perhaps its ability to delay or overcome resistance to trastuzumab [246]. A combination of 17-AAG and the proteasome inhibitor, bortezomib, also yielded similar results in phase I/II studies in multiple myeloma, with anti-tumour activity of 48% in bortezomib-naïve, 22% of bortezomib pretreated and 13% of bortezomib refractory patients [230]. An extension of this study that excluded bortezomib-naïve patients showed an overall response rate of 14% and an additional 10 patients with stable disease [247]. A phase I trial has also demonstrated clinical activity in patients with renal cell carcinoma and melanoma when treated with sorafenib and 17-AAG [248].

17-AAG has also shown synergism when combined with other chemotherapeutic agents in a number of pre-clinical studies. Lung and breast cancer cell lines were sensitized to paclitaxel when co-treated with 17-AAG [249, 250]. Similarly, 17-AAG activity is enhanced by cisplatin *in vitro* due to the blockade of HSF1-induced heat shock response by cisplatin [251]. Despite the encouraging pre-clinical and clinical results, all trials with 17-AAG were suspended for non-clinical reasons by its developer Kosan Biosciences [252].

The substitution of the C-17 methoxy group of GA with N, N-dimethylethylamine led to the formulation of 17-(2- dimethylamino ethylamino)-17-desmethoxygeldanamycin (17-DMAG, alvespimycin), a compound with increased water solubility, improved oral bioavailability and an improved binding affinity to HSP90 compared to that of 17-AAG, with a K_d of 0.35μM [253]. 17-DMAG pharmacokinetic studies in patients with advanced solid tumours showed that a weekly dosage of 80 mg/m^2 led to a sustained induction of HSP70 in peripheral blood mononuclear cells (PBMCs) for 96 hours and client protein degradation (CDK4 and LCK) [254]. Phase I trials of patients with hemangioendothelioma, melanoma and renal cell carcinoma treated with 17-DMAG showed signs of stable disease [255]. Anti-tumour activity was also observed in patients with HER2+ metastatic breast cancer when co-treated with trastuzumab [256]. However, 17-DMAG was also discontinued by its developer Kosan but current trials in haematological malignancies are still being conducted by National Cancer Institute (NCI).

Infinity Pharmaceuticals developed IPI-504 (retaspimycin), a water-soluble hydroquinone derivative of 17-AAG that is more potent [257]. IPI-504 exists in a redox equilibrium with 17-AAG through the action of oxidoreductases. IPI-504 is the most promising compound from the benzoquinone ansamycin class due to its increased inhibitory effect on HSP90 due to a superior binding affinity to HSP90 (EC_{50}=63nM) compared to 17-AAG (EC_{50}=119nM) in a HSP90 competitive binding assay [257]. Pharmacodynamic studies of IPI-504 showed that patients with multiple myeloma receiving ≥ 150mg/m^2 IPI-504 weekly showed an increase in HSP70 induction [258]. Phase I/II trial on patients

with non-small cell lung cancer (NSCLC) pre-treated with EGFR tyrosine kinase inhibitors (TKI) yielded an overall response rate of 7%, with partial response and prolonged stable disease observed in patients with anaplastic lymphoma kinase (ALK) mutation [259]. A monotherapy phase II trial in patients with castrate resistant prostate cancer showed that there was no objective prostate specific antigen (PSA) response in most of the patients and adverse hepatotoxic effect prevented the progression to a phase III trial [260]. A phase II trial of IPI-504 in combination with trastuzumab in patients with HER2+ metastatic breast cancer showed only modest clinical activity with one partial response and one stable disease observed out of 20 subjects enrolled with criteria for trial expansion not being met [261]. Like other benzoquinones, the main dose-limiting toxicity is hepatotoxicity, as shown by on-treatment deaths of patients with gastro-intestinal stromal tumour (GIST) due to hepatic complications [262]. Currently, phase I/II trials of IPI-504 in combination with the mTOR inhibitor, everolimus in patients with KRAS mutant NSCLC are still ongoing.

1.4.2.2 Resorcinol derivatives

Radicicol (RD) is another class of natural HSP90 inhibitor isolated from *Monosporium bonorden* in 1953 that contains a resornicol moiety (Fig. 1.12) [263]. RD binds to the NT of HSP90 with high affinity (K_d=19nM) [264] and interferes with chaperone function but is inactive *in vivo* due to metabolic instability. However, the resorcinol core of the RD scaffold has been retained in the design of many synthetic small molecular inhibitors of HSP90.

STA-9090 (ganetespib) is a resorcinol-based compound developed by Synta Pharmaceuticals. A preclinical study showed that STA-9090 inhibited the binding of HSP90 to biotin-GM at a concentration as low as 0.11 μM, whereas 1 μM of 17-AAG was required to inhibit binding to the same degree, suggesting greater affinity of STA-9090 for HSP90 [265]. In phase I clinical trials of monotherapy, stable disease has been observed in patients with NSCLC, GIST, and renal cell carcinoma [266]. Another phase I dose escalation trial showed one metastatic melanoma patient with partial response and two NSCLC patients with stable disease [267]. No formal response was observed in patients with hematological cancers from a phase I/II trial except for one patient with refractory acute multiple myeloma that showed stable disease [268]. A phase II trial in NSCLC patients was conducted and some adverse effects were reported, such as fatigue, diarrhea, nausea, anorexia and dyspnea. Notably, six out of eight patients with ALK mutation (ALK+) that were not pre-treated with chemotherapy showed tumour shrinkage and other ALK+ patients that had been refractory to prior treatments also showed tumour regression upon STA-9090 monotherapy [269]. In addition, eight out of thirteen patients with KRAS mutation also showed tumour regression. A phase II trial with STA-9090 monotherapy in breast cancer patients that were previously treated with chemotherapy showed

an objective response rate of 9% [270]. Of note, one out of three patients with triple negative breast cancer had a minor response.

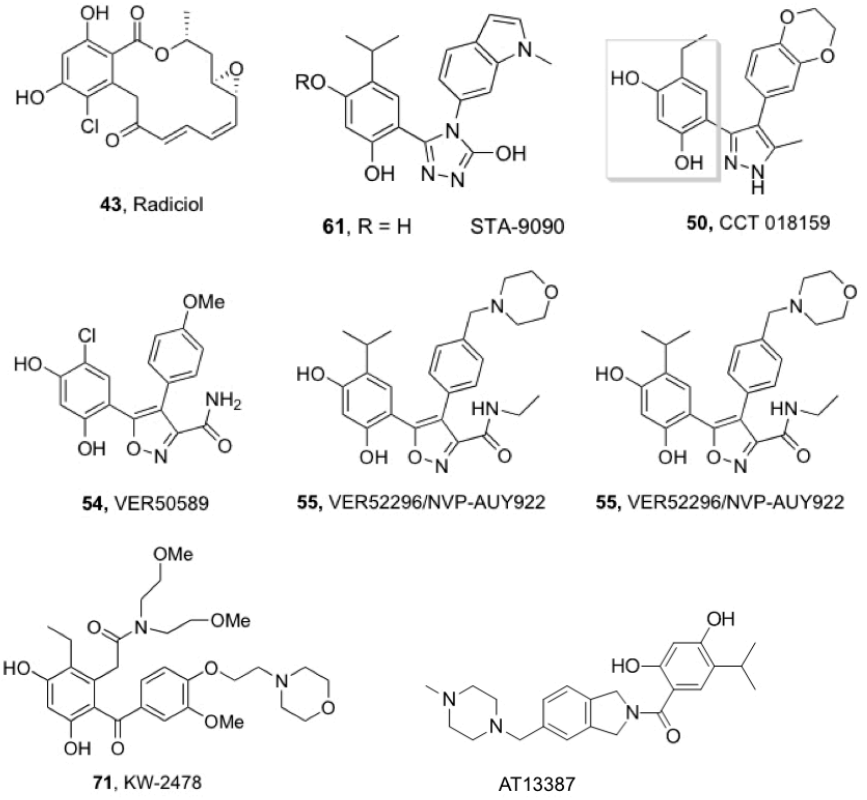


Figure 1.12 Chemical structures of radicicol derivatives [230].

Workman and colleagues first identified a resornicol-based compound CCT018159 with a pyrazole moiety from a high throughput screening of a library of compounds capable of binding to the ATPase domain of yeast HSP90 [271]. Subsequent screens and structure-based modification by Vernalis Ltd resulted in the formulation of VER50589 containing an isoxazole instead of a pyrazole moiety with a K_d of 4.5nM [272]. The same team later reported a new isoxazole compound VER52296, which is currently being developed by Novartis as NVP-AUY-922 [273]. Preclinical studies have shown NVP-AUY-922 to be a highly potent HSP90 inhibitor with a K_d of 1.7nM [274]. Phase I trials on patients with solid tumours to study the dose limiting toxicity revealed adverse effects of the compound such as atrial flutter, diarrhea, fatigue, blurred vision and anorexia [275]. Stable disease and partial response were reported in 16 and 9 patients respectively. This led to a phase II trial of NVP-AUY922 in patients with HER2+ and ER+ metastatic breast cancer and preliminary results revealed that induction of HSP70 was observed in patient-derived peripheral blood cells and metabolic responses were reported [276]. Phase II trials with HER2+ gastric cancer patients in combination with trastuzumab is currently underway [277]. HSP990 is an oral HSP90 inhibitor being developed by

Novartis as a follow-up compound for NVP-AUY-922 but the chemical structure has yet to be reported. Phase I trials are being conducted in patients with solid tumours [278, 279].

Other resorcinol derivatives currently being developed are KW-2478 by Kyowa Hakka Kirin Pharmaceuticals and AT-13387 by Astex. KW-2478 has been assessed in a phase I safety trial in patients with haematological malignancies, which showed dose limiting toxicities such as hypertension and cardiovascular complications [280]. Another phase I/II trial in combination with bortezomib is also being conducted in patients with relapsed and refractory multiple myeloma [281]. ATI-13387 is being evaluated in phase I trials in patients with advanced solid tumours as well as in phase II trials with or without imatinib in patients with gastrointestinal stromal tumor (GIST) [282-285].

1.4.2.3 Purine and purine-like analogs

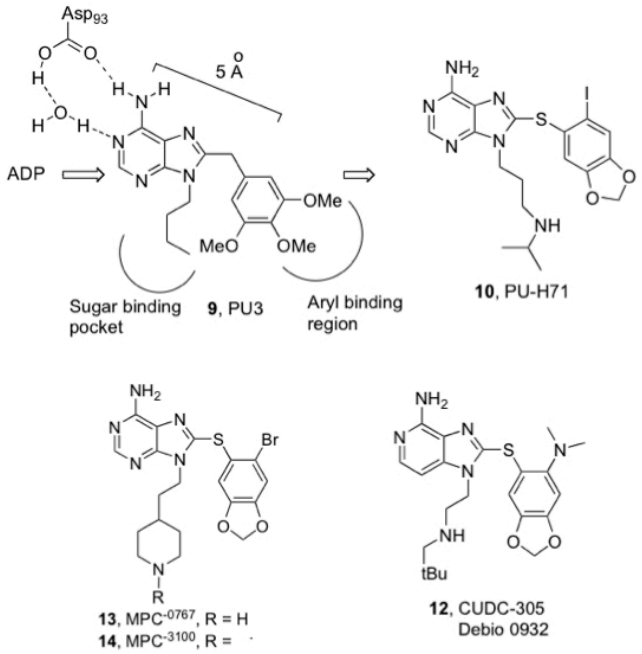


Figure 1.13 Chemical structures of purine derivatives [237].

The ATPase site in HSP90 has a unique fold in comparison to other proteins that contain an ATPase site such that it allows for the binding of small molecule inhibitors over nucleotides [286]. Based on this unique fold, Chiosis and colleagues designed a synthetic HSP90 inhibitor, PU3 that contains a purine scaffold with a bent conformation crucial for ATPase binding [287]. PU3 has been used as a template for the development of selective HSP90 inhibitors with enhanced pharmaceutical properties.

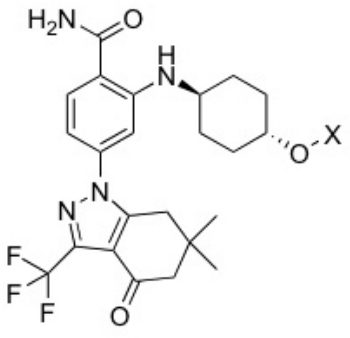
Conforma Therapeutics identified CNF 2024 (BIIB021), a purine-based compound with an aryl moiety, which is currently being developed by Biogen Idec. BIIB021 exhibits competitive binding

with geldanamycin and binds the ATP pocket of HSP90 with an inhibition constant (K_i) of 1.7nM [288]. Phase I trials revealed adverse effects in patients with haematological malignancies and solid tumours, such as fatigue, hyponatremia and hypoglycemia. Notably, one patient with chronic lymphocytic leukemia had a reduction in lymph node size and the majority of patients with solid tumours showed stable disease [289]. Another phase I trial in combination with trastuzumab in patients with HER+ metastatic breast cancer showed dose limiting toxicities like diarrhea and partial seizures. Two patients had a partial response to the treatment and ten more had stable disease out of the 30 patients involved [290]. Despite the promising outcomes, Biogen Idec has suspended trials with BIIB021 and is shifting its focus away from oncology [291].

MPC-3100 and Debio 0932 are also purine-based inhibitors of HSP90 developed by Myrexis and Debiopharm, respectively. Phase I trials with MPC-3100 has been conducted and results from the trial are being awaited. Adverse effects have been observed, such as pre-renal azotemia as well as cardiovascular and respiratory complications [292]. Due to poor solubility and bioavailability, Myrexis has developed a pro-drug, MPC-0767, which is currently being evaluated. Phase I trials of Debio 0932 in patients with advanced solid tumours or lymphoma have been initiated [293].

The most potent purine-based HSP90 inhibitor to date, PU-H71 was developed by the Chiosis group and is currently in phase I trials in patients with advanced solid and hematological tumours [294]. Preclinical studies demonstrated that PU-H71 was a highly potent HSP90 inhibitor with high selectivity for tumour cells in triple negative breast cancer models (700 to 3000-fold) compared to normal cells [295].

1.4.2.4 Benzamide derivatives



37, SNX-5422 X = $COCH_2NH_2$

Figure 1.14. Chemical structure of benzamide compound, SNX-5422 [237].

Using a chemical proteomics approach, Serenex has identified a water soluble and orally active 2-aminobenzamide compound, SNX-5422, which was subsequently acquired by Pfizer. It has high

binding affinity towards HSP90 (K_d=41nM) and displayed high efficacy in preclinical xenograft tumor models with a 67% growth delay in the HT-29 colon cancer model [296]. However, phase I results in patients with refractory solid tumours and lymphomas have shown no objective responses but long lasting disease stabilization was observed [297]. In 2011, the development of SNX-5422 was suspended due to ocular toxicity observed in animal models and a separate clinical study [297]. However, the development of SNX-5422 has been acquired recently by Esanex, a subsidiary of Eli Lilly, and phase I/II trials are set to resume in HER2+ cancers and lung adenocarcinomas.

1.4.2.5 HSP90 C-terminal (CT) targeting compounds

All of the compounds discussed thus far inhibit HSP90 function by binding to the ATPase in the NT of the chaperone. The existence of a potential ATP binding site in the C-terminal (CT) of HSP90 has been reported by several groups [298, 299]. The coumarin antibiotic novobiocin has been shown to interact with the CT of HSP90 and disrupt the binding of the co-chaperones p23 and HSP70 to HSP90. Novobiocin treatment induces the degradation of HSP90 client proteins including HER2, mutant p53 and RAF-1 in SkBr3 breast cancer cells, albeit at a very high concentration (IC_{50} = 700μM) [300, 301]. This has led to the development of more potent HSP90 CT inhibitors such as coumermycin A1 and its derivatives, which have been shown to possess anti-proliferative activity in breast and prostate cancer cell lines, with IC_{50} concentrations (5-9nM) much lower then that of novobiocin [302, 303]. Moreover, this was also associated with the degradation of HSP90 clients, such as HER2, AKT and RAF-1. Targeting the CT may be a potential alternative to some of the drawbacks of NT binding compounds including the induction of the heat shock response that exhibits pro-survival effects [237]. A recent study has revealed that a novel novobiocin-derivative, KU135 exhibited a more potent anti-proliferative affect than that of 17-AAG in human leukemic cells [304]. This is partly due to the lack of a heat shock response by KU135 when compared to 17-AAG [304]. There are currently no HSP90 CT inhibitors in clinical trials and the mechanism of inhibition of these compounds is still being investigated through further study of the CT structure.

1.4.3 Resistance to HSP90 inhibitors

As discussed in the previous section, clinical trials with HSP90 inhibitors on a variety of cancer types have yielded mixed results to date. Single agent activity has been modest in the treatment of many refractory and recurrent cancers in most trials, although more promise has been seen with combination treatments, such as with trastuzumab, showing enhanced degradation of target proteins. To improve the efficacy of HSP90 inhibitors, it is therefore important to understand the mechanisms of potential resistance towards HSP90-targeted compounds in order to design better treatment strategies including improved formulations and better combination treatments.

1.4.3.1 Mechanisms of intrinsic resistance towards HSP90 inhibitors

Drug resistance can be intrinsic due to the existing genotype of the cancer cell that results in its reduced sensitivity towards a first-line therapy. The inhibition of HSP90 has a far-reaching effect due to the involvement of the chaperone in normal cell physiology and multiple deregulated pathways in cancer, as detailed in chapters 1.3.3 and 1.3.4. These complex downstream mechanistic consequences are likely to be dictated by the genotype of tumour cells that will determine the cellular sensitivity to HSP90 inhibitors [305].

P-glycoprotein (P-gp), a member of the ATP-binding cassettes (ABC) transporter family, is a common efflux pump for many chemotherapeutic compounds that results in the multidrug resistance (MDR) phenotype [306]. Intrinsic resistance to GA, linked to the increased expression of P-gp, was described in human breast cancer cell lines in the early 90s, when HSP90 had not been identified as the target of GA [307]. Subsequent studies have shown that MDR cells with high P-gp expression are also resistant to 17-AAG [308]. However, these inherently resistant cells remain sensitive to non-benzoquinone ansamycins, such as resorcinol and purine derivatives, since these compounds are not P-gp substrates [272, 309].

In preclinical studies, intrinsic resistance towards 17-AAG has been observed in cells with a lower endogenous expression of the quinone-metabolising enzyme NAD(P)H: quinone oxidoreductase (NQO1) [310, 311]. NQO1 reduces the quinone moiety in benzoquinone ansamycins to its hydroquinone form, which leads to an increased inhibition of HSP90 due to increased binding affinity to the N-terminal ATPase. Lower endogenous expression of NQO1 in cancer cells has been associated with intrinsic resistance to 17-AAG [310, 311]. However, the level of NQO1 expression is poorly correlated with the inhibitory effect of the hydroquinone form of 17-AAG, IPI-504 [257] and other HSP90 inhibitors without a quinone moiety [312]. This highlights the exclusive role of NQO1 in sensitizing cells to only benzoquinone ansamycins.

As discussed in section 1.2.1, HSP90 is a negative regulator of HSF1 needed for the induction of heat shock response. The inhibition of HSP90 by compounds that bind to its N-terminus ATPase leads to the dissociation of HSF1 monomer from the HSP90 complex resulting in the formation of functional homotrimers. HSF1-dependent induction of heat shock proteins, such as HSP27 and HSP70, has major cytoprotective functions against the effect of HSP90 inhibition. Overexpression of HSP27 has been shown to increase the intrinsic resistance of cancer cell lines to 17-AAG and the down-regulation of HSP27 increased sensitivity [313]. High endogenous levels of HSP70 has been shown to be an important factor in conferring intrinsic resistance towards 17-AAG in human leukemia cells mainly through the inhibition of the pro-apoptotic protein Bax [314]. Furthermore, the inhibition of

HSF1 and the heat shock response by the inhibitor KNK437 abrogated the induction of HSPs by 17-AAG and increased apoptosis [314].

GA is a naturally occuring antibiotic that binds to almost all HSP90 family members except for the HSP90 homolog HtpG of *S.hygroscopicus* and the HSP90 of *C.elegans* [315, 316]. A closer look at the ATPase of *S.hygroscopicus,* HtpG, revealed the loss of charged amino acid side chains resulted in partial GA and 17-AAG resistance in yeast [315]. HSP90 of the fungus *H. fuscoatra* has a low affinity for radicicol, also due to an amino acid change in the ATPase site, although it still remains sensitive to GA [317]. The mutation, when introduced into yeast, resulted in an overall lower binding affinity for radicicol as well as an increased resistance towards the molecule. Notably, only partial resistance towards GA or radicicol was observed in these yeast studies and the HSP90 ATPase activity was not significantly compromised [315, 317]. Furthermore, *S.hygroscopicus* HtpG is still inhibited by radicicol and its derivatives, despite the low binding affinity to GA. Conversely, *H. fuscoatra* HSP90 remains sensitive to GA regardless of the partial resistance towards radicicol. To date, no similar mutation or polymorphism has been reported in human cancer cells.
As discussed in section 1.3.2, post translational modification of HSP90 is important for the normal function of the chaperone. An altered phosphorylation status of HSP90 has been shown to affect the regulation of apoptosis by the chaperone, which leads to an altered sensitivity of cells to chemotherapeutic agents [318]. The acetylation status of HSP90 is tightly controlled by HDACs and histone acetyltransferases (HATs). The hyperacetylation of HSP90, caused by HDAC6 inhibition, has been shown to enhance the binding of 17-AAG and the increased depletion of client proteins in human leukemia cells [319]. In addition, an altered acetylation status of HSP90 via the upregulation of HDACs and the down-regulation of HAT has been observed in cells that are resistant to BCR-ABL TKI [320].

The HSP90 chaperone cycle is tightly regulated by co-chaperones, such as CDC37 and AHA1 (section 1.3.2). The down-regulation of CDC37 has been shown to sensitise cancer cells towards 17-AAG and destabilizes HSP90 client proteins [321]. Another study reported that a dominant negative form of CDC37 that is incapable of interacting with HSP90 impeded proliferation and increased apoptosis [322]. Structural studies have revealed that CDC37 binds to HSP90 near the ATPase pocket and it is thought that this might interfere with the dissociation of HSP90 inhibitors [323]. Increased resistance to 17-AAG in yeast cells expressing mutated HSP90 was found to be mediated through enhanced AHA1 binding to HSP90 [324]. Furthermore, the downregulation of AHA1 in cancer cells increases sensitivity towards 17-AAG and decreased client protein activation [325]. However, alteration in the expressions of co-chaperones in response to HSP90 inhibition has yet to be observed.

The level of client protein expression is also a known determinant of intrinsic resistance. The cytotoxic effects of HSP90 inhibitors are largely attributable to their ability to decrease the stability of oncoproteins within cancer cells. Cells harbouring KRAS mutation have been shown to have increased sensitivity to HSP90 inhibitors [326]. KRAS itself is not a target of HSP90 inhibition, however, its downstream effectors such as the RAF/MEK/ERK kinase cascade are highly dependent on HSP90 for their stability. BRAF mutations also result in improved sensitivity to HSP90 inhibitors due to the increased dependence of BRAF mutants on HSP90 for protein stability [327]. Similarly, cells with high levels of HER2+ expression are exquisitely sensitive to geldanamycin and the client protein is rapidly degraded upon HSP90 inhibition [328]. In contrast, some key HSP90 client proteins seem not to show such depletion upon HSP90 inhibition and this may significantly affect the sensitivity of tumour cells towards HSP90 inhibitors. In fact, HSP90 inhibitors have been shown to activate certain pathways involved in cell growth and survival, such as PKR [329] as well as SRC and its downstream effectors, Akt and ERK [330]. Interestingly, Citri and colleagues showed that HSP90 restrains the catalytic activity of HER2 by limiting its capacity to engage with other HER proteins to form active heterodimers and the inhibition of HSP90 using a sub-lethal dose of GA lifted the restriction on HER2 activation [331]. Similarly, HIF-1α is protected from HSP90 inhibitor induced degradation when in the formation of an active heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT) [332]. ARNT is able to increase the activity of HIF-1α and decrease its sensitivity to HSP90 inhibition through competitive binding of ARNT to HIF-1α with HSP90 [332].

1.4.3.2 Mechanisms of acquired resistance towards HSP90 inhibitors

Prolonged exposure to potent anticancer drugs can result in the rapid acquisition of resistance in cancer cells that are not intrinsically resistant to a specific treatment. Acquired drug resistance is attributable to mutations [333] or the selection of rare clones with an already resistant genotype [334, 335]. These cells may possess a survival and growth advantage after treatment, allowing for the growth and expansion of the tumour. Since repeated administration of HSP90 inhibitors may be necessary for successful outcomes in the clinic, acquired resistance may arise and hence limit the efficacy of targeting HSP90.

In addition to the role of NQO1 in intrinsic resistance, gliobastoma cells with an acquired resistance towards 17-AAG generated by treatment with gradual increasing concentrations of the drug displayed a lower NQO1 expression [336]. The inhibition of NQO1 in parental cells led to decreased sensitivity to 17-AAG but had no effect on NQO1-deficient resistant cells [336]. The resistant cells with lower NQO1 also did not show any cross resistance to HSP90 inhibitors that were structurally distinct to the benzoquinone ansamycins [336]. There was also no cross resistance to structurally distinct HSP90

inhibitors observed in the resistant cells, further highlighting the exclusive role of low NQO1 expression in conferring resistance towards BAs with the quinone moiety.

McCollum and colleagues have shown that P-gp was overexpressed in a lung cancer cell line with acquired resistance to 17-AAG potentially through a chromosomal duplication event [337]. However, pharmacological inhibition of P-gp did not resensitise these resistant cells to HSP90 inhibition [337]. An earlier study using MCF7 breast cancer cells with acquired resistance to GA did not show any expression of P-gp and showed no response to P-gp inhibition as well [307]. Taken together, P-gp may play a minor role in the acquired resistance to HSP90 inhibitors. This is supported by the findings of McCollum and colleagues that the knockdown of either HSP27 or HSP70 was sufficient to increase the sensitivity of resistant cells to 17-AAG and the purine-based HSP90 inhibitor, EC78 despite P-gp overexpression [337]. This finding is supported by an earlier study that demonstrated that the resistance conferred by HSP27 was partly due to the induction of glutathione (GSH), which has also been shown to bind GA and 17-AAG in a non-enzymatic reaction [338]. Direct binding of GSH to these compounds may reduce their binding to HSP90. Besides NQO1, benzoquinone ansamycins are also reduced by cytochrome P450 enzymes leading to the formation of free radicals [339]. GSH may decrease the formation and cytotoxic effects of free radicals by direct binding to the benzoquinones and the oxygen species [313]. HSP70 may contribute to acquired resistance mainly through the inhibition of HSP90-inhibitor-induced apoptotic factors as discussed earlier [314].

1.5 Stress and the bone microenvironment

The skeleton undergoes continual remodeling throughout adult life in order to maintain bone quality and structural integrity. Many factors and pathological conditions can have a negative impact on normal bone remodeling which may result in a sustained loss of bone mass. Such bone loss is often associated with decreased bone strength and thus an increased fracture risk that can significantly diminish the quality of life and increase the rate of mortality [340]. Metastasis and anticancer therapeutics have major roles in causing increased bone loss and bone fractures among cancer patients. Our group has previously identified that 17-AAG increases bone loss in mouse models through the direct induction of osteoclast formation [341]. Furthermore, while 17-AAG proved effective in reducing tumour burden at extraosseous sites, it increased tumour burden within the bone, and caused elevated bone loss even in the absence of tumour cells [341]. Consistent with our findings, Yano *et al* demonstrated that 17–AAG enhanced prostate tumour growth in the bones of mice, which could be abrogated by the administration of inhibitors of osteoclast formation and function [342]. In addition to 17-AAG, we have demonstrated that other structurally unrelated HSP90 inhibitors also enhance osteoclast formation [341, 343]. To date the mechanism by which HSP90 inhibitors stimulate osteoclast formation has not been fully defined. Yano *et al* reported that Src kinase was activated by HSP90 inhibition transiently and its inhibition reduced 17-AAG stimulated tumour growth in bone

[342]. Our group has recently reported that 17-AAG increased the expression of the essential osteoclast transcription factor microphthalmia-associated transcription factor (MITF) [343]. HSP90 inhibition itself is unlikely to be directly critical in 17-AAG actions on osteoclasts, since many of the pathways required for osteoclast formation are HSP90 dependent. Alternatively, the induction of heat shock response via activation of HSF1 by HSP90 inhibition is a likely cause of elevated bone loss, as osteolysis is frequently associated with strong local stress responses. The key players in bone remodeling will be discussed in the following sections as well as factors that lead to osteolysis with a focus on metastasis, anticancer treatments and other forms of stresses.

1.5.1 Physiological bone remodeling

Bone is a dynamic tissue that undergoes continuous remodeling to attain and maintain the structural integrity and mineral homeostasis of the skeleton. Bone remodeling is responsible for the removal and repair of damaged bone through a tightly regulated process of resorption by osteoclasts, followed by bone formation carried out by osteoblasts (Fig. 1.15).

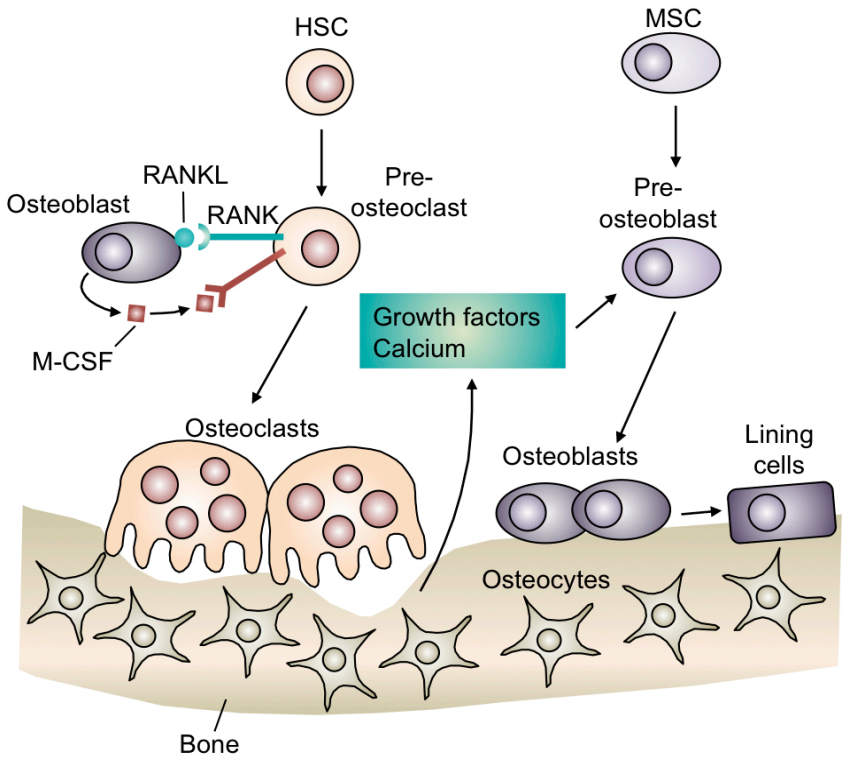


Figure 1.15. Overview of the bone remodeling process. Bone tissue undergoes continuous remodeling to maintain skeletal integrity through a balanced and coupled process. Hematopoietic stem cell (HSC) -derived osteoclasts resorb the bone matrix, releasing growth factors and calcium that in turn induce the differentiation of mesenchymal stem cell (MSC) –derived osteoblasts to replace resorbed bone. Osteoblasts, osteocytes and bone stromal cells also produce RANKL and M-CSF critical for the differentiation of osteoclasts. Osteocytes are bone-embedded osteoblast-derived stromal cells that sense mechanical stress to initiate the remodeling process. Bone lining cells are quiescent osteoblast-derived cells that form a layer over bone surfaces that facilitate the signaling between osteocytes, osteoclasts and osteoblasts. Adapted from [344].

1.5.1.1 Osteoclast differentiation and bone resorption

Osteoclasts are multi-nucleated cells originated from the hematopoietic monocyte-macrophage lineage [345]. The expression of the transcription factor PU.1 is crucial during the early non-specific development of hematopoietic stem cells into macrophage lineage cells, including osteoclasts and macrophages [346] (Fig. 1.16). The maturation of osteoclasts from immature progenitors is dependent on the stimulation by receptor activator of nuclear factor κ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) by osteoblasts and osteoblastic stromal cells. Osteoclast adhesion to substrate via integrin $\alpha_v\beta_3$ constitutes a third necessary stimulus, and autocrine factors that act via immunoreceptor signalling motif (ITAM) domain-containing receptors may also be important. However, it is RANKL expression that is regulated by the actions of osteolytic hormones such as prostaglandins and parathyroid hormone (PTH), and as such is considered the critical controller of osteoclast formation. RANKL binds to the membrane-bound receptor RANK in immature macrophage lineage cells or pre-osteoclasts. M-CSF acts on the early progenitors of osteoclasts via its receptor, c-Fms to initiate signaling pathways required for cell proliferation and survival [347].

The activation of RANK on osteoclast precursors by the tumour necrosis factor (TNF) -related protein RANKL commits the cell to osteoclast fate (Fig. 1.16). Other TNF-family members such as TNF-α [348, 349], FasL [350] have been reported to weakly induce osteoclast formation and/or survival but RANKL and RANK null mice completely lack osteoclasts [351, 352], indicating the essential role of RANKL in osteoclastogenesis. Systemic and local factors like PTH, IL-1 or prostaglandins can induce RANKL expression of osteoblasts and marrow stromal cells [347]. Osteoblasts also express osteoprotegrin (OPG), a secreted decoy receptor for RANKL, which leads to the inhibition of osteoclast differentiation [353]. OPG production is typically reduced by osteolytic factors such as glucocorticoids [354] and prostaglandins [355]. Hence, the proportion of RANKL to OPG is crucial in the regulation of bone resorption and bone strength [347, 356]. Activation of RANK promotes several downstream signaling pathways by the recruitment of TNF receptor-associated factors (TRAFs), which include TRAFs 1, 2, 3, 5 and 6 that act as adaptor proteins to recruit other protein kinases [357]. RANK/TRAF6 signalling activates several key pathways to induce osteoclastogenesis: NF-κB, AP-1 (c-Fos/c-Jun dimer), SRC/ERK, p38/microphthalmia-associated transcription factor (MITF) and nuclear factor of activated T cells cytoplasmic 1 (NFATc1).

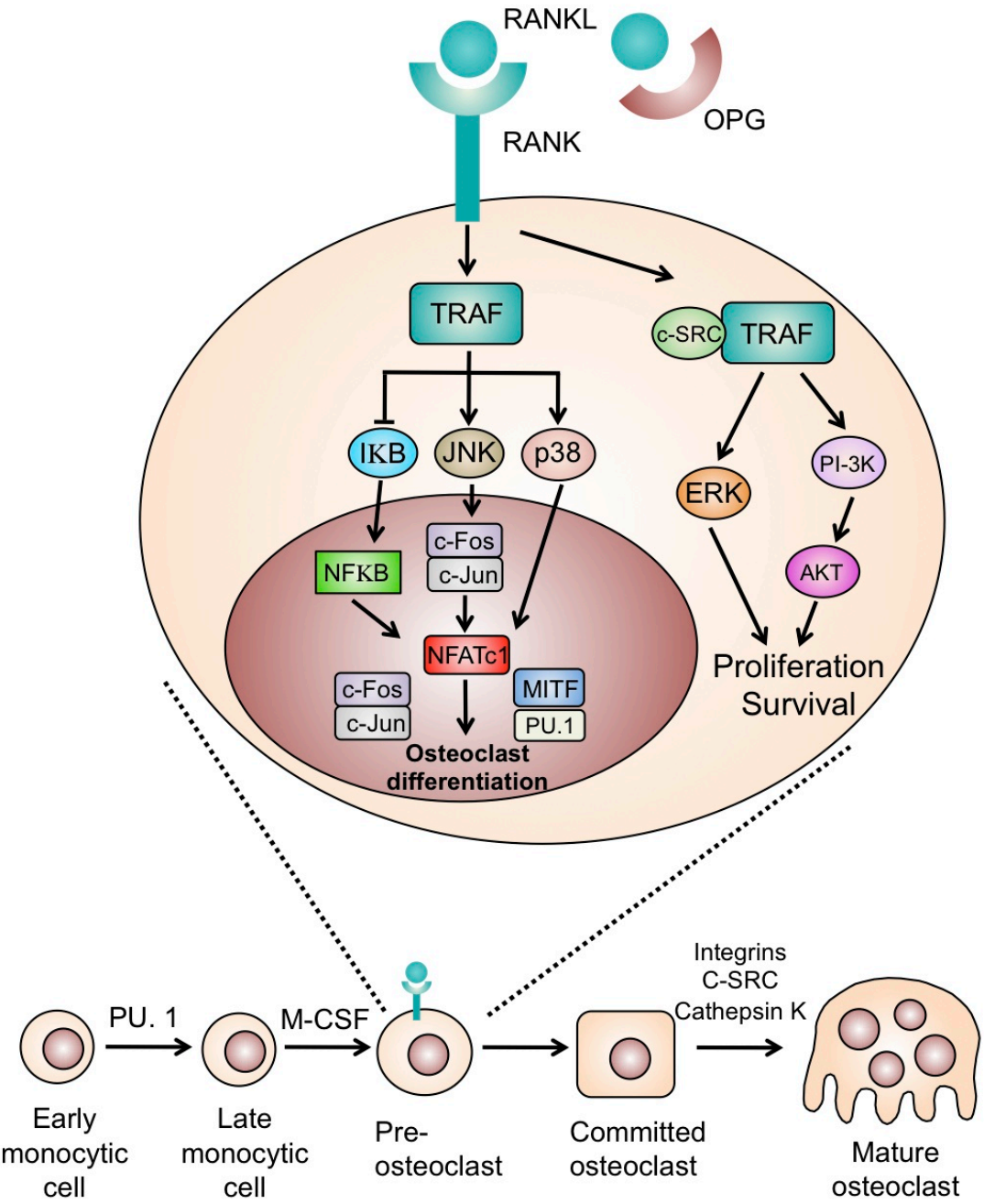


Figure 1.16. Regulation of osteoclastogenesis. The early nonspecific differentiation of monocytic HSCs to osteoclasts is dependent on the transcription factor PU.1 as well as M-CSF that is required for the proliferation and survival of pre-osteoclasts. Activation of the RANKL-RANK pathway commits pre-osteoclasts to the osteoclast fate mediated by downstream signaling molecules that are involved in differentiation, proliferation and survival of osteoclasts. RANKL-RANK pathway is restrained by OPG which suppresses osteoclastogenesis. Adapted from [358].

NF-κB is a family of transcription factors that includes reticuloendotheliosis viral oncogene homolog-A (RelA/p65), p50, Rel B, p52, and c-Rel, which induce expression of genes involved in immune responses, cell division, and differentiation through canonical and non-canonical pathways [359, 360]. The role of NF-kB in osteoclastogenesis was discovered in p50 and p52 double knockout mice in which osteopetrosis developed due to a defect in osteoclast formation [361]. Similarly, hematopoietic cells from mice lacking RelA/p65 exhibited deficiency in osteoclastogenic response to RANKL [362]. A subsequent study showed that the defect in osteoclast formation in p50/p52 knockout mice was rescued by either c-Fos or NFATc1 expression [363], indicating that they act downstream of NF-kB. Indeed, upon RANKL treatment, p50 and p65 are recruited to the promoter of NFATc1 that contains a NF-κB binding element, resulting in NFATc1 expression [364]. In the canonical pathway, activation of RANK leads to the formation of a complex consisting of TRAF6, IKK1, IKK2, TAK1 and IKKγ/NEMO that results in degradation of the inhibitory protein, IκB [365, 366]. This subsequently leads to the rapid release and accumulation of NF-κB subunits, p65 and p50 to form heterodimers and translocate into the nucleus to activate target genes [365, 366]. RANKL also leads to the activation of the non-canonical pathway that involves activation of the NF-κB-inducing kinase (NIK) and IKK1, which subsequently leads to the phosphorylation of p100 and the proteolytic processing of p100 to p52. This pathway generates p52/RelB heterodimers that also translocate into the nucleus to activate a variety of genes [352, 353, [367].

RANK also leads to the activation of the transcription factor AP-1 (c-Fos/c-Jun dimer) via the mitogen-activated protein kinases (MAPK), JNK [368]. c-Fos is essential for the commitment of hematopoietic cells to the osteoclast lineage and differentiation as demonstrated by a lack of osteoclast formation but increased macrophage formation in mice deficient of c-Fos [369]. Similarly, c-Jun is crucial for RANKL-regulated osteoclast formation through its co-operation with NFATc1 [370]. Mice expressing a dominant negative c-Jun showed a decrease in RANKL-dependent osteoclast formation and the overexpression of NFATc1 rescued the effect [370].

The RANKL/RANK pathway also engages p38, another class of MAPK, to induce the formation of functional osteoclasts [371]. RANKL-induced osteoclastogenesis can be decreased by p38 inhibition *in vitro* [371, 372] and p38 null mice have monocytes with impaired differentiation into osteoclasts and decreased osteoclast numbers found on trabecular bones [373]. MEK/ERK and AKT pathways are also induced by RANKL through the interaction of TRAF6 with c-SRC to mediate the survival and actin reorganization of osteoclasts [374, 375].

NFATc1, a member of the NFAT family of transcription factors, is widely regarded as the master regulator of osteoclastogenesis. Mice with *NFATc1*-knockout died of heart defect but when rescued

by Tie-2-promoter driven expression of NFATc1 showed a significant defect in osteoclast formation [376]. Furthermore, mice with conditional deletion of NFATc1 in osteoclasts developed osteopetrosis because of a deficiency in osteoclast formation [377]. The promoter of *NFATc1* contains NFAT binding sites and NFATc1 also acts on its own gene to promote self-amplification [378]. The activation of NFATc1 is mediated by a phosphatase, calcineurin, which is activated by the calcium-calmodulin pathway. In response to the transient increase in intracellular Ca^{2+}, NFATc1 is dephosphorylated by calcineurin and translocated into the nucleus to initiate transcription [379, 380]. Activated NFATc1 subsequently induces a number of genes involved in osteoclast fusion, maturation and resorption, which include tartrate resistant acid phosphatase (TRAP), calcitonin receptor, cathepsin K, osteoclast-associated receptor (OSCAR) and dendritic cell-specific transmembrane protein (DC-STAMP) [364, 381, 382]. NFATc1 has been shown to form a complex with AP-1 for the induction of TRAP, calcitonin receptor and NFATc1 auto-amplification [364].

Directly downstream of p38 and NFATc1 is microphthalmia-associated transcription factor (MITF), a basic helix-loop-helix leucine zipper protein related to the transcription factor E (TFE) family [383]. Strong evidence for a critical role of MITF in osteoclast differentiation was provided by reports showing that the severe osteopetrosis due to impaired osteoclast function in mice homozygous for MITF with a deletion mutation [384-386]. NFATc1 forms a complex with MITF and PU.1 to activate the promoters of cathepsin K, OSCAR, TRAP and V-type ATPase [381]. This suggests that the different components of the NFATc1 transcriptional complex may contribute to the expression of different genes during osteoclastogenesis. The initial step of bone resorption involves the attachment to bone surface, which is mediated by the formation of podosomes containing actin filaments and αvβ3 integrin [387]. Osteoclasts then dissolve bone material by the secretion of hydrochloric acid. This is regulated by a V-type ATPase present in the ruffled membrane of osteoclasts that translocate protons into the resorption area [347]. During the resorption process, calcium levels are increased dramatically at the site, which results in changes in the intracellular calcium level of osteoclasts followed by podosome disassembly [347, 388].

1.5.1.2 Osteoblast differentiation and bone formation

During the remodeling of adult bone, the completion of bone resorption phase is followed by the differentiation of osteoblasts, which are specialised bone forming cells derived from the mesenchymal lineage. The formation of osteoblasts involves differentiation from progenitor cells into proliferating pre-osteoblasts, bone matrix-producing osteoblasts and bone lining cells [389]. The differentiation of progenitor cells into osteoblastic cells requires local factors, such as transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs) and Wnt proteins [344]. These pathways lead to the

expression of key osteoblast-activating transcription factors including Runt-related transcription factor (RunX2), osterix and stabilization of activating transcription factors (ATFs) [390-392]. While osteoblast formation and activity does not depend on prior osteoclast action (as seen in embryonic and modeled bone which form *de* novo), bone formation is typically closely coupled to bone resorption such that factors that inhibit or stimulate bone resorption (e.g., bisphosphonates and PTH respectively) similarly affect bone formation. The mechanism for such coupling is unclear, however many pro-osteoblastic factors are found in the bone matrix and released during the resorption phase, such as BMP, insulin-like growth factors (IGFs), TGF-β and calcium [393]. Activated osteoblasts lay down new bone matrix including collagen, osteocalcin and osteopontin, until the resorbed bone is replaced [347]. Osteoblasts that are embedded in the bone matrix transforming into osteocytes, which are cocooned in fluid-filled cavities within the mineralized bone but maintain cellular contacts with each other and bone lining cells via long processes that extend through canaliculi in the bone; osteocytes are highly abundant, accounting for 90-95% of all bone cells [394]. Osteocytes respond to mechanical stress and bone microdamage by initiating the process of bone remodeling through the induction of osteoclast differentiation and the negative regulation of osteoblasts [347, 395]. Quiescent osteoblasts at the end of the bone formation process form lining cells on the bone surface until the new cycle of bone remodeling is initiated. In addition to their role in bone formation, osteoblasts are also involved in the differentiation of osteoclast by producing RANKL or OPG [396].

1.5.2 Pathophysiology of osteolysis

The mechanism of osteolytic bone loss is multi-factorial but is often associated with pathological conditions such as metastasis, anticancer therapies and inflammatory responses. Moreover, these conditions also induce HSF1-dependent cellular stress locally [55, 397, 398]. A causative link between local stress responses and elevated bone loss if proven can have a significant implication on our understanding of the development of osteolytic lesions. The following sections will focus on the circumstantial evidence of osteolytic bone loss induced by stress-related events that include bone metastasis, anticancer therapy and local stress responses.

1.5.2.1 Bone metastasis

Many common neoplastic, inflammatory, metabolic and iatrogenic conditions are associated with decreased bone mass, either locally or generally, with corresponding increases in fracture propensity and related problems. Bone metastases are a common complication of cancer and occur in 65%-80% of patients with metastatic breast and prostate cancers [344]. Tumour invasion of the bone environment accelerates the bone remodeling process by disturbing the balance between bone degradation by osteoclasts and bone depositing by osteoblasts. Crosstalk between tumour cells and the

bone microenvironment fuels a vicious cycle of tumour growth and bone remodeling, which relies on factors secreted by tumour cells that stimulate maturation of the osteoclasts and osteoblasts, usually leading to a net increase in osteoclast- mediated bone loss [399]. Figure 1.17 summarises the mechanisms of tumour-associated osteolysis.

The preferential homing of tumour cells to the bone marrow signifies the initial key event in bone metastasis. Osteoblasts and bone marrow stromal cells are important for haematopoietic stem cells (HSCs) homing within the bone marrow by providing a niche through the expression of chemokines (e.g. CXCL12), vascular cell adhesion molecule 1 (VCAM1), BMPs, Notch and OPN [400-403]. Metastatic tumour cells are able to use the same physiological mechanisms during localization to bone [344]. The receptor for CXCL12, CXCR4 is highly overexpressed in highly metastatic breast and prostate cancer cell lines [404, 405]. The blocking of CXCL12-CXCR4 binding prevented bone metastasis in numerous metastatic mouse models [406, 407]. Furthermore, studies have also demonstrated a role for CXCL12-CXCR4 in the proliferation and survival of cancer cells to facilitate colonization after arriving in the bone [408-410]. Metastatic tumour cells also express integrins that interact with ECM proteins in the bone microenvironment. The β1 integrins are receptors for fibronectin, collagen I and VCAM1, which have been shown to induce interaction between tumour cells and bone marrow stromal cells [411-413]. The αvβ3 integrin on the other hand interacts with osteopontin (OPN), fibronectin and vitronectin [344]. High expression of αvβ3 is also correlated with increased bone metastasis and tumour-associated osteolysis [344]. In addition, CXCL12-CXCR4 has been associated with the regulation of integrin expression in different cancer cells, suggesting a crosstalk between the two pathways in the homing and colonization of metastatic cancer cells in the bone microenvironment [414].

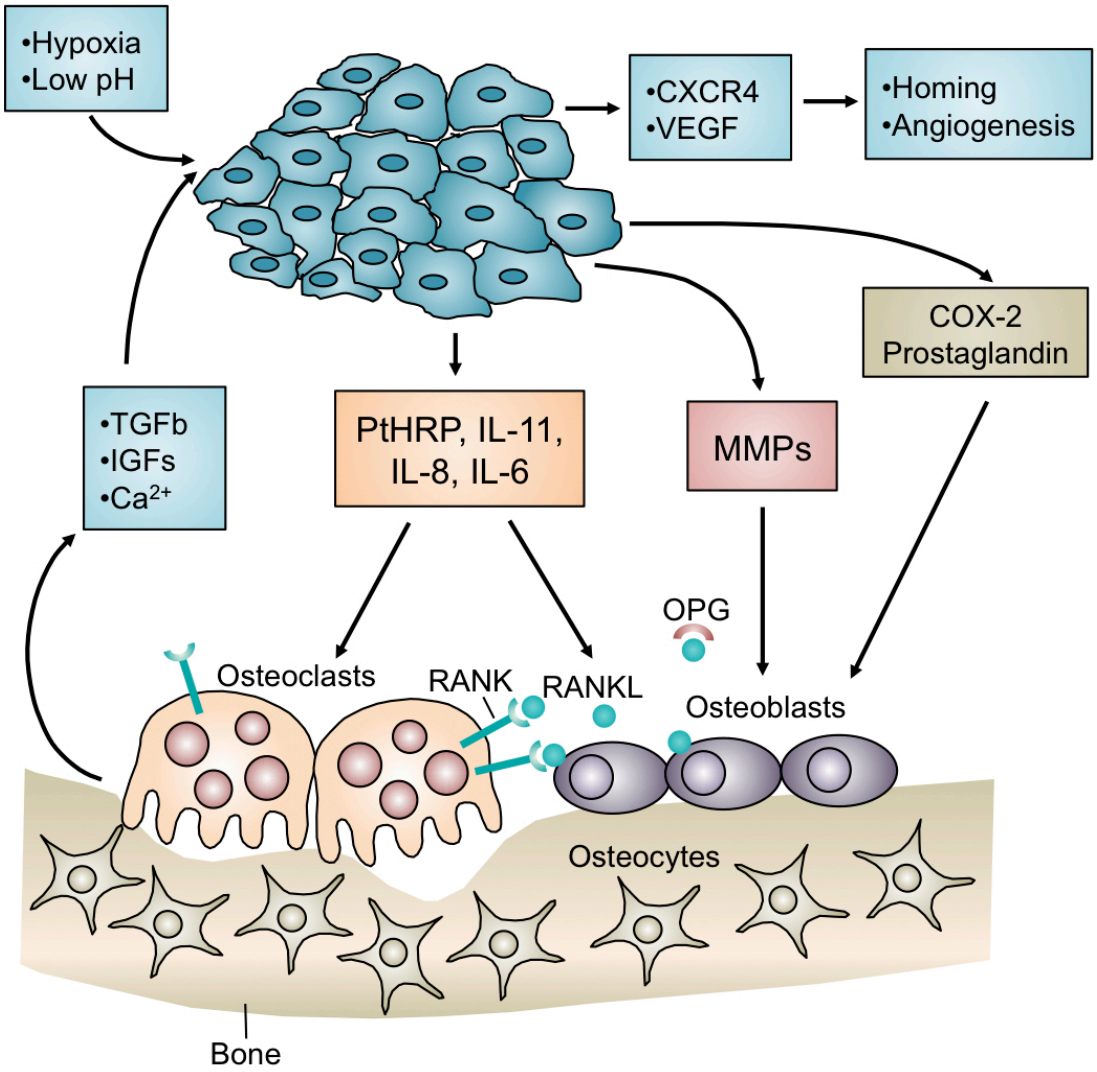


Figure 1.17. The vicious cycle of osteolytic bone metastasis. Tumours secrete osteolytic factors, such as parathyroid hormone-related protein (PTHrP), interleukins, COX-2 and prostaglandins that induce the differentiation of osteoclasts by increasing the ratio of RANKL to OPG secretion of osteoblasts. Bone resorption causes the release of growth factors and calcium ions that are able to stimulate the secretion of tumour-derived osteolytic factors. Tumour-associated hypoxia and acidosis in conjunction with bone-derived factors can increase the expression of factors that lead to bone homing and angiogenesis. Tumour-derived MMPs are involved in the maturation and activation of RANKL. Adapted from [344].

Tumour-derived PTHrP is a major effector in bone metastasis and is expressed in the primary tumours of about 50% of patients and in more than 90% of breast cancer bone metastasis samples [415]. PTHrP expression is regulated by transcription factors, such as HIF-1α, GLI2 and RUNX2 that are activated in response to the hypoxic nature of the bone and tumour microenvironment [416-418]. The increased expression of PTHrP in tumour cells has been shown to enhance osteolytic lesions *in vivo*, and decreased expression reduced the number and size of lesions [419]. PTHrP stimulates RANKL and decrease OPG expression by osteoblast and hence favours osteoclast formation and maturation [420].

In addition to PTHrP, tumour cell-derived cytokines, such as IL-8, IL-6, IL-11 are critical in the induction of osteoclastogenesis. IL-8, the human homolog to murine MIP-2 is overexpressed in metastatic tumour cells and is able to stimulate the formation of osteoclasts through increased RANKL production by osteoblasts or independently of RANKL [421]. The overexpression of IL-6 in cancer cells has been correlated with tumour progression and poor prognosis [422, 423]. IL-6 is expressed in osteoblasts at low levels but when overexpressed it acts to stimulate the expression of RANKL through the activation of STAT3 [424]. Furthermore, mice with high circulating IL-6 levels showed decreased osteoblast and increased osteoclast numbers and activity [425]. IL-11 is an additional osteolytic factor that can also induce osteoclastogenesis by increasing the ratio of RANKL to OPG or directly in a RANKL-independent mechanism [426].

The cyclooxygenase enzymes, COX-1 and COX-2, convert arachidonic acid to prostaglandins and thromboxanes. The signaling pathway involving the prostaglandin, PGE2 and its receptor, EP4, play a critical role in osteolysis by inducing the maturation of osteoclasts. Studies have demonstrated that direct cell-to-cell contact between breast cancer cells and osteoblasts induces the expression of COX-2 in osteoblasts through the activation of the MAPK pathway [427]. The increase of COX-2 results in the increase of PGE2 secretion and its binding to EP4 in osteoblasts, which subsequently leads to increased RANKL production. Increased expression of COX-2 is also correlated with increased production of IL-8 in bone metastatic breast cancer cells, which may further enhance osteolysis [428, 429]. In addition, bone-derived TGF-β released during resorption stimulates COX-2 and subsequently PGE2 expression in tumour cells [430].

Matrix metalloproteinases (MMPs) have been implicated in the metastatic cascade, especially in bone invasion and metastasis [344]. The proteolytic activity of MMPs is not involved in the osteoclast resorption of bone matrix, in which cathepsin K is believed to be the major protease. They act more importantly as regulators of osteolysis by increasing the activity of RANKL. MMP7 has been shown to process RANKL into a soluble form in prostate cancer cells [431]. MMP1 and a disintegrin-like and metalloproteinase with thrombospondin motifs 1 (ADAMTS1) cleave EGF-like growth factors which leads to decreased OPG production by osteoblasts and increase osteoclastogenesis [432]. In addition, MMP13 has been shown to activate MMP9 and TGF-β to increase RANKL expression at the tumour-bone interface, hence favouring the increase in osteoclast formation [433].

During the process of bone resorption, factors from the bone matrix, such as TGF-β, IGFs and calcium are released into the tumour-bone interface. These factors support tumour growth and localization, thus establishing or completing the positive feedback loop between tumour cells and the bone microenvironment (Fig. 1.17). Bone-derived TGF-β is critical for the growth and expansion of

tumour cells in bone through the regulation of tumour cell survival and the production of pro-osteolytic factors. TGF-β also inhibits T-cell proliferation and the activity of natural killer (NK) cells, which assist tumour cells in immune system evasion [434]. Studies have shown that bone-derived TGF-β in the tumour-bone microenvironment induces the expression of PTHrP in tumour cells that results in increased RANKL production by osteoblasts and increased osteoclastogenesis [435]. TGF-β also interacts with HIF-1α in the hypoxic bone microenvironment to enhance tumour metastasis and growth by inducing the expression of VEGF and CXCR4 [436]. The combined treatment of mouse models with pharmacological inhibitors of TGF-β and HIF-1α significantly decrease osteoclastic bone resorption and metastases [436]. Preclinical studies have also demonstrated that the major source of TGF-β in the tumour-bone microenvironment is the bone matrix as a consequence of osteolysis and TGF-β inhibition is a promising approach to treat bone metastases [344]. IGFs released into the bone microenvironment during bone resorption also stimulates tumour cell proliferation and survival, hence further perpetuating the vicious cycle of bone metastasis [437]. IGFs binding also induces the production of M-CSF and RANKL by osteoblasts and the corresponding receptors, C-FMS and RANK in osteoclasts [438]. Furthermore, studies have shown that bone-derived supernatant and IGFs are powerful chemoattractants to stimulate breast cancer cell migration and hence their homing to the bone microenvironment [439, 440].

The large amount of calcium released into the bone microenvironment during resorption has a profound effect on the surrounding tumour cells. The binding of calcium ions to calcium-sensing receptors (CASRs) in tumour cells can lead to the inhibition of apoptosis and increase in proliferation [344, 441]. Furthermore, calcium ions lead to increased PTHrP expression by tumour cells, hence leading to increased osteoclastogenesis, resorption and calcium release [442, 443]. Studies have also shown that calcium ions can act as potent chemoattractants to breast cancer cells to support homing and their localization to the bone through the activation of CASR and its downstream pathway [444].

1.5.2.2 Anticancer therapy-induced bone loss

To inhibit disease progression or prevent disease recurrence, patients with breast cancer are often treated with endocrine treatments, such as gonadotropin-releasing hormone (GnRH), luteinizing hormone-releasing hormone (LHRH) agonists and aromatase inhibitors (AIs) as well as non-hormonal chemotherapeutics [445]. It is increasingly being recognized that these anticancer-treatments can lead to changes in bone remodeling, often resulting in bone loss.

Estrogens play a critical role in the development and growth of breast cancers. Estrogen deprivation achieved by blocking or reducing endogenous levels of estrogen, forms the basis of endocrine

treatments for breast cancer [446]. Estrogens stimulate gene expression and protein production of OPG in osteoblastic cells and suppress RANKL-induced osteoclast formation [447, 448]. Therefore, estrogen deprivation associated with endocrine treatments can lead to increased RANKL production and reduced levels of OPG, which may result in increased levels of osteoclastic precursors and osteoclast maturation. Estrogen deprivation may also have additional effects on bone metabolism by directly modulating the production, storage or activity of bone growth factors, such as TGF-β, which reduces the ability of bone to respond to osteoclastic activity, thus causing an imbalance between bone resorption and formation that leads to bone loss [446, 449]. Indeed, clinical trials have shown that treatments with AIs were associated with a greater prevalence in osteoporosis, loss in bone mass density (BMD) and fracture risk in breast cancer patients [450-453].

While anti-hormone therapies can promote bone loss by inducing hypogonadism, chemotherapeutics can also directly affect bone turnover to cause loss of bone mass and structural integrity. Short stature, low bone mass and increased fracture risks are some of the skeletal side effects of chemotherapy treatments among pediatric patients and adult survivors [454, 455]. Clinically, chemotherapy has been shown to cause osteopenia (low bone mineral density) by direct damage of the osteoblastic precursor cells [456, 457]. Bone formation was reported to be lower and bone resorption was increased in patients with various hematopoetic and solid cancers after chemotherapeutic treatment [458]. Experimental studies in rodent models have also shown that chemotherapeutic drugs such as methotrexate (MTX), etoposide, cyclophosphamide and 5-fluorouracil (5-FU) can cause osteopenia by inhibition of osteoblast formation and increase of osteoclast differentiation [459-462]. Recent reports have shown that MTX chemotherapy induced osteoclast differentiation by increasing cytokine and TNF-α levels as well as NF-κB activation [459, 460]. The underlying molecular mechanisms of chemotherapy-induced bone loss remain largely unclear.

1.5.2.3 Bone loss due to local stress response

Osteolysis is often associated with strong local stress responses seen in skeletal disorders, such as rheumatoid arthritis, osteoporosis, bone metastasis and bacterial infection. Stress factors found in these conditions, such as pro-inflammatory cytokines, oxygen free radicals, low oxygen and low pH are critical in causing bone loss, as well as inducing cellular stress.

In an early study of the effect of pro-inflammatory cytokines as mediators of osteoclastogenesis, bone cultures were exposed to the supernatant from activated human leukocytes, which is characteristic of pro-inflammatory response [463]. Exposure of bone cells to the supernatant resulted in the increase in osteoclast numbers and bone resorption, suggesting that pro-inflammatory factors may induce osteoclast formation and activity. In the autoimmune disease, rheumatoid arthritis (RA), levels of pro-

inflammatory cytokines are dramatically increased in the synovial fluid of affected joints and are associated with osteoclastogenesis and bone erosion [464, 465]. Periodontal disease, an inflammatory condition of the gum caused by bacterial infection, is also associated with osteoclast-dependent destruction of the alveolar bone [466]. As discussed in section 1.5.2, malignancies such as breast cancer and prostate cancer also result in osteolytic bone lesions due to the secretion of pro-inflammatory cytokines by cancer cells, osteoblasts and bone stromal cells in the bone microenvironment. The cytokines involved in many of these conditions have been shown to be capable of stimulating osteoclastogenesis and bone resorption, including IL-1 [467], tumour necrosis factor alpha (TNF-α) [468], IL-6 [469], IL-11 [470], IL-15 [471] and IL-17 [472]. Pro-inflammatory cytokines exert their effect on osteoclast formation through the increase of RANKL production by osteoblast and bone stromal cells. IL-6, IL-11 and TNF-α are also capable of inducing osteoclastogenesis directly in a RANKL-independent manner [349, 426].

Many studies have provided evidence for the positive correlation between oxidative stress and osteolytic conditions. Osteoporosis has been associated with higher oxidative stress index and total plasma oxidant status in patients [473]. Higher levels of oxidative stress was also observed in patients with metastatic prostate cancer that form bone metastasis compared to patients with local prostate cancer [474]. In a study of the gene expression of synovial cells during rheumatoid arthritis, oxidative stress mechanisms are significantly enhanced and this was confirmed by increased cellular reactive oxygen species (ROS) levels [475]. The first study to show a relationship between ROS and the formation of mature osteoclasts was first described by Garrett *et al* in 1990 [476]. A more recent study demonstrated that RANKL-induced osteoclastogenesis requires ROS production in bone marrow precursor cells through the subsequent activation of JNK, p38 and ERK1/2 [477]. ROS also exerts its effect on osteoclastogenesis through the interaction with NF-kB, as shown by the suppression of RANKL-induced osteoclastogenesis in precursor cells by a strong antioxidant, alpha-lipopic acid (ALA), through the inhibition of ROS production and NF-kB activation [478]. One study has shown that RANKL production by osteoblasts was increased upon ethanol treatment through activating transcription factor 3 (ATF-3) following ROS production [479]. Interestingly, another study has found that in human MG63 osteoblast precursor cells, ROS promoted HSF2 binding to the heat shock element found in the human RANKL promoter region [480].

Hypoxia occurs when the blood supply to the tissues is reduced or disrupted. Many potential causes of hypoxia are associated with osteolytic bone loss, including inflammation, infection, fractures, tumours and diabetes [481]. Studies have demonstrated that reduced oxygen levels can lead to the differentiation or activation of marrow precursor cells from the monocyte-macrophage lineage, of which osteoclasts are derived from [482, 483]. Subsequent studies found that hypoxic conditions increased the number and size of RANKL/M-CSF-induced osteoclast formation in mouse bone

marrow cells and human peripheral blood mononuclear cells (hPBMCs) [484, 485]. Interestingly, pre-formed mature osteoclasts from rats showed that hypoxia did not affect the resorptive activity in a pH-controlled condition [484]. This indicates that hypoxia might be involved in the maturation and formation of osteoclasts but not their activity. Another study demonstrated that hypoxia also resulted in the development of osteolytic bone metastasis through the suppression of osteoblast differentiation and the increase in osteoclastogenesis in a HIF-1-dependent mechanism [486]. The effect of hypoxia in osteolysis may involve the upregulation of IL-6 and VEGF, both of which are involved in the formation of osteoclasts [484, 485].

Acidosis is the increase of H^+ concentration and decreased pH in the blood and extracellular fluid due to the disruption of the CO_2/HCO_3^- system [481]. Systemic acidosis can be caused by conditions associated with osteolysis, such as anaerobic exercise, gastroenteritis, excessive protein consumption or acidifying substances, diabetes and aging [481]. Local acidosis can also arise due to hypoxia, tumours, inflammation and fractures [481]. Studies with preformed osteoclasts of rat and human origin showed that low pH has a stimulatory effect on bone resorption activity [487, 488]. Furthermore, acidosis has been shown to be required mainly for the initiation of resorption activity, as osteoclasts can be further stimulated with other factors, such as RANKL, PTH, vitamin D and ATP after exposure to acidic conditions [487, 488]. The initiation of osteoclastic resorption by acidosis involves the upregulation of carbonic anhydrase II and the vacuolar –type H1-ATPase for the regulation of H+ transport critical for bone mineral solubilisation [489, 490]. In addition, expression of cathepsin K, which is required for organic matrix degradation and tartrate-resistant acid phosphatase (TRAP) are also induced by low pH [491, 492]. The effect of acidosis on osteoclast formation on the other hand remains unclear. Low pH has been shown to inhibit osteoclast formation in mouse marrow cells but increase osteoclast formation in cat marrow cells [491, 493].

1.6 Project aims and thesis outline

HSP90 plays a key role in the regulation of multiple oncogenic pathways, hence making it an attractive target for cancer therapeutics. Intrinsic resistance is a common problem in cancer therapy and can significantly limit the potential of HSP90 inhibitors as exemplified by the lack of efficacy as single agents in clinical trials. Moreover, prolonged exposure to HSP90 inhibitors can result in the rapid acquisition of resistance in tumour cells, which may possess survival and growth advantages that allow for proliferation and expansion of tumours. The first hypothesis of the current study is that chronic treatment with HSP90 inhibitors can lead to the selection of cancer cells that harbour multiple molecular changes to circumvent the toxicity of HSP90 inhibition. Chemoresistance is often correlated to metastasis as exemplified by the increased aggressiveness of tumour cells that survive

initial chemotherapeutic treatments. Hence, we also hypothesise that acquired resistance to HSP90 inhibitor will lead to phenotypical alterations in cancer cells that can affect tumour growth and metastasis. We have shown in a previous study that HSP90 inhibitor, 17-AAG reduced tumour growth in soft tissues but increased bone loss and bone metastasis by stimulating osteoclast formation [341]. Our third hypothesis is that the induction of HSF1 activity by HSP90 inhibition is responsible in the increased formation of mature osteoclasts.

The specific aims of this thesis are:

1. To determine the molecular mechanisms of acquired resistance to HSP90 inhibitors.
2. To examine the changes in cell biology of cancer cells with acquired resistance to HSP90 inhibitors.
3. To determine the role of HSF1 in HSP90 inhibitor-induced osteoclastogenesis.

Chapter 2 presents evidence of multiple molecular changes, in particular increased expression of HDACs in HSP90 inhibitor-resistant breast cancer cells that resulted in resistance to different classes of HSP90 inhibitors. Chapter 3 describes the changes in cell growth and metastatic ability of cancer cells that are attributable to changes in gene expression associated with acquired resistance to HSP90 inhibitors. Chapter 4 establishes a role of HSF1 –induced cell stress in the formation of mature osteoclasts by HSP90 inhibitors. Finally, chapter 6 summarises the results and discusses the implications of the findings in this thesis. Overall, this thesis provides strong evidence of a novel molecular signature in breast cancer cells chronically treated with HSP90 inhibitor that led to acquired resistance and altered cell biology. Moreover, the experimental data also establish a novel HSF1-driven mechanism in the induction of osteolysis by HSP90 inhibitors.

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DECLARATION FOR THESIS CHAPTER 2

In the case of Chapter **2**, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Participated in project hypothesis -Designed and performed experiments -Analysed data -Prepared and wrote the manuscript	75

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Jessica L. Vieusseux	Provided technical support, contributed to refinements to manuscript	
Benjamin J. Lang	Provided technical support, contributed to refinements to manuscript	4
Chau H. Nguyen	Provided technical support, contributed to refinements to manuscript	4
Michelle M. Kouspou	Contributed to refinements to manuscript	
John T. Price	Supervision; project co-ordination and development of hypothesis; contributed to writing and refinements to manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to the work.

Candidate's Signature [redacted] **Date 16/12/2013**

Main Supervisor's Signature [redacted] **Date 9/12/2013**

Chapter 2

Molecular Mechanisms of Cancer Cell Acquired Resistance to HSP90 Inhibitors

HDAC Family Members Contribute to Cancer Cell Acquired Resistance Towards Structurally Diverse HSP90 Inhibitors

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ABSTRACT

The molecular chaperone Heat Shock Protein 90 (HSP90) regulates multiple cell signaling pathways critical for tumor growth and progression. As such, pharmacological inhibitors of HSP90 have been shown to act as potent anticancer agents in preclinical tumor models, however, their success within the clinical setting has been less pronounced. As with other anticancer agents, intrinsic and acquired drug resistance may significantly limit the utility and efficacy of HSP90 inhibitors. To examine HSP90 inhibitor resistance, we generated MDA-MB-231 and MDA-MB-435 human cancer cell lines that were resistant towards the HSP90 inhibitor 17-AAG by dose escalation. When cultured in the absence of 17-AAG, cells maintained their respective levels of 17-AAG resistance (7–240x), and were also cross-resistant towards other benzoquinone ansamycin HSP90 inhibitors such as geldanamycin and 17-DMAG. Additionally, the resistant cell lines were cross-resistant to structurally distinct HSP90 inhibitors such as radicicol and other resorcinol-based compounds including CCT018159, VER50589 and NVP-AUY922. Altered expression of NQO1, histone deacetylase 6 (HDAC6) and histone deacetylase 1 (HDAC1) were identified in the resistant cell lines and were considered potential mechanisms of resistance. Consistent with increased HDAC expression, histone 3 acetylation was increased in the resistant cells. Moreover, HDAC inhibition with the pan HDAC inhibitors, TSA and LBH589, as well as a class II HDAC-specific inhibitor, SNDX275, significantly resensitized resistant cells towards 17-AAG. Similarily, HDAC inhibition dramatically resensitized the resistant cells towards 17-DMAG, radicicol and CCT018159. In conclusion, prolonged 17-AAG treatment was found to result in cancer cell resistance towards a spectrum of HSP90 inhibitors to which HDAC upregulation made a significant contribution.

INTRODUCTION

Heat shock protein 90 (HSP90) is a molecular chaperone that is required for the biogenesis, stabilization and folding of many cellular proteins under both physiological and pathophysiological conditions. With respect to the latter, and more specifically in cancer, many client proteins of HSP90 are overexpressed and/or mutated oncoproteins essential for cancer cell survival, growth and invasive potential, and include HER2, RAF-1, Akt, mutated p53, CDK4, MET, ALK and EGFR, [1-8]. Consistent with this, high HSP90 expression is observed in the majority of cancers enabling the cancer cells to maintain functionally active oncoproteins [9]. Therefore, the dependency of cancer cells upon HSP90 for their survival via oncoprotein maintenance has made the chaperone a very attractive therapeutic target for multiple cancer types.

HSP90 contains an ATP-binding/ATPase activity domain within its N-terminal region that mediates binding and hydrolysis of ATP, a functional requirement for the action of the chaperone [10]. Geldanamycin (GA), a member of the benzoquinone ansamycin (BA) family of antibiotics, binds the N-terminal ATPase site, resulting in the inhibition of HSP90 function and the subsequent depletion of oncogenic client proteins via the ubiquitin-proteasome pathway [11]. Although GA was the first molecule identified as a HSP90 inhibitor, the clinical development of GA was limited due to its poor solubility and hepatotoxicity in pre-clinical studies [12, 13]. Thus, the generation of GA derivatives more suitable for clinical use led to the identification of 17-AAG (tanespymycin), which became the first HSP90 inhibitor used in clinical cancer trials, providing 'proof-of-concept' studies for the use of HSP90 inhibitors as anticancer agents [10]. Currently, a number of next-generation HSP90 inhibitors, many generated by rational drug design methodologies, are currently in clinical evaluation, both as single agents and in combination regimes [14-16]. To date, while inhibitors targeting the ATP-binding pocket at the N-terminus region have been the major focus of HSP90 drug discovery and development, compounds that bind the C-terminal region of HSP90, the domain responsible for dimerization and co-chaperone binding, have also been identified [17, 18]. Compounds such as novobiocin and its derivative, coumermycin A1, are known to bind to the C-terminal domain and inhibit HSP90 dimer formation, ultimately leading to HSP90 client protein degradation [19-21]. These compounds are currently being investigated as potential alternatives to N-terminal binding inhibitors.

Studies from early-phase clinical trials of 17-AAG indicated the limitation for its use as a single agent due to the lack of objective clinical outcomes in a number of different cancers [22-24]. Next-generation HSP90 inhibitors, with improved potency such as the resorcinol-based STA9090 and AUY-922 compounds have shown some clinical promise within early-phase monotherapy trials although not to a degree that was initially predicted. However, the use of HSP90 inhibitors within the context of combination therapies, such as with the HER2-specific inhibitor, trastuzumab, have shown more favorable clinical responses [25], potentially via increased target degradation, as well as

impacting upon trastuzumab resistance [26, 27]. To date, pre-clinical and clinical data has highlighted the potential that resistance towards HSP90 inhibitors may be a contributing factor to the efficacy of these agents, although few studies have directly addressed this. Therefore, the prediction and reversal of HSP90 inhibitor resistance may be a potential way of significantly improving the therapeutic efficacy of this anticancer agent class.

Pre-existing or intrinsic resistance towards anticancer drugs is a common feature of many cancer cell types, resulting in a reduced sensitivity of tumours towards first-line chemotherapeutic treatments. For HSP90 inhibitors, some key determinants of intrinsic cellular resistance include the high expression levels of oncoproteins such as HER2 [28], the heat shock transcription factor 1 (HSF1) [29], other heat shock proteins and HSP90 co-chaperones [30-32], as well as cell cycle and apoptotic regulators, such as retinoblastoma protein (RB), p53, BAGs and BAX [33]. Intrinsic resistance to BAs, including GA and its derivatives has also been linked to the multiple drug resistance (MDR) phenotype due to the up-regulation of the drug efflux pump, p-glycoprotein (P-gp or MDR1) [34]. In addition, resistance towards the quinone containing BAs could be attributed to low levels of NAD(P)H: quinone oxidoreductase (NQO1), responsible for metabolising drugs such as 17-AAG, to their more potent semiquinone and hydroquinone forms leading to increased HSP90 inhibition [35, 36]. Moreover, post-translational modification of HSP90, such as acetylation, has been shown to be critical in maintaining normal chaperone function, in particular client protein and co-chaperone binding [37, 38]. Histone deacetylase 6 (HDAC6) has been reported to deacetylate Lys294 within the middle domain of HSP90 [37, 38] and inhibition of HDAC6 has been shown to result in the increased depletion of client proteins and apoptosis induced by 17-AAG in human leukemia cells [39, 40].

As well as intrinsic resistance, prolonged exposure of cancer cells to an anticancer drug can result in the acquisition of a chemoresistant phenotype. This can occur due to induced genetic mutations due to drug pressure that results in acquired drug resistance [41, 42], or the active selection and outgrowth of a rare population of cells that possess a resistant genotype [43]. Furthermore, epigenetic changes can be a crucial driving force behind acquisition of drug resistance [44]. Indeed, studies of drug resistant cell line models have shown that multiple changes in histone acetylation and CpG island methylation are present and can be induced by drug treatment [45, 46]. These changes may not only generate cells that are resistant to a particular drug but may also confer growth and survival advantages after drug treatment allowing for a more aggressive and drug resistant tumor phenotype.

As the repeated administration of HSP90 inhibitors will be required for treatment efficacy, exemplified by early phase trials of 17-AAG and second generation HSP90 inhibitors that required weekly administration for up to 6 months [14-16, 22-24], it is imperative to evaluate whether chronic HSP90 inhibitor treatment results in cancer cell resistance. Previously, a lung cancer cell line with an acquired resistance towards GA and 17-AAG has been described with the induction of heat shock

proteins being shown to have an important role in conferring resistance towards 17-AAG [47]. Interestingly, low NQO1 levels also plays a significant role in the underlying mechanism in the acquired resistance towards 17-AAG in glioblastoma and melanoma cell lines [48]. Many of the acquired resistant cell line models reported to date have been generated through continuous but rapid dose escalation of GA or 17-AAG treatment. These models are highly resistant to BAs and the mechanisms described are limited to this class of HSP90 inhibitors with no cross-resistance to other classes of compounds reported [30, 47, 48]. While the GA analogue IPI-504 is still currently in clinical trials, second-generation HSP90 inhibitors such as the resorcinol and purine-based compounds have a higher likelihood of successfully translating to the clinic due to their improved efficacy and toxicity profiles. However, it is still to be determined whether acquired resistance towards these second-generation compounds may also occur and if so, the mechanism by which this may be mediated.

In the current study, we have generated two cancer cell lines with acquired resistance towards 17-AAG achieved by gradual dose escalation treatments. We have shown that all 17-AAG-resistant cell lines are also cross-resistant to other BAs as well as other HSP90 inhibitors that are structurally unrelated to that of 17-AAG, namely radicicol and resorcinol-based compounds. Acquired resistance towards 17-AAG is partly attributable to reduced NQO1 expression in one of the resistant cell models, MDA-435R. Increased expression of histone deacetylases (HDACs) was identified in the MDA-231R cell line model and pharmacological inhibition of HDACs re-sensitized the cells to 17-AAG and other structurally unrelated HSP90 inhibitors. Collectively, our data suggest that prolonged exposure to 17-AAG leads to the development of an acquired resistance phenotype towards structurally diverse HSP90 inhibitor compounds and that HDAC's play a contributing role.

MATERIALS AND METHODS

Cell lines and cell culture

Human cancer cell lines MDA-MB-435 was a kind gift from Dr. Janet Price (MD Anderson Cancer Center, University of Texas, Houston, TX, USA) and MDA-MB-231 was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). MDA-MB-435 is a misidentified breast cancer cell line identical to the M14 melanoma cell line and is thus melanocytic in origin. MDA-MB-231 is a metastatic breast cancer cell line. Both cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Rockford, IL, USA), anti-biotic/anti-mycotic (Life Technologies). All assays utilised this medium (DMEM/FBS). The cell lines are referred to as MDA-435 and MDA-231, respectively from hereon.

Compounds

17-AAG was obtained from (Merck-Calbiochem, Darmstadt, Germany); geldanamycin (GA), 17-DMAG, radicicol, LBH589 and SNDX275 were obtained from LC Labs (Woburn, MA, USA). Coumermycin A1, novobiocin and trichostatin A (TSA) were obtained from Sigma Aldrich (St. Louis, MO, USA). AUY922, CCT018159 and VER50589 were obtained from Cayman Chemicals (Ann Arbor, MI, USA). IGF-1R inhibitor (PPP), Akt inhibitor (AktX), p-gp inhibitor (verapamil) and chemotherapeutic agents paclitaxel, doxorubicin and 5-Fluorouracil (5-FU) were obtained from Merck-Calbiochem.

Growth inhibition studies

Growth inhibition was determined using the sulforhodamine B (SRB) assay as described by Skehan *et al* [49]. Briefly, 5 x 10^3 cells were seeded into 96-well plates in triplicates, allowed to attach overnight, after which time drug(s) was added to the wells. After 3 days of exposure to the drug, cells were fixed with cold 50% trichloroacetic acid (TCA) (Sigma-Aldrich) for 1 hour at 4ºC and stained with 0.4% SRB (Sigma-Aldrich) in 1% acetic acid for 10 minutes at room temperature. Unbound SRB stain was then rinsed with 1% acetic acid after which the plates were left to air-dry overnight. SRB stain was then solubilised in 150µl of Tris-HCl (pH 10.5). Absorbance at 550nm was measured using a Multiskan FC Absorbance plate reader (Thermo Fisher Scientific). The IC_{50} was calculated using Graphpad Prism (San Diego, CA, USA) as the drug concentration that inhibited cell growth by 50% compared to control cell growth.

Development of 17-AAG acquired resistant cell lines

Early passage MDA-435 and MDA-231 cells were incubated in T75 flasks at 1xIC_{50} concentrations of 0.05µM and 1µM 17-AAG, respectively, as previously determined. Surviving MDA-435 cells were allowed to grow to confluence and the concentration of 17-AAG was increased gradually to 0.1, 0.15, 0.3, 0.35, 0.4, 0.45, 0.5, 0.6, 0.8, 1, 1.8 and 2 µM until cells maintained stable growth over a period of 20 weeks. Similarly, 17-AAG concentration was increased gradually in MDA-231 cells to 1, 1.2, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.2, 2.6, 2.8, 4, 5 and 6µM over a period of 15 weeks until stable growth was achieved. Untreated cells and cells treated with vehicle control were serially passaged along the 17-AAG treated cells as controls with no changes in phenotype or sensitivity to 17-AAG observed in these cells. After cells were confirmed to be stably resistant to 17-AAG by growth inhibition assays, cells were grown in 17-AAG-free DMEM/FBS. The consistency of cellular genotypes and identities was confirmed by short-tandem repeat (STR) profiling performed at Cell Bank Australia (Westmead, NSW, Australia).

Western blot analysis

Western blot was performed as described previously [50]. Briefly, cells were plated in 6-well plates, allowed to adhere overnight, and then treated as described. Cells were rinsed twice with ice-cold PBS, lysed and lifted from the plate by scraping in modified RIPA lysis buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.25% Sodium Deoxycholate) containing protease inhibitors (Sigma-Aldrich) and phosphatase inhibitors (Sigma-Aldrich). After 2-min sonication at 4°C in a sonicating water bath, the lysate was clarified by centrifugation (13,000 rpm) for 15 min at 4°C. Total protein concentration was determined using bicinchoninic acid (BCA) assay according to the manufacturer's protocol (Thermo Fisher Scientific). To prepare samples for SDS-PAGE, SDS sample buffer containing reducing agent (Life Technologies) was added to the protein samples which were then boiled for 5 minutes and centrifuged. Protein samples were then separated by one-dimensional SDS-PAGE on a 4%-12% gradient acrylamide gel (Life Technologies). The separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Merck-Millipore), blocked with 3% skim milk and probed with primary antibodies at 4°C overnight and then at room temperature with peroxidase-conjugated secondary antibodies (Thermo Fisher Scientific) for 1 hour. Proteins were visualised by an ECL detection system according to manufacturer's protocol (Thermo Fisher Scientific). All antibodies were purchased from commercial sources. Antibodies that detected IGF-1R (#3027), AKT (#9272), phosphorylated-AKT (#4058), EGFR #2232), PDK (#3062), HDAC1 (#5356), HDAC3 (#5113), HDAC4 (#7628), HDAC5 (#2082), HDAC6 (#7558), Acetylated-Histone H3 (Lys27) (#4353) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies that detected cyclin B1 (554176), CDK2 (610145) and actin (612656) were purchased from BD Pharmingen (CA, USA) and antibodies that detected HSP27 (ADI-SPA-803) and HSP70 (ADI-SPA-812) were purchased from Enzo Life Science (San Diego, CA, USA). Anti-mouse IgG, anti-rabbit IgG and anti-goat IgG HRP-conjugated secondary antibodies were obtained from Thermo Fisher Scientific.

Immunoprecipitation

Cell lysates were prepared as described previously and pre-cleared with Protein A Sepharose beads (Merck-Millipore) for 1 hour at 4°C after which the protein concentration was measured as described previously. Pre-cleared lysate (0.5mg) was added to 50µL of antibody-bead slurry and rotated overnight at 4ºC. Antibody complexes were washed thrice with lysis buffer and then resuspended in sample buffer. Acetylated lysine residues of a number of HSP90 isoforms was determined by SDS-PAGE and western blotting. Antibodies used included HSP90 (ADI-SPA-835), GRP94 (ADI-SPA-850) and TRAP1 (ADI-SPA-971) obtained from Enzo Life Science and acetylated-lysine (#9441) was obtained from Cell Signaling.

Semi quantitative qPCR and primers

Total RNA was isolated using the Qiagen RNeasy kit according to the manufacturer's instructions (Qiagen, CA, USA). One to two micrograms of total RNA was used to synthesize cDNA using the superscript VILO cDNA synthesis kit according to the manufacturer's instructions (Life Technologies). The synthesized cDNA underwent PCR amplification using the Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN, USA). The primers used were: NQO1 (FWD): 5'-GGGATCCACGGGGACATGAATG-3', NQO1 (REV): 5'-ATTTGAATTCGGGCGTCTGCTG-3'. Amplifications were performed with the following profile: 95ºC 3mins; 30x (95ºC 30s, 60ºC 30s, 72ºC 30s); 72ºC 7 mins; Ended at 25ºC. The PCR products were run on 2% agarose gel.

Statistical analysis

Data are presented as mean ± SD or mean ± SEM as stated. All assays were analysed by student's t-test. Significance is represented as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

RESULTS

Chronic exposure of cancer cells to 17-AAG leads to an acquired resistance towards 17-AAG and other benzoquinone ansamycin HSP90 inhibitors

To investigate if prolonged treatment with 17-AAG would lead to an acquired resistance towards this agent, human cancer cell lines MDA-435 and MDA-231 were treated with increasing concentrations of 17-AAG over a period of 20 weeks and 15 weeks, respectively, as detailed in materials and methods. SRB assays revealed that the parental cells, MDA-435 and MDA-231 were sensitive to HSP90 inhibition by 17-AAG with IC_{50} concentrations of 0.03 ± 0.01 μM and 1.52 ± 0.81 μM, respectively (Fig. 2.1A and Fig. 2.2A). Cells that survived successive 17-AAG treatments at increasing concentrations, designated MDA-435R and MDA-231R required much higher 17-AAG concentrations to result in growth inhibition with IC_{50} concentrations of 7.12 ± 1.47 μM and 10.35 ± 3.57 μM, respectively (Fig. 2.1A and 2.2A). Thus, both MDA-435R and MDA-231R cell lines showed high levels of resistance towards 17-AAG with resistance index (RI = IC_{50} resistant line / IC_{50} parental line) values of 195 ± 40 and 7.2 ± 3.9, respectively. The resistant cells also exhibited significant resistance to other HSP90 inhibitors that were structurally related and members of the BA family. For example, towards 17-DMAG the MDA-435R and MDA-231R had RI's of 12 ± 5.6 and 24 ± 6.4, respectively (Fig. 2.1B and Fig. 2.2B). Moreover, the resistant cells also showed a low but significant level of resistance towards GA with RI's of 3.8 ± 0.5 and 5.3 ± 1.4 for MDA-435R and

MDA-231R, respectively (Fig. 2.1C and Fig. 2.2C). The IC_{50} values for all cell lines to the structurally related drugs are illustrated in Table 2.1. These data demonstrate that chronic treatment with 17-AAG leads to not only resistance towards 17-AAG, but also the development of cross resistance towards structurally related BA HSP90 inhibitors in the cancer cell lines tested.

17-AAG resistant cells are cross-resistant to HSP90 inhibitors structurally unrelated to the benzoquinone ansamycins

In light of the significant level of resistance to BAs, we then examined the expression of NQO1 in the resistant cells as this has previously been shown to be a major mediator of resistance towards BA HSP90 inhibitors [48]. NQO1 mRNA expression was determined to be significantly lower in the MDA-435R cells when compared to the parental cells as shown by semi-quantitative RT-PCR (Fig. 2.3A). However, no difference in the mRNA expression levels of NQO1 was observed between MDA-231 and MDA-231R cells. These results demonstrated that decreased NQO1 expression may be a key determinant of acquired resistance towards 17-AAG and other BAs in the MDA-435R cells but does not appear to be a major mode of resistance in the MDA-231R cells. Since decreased NQO1 expression is limited to conferring resistance towards BAs [48, 51, 52], we sought to investigate whether cross resistance in MDA-435R and MDA-231R was extended to other HSP90 inhibitors that were structurally unrelated to 17-AAG. Interestingly, both MDA-435R and MDA-231R cells were also resistant to radicicol, an antibiotic with a resorcinol scaffold and structurally unrelated to that of the BAs, with RI's of 2.9 ± 0.7 and 2.6 ± 0.1, respectively (Fig. 2.3B and 2.3C). We also tested the sensitivity of the resistant cell lines to the resorcinylic compound, CCT018159, and the structure-based designed pyrazole resorcinols VER50589 and NVP-AUY922, which have been shown to be more potent than GA derivatives in inhibiting HSP90 and their cellular potency being independent of NQO1 expression [51-53]. Interestingly, both MDA-435R and MDA-231R cells showed significant resistance to CCT018159 and VER50589 and to a lesser extent NVP-AUY922 (Fig 2.3B and 2.3C). To our knowledge, this is the first report that demonstrates acquired resistance towards HSP90 inhibitors that are structurally unrelated to BAs in cancer cells. Notably, MDA-435R cells that showed lower expression levels of NQO1 were also significantly resistant to the resorcinylic compounds, indicating that additional mechanisms are in operation conferring resistance of non-BA HSP90 inhibitors in the MDA-435R cells.

In contrast to the resistance towards N-terminal ATPase targeting HSP90 inhibitors, the resistant cells remained sensitive towards HSP90 inhibitors that acted through the binding of the C-terminus of HSP90, namely novobiocin and its derivative coumermycin A1 (Fig. 2.3B and 2.3C). Cisplatin has also been shown to inhibit HSP90 activity through the binding of the C-terminus of the molecule [18]. Consistent with the data of novobiocin and coumermycin A1, no cross-resistance was observed in the resistant cells to cisplatin as well as other chemotherapeutic agents, namely paclitaxel, doxorubicin

and 5-fluorouracil (5-FU) (Fig. 2.3B and 2.3C). This also highlighted the lack of involvement of the drug efflux pumps in conferring 17-AAG resistance, as paclitaxel and doxorubicin are known substrates of P-gp (MDR1) [54, 55] and cisplatin is a known substrate of MRP2 [56] and MRP3 [57]. Furthermore, verapamil, a specific P-gp inhibitor did not sensitize the resistant cells towards 17-AAG as shown in supplementary data (Fig. 2.S1). The IC_{50} values of the cell lines towards non-BA HSP90 inhibitors and chemotherapeutic agents are summarized in Tables 2.2 and 2.3, respectively. The cross resistance of the 17-AAG-resistant cells towards structurally unrelated HSP90 inhibitors suggests a potential common mechanism of resistance that is not exclusive to any one specific class of HSP90 inhibitor.

Sustained expression of HSP90 client proteins upon 17-AAG treatment in resistant cells

To investigate the molecular effects of acquired resistance towards 17-AAG, we compared the changes in the steady-state protein levels of HSP90 client protein induced by 17-AAG treatment in the parental and resistant cell lines. The parental MDA-435 and resistant MDA-435R cells were treated with 17-AAG at 5 x IC_{50} of the parental line (0.1 μM) and 5 x IC_{50} of the resistant cell line (30 μM). Treatment with 0.1 μM 17-AAG (5 x IC_{50} of parental cells) resulted in the depletion of the HSP90 client proteins IGF-1R, Akt, cyclin B1 and PDK in the parental MDA-435 cells by 24 hours (Fig. 2.4A). This was accompanied by the induction of HSP70 and HSP27 in both the parental and resistant cell lines, however, notably in the MDA-435R cells lower levels of induction were observed (Fig. 2.4A). A transient increase in the phosphorylation of AKT after 6 hours was observed in the parental cells before being depleted by 24 hours. In the MDA-435R cells treated with 5 x IC_{50} of the parental line, no significant depletion of the client proteins was observed. As with the parental cells, although to a lesser degree, phosphorylation of Akt was initially increased and then reduced with a recovery to baseline levels by 72 hours. When treated with 30 μM 17-AAG (5 x IC_{50} of resistant cells), depletion of the client proteins was observed in the MDA-435R cells but was more delayed compared to the parental cells (Fig. 2.4B). The induction of HSPs was observed in both cell lines treated with 30 μM 17-AAG, however, the overall level was again lower in MDA-435R when compared to the parental cells (Fig. 2.4B).

The parental MDA-231 and resistant MDA-231R lines were also treated with 17-AAG at both 5 x IC_{50} of the parental line (7.5 μM) and 5 x IC_{50} of the resistant line (50 μM). Similar to the MDA-435 model, MDA-231 cells showed a depletion of HSP90 client proteins IGF-1R, EGFR, Akt, CDK2 and PDK at different time points of treatment with 7.5 μM 17-AAG, along with an induction of HSP70 and HSP27 (Fig. 2.4C). However, the MDA-231R cells showed no depletion of client proteins, except for a decrease in the phosphorylation of Akt by 12 hours of treatment. When treated with 50 μM 17-AAG, depletion of client proteins was observed in both the parental and resistant lines (Fig. 2.4D).

The effect of 17-AAG in the MDA-231R cells was not as pronounced compared to the parental line as shown by the overall delay in depletion of the client proteins as with the MDA-435 resistant cell lines (Fig. 2.4D). Notably, the overall level of HSP27 was lower in MDA-231R cells. These data demonstrated that HSP90 client protein levels are more stable in resistant cells upon 17-AAG treatment as a consequence of acquired resistance.

Inhibition of selected client proteins does not sensitise 17-AAG resistant cells to HSP90 inhibition

Drug induced regulation of target and target-associated proteins may be implicated in the development of resistance. This is supported by studies that showed drug-induced overexpression of target proteins which obstruct the clinical efficacy of chemotherapies [58, 59]. The sustained levels of client proteins upon 17-AAG treatment may therefore play a role in the reduced sensitivity observed in 17-AAG resistant cells. Furthermore, the resting levels of client proteins such as Akt were up-regulated in the MDA-435R and MDA-231R cells compared to the parental cells (Fig. 2.4). The activation of Akt, as indicated by phosphorylated Akt levels, was also increased at resting levels and was more sustained during 17-AAG treatment in the resistant cells (Fig. 2.4). This is consistent with the findings of Koga *et al*, which showed Akt to be transiently activated by HSP90 inhibition [60]. In addition, the role of Akt in conferring resistance to chemotherapeutic agents through activation of survival pathways is well documented [61]. We therefore investigated the effect of Akt inhibition upon 17-AAG sensitivity using a specific pharmacological inhibitor of Akt, Akt X at a sublethal concentrations that have been previously shown to be effective in inhibiting phosphorylation of Akt (Fig. 2.S2A and 2.S2B) [62]. AktX did not significantly improve the sensitivity of the parental or resistant cells as shown by the comparable dose response curves and RI values between untreated and treated cells of both models (Fig. 2.S2C and 2.S2D).

The expression of another HSP90 client protein, IGF-1R was also significantly upregulated in MDA-231R at resting level (Fig. 2.4C and 2.4D) and a sustained protein level was observed upon 17-AAG treatment compared to the parental cells (Fig. 2.4B and 2.4C). IGF-1R has also been implicated in drug resistance [63] through the activation of downstream pathways involved in cell survival such as PI-3K-Akt and MAPK/ERK [64]. The inhibition of IGF-1R with a specific pharmacological inhibitor, PPP at a sublethal concentrations that have been previously shown to be effective in inhibiting phosphorylation of IGF-1R and its downstream proteins, (Fig. 2.S3A and 2.S3B) [65] was not found to sensitise the MDA-231R cells to 17-AAG as shown by the lack of change in growth inhibition and RI compared to the parental cells (Fig. 2.S3C and 2.S3D).

These results indicate that the increased level of some client proteins including Akt and IGF-1R prior to and during HSP90 inhibition might serve as good biomarkers of resistance to HSP90 inhibitors but do not play a significant role individually in conferring resistance.

HDACs are overexpressed in 17-AAG-resistant cells

The regulation of HSP90 acetylation by histone deacetylases (HDACs), such as HDAC6 and HDAC1, as well as histone acetylases (HAT) is important in the regulation of HSP90 activity [37, 38, 66]. Recent studies have shown that HDAC inhibition partially reversed intrinsic resistance of leukemia cells towards 17-AAG through the hyperacetylation of HSP90 that resulted in increased 17-AAG binding and increased client protein degradation [39, 67]. In light of these findings, we sought to examine whether there was a potential role of HDACs in the underlying mechanism of acquired resistance towards HSP90 inhibitors. Our results from western blot analysis demonstrated that both HDAC6 and HDAC1 protein levels were up-regulated in 17-AAG-resistant MDA-231R cells compared to the parental cell line (Fig. 2.5A). HDAC3 and HDAC4 remained unchanged and HDAC5 was decreased (Fig. 2.5A). To further investigate the expression of HDAC proteins during HSP90 inhibition, MDA-231 and MDA-231R cells were treated with 17-AAG for 24 hours (Fig. 2.5B). In MDA-231 cells, HDAC6 was depleted by 17-AAG in a dose-dependent manner. This is consistent with the fact that HDAC6 is a known HSP90 client protein [39]. The depletion of HDAC6 in MDA-231R cells on the other hand was less rapid and remained stable even at 10μM of 17-AAG (Fig. 2.5B). HDAC1, which previously has not been shown to be a HSP90 client protein, was partially depleted by 17-AAG in the parental cells, but remained stable in the resistant cells (Fig. 2.5B). Interestingly, HDAC5 showed a rapid degradation profile with 17-AAG treatment in both the parental and resistant cells but remained lower overall in the resistant cells. The expression of HDAC3 and 4 remained unchanged by 17-AAG (Fig. 2.5B). In contrast to the MDA-231R model, none of the HDACs were altered in the MDA-435R cell line in either untreated or 17-AAG treated cells (data not shown), suggesting an alternative mechanism of resistance towards HSP90 inhibitors in this resistant cell line model.

17-AAG resistance alters the acetylation status of HSP90 and histone H3

To evaluate the effect of HDAC6 and HDAC1 up-regulation in the MDA-231R cell line, the acetylation status of cytosolic HSP90 was assessed by immunoprecipitation and western blot analysis in the absence or presence of 17-AAG. Interestingly, MDA-231R cells have an increased level of HSP90 acetylation compared to the parental cells without 17-AAG (Fig. 2.5C). 17-AAG treatment caused a decrease in HSP90 acetylation in both the parental MDA-231 cells and the resistant MDA-231R. However, the overall level of acetylation remained higher in the resistant cell line. No change was observed in the acetylation status of the endoplasmic reticulum (ER) and mitochondria isoforms

of HSP90, namely GRP94 (HSPC4) and TRAP1 (HSPC4), respectively (Fig. 2.S4). Consistent with the up-regulation of HDAC6 and HDAC1, acetylation of histone H3 in the resistant cells was suppressed under normal conditions and 17-AAG treatment (Fig. 2.5D). 17-AAG also resulted in increased levels of acetylation of histone H3 in a dose dependent manner in both cell lines potentially due to the inhibition and degradation of HDAC6. The results demonstrate that increased HDAC6 and HDAC1 expression may play an important role in acquired resistance through histone modification and not through HSP90 modification.

Inhibition of HDACs in resistant cells increases sensitivity towards HSP90 inhibitors

In light of the increased expression and activity of HDACs in the resistant cells, we investigated the effect of HDAC inhibition upon 17-AAG sensitivity. A pan-HDAC inhibitor, trichostatin A (TSA) inhibited HDAC activity as shown by the induction of histone H3 acetylation in both the parental and resistant cells (Fig. 2.S3A). TSA significantly improved the sensitivity of MDA-231R cells to 17-AAG treatment as demonstrated by a decrease in the survival and RI of the resistant cells in combination with 17-AAG treatment compared to 17-AAG alone (Fig. 2.6A). LBH589 (panobinostat) is another potent pan-HDAC inhibitor that is currently in clinical trials [68, 69], which potently induced the hyperacetylation of histone H3 (Fig. 2.S2B). Co-treatment of 17-AAG with LBH589 resulted in an overall decrease in the resistance of the MDA-231R cells towards 17-AAG as shown by decreased cell survival and RI (Fig. 2.6B). Another HDAC inhibitor that is currently under clinical assessment, SNDX-275 (entinostat; formerly MS-275) inhibits class I HDACs, which include the up-regulated HDAC1 (Fig. 2.5) as well as HDAC2, HDAC3 and HDAC8, however it does not inhibit HDAC6 [70, 71]. SNDX-275 also induced histone H3 acetylation (Fig. 2.S2C) and decreased cell survival and RI of the resistant MDA-231R cells when used in combination with 17-AAG (Fig. 2.6C). Notably, co-treatment with each of the HDAC inhibitors did not affect the sensitivity of the parental MDA-231 cells towards 17-AAG (Fig. 2.6A-2.6C). The results are consistent with the up-regulation of HDACs contributing to mediating resistance in the MDA-231R cells towards 17-AAG. The IC_{50} values for the differing treatment combinations of parental and resistant cells are recorded in Table 2.4.

We then assessed whether HDAC inhibition would also reverse the resistance of the MDA-231R cells to other HSP90 inhibitors, both BA and non-BA based agents. Co-treatment with LBH589 significantly increased the sensitivity of MDA-231R cells towards 17-DMAG (Fig. 2.7A), CCT018159 (Fig. 2.7B) and radicicol (Fig. 2.7C) without affecting the sensitivity of the parental cells. The IC_{50} values of the different treatment combinations are summarized in Table 2.5. The results demonstrate that inhibition of the HDACs can reverse the acquired resistance towards different classes of HSP90 inhibitors in the MDA-231R cell line, indicating a role for HDACs in conferring acquired resistance towards diverse classes of HSP90 inhibitors.

DISCUSSION

HSP90 inhibitors have thus far shown only a modest activity as a single agent in the treatment of recurrent and refractory tumours in the majority of clinical trials [22-24]. The lack of efficacy of HSP90 inhibitors may be due to a number of reasons, one of which could be intrinsic or acquired drug resistance, a common problem in the treatment of all cancer types [72]. The current study aimed to understand the potential mechanisms of acquired resistance towards HSP90 inhibitors and in doing so, identify ways in which resistance could be potentially overcome for improved clinical outcomes. Our data demonstrated that chronic HSP90 inhibition with 17-AAG treatment led not only to the development of acquired resistance towards this HSP90 inhibitor but also towards a number of structurally related and diverse clinically relevant HSP90 inhibitors. The resistant cell lines generated in this study were found to be stably resistant towards 17-AAG even when cultured for extended periods in the absence of the drug. Our approach of gradual 17-AAG-dose escalation over an extended period mirrors the treatment schedules and clinical trial length that have been conducted with 17-AAG and other second generation HSP90 inhibitors to date [14-16, 22-24]. Acquired resistance towards HSP90 inhibitors have been previously assessed in lung [47], glioblastoma and melanoma cell lines [48]. The current study employed MDA-435 and MBA-231 cell lines, which harbour oncoproteins that are classically dependent on HSP90 for stability, such as B-RAF and p53 [73]. Furthermore, MDA-231 cell line harbours mutations in the oncogene KRAS, which has downstream effectors that are highly dependent on HSP90, such as RAF/MEK/ERK kinase cascade.

Interestingly, the 17-AAG-resistant cells showed cross-resistance to other members of the BAs, such as 17-DMAG and GA. Consistent with this, the expression of the quinone-reducing enzyme, NQO1, which metabolizes the HSP90 inhibitors from the BA family to the more potent hydroquinone forms, was significantly lower in the MDA-435R. However, NQO1 expression remained unchanged in the MDA231R, suggesting an alternative mechanism of resistance towards the BAs in the MDA-231 resistant cell line. Notably, both the MDA-435R and the MDA-231R were also cross-resistant to compounds that were structurally unrelated to the BAs, such as radicicol, CCT018159, VER50589 and NVP-AUY922. To our knowledge, this is the first report of cell lines with a resistance profile that spans different classes of HSP90 inhibitors. The cross-resistance towards the non-BA HSP90 inhibitors cannot be explained by the decrease in NQO1 expression as these compounds lack the quinone-moiety. Moreover, only the MDA-435R cell line model had decreased NQO1. The cross-resistance confirmed that other novel molecular mechanisms of resistance that are common to different classes of HSP90 inhibitors must exist.

A notable drawback of the N-terminal inhibitors including BAs and second generation HSP90 inhibitors is their potent induction of the heat shock response via HSF1 activation leading to the enhanced expression of heat shock proteins (HSPs) and increased cell survival [30, 31, 47, 52, 74]. In

fact, the HSPs have been implicated in both intrinsic and acquired resistance towards BAs [18, 19, 29]. However, in our study, although 17-AAG treatment induced the expression of HSPs in the resistant cells, their overall expression was noticably lower than that of the parental cells. This suggests that the induction of the heat shock response does not contribute significantly to the acquired resistance observed in these cell line models. Furthermore, the resistant cells remained sensitive towards C-terminus HSP90 inhibitors that were not found to elicit a heat shock response. These compounds may be potential alternatives to N-terminal HSP90 inhibitors to circumvent the pro-survival effect of heat shock response.

A major cause of drug resistance in cancer cells is due to the expression of drug efflux pumps of the ATP binding cassette (ABC)-containing family of proteins [75]. In previous studies, BAs such as GA and 17-AAG have been shown to be substrates of the ABC transporter, P-gp. [47, 76]. We therefore addressed whether the broad resistance towards HSP90 inhibitors in the cell lines was due to altered expression of drug efflux pumps. However, in contrast to this being a mechanism, qRT-PCR did not detect P-gp expression in any of the cell lines (data not shown). In addition, dose response growth assays using the P-gp substrates, paclitaxel and doxorubicin, showed no difference in their respective IC_{50} and resistance index between the parental and resistant cells for these agents. Furthermore, pharmacological inhibition of P-gp with verapamil did not alter the sensitivity of the resistant cells towards 17-AAG (Fig. S1). Our findings are consistent with previous investigations that demonstrated that although P-gp was overexpressed in cell lines resistant to geldanamycin and 17-AAG, its inhibition with verapamil was not able to reverse the acquired resistance profile [47, 76]. It should also be noted that although many tumour cells naturally overexpress P-gp, these are still very much susceptible to second generation HSP90 inhibitors such as the purine-based BIIB021 [77] and resorcinol-based compounds [51]. Collectively, these results indicated that drug efflux pumps play a minimal role in acquired resistance to HSP90 inhibitors.

The expression level of client proteins can have a profound effect on cell sensitivity to HSP90 inhibition. Smith and colleagues have shown that the overexpression of the HSP90 client protein, HER2 resulted in the increased sensitivity of cancer cells to HSP90 inhibition [28]. Conversely, the overexpression of drug targets can also lead to the development of resistance to chemotherapeutic agents, as exemplified by the up-regulation of thymidylate synthase by its antagonist 5-fluorouracil that leads to therapy resistance [59]. In the current study, we observed that the protein level of HSP90 clients namely Akt and IGF-1R were increased at resting levels as well as during 17-AAG treatment in the resistant cells. This could be due to the selection of pre-existing cell populations that harbour increased endogenous levels of client proteins or drug targets and were selected out during the course of treatment, as reported in the development of drug resistance in NSCLC and CML [43, 78]. Our result is also consistent with a previous report that showed transient activation of survival and

proliferation pathways, such as Src and Erk kinases by HSP90 inhibition [60], promotes tumour survival and progression. However, pharmacological inhibition of Akt or IGF-1R in isolation did not significantly reverse the resistance towards 17-AAG in the resistant cells. However, this does not preclude the possibility that the concomitant increase in expression of multiple client proteins in the resistant cell lines may act in concert to provide an overall increased resistance index.

The resistance of the cells towards different classes of N-terminal HSP90 inhibitors led us to hypothesise that the mechanism may involve modifications to the actual molecular target HSP90. Previous reports have shown that reversible hyperacetylation of HSP90 lysine residues affects co-chaperone association, ATP binding and chaperone function, which resulted in increased binding of 17-AAG to HSP90 and client protein degradation [39]. The mainly cytosolic class IIb HDAC family member HDAC6 has been shown to be the main deacetylase of HSP90 [67, 79]. Studies also describe that the class I HDAC family member, HDAC1, also has the potential to regulate HSP90 acetylation [66, 80]. We have shown that both HDAC6 and HDAC1 are both increased at the protein level in the MDA-231R cells. This is consistent with previous findings that correlates increased HDAC1 and HDAC6 expression with chemotherapeutic resistance and tumour progression [81, 82]. However, we did not observe any change in HDAC expression in the MDA-435R cells, suggesting an alternative mechanism exists in these cells that warrant further investigation. This also highlights that the underlying regulatory pathways that mediate HSP90 inhibitor resistance may also be dependent upon the cellular context.

Contrary to our expectation, HSP90 was actually hyperacetylated in the MDA-231R cells despite increased HDAC6 and HDAC1 expression. No significant changes in the acetylation status of other HSP90 isoforms, namely GRP94 and TRAP1, which are localised in the endoplasmic reticulum and mitochondria, respectively, was noted (Fig. S4). This is consistent with the fact that HDAC6 and HDAC1 are localised within the cytoplasm and/or nuclei [83, 84]. We speculate that the increased acetylation of HSP90 in the resistant cells may be due to an increase in histone acetyltransferase (HAT) activity towards HSP90 and that the increased expression of HDACs may act as a compensatory mechanism to maintain HSP90 acetylation homeostasis. Indeed, acetylation homeostasis in cells is a tightly regulated process with HDAC and HAT levels maintained in a fine balance [85]. HATs that target HSP90 remain largely unknown to date. A study has shown that acetyltransferase p300 was responsible for acetylating HSP90 at multiple sites but notably the target residue of HDAC6, Lys294 was not affected [86]. The possible role of p300 or other HATs in the regulation of HSP90 acetylation and resistance to HSP90 inhibitors requires further study.

The treatment with pan-HDAC inhibitors, TSA and LBH589 significantly increased the sensitivity of MDA-231R cells to 17-AAG. Selective inhibitors of class I HDACs, SNDX-275 also significantly improved the sensitivity of resistant cells towards 17-AAG. Furthermore, HDAC inhibition by

LBH589 also significantly improved the sensitivity of resistant cells towards radicicol and the resorcynylic compound, CCT018159. Consistent with our data, the use of HDAC inhibitors has been shown to be effective in overcoming resistance to other chemotherapeutic agents such as DNA-damaging and anti-microtubule compounds [87-89]. This strongly suggests that HDACs are involved in the underlying mechanism of resistance towards multiple classes of HSP90 inhibitors. Notably, none of the HDAC inhibitors affected the sensitivity of the parental cells to HSP90 inhibitors. This highlights the specificity of the role of HDACs in cells with acquired rather than intrinsic resistance towards HSP90 inhibitors.

In addition to the regulation of HSP90 activity, HDACs are also associated with transcriptional regulation through their chromatin modifying activity [90, 91]. HDAC1 localisation is predominantly nuclear and has been implicated in the regulation of various gene expressions [84, 92]. HDAC6 on the other hand is known to be cytoplasmic but recent evidence is mounting that it shuttles between the cytoplasm and nucleus and does play a critical role in the regulation of gene expression [93]. Consistent with the increased expression of HDAC6 and HDAC1, histone 3 in the resistant cells showed a significant decrease in acetylation. In transcriptionally active genes, lysine residues on the N-terminal of core histones are acetylated [94]. This results in the decompression of DNA allowing for increased binding of transcription factors and polymerases to coding sequences [94]. Consequently, HDACs and hypoacetylation of histone proteins are associated with gene repression [94, 95]. Increased expression of HDACs in cancer cells was found to be associated with decreased response to chemotherapy [96], potentially through epigenetic silencing of cell cycle regulators, tumour suppressor and pro-apoptotic genes affecting growth and survival of cancer cells [97]. Studies have shown that HDAC inhibition results in the de-repression and increase of p21 expression (in a wt p53-independent manner), a CDK inhibitor that induces cell cycle arrest at G1 phase [98, 99]. HDAC inhibition also downregulates pro-survival gene expression, such as Bcl-2 and Bcl-I_L, and induces pro-apoptotic genes, such as Bim, Bak and Bax [100-102]. We therefore postulate that 17-AAG resistance could arise partly due to HDAC-mediated alterations in apoptotic and survival gene expression. The sensitisation of resistant cells to HSP90 inhibitors by HDAC inhibition is potentially mediated through the modulation of some of these genes. Further study to determine the genes downstream of HDACs will shed more light on the pathways involved in acquired resistance to HSP90 inhibitors. The partial reversal of resistance to HSP90 inhibitors by HDAC inhibition indicates that additional mechanisms are involved. This could be a result of a complex regulatory pathways or a reflection of the fact that the cells were generated in a gradual and multi-step manner, which can result in distinct mechanisms at each step.

In summary, our data demonstrate that the chronic treatment of cancer cells with 17-AAG results in the development of acquired resistance towards HSP90 inhibitors of different structural classes

including the BAs and resorcinol-based compounds. This broad spectrum resistance is partly attributable to the upregulation of HDACs in the resistant cells that potentially leads to epigenetic changes affecting the expression of apoptotic and survival genes. Our results also support the use of HDAC inhibitors in combination with HSP90-directed compounds to improve therapeutic efficacy and potentially prevent the development of acquired resistance.

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Table 2.1. Sensitivity of the parental and resistant lines of human breast cancer cells to benzoquinone ansamycins, as represented by IC_{50} values (μM).

	17-AAG	*P*	17-DMAG	*P*	GA	*P*
MDA-435	0.03±0.01	0.001	0.054±0.009	0.026	0.032±0.003	0.001
MDA-435R	7.12±1.47		0.66±0.31		0.12±0.02	
MDA-231	1.52±0.81	0.003	0.07±0.01	0.005	0.07±0.03	0.008
MDA-231R	10.35±3.57		1.44±0.42		0.37±0.1	

Note: Values are mean ± SEM of at least three independent experiments. P-values were derived from student t-test of parental and resistant celll lines.

Table 2.2. Sensitivity of parental and resistant lines of human breast cancer cells to non-ansamycin-based HSP90 inhibitors, as represented by as represented by IC50 values (μM).

	Radicicol	*P*	CCT018159	*P*	VER50589	*P*	NVP-AUY922	*P*	Novobiocin	*P*	Coumermycin A1	*P*
MDA-435	0.22±0.01	0.0113	8.02±2.21	0.0003	0.1±0.02	0.0019	0.013±0.003	0.0495	293.23±44.13	0.9621	14.62±3.04	0.1558
MDA-435R	0.8±0.16		21.08±2.36		0.34±0.05		0.021±0.003		351.53±37.4		14.49±3.1	
MDA-231	0.31±0.08	0.0154	9.08±1.64	0.0005	0.15±0.03	0.0109	0.028±0.007	0.0263	353.73±75.16	0.4562	24.42±9.07	0.4934
MDA-231R	0.83±0.04		31.25±3.25		0.7±0.21		0.042±0.003		316.57±40.75		24.5±3.82	

Note: Values are mean ± SEM of at least three independent experiments. P-values were derived from student t-test of parental and resistant celll lines.

Table 2.3. Sensitivity of parental and resistant lines of human breast cancer cells to chemotherapeutic agents, as represented by as represented by IC50 values (μM).

	Cisplatin	*P*	Paclitaxel	*P*	Doxorubicin	*P*	5-FU	*P*
MDA-435	9.23±0.84	0.1812	0.0027±0.00063	0.5682	0.15±0.03	0.6114	5.98±0.74	0.09
MDA-435R	12.26±2.6		0.003±0.00062		0.17±0.06		9.39±2.55	
MDA-231	12.04±1.94	0.0826	0.0095±0.004	0.1445	0.12±0.02	0.014	17.59±4.87	0.0861
MDA-231R	6.89±1.09		0.0045±0.0017		0.07±0.01		26±4.2	

Note: Values are mean ± SEM of at least three independent experiments. P-values were derived from student t-test of parental and resistant celll lines.

Table 2.4. Sensitivity to 17-AAG of MDA-231 and MDA-231R and the effects of HDAC inhibitors, as represented by as represented by IC50 values (μM).

	IC_{50}			
	MDA-231	***P***	**MDA-231R**	***P***
17-AAG + Veh.	1.20±0.56	0.3	8.84±0.58	0.003
17-AAG + TSA	0.54±0.21		3.29±0.87	
17-AAG + Veh.	0.45±0.09	0.9	10.15±1.07	0.009
17-AAG + LBH589	0.47±0.15		5.38±0.9	
17-AAG + Veh.	0.44±0.12	0.9	10.7±1.17	0.035
17-AAG + SNDX275	0.47±0.13		6.84±0.82	

Note: Values are mean ± SEM of at least three independent experiments. P-values were derived from student t-test of parental and resistant celll lines.

Table 2.5. Sensitivity to other HSP90 inhibitors of MDA-231 and MDA-231R and the effects of LBH589

	IC_{50} (μM)			
	MDA-231	**P-value**	**MDA-231R**	**P-value**
17-DMAG + Veh.	0.03±0.004	0.6	0.58±0.06	0.013
17-DMAG + LBH589	0.02±0.003		0.08±0.003	
CCT018159 + Veh.	2.75±0.46	0.7	20.92±1	0.008
CCT018159 + LBH589	2.52±0.36		9.78±0.02	
Radicicol + Veh.	0.06±0.01	0.4	0.51±0.13	0.047
Radicicol + LBH589	0.05±0.0007		0.24±0.03	

Note: Values are mean ± SEM of at least three independent experiments. P-values were derived from student t-test of parental and resistant celll lines.

Figure legends

Figure 2.1. Acquired resistance of MDA-435 cell lines to 17-AAG and other benzoquinone anasamycins. Dose response and resistance index (RI = IC_{50} ratio relative to parental cell line) of MDA-435 parental and MDA-435R resistant cells treated with **(A)** 17-AAG, **(B)** 17-DMAG and **(C)** GA were assessed with SRB assay as described in materials and methods. *Growth curves*, representative of at least three independent experiments; *bars*, SD. *Columns*, mean of at least three independent experiments; *bars*, SEM. *, p-value < 0.05.

Figure 2.2. Acquired resistance of MDA-231 cell lines to 17-AAG and other benzoquinone anasamycins. Dose response and resistance index (RI = IC_{50} ratio relative to parental cell line) of MDA-231 parental and MDA-231R resistant cells treated with **(A)** 17-AAG, **(B)** 17-DMAG and **(C)** GA were assessed with SRB assay as described in materials and methods. *Growth curves*, representative of at least three independent experiments; *bars*, SD. *Columns*, mean of at least three independent experiments; *bars*, SEM. *, p-value < 0.05.

Figure 2.3. NQO1-independent cross resistance to HSP90 inhibitors that are structurally unrelated to benzoquinones. (A) NQO1 mRNA expression of parental and resistant cell lines was determined by semi-quantitative RT-PCR. Resistance index (RI) of parental and resistant lines of **(B)** MDA- 435 and **(C)** MDA- 231 to other HSP90 inhibitors and chemotherapeutic agents, as determined by IC_{50} values from SRB dose response growth assay. *Columns,* means of at least three independent experiments; *bars*, SEM. *, p-value < 0.05.

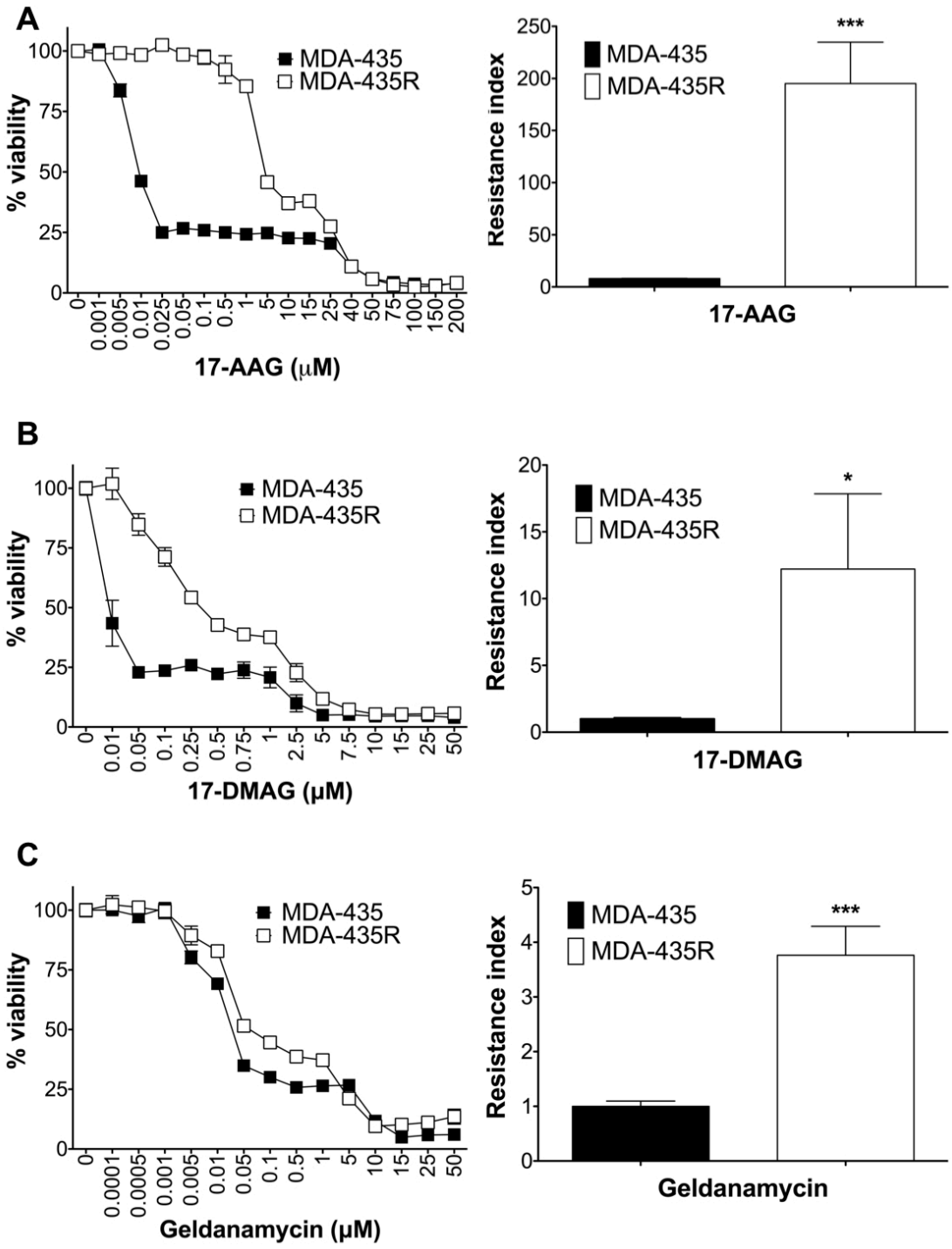
Figure 2.4. Molecular effects of HSP90 inhibition by 17-AAG. Parental and resistant MDA-435 cells were treated with 5 x 17-AAG IC_{50} concentrations of the **(A)** parental line (0.1μM) and **(B)** resistant line (30μM). Parental and resistant lines of MDA-231 were treated with 5 x 17-AAG IC_{50} concentrations of the **(C)** parental line (7.5μM) and **(D)** resistant line (50μM). Total cell lysates were collected at the indicated time-points and analysed by western blotting.

Figure 2.5. HDAC6 and HDAC1 are up-regulated in 17-AAG resistant cells. (A) Expression of different HDAC isoforms in MDA-231 and MDA-231R was assessed by western blot using total cell lysates. (**B)** HDAC protein expression of MDA-231 and MDA-231R treated with indicated concentrations of 17-AAG for 24hrs was assessed by western blotting with total cell lysate. **(C)** Immunoprecipitation of cytosolic HSP90 was performed with total cell lysate protein from MDA-231 and MDA-231R treated with the indicated concentrations of 17-AAG for 24hrs, as described in materials and methods. Acetylated lysine residues were detected by western blotting. **(D)** Acetylated histone 3 was detected by western blotting using total cell lysate from MDA-231 and MDA-231R treated with the indicated concentrations of 17-AAG for a period of 24hrs.

Figure 2.6. Inhibition of HDACs re-sensitises resistant cells to 17-AAG. Dose response and resistance index (RI) of MDA-231 and MDA-231R cells treated with 17-AAG or 17-AAG in combination with **(A)** 75nM TSA, **(B)** 10nM LBH589 or **(C)** 1μM SNDX275 for 24h. *Growth curves*, representative of at least three independent experiments; *bars*, SD. *Columns*, mean of at least three independent experiments; *bars*, SEM. *, p-value < 0.05.

Figure 2.7. Inhibition of HDACs re-sensitises resistant cells to HSP90 inhibitors that are structurally unrelated to 17-AAG. (A) Dose response and resistance index of cells treated with **(A)** 17-DMAG, **(B)** CCT018159 and **(C)** radicicol alone or in combination with 10nM LBH589 for 24h. *Growth curves*, representative of at least three independent experiments; *bars*, SD. *Columns*, mean of at least three independent experiments; *bars*, SEM. *, p-value < 0.05.

Figure 2.1


A
% viability
MDA-435
MDA-435R
17-AAG (μM)
Resistance index
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17-AAG
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MDA-435
MDA-435R
17-DMAG (μM)
Resistance index
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MDA-435
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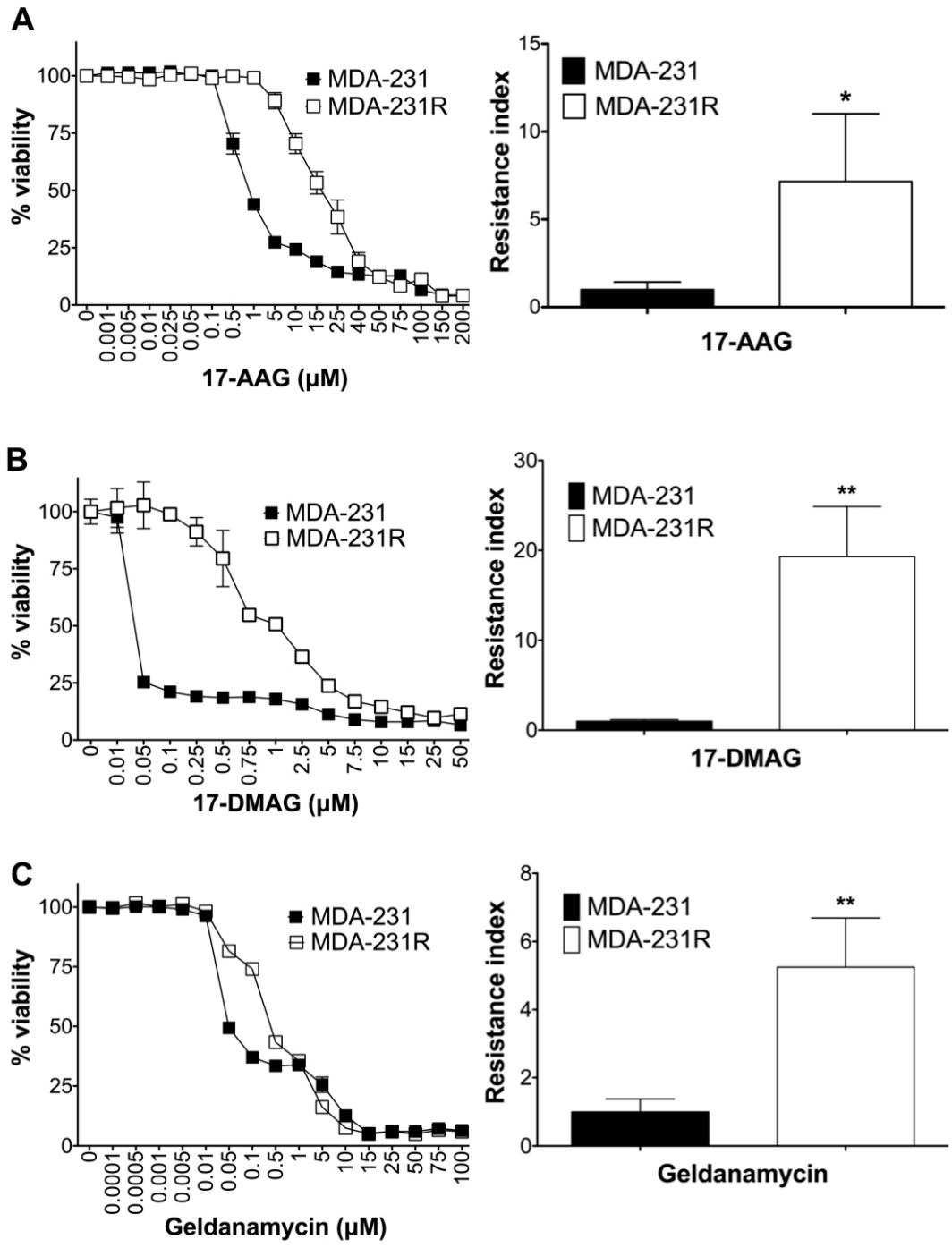
Geldanamycin (μM)
Resistance index
***
Geldanamycin

Figure 2.2


A
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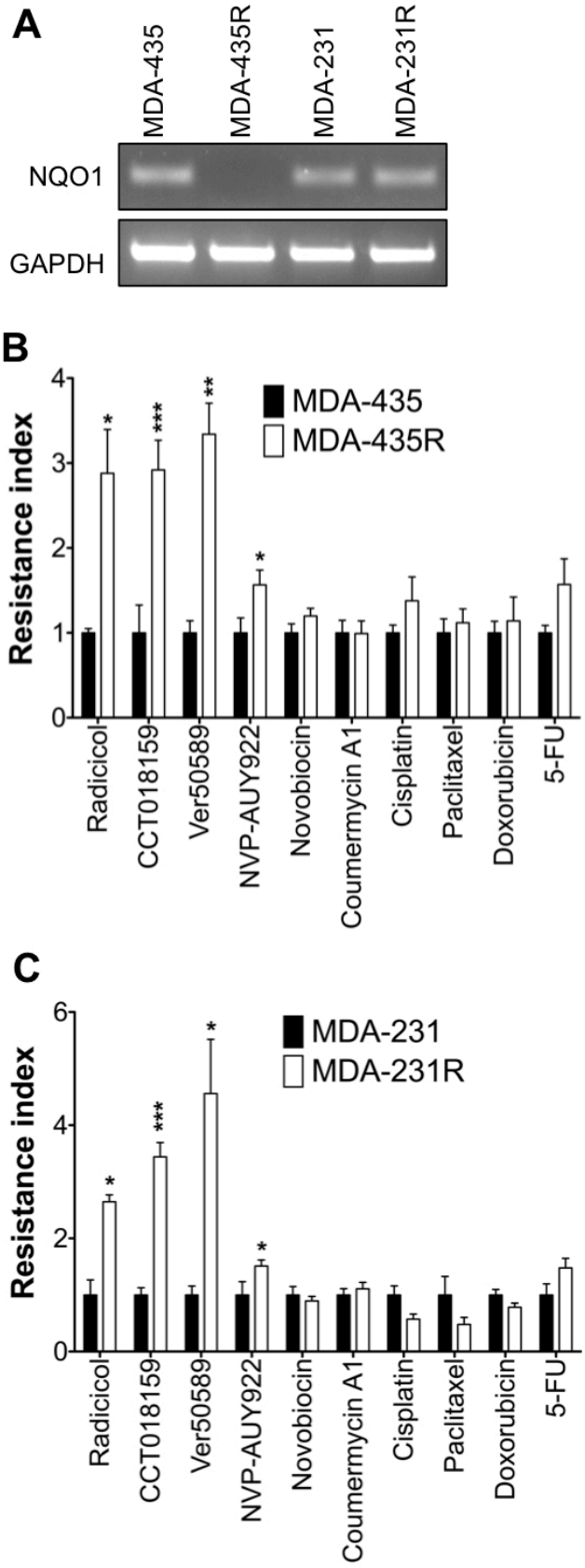
Geldanamycin (μM)
Geldanamycin

Figure 2.3


A
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Resistance index
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Radicicol
CCT018159
Ver50589
NVP-AUY922
Novobiocin
Coumermycin A1
Cisplatin
Paclitaxel
Doxorubicin
5-FU
C
Resistance index
MDA-231
MDA-231R
Radicicol
CCT018159
Ver50589
NVP-AUY922
Novobiocin
Coumermycin A1
Cisplatin
Paclitaxel
Doxorubicin
5-FU

Figure 2.4

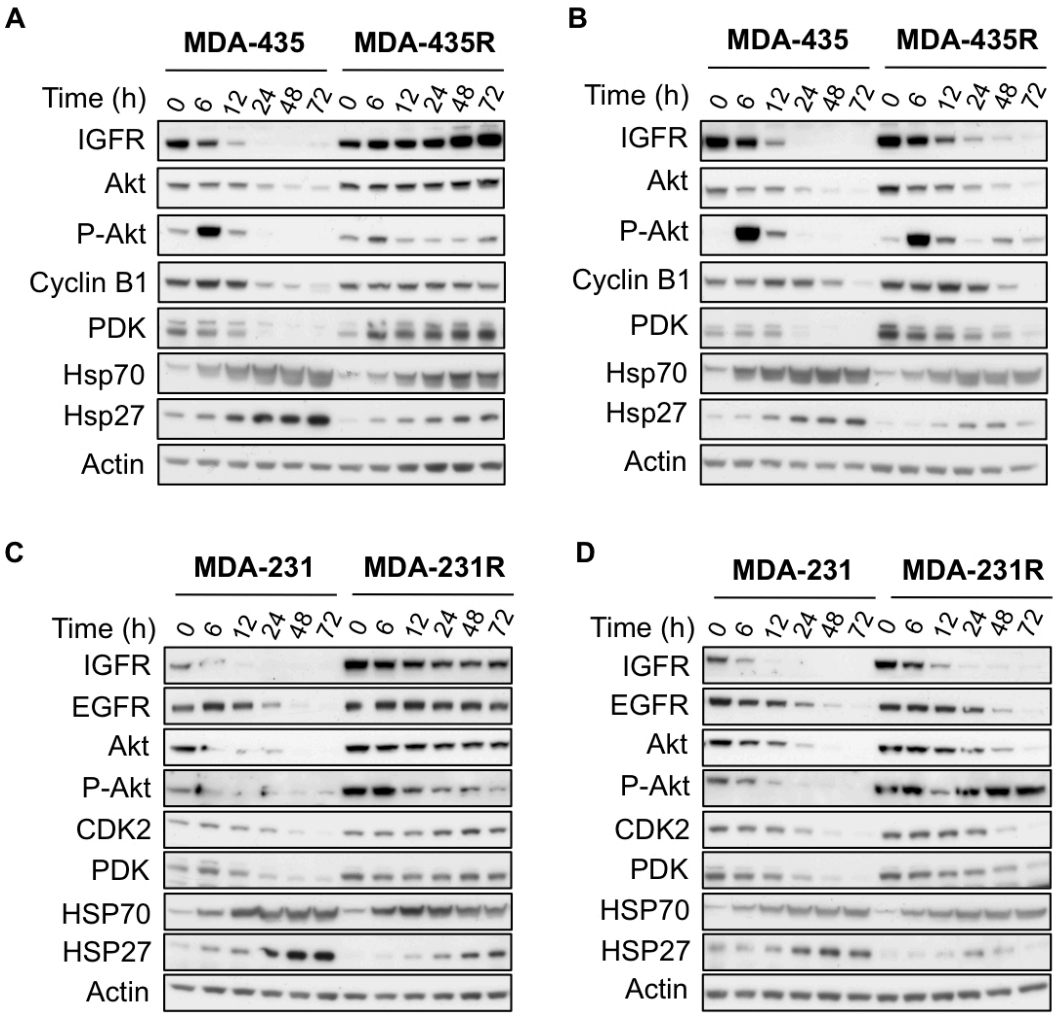

A
MDA-435
MDA-435R
Time (h)
0 6 12 24 48 72 0 6 12 24 48 72
IGFR
Akt
P-Akt
Cyclin B1
PDK
Hsp70
Hsp27
Actin
B
MDA-435
MDA-435R
Time (h)
0 6 12 24 48 72 0 6 12 24 48 72
IGFR
Akt
P-Akt
Cyclin B1
PDK
Hsp70
Hsp27
Actin
C
MDA-231
MDA-231R
Time (h)
0 6 12 24 48 72 0 6 12 24 48 72
IGFR
EGFR
Akt
P-Akt
CDK2
PDK
HSP70
HSP27
Actin
D
MDA-231
MDA-231R
Time (h)
0 6 12 24 48 72 0 6 12 24 48 72
IGFR
EGFR
Akt
P-Akt
CDK2
PDK
HSP70
HSP27
Actin

Figure 2.5

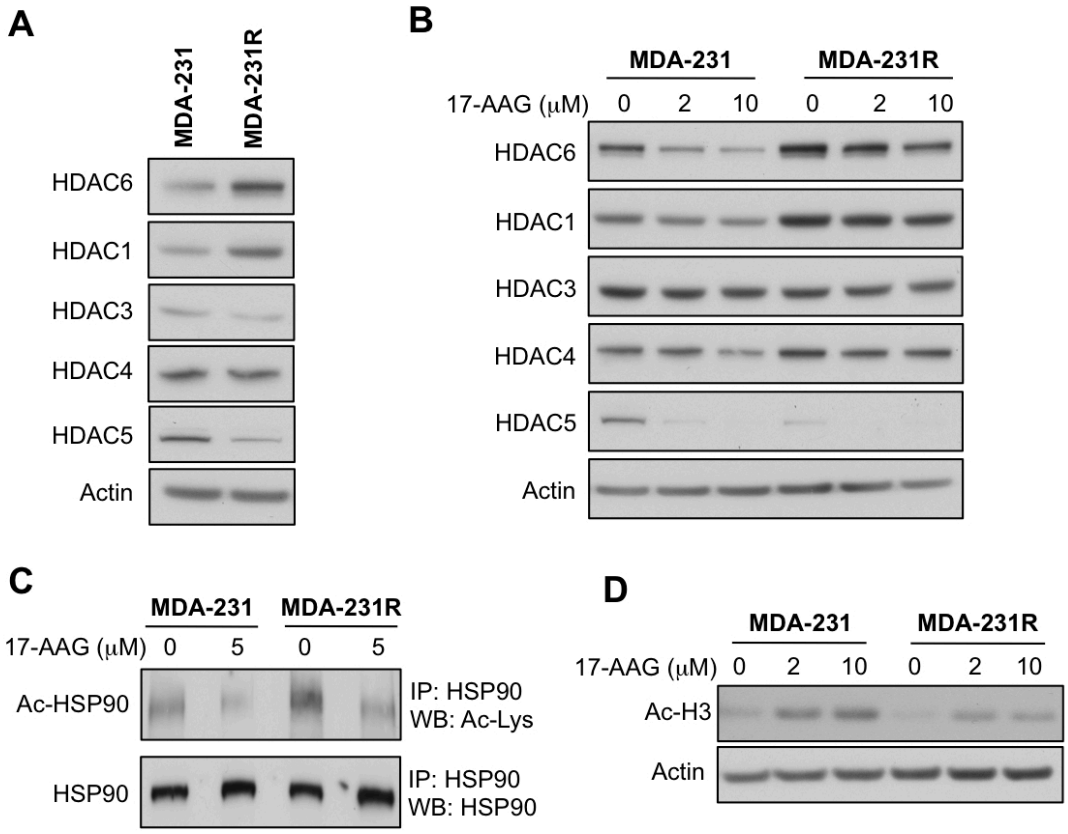

A
MDA-231
MDA-231R
HDAC6
HDAC1
HDAC3
HDAC4
HDAC5
Actin
B
MDA-231
MDA-231R
17-AAG (μM) 0 2 10 0 2 10
HDAC6
HDAC1
HDAC3
HDAC4
HDAC5
Actin
C
MDA-231
MDA-231R
17-AAG (μM) 0 5 0 5
Ac-HSP90
IP: HSP90
WB: Ac-Lys
HSP90
IP: HSP90
WB: HSP90
D
MDA-231
MDA-231R
17-AAG (μM) 0 2 10 0 2 10
Ac-H3
Actin

Figure 2.6

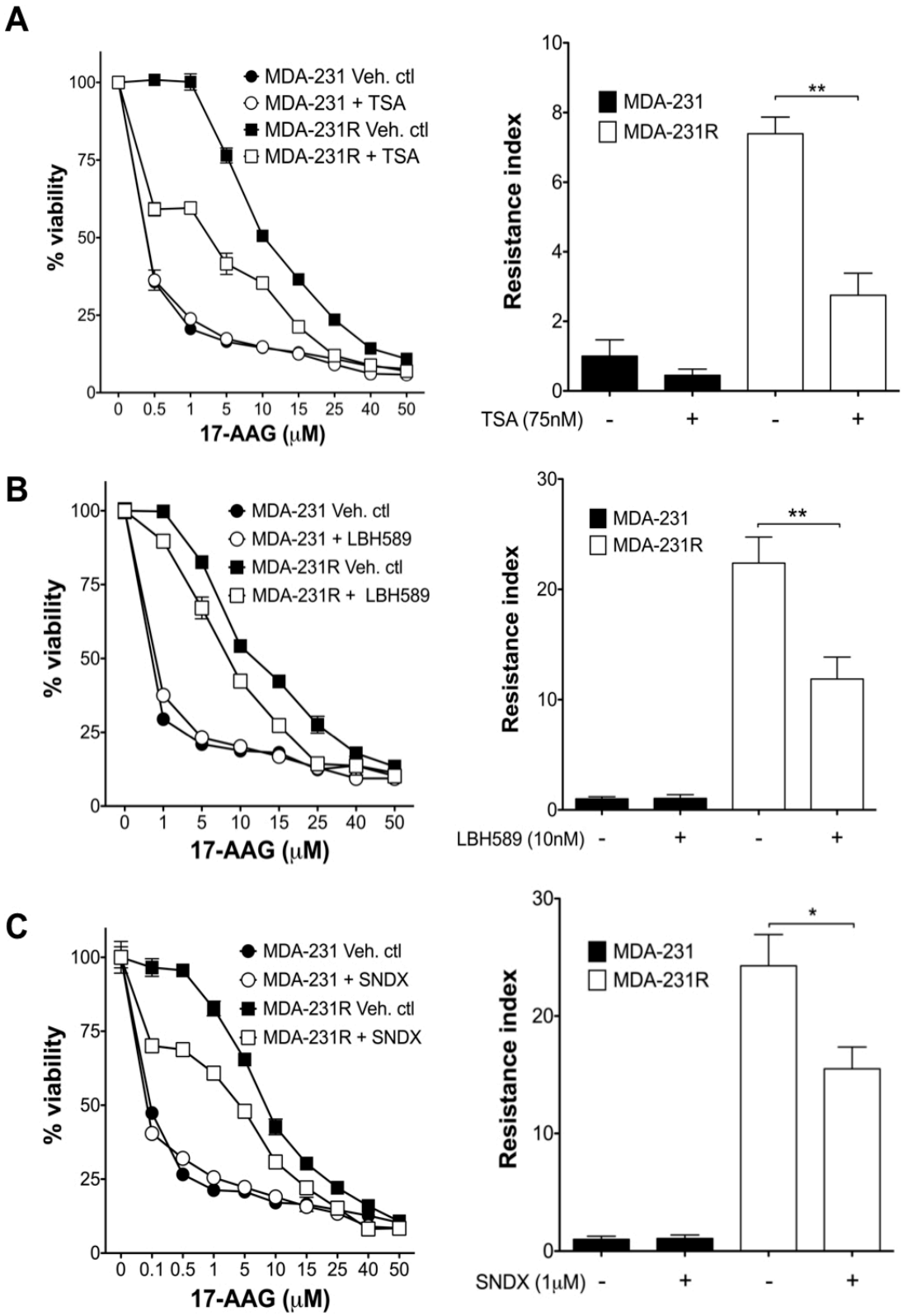
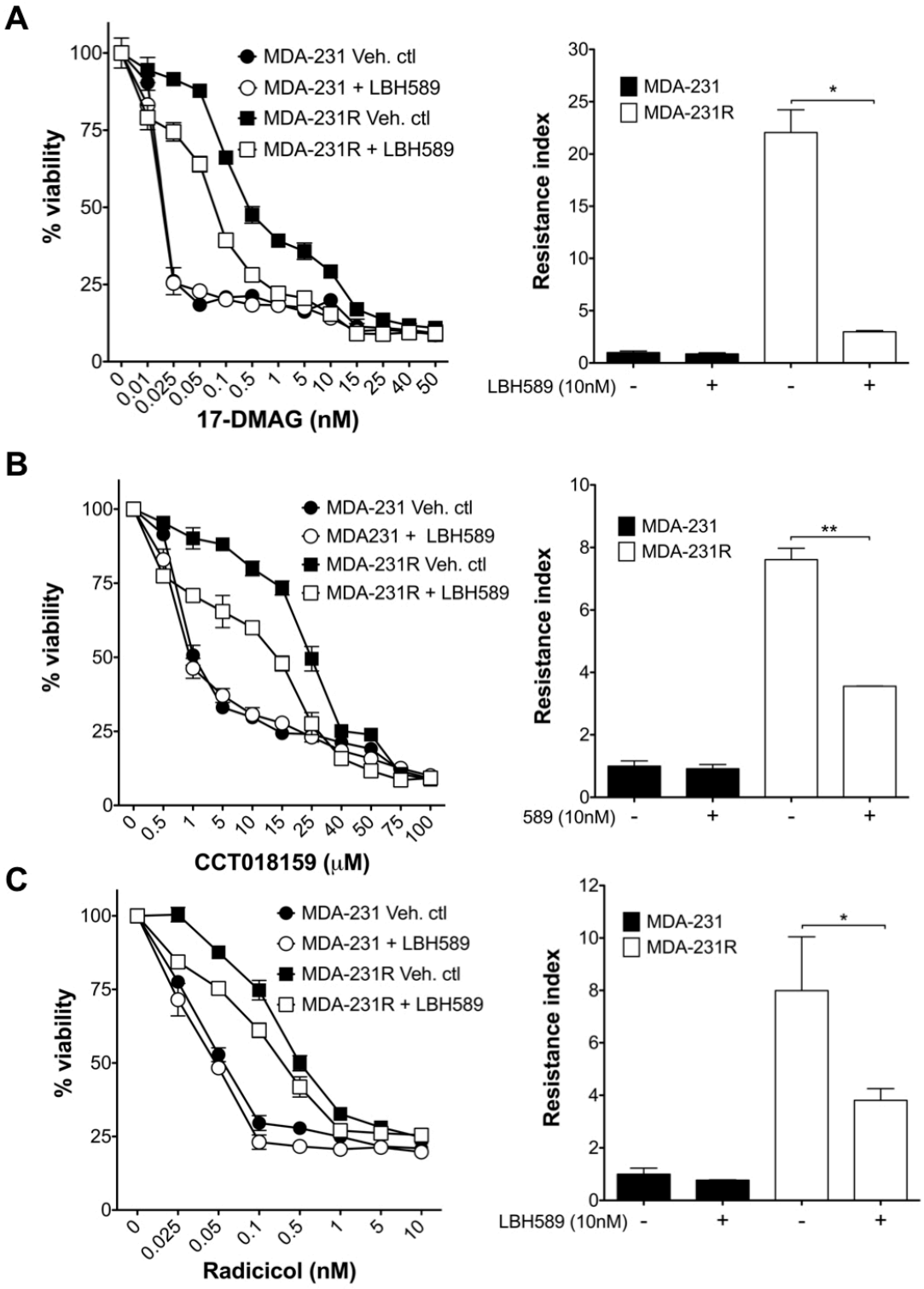

A
MDA-231 Veh. ctl
MDA-231 + TSA
MDA-231R Veh. ctl
MDA-231R + TSA
% viability
17-AAG (μM)
Resistance index
MDA-231
MDA-231R
**
TSA (75nM)
B
MDA-231 Veh. ctl
MDA-231 + LBH589
MDA-231R Veh. ctl
MDA-231R + LBH589
% viability
17-AAG (μM)
Resistance index
MDA-231
MDA-231R
**
LBH589 (10nM)
C
MDA-231 Veh. ctl
MDA-231 + SNDX
MDA-231R Veh. ctl
MDA-231R + SNDX
% viability
17-AAG (μM)
Resistance index
MDA-231
MDA-231R
*
SNDX (1μM)

Figure 2.7


A
% viability
MDA-231 Veh. ctl
MDA-231 + LBH589
MDA-231R Veh. ctl
MDA-231R + LBH589
17-DMAG (nM)
Resistance index
MDA-231
MDA-231R
*
LBH589 (10nM)
B
% viability
MDA-231 Veh. ctl
MDA231 + LBH589
MDA-231R Veh. ctl
MDA-231R + LBH589
CCT018159 (μM)
Resistance index
MDA-231
MDA-231R
**
589 (10nM)
C
% viability
MDA-231 Veh. ctl
MDA-231 + LBH589
MDA-231R Veh. ctl
MDA-231R + LBH589
Radicicol (nM)
Resistance index
MDA-231
MDA-231R
*
LBH589 (10nM)

APPENDIX: Supporting information

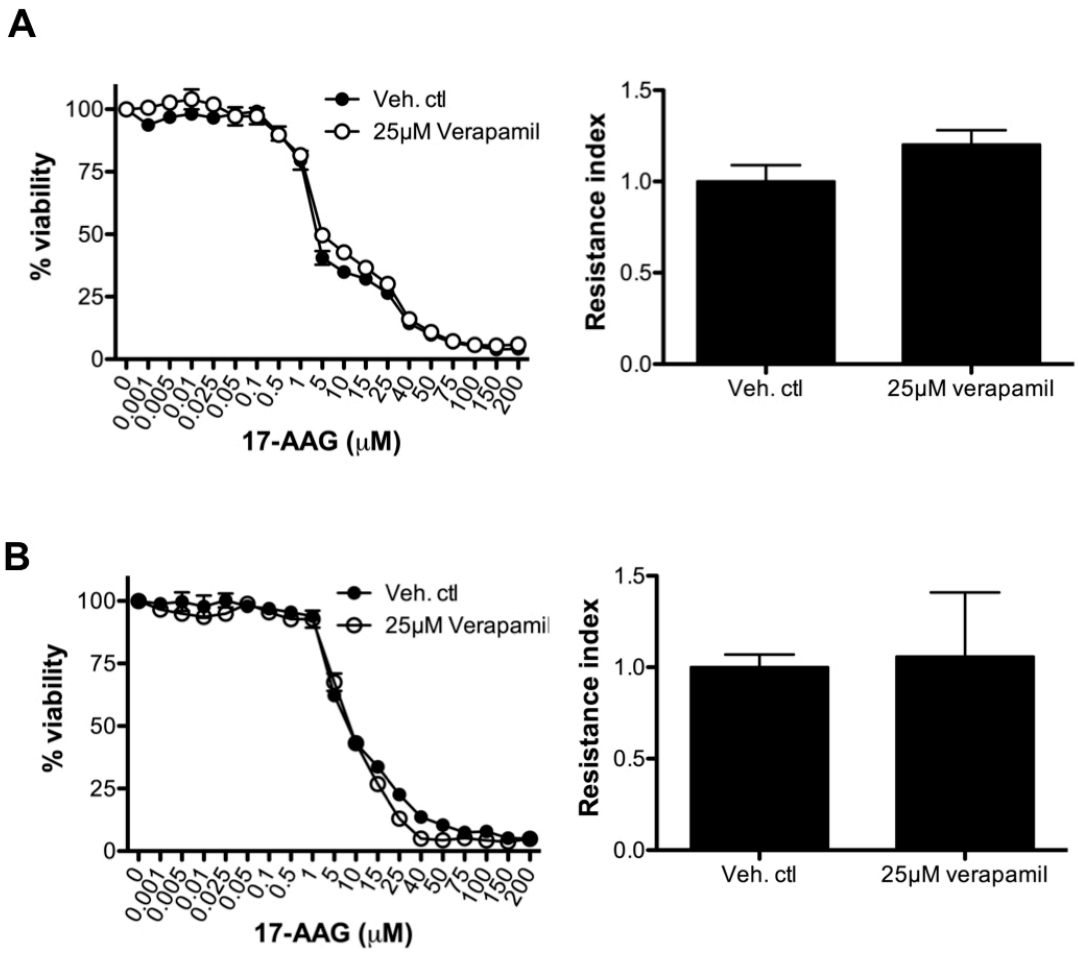


Figure 2.S1. Inhibition of p-gp by verapamil did not re-sensitise resistant cells to 17-AAG. Survival curve (top) and resistance index (bottom) of **(A)** MDA-435R and **(B)** MDA-231R to 17-AAG, co-treated with vehicle or 25mM verapamil were determined by SRB assay after 3 days. *Survival curves*, representative of at least two independent experiments; *bars*, SD. *Columns*, mean of at least two independent experiments; *bars*, SEM. *, $p<0.05$ using student t-test.

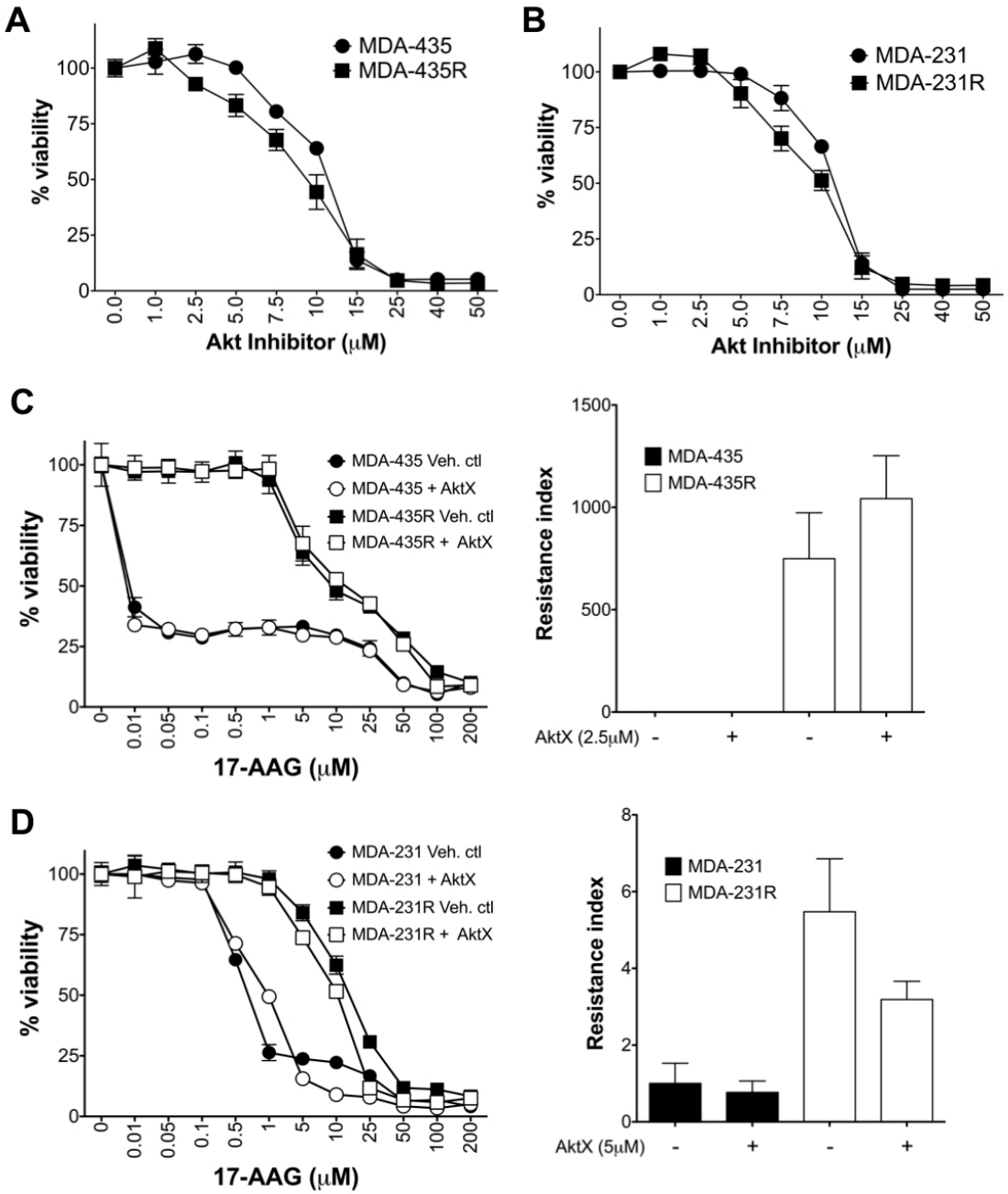


Figure 2.S2. Inhibition of Akt did not re-sensitise resistant cells to 17-AAG. Dose response of **(A)** MDA-435 and MDA-435R, **(B)** MDA-231 and MDA-231R cells treated with Akt inhibitor, Akt X to determine sublethal doses (2.5μM and 5μM respectively) for combination treatment with 17-AAG. Dose response and resistance index of **(C)** MDA-435 and MDA-435R cells treated with 17-AAG alone or 17-AAG with 2.5mM AktX were determined by SRB assay after 3 days. **(D)** Dose response and resistance index of MDA-231 and MDA-231R cells treated with 17-AAG alone or 17-AAG with 5mM AktX were determined by SRB assay after 3 days. *Curves*, representative of at least three independent experiments; *bars*, SD. *Columns*, mean of at least three independent experiments; *bars*, SEM. *, $p<0.05$ using student t-test.

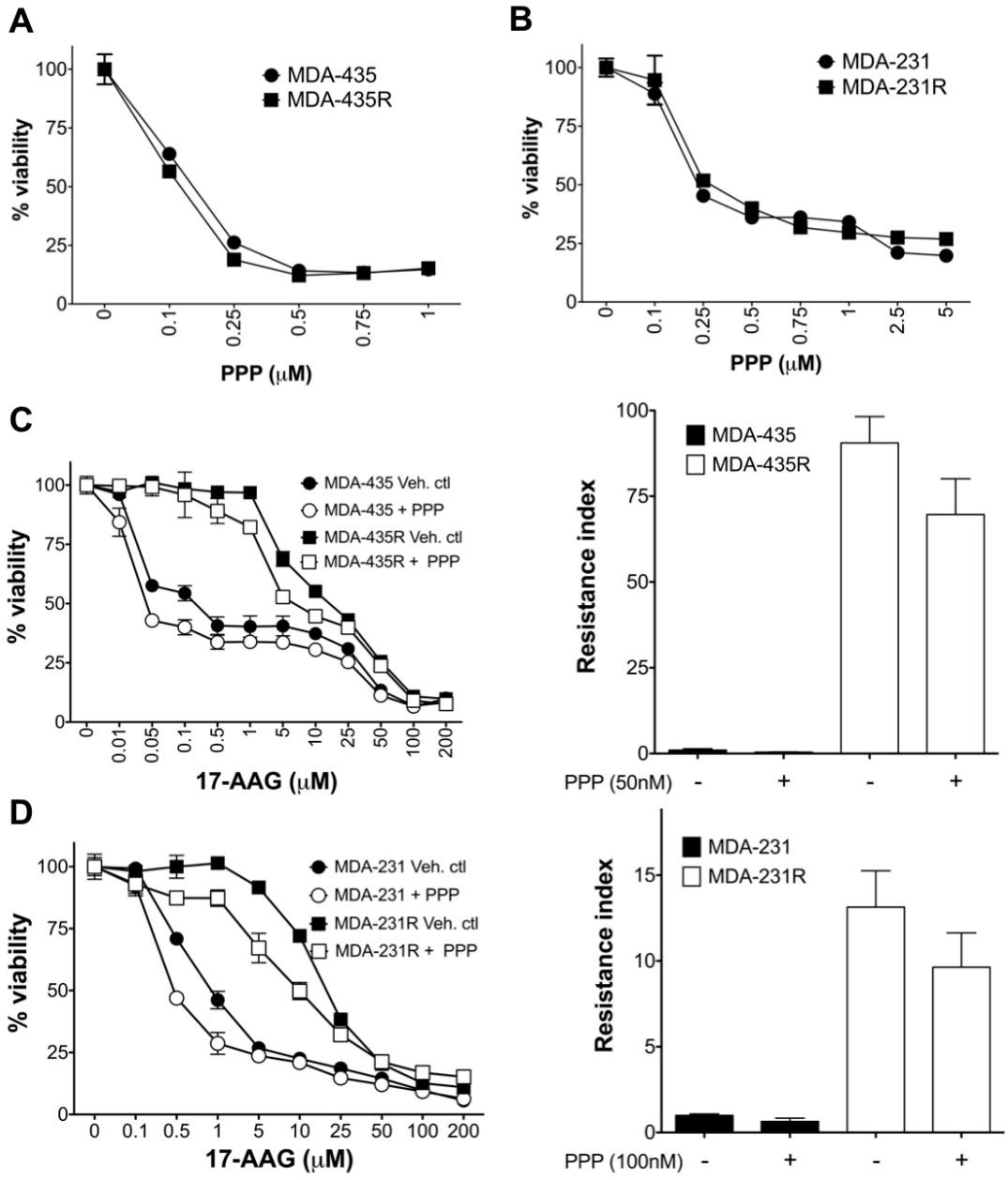


Figure 2.S3. Inhibition of IGF-1R did not re-sensitise resistant cells to 17-AAG. Dose response of **(A)** MDA-435 and MDA-435R, **(B)** MDA-231 and MDA-231R cells treated with IGF-1R inhibitor, PPP to determine sublethal doses (50nM and 100nM respectively) for combination treatment with 17-AAG. **(C)** Dose response and resistance index of MDA-435 and MDA-435R cells treated with 17-AAG alone or 17-AAG with 50nM PPP were determined by SRB assay after 3 days. (D) Dose response and resistance index of MDA-231 and MDA-231R cells treated with 17-AAG alone or 17-AAG with 100nM PPP were determined by SRB assay after 3 days.*Curves*, representative of at least three independent experiments; *bars*, SD. *Columns*, mean of at least three independent experiments; *bars*, SEM. *, $p<0.05$ using student t-test.

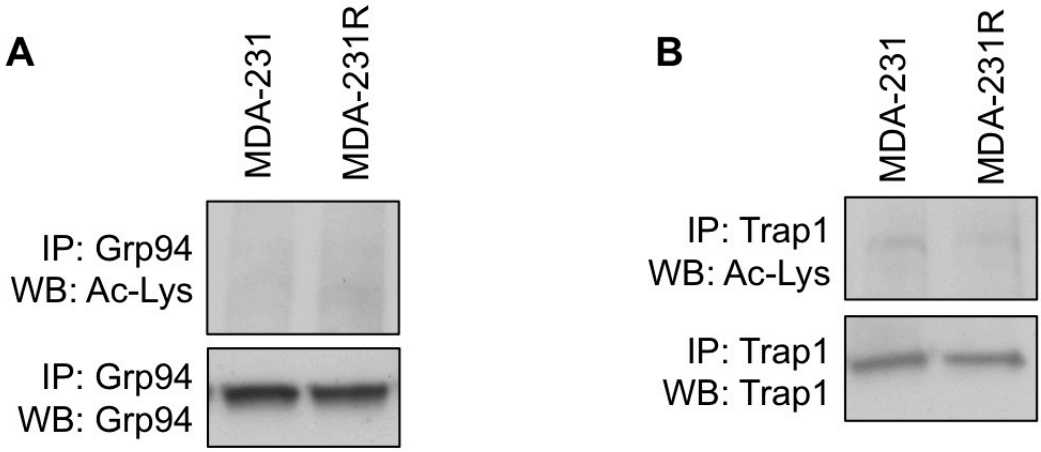


Figure 2.S4. Acetylation of other Hsp90 isoforms were not altered. Immunoprecipitation was performed using antibodies towards **(A)** Grp94 or **(B)** Trap1 in MDA-231 and MDA-231R total cell lysates prepared as described in materials and methods. Acetylated lysine residue was detected by western blotting.

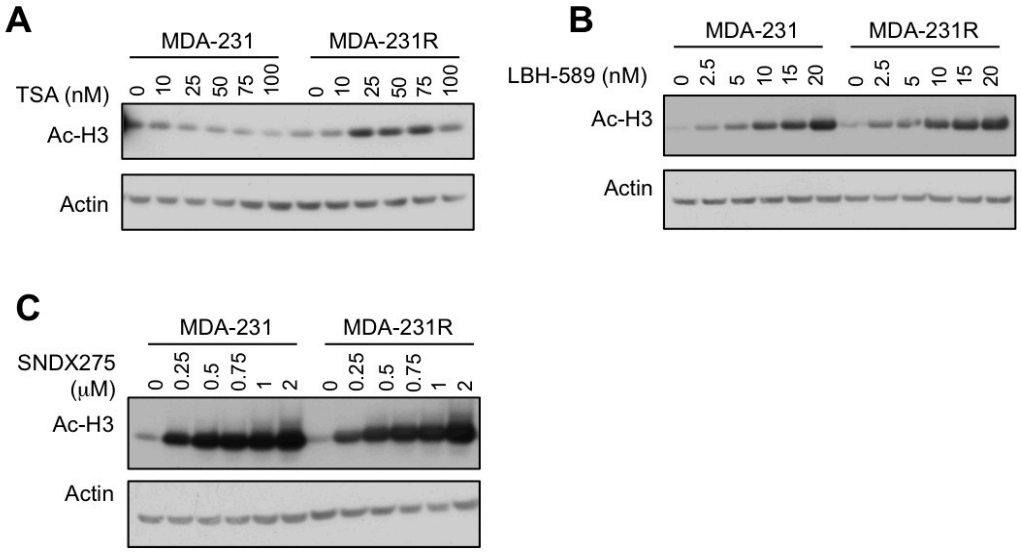


Figure 2.S5. HDAC inhibitors induced histone acetylation in a dose-dependent manner. MDA-231 and MDA-231R cells were treated with indicated concentrations of **(A)** TSA, **(B)** LBH-589 and **(C)** SNDX-275 for 24hrs. Acetylation of histone protein 3 was assessed by western blot.

DECLARATION FOR THESIS CHAPTER 3

In the case of Chapter **3**, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Participated in project hypothesis -Designed and performed experiments -Analysed data -Prepared and wrote the manuscript	70

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Jessica L. Vieusseux	Provided technical support, contributed to refinements to manuscript	
Chau H. Nguyen	Provided technical support, contributed to refinements to manuscript	5
Benjamin J. Lang	Provided technical support, contributed to refinements to manuscript	4
Narelle E. McGregor	Provided technical support	
Natalie A. Sims	Provided technical support	
Kara L. Britt	Provided technical support	
Michelle M. Kouspou	Contributed to refinements to manuscript	
John T. Price	Supervision; project co-ordination and development of hypothesis; contributed to writing and refinements to manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to the work.

Candidate's Signature [redacted] **Date 16/12/2013**

Main Supervisor's Signature [redacted] **Date 9/12/2013**

Chapter 3

The Effect of Acquired Resistance Towards HSP90 Inhibitors on Cancer Cell Biology

Acquired Resistance towards HSP90 Inhibitors is Associated with Decreased Metastatic Potential but an Enhanced Osteolytic Phenotype.

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Running title: HSP90 Inhibitor Resistance and Osteolysis

Keywords: Hsp90, acquired resistance, 17-AAG, metastasis, bone, osteolysis

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Word count:

5 total figures

ABSTRACT

Heat shock protein 90 (HSP90) inhibitors are a promising class of anticancer therapeutic drugs due to the central role of HSP90 in the stabilization and maturation of oncoproteins. However, their therapeutic efficacy may be limited due to intrinsic and acquired cell drug resistance. Chemoresistance frequently associates with a more aggressive and metastatic cancer cell phenotype, although, this has yet to be determined with respect to HSP90 inhibitor resistance. To address this, we examined whether MDA-MB-231 and MDA-MB-435 cancer cell lines that had been generated to be resistant towards HSP90 inhibitors displayed altered growth, survival and metastatic properties. Despite both cell lines being significantly resistant to HSP90 inhibitors, each displayed reduced growth and anchorage-independent colony formation. Consistent with these *in vitro* findings, when HSP90 inhibitor resistant cells were examined for their ability to form tumours at the orthotopic site in mice, the resistant cells formed smaller, slower growing tumours than the parental cells. Despite decreased *in vitro* and *in vivo* growth and survival, the migratory capacity of the resistant cells was found to be significantly greater than that of the matched parental cells. However, in contrast to this, the metastatic potential of the resistant cells was found to be markedly decreased, developing less metastatic tumour burden at both visceral and skeletal sites. Interestingly, within the bone, although the tumour burden was significantly lower with respect to the resistant cells, x-ray and bone morphometric analysis demonstrated that the extent of osteolytic lesions to be similar, indicating an enhanced pro-osteolytic phenotype in the resistant cells. Consistent with the lower growth and survival observed in the resistant cells, gene expression profiling revealed that genes involved in cell cycle progression were downregulated and markers of a cancer stem cell (CSC) phenotype, associated with slower growth were upregulated in the resistant cells. Moreover, the expression of epithelial to mesenchymal transition (EMT) markers, associated with increased cellular migration, were also upregulated. Gene set enrichment analysis (GSEA) revealed that the pathways that were most significantly upregulated in the MDA-435R cells are involved in cellular migration, cytoskeletal organisation and apoptosis. Pathways involved in protease inhibition, immune response and histone deacetylase (HDAC) activity were upregulated in MDA-231R cells. Taken together, this study is the first to demonstrate that acquired resistance towards HSP90 inhibitors results in phenotypical changes that have a negative impact upon tumour growth and metastasis, yet paradoxically, can enhance the pro-osteolytic phenotype, potentially through multiple molecular changes observed in the resistant cells. These findings are crucial in highlighting the potential benefits and complications of prolonged treatment with HSP90 inhibitors.

INTRODUCTION

HSP90 is a molecular chaperone that plays an important role in facilitating the folding, maturation and activity of a wide-variety of client proteins in an ATP-dependent manner [1, 2]. Many HSP90 client proteins are central to the signaling pathways and cellular processes important for oncogenic growth and progression. In fact, HSP90 is overexpressed in multiple cancer types primarily due to the increased dependency of cancer cells on the chaperoning of oncoproteins that can include ErbB2, Src, Akt, Raf-1, mutated p53 and hypoxia-inducible factor 1α (HIF1α) [1, 3-8]. As a result, HSP90 has emerged as a major therapeutic target in cancer and multiple HSP90 inhibitors are currently under clinical evaluation.

The benzoquinone ansamycin antibiotic, geldanamycin and its derivative, 17-allylamino-17 demethoxygeldanamycin (17-AAG) have been crucial in providing 'proof-of-concept' pre-clinical and clinical studies for HSP90 inhibition in human cancers [9]. These compounds bind to the ATP-binding site of HSP90 with a higher affinity than that of ATP and ADP [10], subsequently leading to the dissociation and degradation of client proteins via the proteasome machinery. High throughput drug screening and structure-based drug design have resulted in the generation of synthetic HSP90 inhibitors, including the resorcinol-based and purine-based compounds that have improved pharmacological properties and toxicity profiles over first-generation inhibitors [11, 12]. HSP90 inhibitors are unique in that, although they are directed toward a specific molecular target, they simultaneously inhibit multiple pathways that frequently interact to promote cancer survival and progression [13]. Recent studies using gene expression profiling and proteomics have provided key insights into the far-reaching effect of HSP90 inhibition on multiple deregulated pathways in cancer, including signal transduction, RNA processing, metabolism, DNA repair, chromatin modification and apoptosis, coupled by an increased expression of the heat shock proteins [14-16].

Despite the many promising results in pre-clinical studies, HSP90 inhibitors have thus far only shown clear clinical activity in ErbB2 positive breast cancers and EML-ALK translocated non-small cell lung carcinoma (NSCLC) [17, 18]. These data demonstrate that although HSP90 oncogenic client proteins are important for treatment success in some cancer types, others, such as NSCLC with mutated EGFR and KRAS do not demonstrate the same degree of HSP90 inhibitor sensitivity [18, 19]. This indicates that other factors, such as intrinsic and acquired resistance towards HSP90 inhibitors, may be limiting the efficacy of HSP90 inhibitors. Although it was initially thought that as HSP90 inhibitors targeted multiple oncogenic pathways that the development of resistance towards these agents would be rare [20, 21], recent studies from our group and others have shown that this may not be the case. Both acute and prolonged exposure of cancer cells to HSP90 inhibitors can result in the emergence of cells that are stably resistant to a variety of HSP90 inhibitors *in vitro* [22, 23] and (chapter 2). A variety of molecular changes have been identified as contributing to the underlying

mechanisms of resistance, including altered expression of NQO1, heat shock proteins (HSPs) and histone deacetylases (HDACs) [22, 23] (chapter 2). However, the implications of the molecular changes associated with acquired resistance on other aspects of tumour cell biology are largely unknown.

Cancer cell drug resistance and metastasis are closely linked and are the major causes of mortality in cancer patients [24]. In addition to treatment resistance, prolonged drug pressure can also result in phenotypical changes that are associated with increased metastasis [25-27]. Drug resistant tumour cells that survive initial chemotherapeutic treatment have been shown to be more aggressive leading to increased risk of local recurrence and metastasis to distal organs [28-30]. Lung carcinomas with acquired resistance towards cisplatin exhibit an increased aggressiveness, displaying an enhanced migratory and invasive potential *in vitro* and increased metastatic growth *in vivo* [28]. Similarly, prostate cancer cells with acquired resistance to paclitaxel became highly motile and invasive *in vitro* forming larger tumours in xenograft studies [29]. Clinically, patients who develop resistance towards chemotherapeutics relapse within 2 years of first-line therapy [31], which in association with metastasis, results in a poor prognosis and lower overall survival [32].

A growing body of evidence now supports a link between drug resistance and phenotypical changes through the acquisition of cancer stem cell (CSC) –like features. CSC is a subpopulation of self-renewing cells which have been identified in a variety of cancers and possess the ability to drive the continued expansion of malignant cells with increasing resistance to therapy and metastatic propensity [33]. CSCs in mammary tumours have been shown to contribute to cisplatin and paclitaxel resistance [34, 35]. Similarly, a population of CSC-like cells in glioblastoma showed increased resistance towards a range of chemotherapeutic agents [36]. Furthermore, pancreatic CSCs have been reported to be highly resistant to chemotherapy as well as highly migratory and invasive [37].

Emerging evidence also suggests that the epithelial-to-mesenchymal transition (EMT) switch in cancer cells is also linked to chemoresistance and the acquisition of increased motility and invasive properties [38]. EMT is characterised by changes in cell-cell adhesion, cytoskeletal reorganization, expression of mesenchymal markers and an increased migratory potential [39]. Recent studies have shown that the EMT phenotype is closely linked to resistance against conventional chemotherapeutics such as taxol [40] and targeted compounds such as EGFR inhibitors [41]. Moreover, phenotypical changes associated with EMT also result in tumour progression and metastasis [39]. Interestingly, evidence has indicated that the relative resistance and invasive nature of cancer cells that have undergone an EMT are consistent with a CSC phenotype [38], hence making the elimination of these cells an attractive approach of overcoming drug resistance and tumour progression.

Therefore, this prompted us to investigate whether cancer cells that develop resistance towards HSP90 inhibitors will possess similarly altered tumour growth properties. Due to the multi-targeted capacity

of HSP90 inhibitors, acquired resistance to this class of drug will likely arise due to a combination of molecular aberrations as reported previously [22, 23] and (chapter 2). These molecular alterations associated with acquired drug resistance may also lead to unexpected changes in growth and metastatic properties of cancer cells. It is well accepted that HSP90 inhibition leads to client protein degradation but the downstream effects are not well understood. This is exemplified by the fact that HSP90 inhibition efficiently impedes invasion and/or metastasis through the simultaneous impairment of multiple pathways [42-44] but reports from our group and others have shown that 17-AAG treatment increases formation of osteolytic bone loss and augments bone metastasis through the upregulation of osteoclast formation [45-48]. These data highlight the complicated implications of HSP90 inhibition and underscore the importance of unraveling the effect of long-term treatment with HSP90 inhibitors on tumour progression in order to anticipate potential complications that may arise in the clinic.

In the current study, we assessed the biological properties commonly associated with metastasis and analysed the gene expression in 17-AAG resistant breast cancer cell lines that were established by prolonged 17-AAG treatment with gradual dose escalation as described previously (paper 1). The resistant cells demonstrated a significant decrease in growth in both adhesion-dependent and –independent conditions, coupled by an increase in chemotactic migration *in vitro*. *In vivo* studies using xenograft mouse models showed decreased mammary tumour formation and decreased metastasis to visceral organs and skeletal sites. However, nude mice inoculated with resistant cells had similar levels of hindlimb bone lesions compared to the parental group despite a lower tumour burden, indicating an enhanced pro-osteolytic phenotype in the resistant cells. Microarray analysis revealed multiple molecular changes in the resistant cells consistent with the decreased growth, increased cellular migration and decreased metastatic potential in the resistant cells. Collectively, these data indicated that acquired resistance to 17-AAG leads to the emergence of tumour cells with impaired tumour formation, decreased metastasis yet an enhanced osteolytic potential as a result of multiple molecular changes.

MATERIALS AND METHODS

Cell lines

Human cancer cell lines MDA-MB-435 was a kind gift from Dr. Janet Price (MD Anderson Cancer Center, University of Texas, Houston, TX, USA) and MDA-MB-231 was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). MDA-MB-435 is a misidentified breast cancer cell line identical to the M14 melanoma cell line and is thus melanocytic in origin. MDA-MB-231 is a metastatic breast cancer cell line. Both cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Rockford, IL, USA), anti-biotic/anti-mycotic (Life

Technologies). All assays utilised this medium (DMEM/FBS). The cell lines are referred to as MDA-435 and MDA-231, respectively from hereon.

Microscopy

All cells were cultured on 6-well culture plate in a concentration of $5x10^3$ cells/ml for 24 hrs before image acquisition. Phase contrast images were obtained using a Nikon Eclipse microscope at 100x magnification.

Two-dimensional standard growth assay

Cell growth was determined by the sulforhodamine B assay in a 96-well format as described previously described by Skehan *et al* [49]. Briefly, cells were plated in triplicate at $2.5x10^3$ cells/well in 100µl medium, grown and fixed each day for 6 days in 50% tricloroacetic acid (TCA) at 4ºC for 1 hour, followed by five washes in 1% acetic acid. Cells were stained with SRB (Sigma Aldrich, USA) and solubilised in 150µl of 10mM Tris-HCl pH10.5. Absorbance at 550nm was measured using a Multiskan FC Absorbance plate reader (Thermo Fisher Scientific).

Three-dimensional adhesion-independent clonogenic growth assay

Cells were suspended in 1.5ml 0.5 % top agar, comprised of 2x DMEM/FBS and Bacto agar (BD, NJ, USA), and added to 2ml of pre-set 1% base agar comprised of 2x DMEM/FBS and Bacto agar in 6-well plates. 1ml of medium was added to the plates and replenished twice a week. Cells were incubated at 37ºC for 4 weeks until colonies were visible. Colonies were stained with 0.005% crystal violet and counted manually.

Chemotactic migration assay

The assay was performed as previously described using 8µM collagen IV-coated membranes and a 48 well microchemotaxis chamber (Neuroprobe, Gaithersburg, MD, USA) [45, 50]. Briefly, cells at a 70%-80% confluence were lifted non-enzymatically by incubation in PBS-EDTA for 15 mins, washed thrice with 0.1% bovine serum albumin (BSA; Sigma-Aldrich) in FBS-free DMEM and resuspended at concentrations of $2x10^6$ cells/ml for MDA-435 and MDA-435R, and $1x10^6$ cells/ml for MDA-231 and MDA-231R. Human fibroblast conditioned-medium (FbCM) and 10ng/ml of insulin-like growth factor (IGF) (BD Biosciences) in 0.1% BSA DMEM were used as chemoattractants and 0.1% BSA DMEM was used as a negative control. Cells were loaded into he upper wells of the chamber and incubated for 2-4 hrs in a 37ºC humidified incubator with 5% CO_2. After incubation the membranes were fixed in methanol, followed by staining in Quickdip I for 1 min and Quickdip II (Thermo Fisher Scientific) for 2 mins. The membrane was then mounted on a glass slide with the non-migrated cells being removed with a wet tissue. Migrated cells were manually

counted at 100x magnification in 4 random fields of each well (each condition was performed in triplicate for each assay) on a CKX41 microscope (Olympus).

Mammary fat pad tumour model

Mammary fat pad injection of cells was performed as previously described [45]. Mice (5-6 week-old BALB/c *nu*/*nu*) were obtained from Laboratory Animal Services (Adelaide, SA, Australia) and were maintained in the Animal Resource Laboratories of Monash University (Clayton, VIC, Australia) according to procedures approved by Monash University Animal Ethics Committee (Clayton, VIC, Australia), authorization SOBS/B/2006/52. Mice were injected with MDA-435 and MDA-435R cells, respectively at both left and right flanks of mammary fat pads in a mixture (1:1) of PBS and Matrigel (BD, NJ, USA) at $1.5x10^6$/15µl. Tumour growth was assessed by measuring the length and width of tumours with electronic calipers every 3 to 4 days continuously after the tumours became palpable. Volumes were calculated using the formula (length) x $(width)^2$ /2, where the length was determined as the larger measurement. Mice were humanely killed when tumours approached $200mm^3$.

Generation of cells stably expressing mCherry-Luc2

The retroviral pBABE-Luc2-IRES-mCherry construct was transiently co-transfected with pCL-Ampho packaging vector (Imgenex, San Diego, CA, USA) into HEK293T cells using Lipofectamine LTX according to the manufacturer's instructions (Life Technologies). Fresh media was replaced after 16 hrs. After a further 24 hrs the retroviral-conditioned medium was collected and filtered using a 0.45µM filter. MDA-435 or MDA-435R cells were transduced by the addition of the retroviral-conditioned medium for 24 hrs with the addition of 10µg/ml of polybrene. Cells were then grown in standard growth conditions and the transduced cells were selected based upon their mCherry expression using FACS (Flowcore Platform, Monash University). Selection gates were set to normalise mCherry fluorescence intensity between the transduced MDA-435 and MDA-435R cell lines.

Knockdown of BST2

For BST2 knockdown GIPZ lentiviral shRNAmir constructs (V3LHS_310324, V2LHS_15177, V3LHS_310325, V3LHS_310323, V3LHS_310326) targeted towards human *Hsf1*, and a GIPZ non-silencing control lentiviral shRNAmir construct (RHS4346) were purchased from Thermo Fisher Scientific. The non-silencing control and the targeted human GIPZ shRNA constructs were transiently co-transfected with psPAX2 and pMD2.G packaging constructs into HEK293T cells using Lipofectamine LTX according to the manufacturer's instructions (Life Technologies). Media was replaced 16hrs later and after a further 24hrs the lentiviral-conditioned media was collected and filtered using a 0.45mm filter. MDA-435R and MDA-231R cells were transduced by the addition of

the lentiviral-conditioned media for a period of 24hrs with the addition of 10μg/ml of polybrene. Cells were then grown in standard media and transduced cells were selected based upon GFP expression using FACS (Flowcore Platform, Monash University) with the selection gates being set to normalise GFP fluorescence intensity between the non-silencing and *BST2* silencing shRNAmir expressing cells. The most efficient knockdowns were achieved by using V2LHS_15177 and V3LHS_310326 shRNAmirs, which were used for subsequent experiments and referred to as mir2 and mir5, respectively.

Intracardiac experimental metastasis model

Intracardiac injection of the MDA-435-Luc2-mCherry and MDA-435R-Luc2-mCherry cells was performed as previously described [45, 51, 52]. Briefly, cells previously supplied with fresh media 24h prior to intracardiac injections, were lifted non-enzymatically and resuspended in DMEM/FBS, washed thrice with PBS and then resuspended in PBS at $1x10^6$ cells/ml. Female BALB/c *nu/nu* mice (4-5-weeks old) were anesthetized with ketamine (40mg/kg/mouse)/xylazine (16mg/kg/mouse) and $1x10^5$ cells were inoculated into the left-ventricle of the heart using a 27-gauge needle. Anesthetized mice were then placed in the IVIS 200™ Imaging System (Perkin Elmer, Waltham, MA, USA) and imaged approximately 15 mins after intra-peritoneal injection of D-luciferin (Thermo Fisher Scientific). A successful intracardiac injection was indicated by a whole-body distribution of bioluminescence signal. Evaluation of metastatic tumour progression was monitored once a week for up to 6 weeks. Organ-specific metastasis was assessed *ex vivo* at the end point of the experiment (week 5 and week 6 for mice inoculated with MDA-435-Luc2-mCherry and MDA-435R-Luc2-mCherry cells respectively) and a flux reading of 1×10^5 photon/s was set as the minimum threshold as determined previously [53] for the analysis of metastasis incidence. The bone integrity of tumour-bearing mice by intracardiac injection was assessed by digitized radiography using a μB1600 x-ray inspection system (Matsusada Precision, Kusatsu, Shiga, Japan) once a week for up to 6 weeks.

Three-dimensional micro-computed tomography (micro-CT)

The hindlimbs of the mice were fixed in formaldehyde for 24 hrs and stored in 70% ethanol. Muscle and skin were removed prior to further analysis. To obtain quantitative measurements of tumour-induced osteolytic bone lesions, the bone architecture of the hindlimbs was analysed using micro-computed tomography (micro-CT) (Skyscan 1076 X-ray Microtomograph, Skyscan, Kontich, Belgium). All bone samples were scanned at 50kV/100mA with isometric resolution of 8.7μM per pixel using a 0.5mm aluminium filter and one-frame averaging. Reconstruction of the original scan data was performed using NRecon (SkyScan) with the following settings: beam-hardening correction 35%, ring artefact correction 6, smoothing 1 and 5% defect pixel masking. Reconstructed images were straightened with Dataviewer (SkyScan). Analysis of bone volume was performed using CT-Analysis (SkyScan). For the analysis of the femur, the region of interest (ROI) was defined as a 1mm

region (110 slices) commencing at 2mm below the growth plate. For the analysis of the tibia, the ROI was defined as a 1mm region (110 slices) commencing at 0.5mm below the growth plate. The threshold used for the analysis was from 124 to 255 for the femurs and 112 to 255 for the tibias. In our protocol, the bone tissue component included both cortical and trabecular bones without distinction.

Microarray analysis

Isolation of RNA for microarray was performed using the RNeasy Miniprep kit (Qiagen, CA, USA) according to the manufacturer's instructions. RNA was submitted to the Australian Genome Research Facility for gene expression profiling processing and analysis was performed using the Illumina platform. Bioinformatic analysis was conducted to assess differential gene expression using GeneSpring GX Software (Agilent Technologies, CA, USA). For determination of the most significant gene ontology pathways and networks altered between samples, gene set enrichment analysis (GSEA) was performed as described previously [54].

Semi quantitative PCR and primers

Total RNA was isolated using the Qiagen RNeasy kit according to the manufacturer's instructions (Qiagen, CA, USA). One to two micrograms of total RNA was used to synthesize cDNA using the superscript VILO cDNA synthesis kit according to the manufacturer's instructions (Life Technologies). The synthesized cDNA underwent PCR using the Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN, USA). The primers used were:

BST2 (FWD): 5'–AGGAAGCTGGCACATCTTGGAAGGT-3', BST2 (REV): 5'-CCCGTGCTCTCCCCGCTAAC-3'

Amplifications were performed with the following profile: 95ºC 3mins; 30x (95ºC 30s, 60ºC 30s, 72ºC 30s); 72ºC 7 mins; Ended at 25ºC. The PCR products were run on 2% agarose gel.

Statistical Analysis

Data was analysed using Prism 5 software (GraphPad, San Diego, CA, USA) and statistical significance determined using unpaired *t*-test. Data were presented as mean ± SEM of three or more pooled experiments and significance is represented graphically by * $p < 0.05$, ** $p<0.01$ and *** $p<0.001$.

RESULTS

17-AAG-resistant cells have undergone minimal morphological changes

Human cancer cell lines MDA-435 and MDA-231 with acquired resistance towards 17-AAG, designated MDA-435R and MDA-231R, were developed as previously described in chapter 2. The resistant cells exhibited a resistance profile that spanned a broad spectrum of HSP90 inhibitors, including other benzoquinone ansamycins, such as 17-DMAG and geldanamycin, as well as resorcinylic HSP90 inhibitors, such as radicicol, CCT018159, VER50589 and NVP-AUY922 (chapter 2). As shown in figure 3.1A, the morphology of MDA-435R cells was identical to that of the parental cells. MDA-231R on the other hand show a more spindle-shaped morphology compared to the parental cells (Fig. 3.1A). The 17-AAG-resistant cells maintained their morphologies through 20 passages after 17-AAG withdrawal.

17-AAG-resistant cells have decreased growth potential both in vitro and in vivo

To characterise the growth properties of the cells, we investigated *in vitro* growth of the parental and resistant cells in a standard two-dimensional growth assay and three-dimensional adhesion–independent colony forming growth assay. A significant decrease in growth was observed in both the MDA-435R and MDA-231R when compared to their respective parental cells (Fig. 3.1B). Likewise, in the three-dimensional adhesion-independent colony growth assay, a significant decrease in the number of colonies formed by MDA-435R and MDA-231R in comparison to parental cells was observed (Fig. 3.1C). To determine whether these observations translated to *in vivo* tumour growth potential, parental MDA-435 and MDA-435R cells were injected into the mammary-fat pad (mfp) of nude mice (Balb/c *nu/nu*). Mean external calliper measurements of the mammary tumours indicated that MDA-435 parental cells grew at a significantly faster rate than of the MDA-435R cells (Fig. 3.1D). After four weeks of growth in nude mice, MDA-435 tumours had reached a volume three-times larger than the tumours of MDA-435R (Fig. 3.1D). MDA-435R tumours took another five weeks to grow to the volume comparable to MDA-435 tumours at week four. These results indicated that MDA-435 cells with acquired resistance towards 17-AAG decreased the growth potential of these cancer cells.

17-AAG-resistant cells have an increased migratory potential in vitro

Increased cell migration is a major cellular attribute associated with not only increased metastatic propensity but is also associated with drug resistance. To examine this, parental and resistant cells were tested for their migratory capacity in a microchemotactic migration assay using IGF-I or fibroblast-conditioned media (FbCM) as chemoattractants. A significant increase in migratory potential was observed in the MDA-435R cells when compared to the parental cells, with a 2-fold and 1.5-fold increase in migration towards IGF-1 and FbCM, respectively (Fig. 3.2A). Similarly, MDA-

231R cells showed a 2.5-fold increase in IGF-I and FbCM induced chemotactic migration when compared to the MDA-231 parental cells (Fig. 3.2B). These results indicate that acquired resistance towards 17-AAG affects the migratory potential of these cell lines and potentially may be associated with an increased metastatic potential.

17-AAG-resistant cells are less metastatic than parental cells

Upon termination of the mice inoculated with MDA-435 and MDA-435R cells in the mfp, metastasis to soft organs and bone was examined by visual inspection and x-ray, respectively. However, no metastatic lesions were observed in either group of mice, possibly due to the relatively short amount of time until termination of the experiment after inoculation of the tumour cells (data not shown). To definitively demonstrate the metastatic potential of the resistant cells, we then utilised the intracardiac inoculation experimental metastasis model. MDA-435 parental and MDA-435R cells that had been previously generated to stably express luciferase were seeded into the circulation of the mice via the left ventricle. Metastatic tumour growth was monitored by the longitudinal measurement by whole body bioluminescence imaging (BLI) once a week for up to six weeks in the dorsal (Fig. 3.2C) and ventral (Fig. 3.2D) positions [45, 51, 52]. Within three weeks post inoculation, bioluminescence signals started to appear at multiple sites in both groups of mice inoculated with MDA-435 and MDA-435R, respectively (Fig. 3.2C and 3.2D). Four to five weeks after inoculation, mice inoculated with MDA-435 parental cells exhibited a higher metastatic tumour burden at multiple sites including the head, thorax, abdomen and hind limbs as compared to mice inoculated with MDA-435R cells (Fig. 3.2C and 3.2D). Quantitative measurement of whole body bioluminescence signal confirmed that the overall metastatic tumour burden was significantly lower in mice inoculated with MDA-435R cells compared to MDA-435 parental cells (Graphs in Fig. 3.2C and 3.2D).

To evaluate metastases in soft tissues, *ex vivo* bioluminescence imaging was performed with visceral organs of nude mice inoculated with MDA-435 and MDA-435R in weeks 5 and 6, respectively. Measurements of bioluminescence signal indicated that metastatic tumour burden in the lungs (Fig. 3.5A) and spleen (Fig. 3.5B) was significantly lower in mice inoculated with MDA-435R cells when compared to parental cells. The incidence of metastatic tumour growth in the lungs and spleen was 100% for both groups of mice (Fig. 3.5A and Fig. 3.5B). A trend of lower metastatic tumour growth in the kidneys was also observed in most mice with MDA-435R although this did not reach statistical significance. However, the metastatic tumour incidence in kidneys of mice bearing MDA-435R cells (14/16) was lower compared to the parental group (14/14). No difference in brain metastatic tumour growth or incidence was detected between the two groups.

These data collectively demonstrate that cancer cells with acquired resistance to 17-AAG have an overall decreased metastatic growth potential within visceral organs.

Acquired resistance to 17-AAG is associated with enhanced osteolytic bone lesions *in vivo*

To evaluate bone metastasis and tumour-induced osteolysis lesions in mice inoculated with parental or resistant MDA-435 cells, x-ray imaging was performed subsequent to each BLI session. In week 5, the metastatic tumour burden in the hind limbs of mice inoculated with MDA-435R cells was lower as indicated by BLI imaging (Fig. 3.6A, B). Despite the lower tumour burden, x-ray imaging revealed that the extent of osteolytic lesions in the hind limbs of mice bearing MDA-435R cells was equal to, or in some cases more severe than that seen in mice bearing MDA-435 parental cells (Fig. 3.6C). To evaluate bone volume more accurately, micro-computed tomography (micro-ct) analysis was performed on the tibiae (Fig. 3.6D) and femurs (Fig. 3.6E) of the mice with a pre-defined 1mm region-of-interest (ROI). The bone volume of the ROI in the right tibiae of mice bearing MDA-435R cells was significantly lower, indicating enhanced osteolysis (Fig. 3.6F). The bone volume of the ROI of the left tibiae was not significantly different (Fig. 3.6F). Bone volume of the ROI in femurs of both groups was not significantly different (Fig. 3.6G). These results demonstrate that 17-AAG-resistant cells are significantly more pro-osteolytic, which leads to bone lesions despite having a decreased ability to form metastatic tumour in bone.

Identification of a molecular signature associated with acquired 17-AAG resistance

We next sought to identify gene expression changes, using gene expression microarray analysis, that may contribute to the acquired resistance and the altered cell biology observed in the MDA-435R and MDA-231R cell lines. We initially identified 134 and 1183 genes that were upregulated or downregulated by >1.5 fold (p-value < 0.05) in MDA-435R and MDA-231R cells, respectively when compared to their corresponding parental cells. A list of 14 genes was obtained, which constituted a molecular signature of acquired resistance to 17-AAG in breast cancer cells (Table 3.1). Among the 14 genes, 6 were upregulated and 8 were downregulated. Genes showing increased expression in the resistant cell lines included *BST2*, *ASAP3*, *IGFBP7*, *SPRY1*, *PRMT2* and *LAMA5*, while genes that were decreased in expression included *MT1E*, *COL8A1*, *KRT7*, *CDC42EP5*, *POLR1E*, *PTGR1*, *RPS15* and *TRIB3*.

BST2 overexpression is not involved in acquired resistance or morphological changes

Among the commonly altered genes, bone marrow stromal cell antigen 2 (BST2) was the most significant gene upregulated in the resistant cells of both models (>2-fold in MDA-435R and >13-fold in MDA-231R). Results from semi-quantitative PCR confirmed the increase of BST2 at the mRNA level in the resistant cells (Fig. 3.5A). Consistent with the increase in mRNA expression, BST2 protein levels were also increased in the MDA-435R and MDA-231R cells as determined by immunoblot analysis (Fig. 3.5B). Upon treatment of the cell lines with 17-AAG, BST2 protein expression was also observed to be higher in both of the resistant cells when treated with 17-AAG

compared to the parental cells (Fig. 3.S2A and 3.S2B). Moreover, BST2 expression was induced in a dose-dependent manner by 17-AAG (Fig. S2A and S2B). It is well known that the inhibition of HSP90 by 17-AAG results in a heat shock response via activation of the transcription factor, Heat Shock Factor 1 (HSF1), the master regulator of the heat shock protein family [55]. This led us to hypothesise that BST2 may be a novel heat shock regulated gene. Bioinformatic analysis of the promoter region of the human BST2 gene revealed the presence of putative heat shock elements (HSEs), potential binding sites for HSF1 (Fig. 3.S2C). However, further investigation is needed to define BST2 as a HSF1 regulated gene, which is beyond the scope of this current study.

To investigate the role of BST2 in conferring resistance towards HSP90 inhibitors and/or contributing to the alterations in the cell biology of the cancer cell line models, we reduced BST2 expression by RNA interference using a shRNAmir approach. MDA-435R and MDA-231R cells were transduced using lentiviral constructs expressing either a non-silencing shRNAmir or shRNAmirs with specificity towards human BST2. Immunoblot analysis confirmed BST2 knockdown at both the resting level and during 17-AAG treatment in MDA-435R (Fig. 3.5C) and MDA-231R (Fig. 3.5D). However, BST2 knockdown did not alter the sensitivity of the resistant cells towards 17-AAG (Fig. 3.S3). Analysis of *in vitro* growth in standard 2-D growth assays overall demonstrated little impact of BST2 knockdown upon growth in both the MDA-435R (Fig. 3.5E) and MDA-231R (Fig. 3.5F) cells. Although there was a small reduction in growth in the MDA-435R cells expressing BST2 shRNAmir5 compared to the non-silencing control, this was not observed in cells expressing shRNAmir2 (Fig. 3.5E). Furthermore, BST2 knockdown had no effect on the *in vitro* chemotactic migration in both resistant models (Fig. 3.5G and 3.5H). These results showed that although BST2 may be a potential biomarker of 17-AAG-resistance and a novel putative heat shock regulated gene, it is not mechanistically involved in conferring resistance to 17-AAG or in the observed cell biological alterations in the 17-AAG resistant cells *in vitro*.

Altered expression of genes associated with cell cycle regulation, CSCs and EMT in 17-AAG-resistant cells

Although there were a limited number of common genes that were altered between the MDA-435R and MDA-231R cells, many genes that were involved in the cell cycle, and mediating a CSC and/or EMT phenotype were altered in both models. Consistent with decreased tumour growth in the resistant cell lines a number of genes involved in cell cycle regulation were downregulated. In the MDA-435R cells, genes encoding for cyclins, such as *CCND1*, *CCNC*, *CCNB2*, *CCNG1* and *CCNT1* were downregulated (Table. 3.S1), while *CCND2*, *CDK10*, *CCNH*, *CDK9*, *CDK8*, *CCNE1*, *CCNH* and *CCNM* were downregulated in the MDA-231R cells (Table. 3.S3).

Interestingly, key genes involved in the CSC phenotype were also upregulated in the resistant cells. These include *FZD2* in the MDA-435R (Table. 3.S2), *FZD1* and *FZD8* in the MDA-231R (Table.

3.S4), and *CTNNB1* in both resistant lines (Table. 3.S2 and Table. 3.S4). In the MDA-231R cells, many biomarkers of EMT were also upregulated, namely *ZEB1*, *CDH2* (N-cadherin), *VIM* (vimentin) and *Snai2* (SNAIL homolog 2) (Table. 3.S4).

Analysis of biological pathways associated with 17-AAG resistance and morphological changes

To assess the biological pathways represented by the genes altered in 17-AAG resistant cells, we used Gene Set Enrichment Analysis (GSEA) [54] to examine ranked lists of genes for enrichment of biological pathways contained within the C5 database of the Gene Ontology (GO) project, The Gene Ontology Consortium. The top ranked gene sets associated with 17-AAG resistance in the MDA-435 and MDA-231 models are shown in Table 3.2 and Table 3.3, respectively. The predominant gene sets enriched in the MDA-435R cells compared to the parental cells were involved in cellular migration and cytoskeletal organization, which include the following networks: *leukocyte migration*, *actin filament organization*, *spindle microtubule*, *contractile microfiber* and *spindle pole organization*. This is consistent with the observation of increased chemotactic migration in the MDA-435R cells. There was also an enrichment of multiple gene sets involved in the activation of c-Jun N-terminal kinase (JNK) including *positive regulation of JNK activity*, *activation of JNK activity*, *regulation of JNK activity* and *JNK cascade*. Pathways involved in the regulation of apoptosis were also enriched, including *caspase activation*, *positive regulation of caspase activity*, *apoptotic program* and *activation of NF-κB*. Interestingly, the network *regulation of cytokine production* was also enriched, which may be an underlying mechanism for the pro-osteolytic phenotype of the MDA-435R cells observed *in vivo*.

The predominant gene sets enriched in the MDA-231 model were those involved in protease inhibition, which include *protease inhibitor activity* and *serine type endopeptidase inhibitor activity.* There was also enrichment of gene sets involved in immune cell activation namely *positive regulation of T-cell activation*, *positive regulation of lymphocyte activation*, *regulation of defense response*, *regulation of immune effector process* and *humoral immune response*. Gene sets involved in metabolic programs were also enriched, such as *icosanoid metabolic process* and *lipid homeostasis*. Consistent with our previous finding of HDACs in conferring resistance to HSP90 inhibitors, the gene set of *HDAC activity* was enriched in MDA-231R cells, which included genes such as *HDAC1*, *HDAC2*, *HDAC4*, *HDAC6*, *HDAC8* and *HDAC11*. Another notable enriched gene set was *response to hypoxia* including genes such as *HIF-1α*, *HSP90B1*, *CD24* and *SMAD3*, all of which may be important in enhancing survival of the resistant cells exposed to the HSP90 inhibitors.

Taken together, the microarray data indicated that the acquired resistance towards the HSP90 inhibitors can lead to multiple molecular changes in a diverse set of pathways involved in cellular processes such as migration, regulation of apoptosis, cell survival, protease inhibition, immune

response, regulation of cytokine and cell metabolism. These molecular changes in turn led to enhanced survival upon HSP90 inhibition but decreased their growth and metastatic potential.

DISCUSSION

HSP90 plays a critical role in cancer maintaining the stable and functionally active conformation of a wide array of client proteins. Despite the inhibitory effect of HSP90 inhibitors on a broad-spectrum of oncogenic client proteins, our group and several others have reported that chronic HSP90 inhibitor treatment of cancer cells can lead to resistance [22, 23] (chapter 2). Due to the impact that HSP90 inhibitors have upon multiple pathways, acquired resistance can be attributed to a variety of mechanisms including decreased expression of NQO1 as well as increased expression of client proteins, HSPs and HDACs [22, 23] (chapter 2). These and other yet-to-be defined molecular changes associated with acquired resistance towards HSP90 inhibitors may also lead to unexpected alterations in the growth and metastatic properties of the cancer cells. In fact, tumour cells that have survived initial chemotherapeutic treatment have been shown to be more aggressive, leading to local recurrence and metastatic spread [28-30]. Thus, the current study aimed to identify consequences of acquired resistance towards HSP90 inhibitors upon cancer cell biology and metastasis, as well as gaining insights into the underlying molecular changes at the level of gene expression. Cells with an acquired resistance to 17-AAG displayed a lower growth and survival potential *in vitro* and xenograft tumour growth was also impaired. This may be due to increased quiescence and/or a lower proliferation rate via increased cell cycle length of the resistant cell populations, which has been previously associated as a mechanism of chemoresistance [56-60]. Many conventional chemotherapeutics target proliferating cells, including HSP90 inhibitors, which impede the cell cycle through the inhibition of multiple cell cycle regulators [61] leading to decreased proliferation rates. Thus, if the cancer cell is not killed by drug treatment it may result in that cell having a reduced proliferation and being less sensitive to HSP90 inhibitors. Concordant with this, microarray analysis has identified the downregulation of many genes involved in cell cycle regulation in the 17-AAG resistant cells, such as cyclins and cyclin dependent kinases (CDKs). Myung and colleagues showed that quiescent hepatic stellate cells (HSCs) with low mitotic activity were less susceptible to 17-AAG-mediated apoptosis than activated HSCs that were highly proliferative [62]. Similarly, another study involving other chemotherapeutics also demonstrated that a lower proliferative index was observed in renal clear cell (RCC) carcinoma cells that were resistant to treatment compared to sensitive cells [63]. In addition, Ki-67, a proliferation marker, has a lower labelling index in tumours of patients with RCC carcinoma that did not respond to chemotherapy compared to tumours from patients that responded well [63]. The effect of increased cell cycle length on drug responses has also been demonstrated in a study in which cancer cells pre-treated with cisplatin, causing a G2 arrest, had reduced sensitivity to subsequent paclitaxel treatments [64].

The lower growth potential of 17-AAG-resistant cells observed in the present study is also consistent with the hallmark of CSCs [65, 66]. The slow-cycling and quiescent nature of CSCs results in resistance to many therapies due to their variations in cell cycle control [65, 67, 68]. The canonical Wnt signalling pathway is crucial in cell fate determination and the maintenance of the CSC phenotype [69]. In the 17-AAG-resistant cells, microarray analysis identified the upregulation of several members of the Frizzled (FZD) family of G-protein coupled receptors, adaptor proteins that are crucial in the transduction of Wnt signals [70]. These include *FZD2* in MDA-435R as well as *FZD1* and *FZD8* in MDA-231R cells. Furthermore, β-catenin (*CTNNB1*), another key mediator of the canonical Wnt signalling pathway [71] was also up-regulated at the transcriptional level in MDA-435R and MDA-231R cells. The presence of these potentially CSC-like resistant cells may be attributable to the enrichment of rare populations or the de-differentiation of tumour cells induced by the selection pressure of 17-AAG. Further studies to characterise CSC markers and the associated cell biology in the 17-AAG resistant cells are warranted.

Both MDA-435R and MDA-231R cell lines exhibited a significant increase in chemotactic migration *in vitro*, which is consistent with the observations of other chemoresistant models of various therapies [60, 72-74]. GSEA identified the enrichment of many gene sets involved in cellular migration and cytoskeletal modification in MDA-435R cells. Furthermore, gene sets involved in the activation of JNK pathway were also significantly increased in the MDA-435R cells. The JNK pathway has an evolutionarily conserved role in cell migration in developmental events as demonstrated in drosophila [75]. In the context of cancer, the JNK pathway has been implicated in the regulation of cell migration through the activation of the adaptor protein paxillin and IGFBP2 in cancer cells [76, 77]. In a previous study, we have found that the expression of histone deacetylases (HDACs) was significantly increased in MDA-231R cells (chapter 2). The inhibition of HDACs resulted in a partial resensitisation of the resistant cells to HSP90 inhibitors (chapter 2). In line with this observation, the HDAC pathway was significantly upregulated in the MDA-231R cells as demonstrated by microarray and subsequent GSEA pathway analysis. This included the upregulated expression of *HDAC6* and *HDAC1* genes, which have previously been implicated in the regulation of cell-cell interaction and cell migration [78, 79]. The increase in migration could also be attributable to EMT, which is closely linked to the CSC phenotype and chemoresistance [80, 81]. Consistent with this notion, the morphology of the highly motile MDA-231R cells were more mesenchymal or spindle-like compared to parental cells. We also observed a significant increase in the mRNA expression of Snail, an important mediator of EMT, in both resistant cell lines. Snail is instrumental in the induction of EMT through the induction of mesenchymal markers including vimentin and fibronectin [82] as well as molecules involved in invasion such as metalloproteinase 2 (MMP-2) [83] and other transcription factors such as ZEB-1 and LEF-1 [84]. Consistent with this, vimentin, ZEB-1 and N-cadherin mRNA levels were also upregulated in the MDA-231R, potentially through the action of Snail. Interestingly,

a study has shown that Snail is also involved in the attenuation of cell cycle through the repression of cyclin D2 expression and the increase in p21 [85]. This is consistent with the observation of a lower proliferative rate in the 17-AAG resistant cells and a decrease in the expression of a number of different cyclins in both resistant cell lines, as well as an increase in p21 expression in the MDA-231R cells.

The intracardiac inoculation model was used as a model of experimental metastases that models the latter steps of the metastatic cascade [52]. The metastatic spread of 17-AAG resistant cells to visceral organs such as lungs, spleen and kidneys was impaired when compared to the parental cells. Metastatic tumour growth at skeletal sites, in particular the hind limbs, was also significantly lower in mice bearing 17-AAG-resistant cells. Once disseminated cells are located at the secondary site, the volume of metastases is determined by the growth and survival of the disseminated cancer cells [86-88]. A low proliferation rate is most commonly observed in disseminated tumour cells (DTCs), which are survival-driven cells that have exited the cell cycle [89]. This is consistent with the low proliferative potential of the 17-AAG-resistant cells observed *in vitro* and the downregulation of cell cycle regulators observed at the transcriptional level. Furthermore, tumour cells are subjected to a variety of apoptotic stimuli during the metastatic process and decreased sensitivity towards these stimuli contributes to the successful establishment of a metastatic growth [90]. In the MDA-435R cells, many gene sets associated with caspase activation and initiation of apoptotic pathways were upregulated, which included genes such as caspase-8, DIABLO and Bcl-2/adenovirus E1B 19 kDa interacting protein-3 (BNIP3). This is consistent with the observation of decreased metastatic growth from the resistant cells *in vivo* possibly due to an increased susceptibility to apoptosis affecting cell survival at secondary sites. Caspase-8 is an initiator of apoptosis through the cleaving of downstream caspases that triggers cell death. Stupack *et al* showed that the suppression of caspase-8 expression occurs *in vivo* during the establishment of neuroblastoma metastases, and the reconstitution of caspase-8 expression suppressed metastases [91]. DIABLO, a pro-apoptotic mitochondrial protein that is released to the cytosol in response to apoptotic stimuli, has been shown to have an inverse correlation with metastatic disease in cancer patients [92]. Similarly, another study showed that DIABLO expression decreased significantly during lung cancer progression [93]. A member of the Bcl-2 protein family, BNIP3 is a target for the hypoxia-inducible transcription factor-1 (HIF-1) and mediates cell death under hypoxic condition [94]. Manka *et al* found that BNIP-3 expression is elevated under hypoxic condition in 4T1 cells, a highly metastatic mouse breast cancer cell line, compared to 67NR cells, the non-metastatic counterpart [95]. Taken together, the impaired growth properties coupled with decreased survival due to activation of apoptotic pathways are the main limiting steps affecting the metastatic potential of the resistant cells despite the enhanced migratory ability observed *in vitro*.

A very surprising finding from x-ray and bone morphometric analyses was that there was a similar level of osteolytic lesions in the hind legs of both groups of mice, despite a significantly reduced skeletal tumour burden in mice inoculated with the resistant cells. This indicated that 17-AAG-resistant cells were more pro-osteolytic and able to induce bone lysis more efficiently. Bone metastasis occurs frequently in many cancer types, including breast cancer, whereby the tumour cells secrete hormones, cytokines and growth factors, that stimulate bone resorption which in turn releases factors e.g. TGF-β from the bone matrix stimulating further bone resorption and tumour growth [96]. Key players in the resorption of bone matrix are the osteoclasts, multinucleated cells of hematopoietic origin found in the bone. Inflammatory cytokines produced by immune cells in the tumour microenvironment or by the tumour cells themselves can lead to osteolytic bone metastasis through the differentiation and activation of osteoclasts [97]. Consistent with this, GSEA analysis revealed that gene sets involved in the regulation of cytokine production were upregulated in the MDA-435R cells, which include osteoclastogenic cytokines such as interleukins (IL-8, IL-25) and chemokines (CXCL16, CXCL28 and CXCL24) were overexpressed at the transcriptional level. This may in turn lead to the production of RANKL, an essential pro-osteoclastic factor by osteoblasts and stromal cells or the direct activation of the pre-osteoclastic cells. Furthermore, multiple gene sets that are involved in immune cell activation were also enriched in the MDA-231R cells, which may also lead to the increased activation of myeloid cells that include the osteoclasts. BST2 has been previously shown to be upregulated in bone metastatic breast cancer cells [98]. Despite the lack of involvement in conferring resistance to HSP90 inhibitors or morphological changes in vitro, BST2 may have a role in bone remodelling. In fact, the promoter of BST2 contains a number of elements that would suggest transcriptional regulation by cytokines, such as interferons (IFNs) and IL-6 through transcription factors, including STAT1 and STAT3 [99, 100]. Furthermore, we have shown that BST2 may be a putative heat shock gene and its expression could potentially be used as a biomarker for treatment response and acquired resistance to HSP90 inhibitors. Taken together, we postulate that 17-AAG resistant cells possess a more pro-osteolytic phenotype. However, due to the reduced growth and survival potential, the 17-AAG resistant cells are limited in their ability to grow extensively in the bone. Previous work by our group and others has shown that HSP90 inhibition increased the incidence of bone loss and tumour invasion through the activation of osteoclasts [45, 46]. Our recent studies have further shown that 17-AAG induced the activation of osteoclasts through the up-regulation of MITF in a heat shock-dependent manner [47, 48] (chapter 4). Findings from the current study suggests that 17-AAG or other HSP90 inhibitors may potentially act on the tumor cells to generate a resistant phenotype in the cells but also directly impacts upon osteoclast resorption/differentiation which leads to increased bone loss. However, more studies are needed to elucidate if increased pro-osteolytic potential and bone loss is a common feature of tumour cells subjected to chronic HSP90 inhibition.

In summary, we have identified that 17-AAG resistant cells have impaired growth properties and enhanced chemotactic migration, associated with gene expression alterations that may be associated with CSC-like and mesenchymal phenotypes. In addition, the 17-AAG-resistant cells developed less metastases in visceral organs and skeletal sites but were more potent in inducing osteolytic bone lesions. Collectively, these data suggest that chronic treatment of tumour cells with HSP90 inhibitors can lead to tumour cells with impaired growth and metastatic potential, but are potentially more pro-osteolytic and resistant to treatments. This finding is crucial for the clinical outcome of HSP90 inhibitors and highlights the potential secondary effects of prolonged treatment of patients with this class of drugs.

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Table 3.1. List of genes that are altered in 17-AAG resistant cells compared to parental cells

Genes up-regulated in resistant cells

		Fold change		p-value	
Gene name	**Description**	**435R vs 435**	**231R vs 231**	**435R vs 435**	**231R vs 231**
BST2	bone marrow stromal cell antigen 2 (BST2)	2.0283	13.6317	1.15E-08	3.49E-13
ASAP3	ArfGAP with SH3 domain, ankyrin repeat and PH domain 3 1 (ASAP3)	1.69397	1.58224	2.08E-05	5.67E-05
IGFBP7	insulin-like growth factor binding protein 7 (IGFBP7)	1.53691	2.9561	2.18E-05	1.79E-08
SPRY1	sprouty homolog 1, antagonist of FGF signaling (Drosophila) (SPRY1)	1.57634	1.59765	0.00035141	0.00028934
PRMT2	protein arginine methyltransferase 2 (PRMT2), transcript variant 2	1.5301	1.68786	0.00088311	0.00055207
LAMA5	laminin, alpha 5 (LAMA5)	1.51654	2.99905	0.00439009	6.56E-06

Genes down-regulated in resistant cells

		Fold change		p-value	
Gene name	**Description**	**435R vs 435**	**231R vs 231**	**435R vs 435**	**231R vs 231**
MT1E	metallothionein 1E (MT1E)	8.58256	1.97348	1.07E-08	6.97E-05
COL8A1	collagen, type VIII, alpha 1 (COL8A1), transcript variant 2	1.62062	3.21216	1.02E-05	1.11E-08
KRT7	keratin 7 (KRT7)	1.57372	1.67729	1.90E-05	7.15E-06
CDC42EP5	CDC42 effector protein (Rho GTPase binding) 5 (CDC42EP5)	1.59752	2.86594	2.13E-05	4.32E-08
POLR1E	polymerase (RNA) I polypeptide E, 53kDa (POLR1E)	1.87371	1.73002	2.48E-05	6.86E-05
PTGR1	prostaglandin reductase 1 (PTGR1)	1.50976	1.51144	3.19E-05	0.00049377
RPS15	ribosomal protein S15 (RPS15)	1.55615	1.60418	0.00031984	0.029726
TRIB3	tribbles homolog 3 (Drosophila) (TRIB3)	1.6927	1.68152	0.00394893	0.00423808

Table 3.2. Top gene sets enriched in MDA-435R cells compared to parental cells

GSEA rank	C5 Gene set name (C5 dataset)	GO ID	Functional description	Normalised Enrichment Score (NES)	FDR q-value	FWER p-value
1	LEUKOCYTE_MIGRATION	0050900	The movement of leukocytes within or between different tissues and organs of the body.	1.97	0.054	0
2	ACTIN_FILAMENT_ORGANIZATION	0007015	Control of the spatial distribution of actin filaments	1.67	0.474	0.367
3	RIBONUCLEOTIDE_METABOLIC_PROCESS	0009259	The chemical reactions and pathways involving a ribonucleotide	1.67	0.36	0.417
4	POSITIVE_REGULATION_OF_JNK_ACTIVITY	0043507	Any process that activates or increases the frequency, rate or extent of JUN kinase activity.	1.66	0.32	0.417
5	REGULATION_OF_JNK_ACTIVITY	0043506	Any process that modulates the frequency, rate or extent of JUN kinase activity.	1.64	0.287	0.458
6	ACTIVATION_OF_JNK_ACTIVITY	0007257	The initiation of the activity of the inactive enzyme JUN kinase by phosphorylation by a JUN kinase kinase (JNKK).	1.62	0.325	0.506
7	GLUTATHIONE_TRANSFERASE_ACTIVITY	0004364	Catalysis of the reaction: R-X + glutathione = H-X + R-S-glutathione. R may be an aliphatic, aromatic or heterocyclic group; X may be a sulfate, nitrile or halide group.	1.58	0.457	0.564
8	SPINDLE_MICROTUBULE	0005876	Any microtubule that is part of a mitotic or meiotic spindle; anchored at one spindle pole.	1.56	0.506	0.564
9	JNK_CASCADE	0007254	A cascade of protein kinase activities, culminating in the phosphorylation and activation of a member of the JUN kinase subfamily of stress-activated protein kinases	1.56	0.487	0.613
10	CASPASE_ACTIVATION	0006919	Upregulation of the activity of a caspase, any of a group of cysteine proteases involved in apoptosis.	1.55	0.465	0.613
11	CONTRACTILE_FIBER_PART	0044449	Any constituent part of a contractile fiber composed of actin, myosin, and associated proteins.	1.53	0.509	0.665
12	STRESS_ACTIVATED_PROTEIN_KINASE_SIGNALING_PATHWAY	0031098	A series of molecular signals in which a stress-activated protein kinase (SAPK) cascade relays one or more of the signals.	1.53	0.502	0.665
13	POSITIVE_REGULATION_OF_CASPASE_ACTIVITY	0043280	Any process that activates or increases the activity of a caspase, any of a group of cysteine proteases involved in apoptosis.	1.52	0.481	0.665
14	SPINDLE_POLE	0000922	Either of the ends of a spindle, where spindle microtubules are organized; usually contains a microtubule organizing center and accessory molecules, spindle microtubules and astral microtubules.	1.51	0.484	0.665
15	VESICLE_MEMBRANE	0012506	The lipid bilayer surrounding any membrane-bounded vesicle in the cell.	1.51	0.472	0.665
16	CYTOPLASMIC_VESICLE_MEMBRANE	0030659	The lipid bilayer surrounding a cytoplasmic vesicle.	1.51	0.465	0.665
17	CYTOPLASMIC_VESICLE_PART	0044433	Any constituent part of cytoplasmic vesicle, a vesicle formed of membrane or protein, found in the cytoplasm of a cell.	1.51	0.441	0.665
18	REGULATION_OF_CYTOKINE_PRODUCTION	0001817	Any process that modulates the frequency, rate, or extent of production of a cytokine.	1.51	0.428	0.665
19	APOPTOTIC_PROGRAM	0008632	The intracellular signaling cascade that results when a cell is triggered to undergo apoptosis.	1.5	0.415	0.665
20	ACTIVATION_OF_NF_KAPPAB_TRANSCRIPTION_FACTOR	0051092	Any process that activates or increases the frequency, rate or extent of activity of the transcription factor NF-kappaB.	1.5	0.402	0.665

Table 3.3. Top gene sets enriched in MDA-231R cells compared to parental cells

GSEA rank	C5 Gene set name (C5 dataset)	GO ID	Functional description	Normalised Enrichment Score (NES)	FDR q-value	FWER p-value
1	PROTEASE_INHIBITOR_ACTIVITY	0030414	Stops, prevents or reduces the activity of a protease, any enzyme catalyzes the hydrolysis peptide bonds.	1.87	0.65	0.292
2	ICOSANOID_METABOLIC_PROCESS	0006690	The chemical reactions and pathways involving icosanoids, any of a group of C20 polyunsaturated fatty acids.	1.82	0.382	0.401
3	INSULIN_RECEPTOR_SIGNALING_PATHWAY	0008286	The series of molecular signals generated as a consequence of the insulin receptor binding to insulin.	1.73	0.518	0.455
4	POSITIVE_REGULATION_OF_T_CELL_ACTIVATION	0050870	Any process that activates or increases the frequency, rate or extent of T cell activation.	1.72	0.439	0.455
5	POSITIVE_REGULATION_OF_LYMPHOCYTE_ACTIVATION	0051251	Any process that activates or increases the frequency, rate or extent of lymphocyte activation.	1.71	0.362	0.455
6	REGULATION_OF_DEFENSE_RESPONSE	0031347	Any process that modulates the frequency, rate or extent of a defense response.	1.67	0.432	0.455
7	SERINE_TYPE_ENDOPEPTIDASE_INHIBITOR_ACTIVITY	0004867	Stops, prevents or reduces the activity of serine-type endopeptidases	1.65	0.41	0.455
8	REGULATION_OF_IMMUNE_EFFECTOR_PROCESS	0002697	Any process that modulates the frequency, rate, or extent of an immune effector process.	1.64	0.393	0.455
9	VIRAL_GENOME_REPLICATION	0019079	Any process involved directly in viral genome replication, including viral nucleotide metabolism.	1.63	0.377	0.455
10	LIPID_HOMEOSTASIS	0055088	Any of the processes involved in the maintenance of an internal equilibrium of lipid within an organism or cell.	1.61	0.373	0.455
11	GENERATION_OF_A_SIGNAL_INVOLVED_IN_CELL_CELL_SIGNALING	0003001	The cellular process by which a physical entity or change in state, a signal, is created that originates in one cell and is used to transfer information to another cell.	1.6	0.386	0.455
12	HUMORAL_IMMUNE_RESPONSE	0006959	An immune response mediated through a body fluid.	1.59	0.39	0.455
13	HISTONE_DEACETYLASE_COMPLEX	0000118	Complex that possesses histone deacetylase activity.	1.56	0.481	0.493
14	RESPONSE_TO_HYPOXIA	0001666	A change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a stimulus indicating lowered oxygen tension.	1.55	0.467	0.493
15	REGULATION_OF_T_CELL_ACTIVATION	0050863	Any process that modulates the frequency, rate or extent of T cell activation.	1.54	0.464	0.493
16	ENZYME_INHIBITOR_ACTIVITY	0004857	Stops, prevents or reduces the activity of an enzyme.	1.54	0.464	0.493
17	ANGIOGENESIS	0001525	Blood vessel formation when new vessels emerge from the proliferation of pre-existing blood vessels.	1.53	0.456	0.493
18	HORMONE_SECRETION	0046879	The regulated release of hormones, substances with a specific regulatory effect on a particular organ or group of cells.	1.52	0.458	0.493
19	TRANSMEMBRANE_RECEPTOR_PROTEIN_PHOSPHATASE_ACTIVITY	0019198	The catalysis of phosphate removal from a phosphotyrosine using aspartic acid as a nucleophile in a metal-dependent manner.	1.52	0.45	0.493
20	EXOCYTOSIS	0006887	The release of intracellular molecules (e.g. hormones, matrix proteins) contained within a membrane-bounded vesicle by fusion of the vesicle with the plasma membrane of a cell.	1.52	0.45	0.493

Figure legends

Figure 3.1. Morphology and *in vitro* growth properties of 17-AAG resistant cells. (A) Phase contrast microscopy showed no morphological changes in the MDA-435R cells compared to the MDA-435 parental cells. MDA-231R cells were more spindle-like or mesenchymal compared to MDA-231 parental cells. Anchorage-dependent cell proliferation of the **(B)** MDA-435R and MDA-231R cells was decreased compared to their parental counterparts as identified by SRB growth assay at the indicated time-points. **(C)** Three-dimensional anchorage-independent colony formation of MDA-435R and MDA-231R was decreased compared to the parental cells as assessed by incubating cells in bacto agar containing medium for 28 days to allow colony formation. Data presented in the graphs are mean from at least two independent experiments ± s.e.m, *P<0.05 by student's t-test. **(D)** Mice were orthotopically inoculated in both sides of mammary fat pads with MDA-435 (n=12) or MDA-435R (n=10) and were kept for 4 and 9 weeks, respectively. Tumour formation was significantly decreased in mice inoculated with MDA-435R compared to parental cells. Mean ± s.e.m

Figure 3.2. 17-AAG-resistance is associated with increased *in vitro* chemotactic migration but decreased metastatic tumour formation. Cells were examined in a microchemotactic migration assay with chemoattractants that included 0.1% bovine serum albumin (BSA) as background control, 10ng/ml insulin-like growth factor (IGF) and human fibroblast conditioned-media (FbCM). Significant increases in migration was observed in **(A)** MDA-435R and **(B)** MDA-231R cells compared to their respective parental cells. Data presented in the graphs are mean from two independent experiments ± s.e.m, *P<0.05 by student's t-test in comparison with each control. **(C-D)** Cells were virally transduced with pBABE-mCherry construct containing luciferase gene and inoculated into mice intracardiacally; MDA-435 (n=9), MDA-435R (n=8). Whole body bioluminescence imaging (BLI) was performed weekly three weeks post inoculation. **(B)** BLI with dorsal and ventral views showed that mice inoculated with MDA-435R had significantly lower metastatic tumour burden compared to parental cells throughout the course of the experiment. **(C)** Quantitative measurement of BLI signal, expressed as the sum of integrated photons over the period of imaging, confirmed that metastatic tumour progression was lower in mice inoculated with MDA-435R cells compared to parental cells. Mean ± s.e.m, *p<0.05 by student's t-test.

Figure 3.3. Lower metastatic tumour burden and incidence in soft organs of mice bearing 17-AAG-resistant cells. Presence of metastases in soft organs of mice inoculated intra-cardially with MDA-435 (n=7) or MDA-435R (n=8) were assessed in weeks 6 and 8 respectively post inoculation by *ex vivo* measurement of bioluminescence signal. A flux signal of at least $1x10^5$ photon/s (Log value=5) as indicated by dashed line in graphs, was considered the minimum threshold as determined previously. Lower metastatic tumour growth was observed in **(A)** lungs and **(B)** spleen of mice bearing MDA-435R cells. A higher metastatic incidence of MDA-435 parental cells compared to the

MDA-435R cells in the spleen was observed. No difference in metastatic tumour growth in the **(C)** kidneys or **(D)** brain was observed between the two groups of mice. The metastatic incidence of MDA-435 parental cells to the kidneys was higher compared to the MDA-435R cells. Mean ± s.e.m, *p<0.05 by student's t-test.

Figure 3.4. Enhanced bone lesions in hind limbs of nude mice inoculated with 17-AAG resistant cells despite a lower metastatic tumour burden. (A) Representative images of bioluminescence imaging for the assessment of tumour burden in the hind legs of tumour bearing mice five weeks post-inoculation. **(B)** Bioluminescence signal of left and right hind legs was quantified by measuring bioluminescent signal and expressing it as the sum of integrated photons over the period of imaging. Lower metastatic tumour burden was observed in both hind legs of mice inoculated with MDA-435R compared to mice with MDA-435 parental cells. MDA-435, n=9; MDA-435R, n=8. Mean ± s.e.m, P<0.05 by student's t-test. **(C)** The presence of osteolytic bone lesions was monitored by x-ray imaging. Bone lesions were observed as opaque regions on the representative images acquired at 5 weeks post-inoculation (arrows). Serial 2D cross-sectional micro-computed tomographic (micro-CT) images covering the 1mm region-of-interest (ROI) in the **(D)** tibia and **(E)** femur were acquired as indicated in the 2D longitudinal images in week 5 for mice bearing MDA-435 cells (n=9) and in week 6 for mice bearing MDA-435R cells (n=8). Bone volume of was measured and quantified with micro-CT analysis. **(F)** Bone volume of the right tibia from the MDA-435R bearing mice was lower compared to the parental group and the bone volume of the left tibia was not significantly different. **(G)** Bone volume of femurs from both groups was not significantly different. Mean ± s.e.m, *p<0.05 by student's t-test.

Figure 3.5. BST2 is not involved in growth and migration of 17-AAG resistant cells *in vitro*. BST2 expression was increased in the resistant cell lines at the **(A)** transcriptional level as assessed by semi-quantitative RT-PCR and at the **(B)** protein level as assessed by immunoblot analysis. **(C-D)** Lentivirus-transduced resistant cells of the **(C)** MDA-435 line and **(D)** MDA-231 line with non-silencing or BST2-shRNA constructs were treated with indicated concentrations of 17-AAG for 24 hrs. Immunoblot analysis showed that BST2 protein levels were decreased and the induction of BST2 protein by 17-AAG treatment was impaired in BST2-knockdown cells. **(E-F)** Stable knockdown of BST2 resulted in minimal decrease in the two-dimensional growth of **(E)** MDA-435R with no change in **(F)** MDA-231R cells at the indicated time-points. **(G-H**) Cells were tested in a microchemotactic assay with human fibroblast conditioned-media (FbCM) as the chemoattractant. No significant change was observed in **(G)** MDA-435R and **(H)** MDA-231R with stable BST2 knockdown compared to control cells. Data presented in the graphs are mean from two independent experiments ± s.e.m, *p<0.05 by student's t-test in comparison with each control.

Figure 3.1

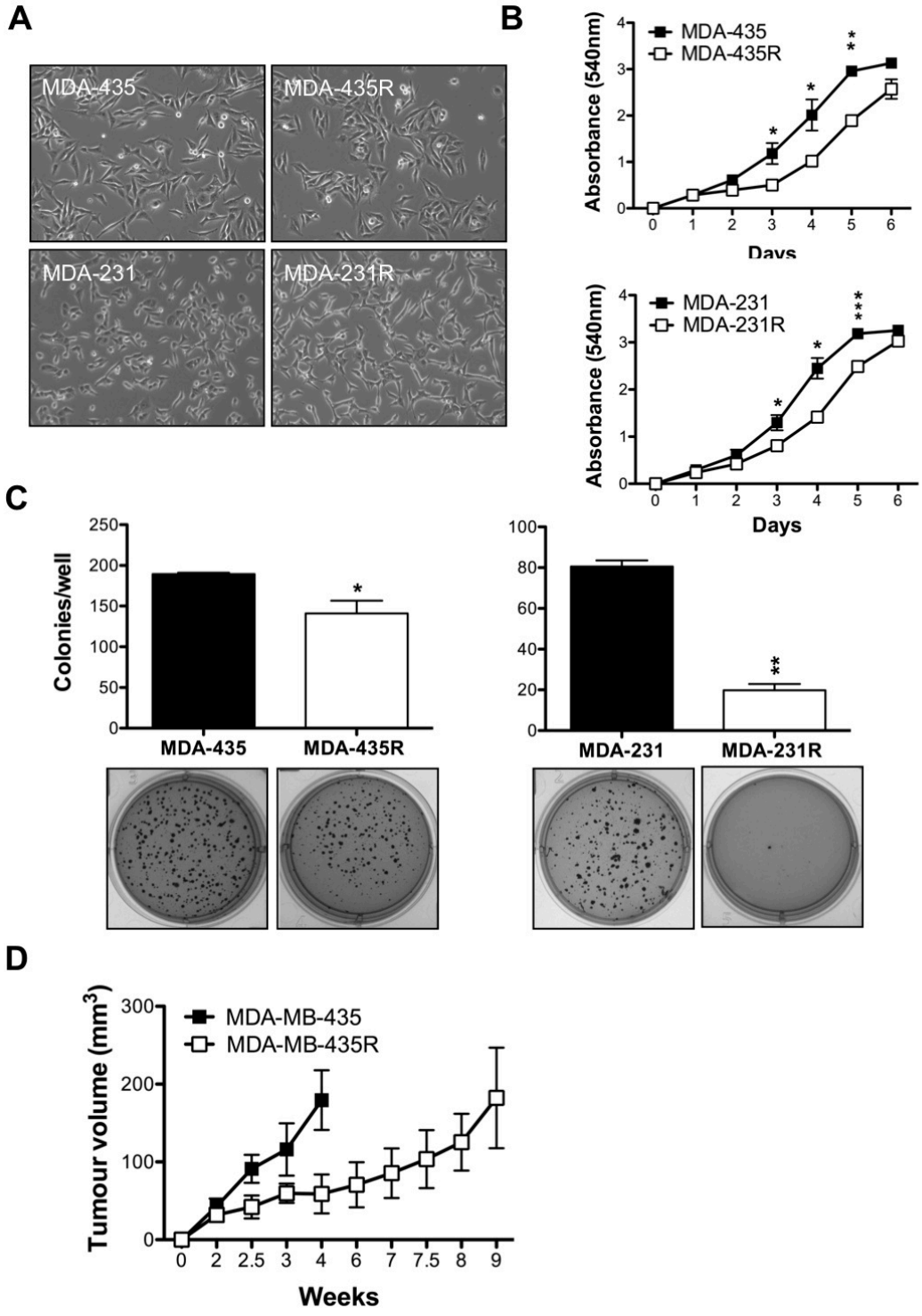

A
MDA-435
MDA-435R
MDA-231
MDA-231R
B
MDA-435
MDA-435R
Absorbance (540nm)
Days
MDA-231
MDA-231R
Absorbance (540nm)
Days
C
Colonies/well
MDA-435
MDA-435R
MDA-231
MDA-231R
D
MDA-MB-435
MDA-MB-435R
Tumour volume (mm3)
Weeks

Figure 3.2

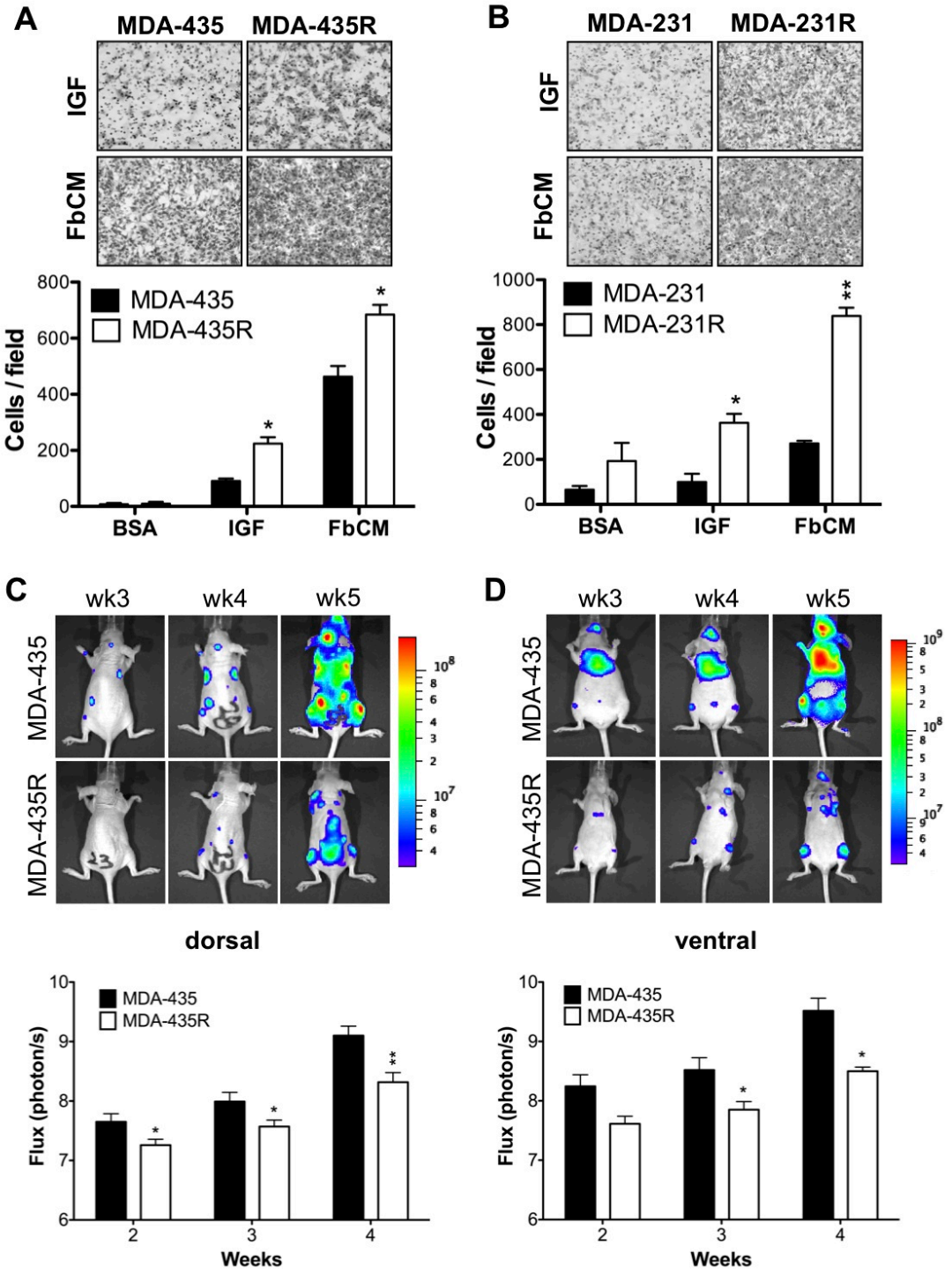

A
MDA-435
MDA-435R
IGF
FbCM
Cells / field
MDA-435
MDA-435R
BSA
IGF
FbCM
B
MDA-231
MDA-231R
C
wk3
wk4
wk5
dorsal
Flux (photon/s)
Weeks
D
ventral

Figure 3.3

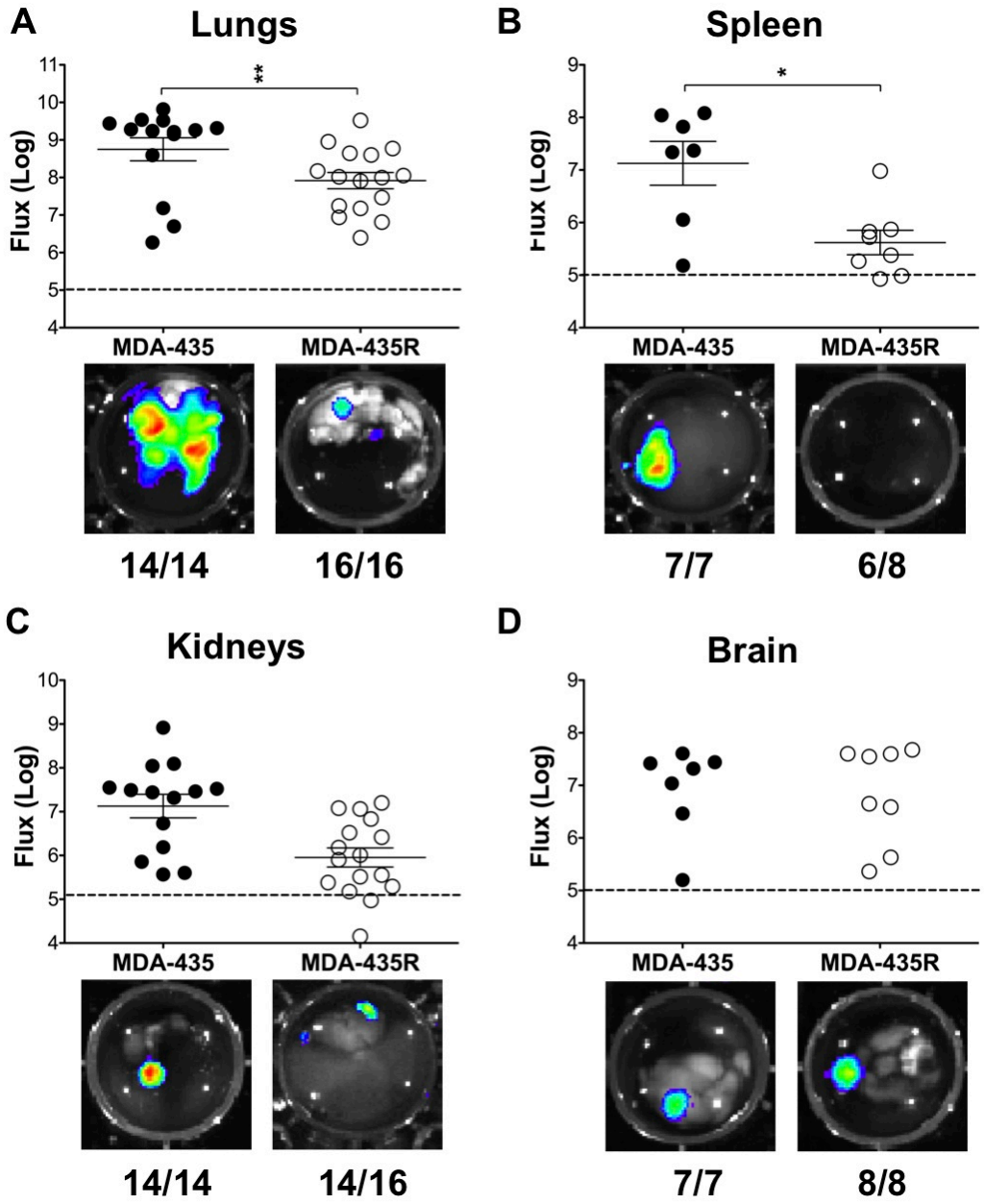

A
Lungs
Flux (Log)
**
MDA-435
MDA-435R
14/14
16/16
B
Spleen
*
MDA-435
MDA-435R
7/7
6/8
C
Kidneys
Flux (Log)
MDA-435
MDA-435R
14/14
14/16
D
Brain
Flux (Log)
MDA-435
MDA-435R
7/7
8/8

Figure 3.4

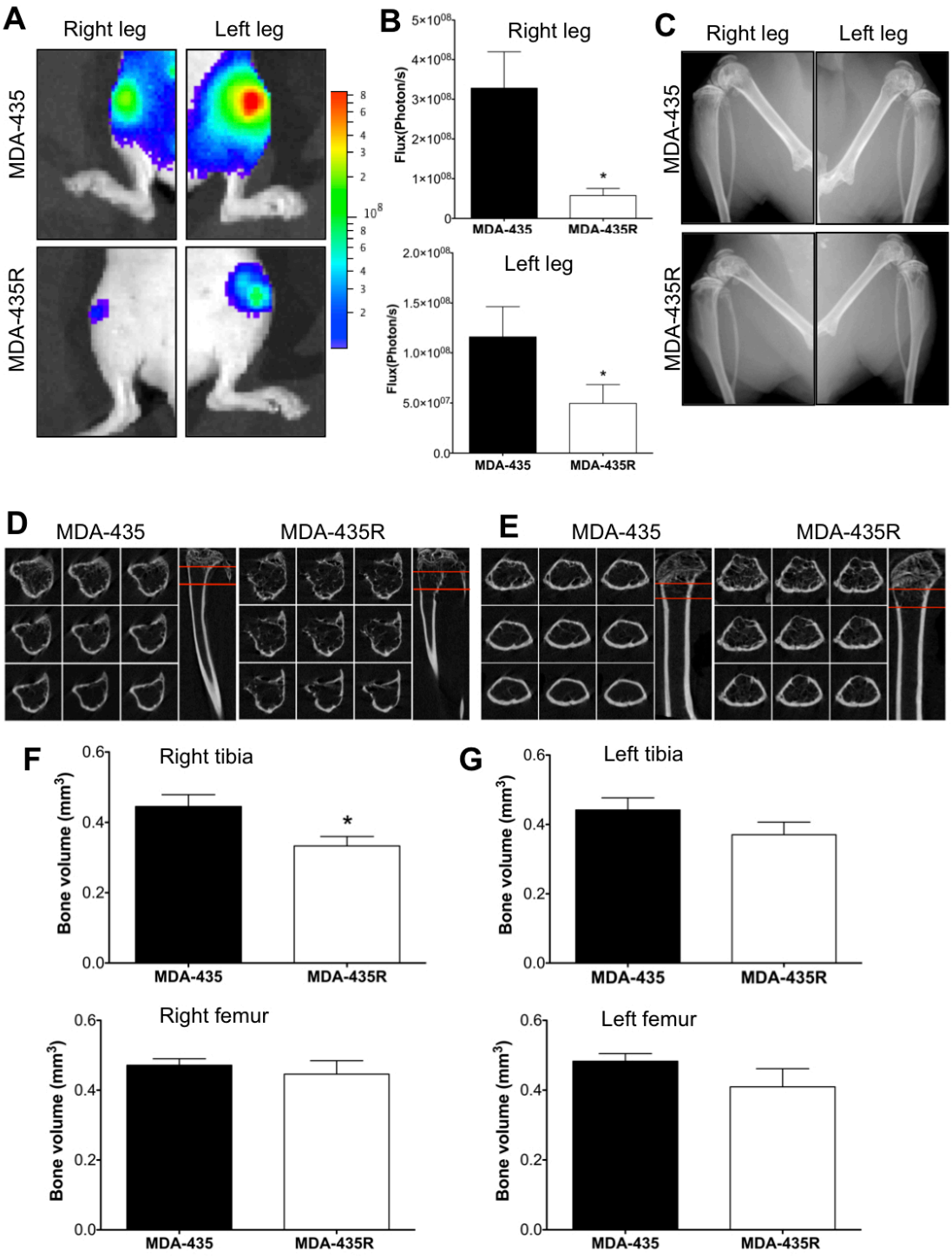
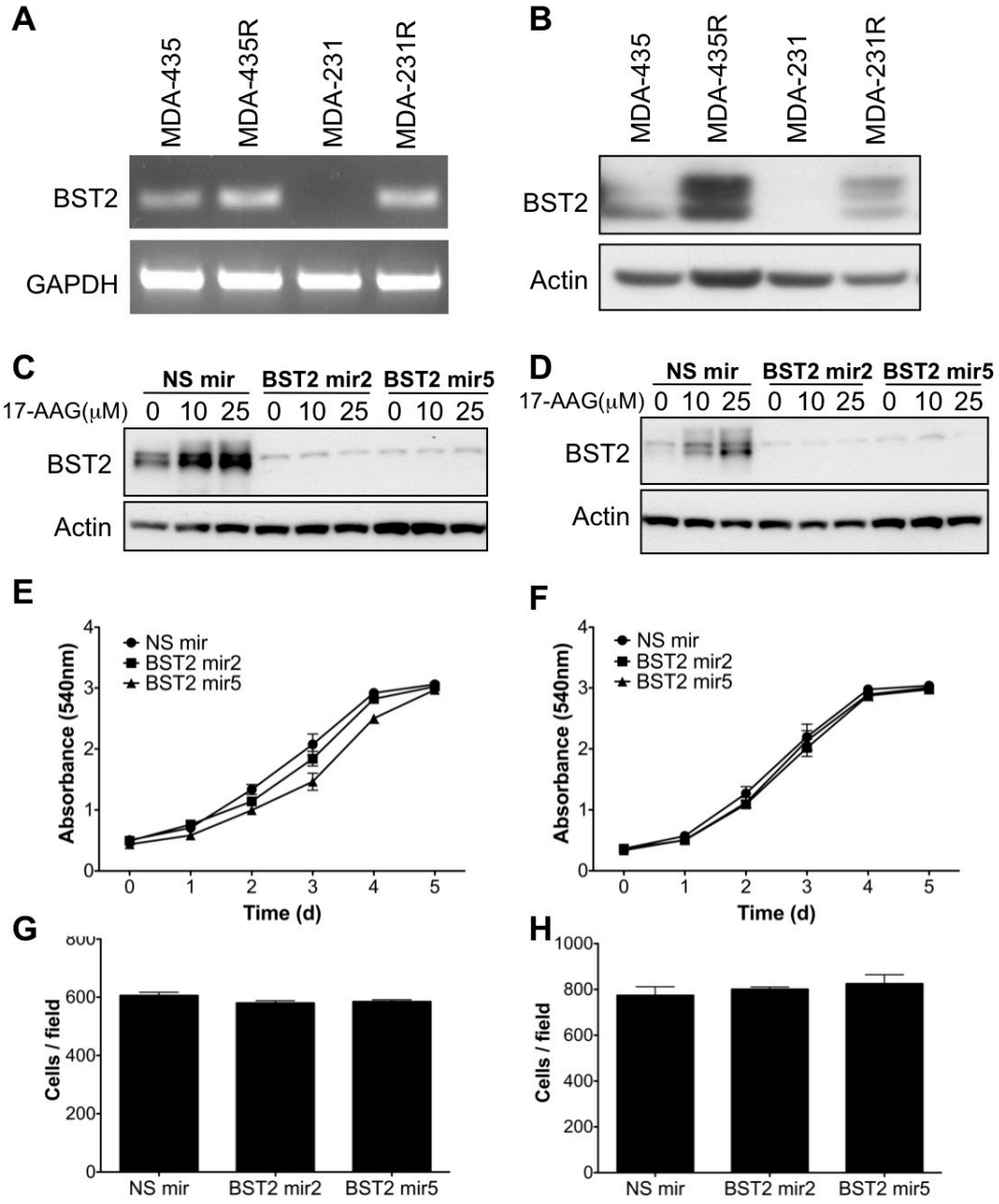

A
Right leg
Left leg
MDA-435
MDA-435R
B
Right leg
Flux(Photon/s)
MDA-435
MDA-435R
Left leg
C
Right leg
Left leg
MDA-435
MDA-435R
D
MDA-435
MDA-435R
E
MDA-435
MDA-435R
F
Right tibia
Bone volume (mm3)
Right femur
G
Left tibia
Left femur

Figure 3.5


A
MDA-435
MDA-435R
MDA-231
MDA-231R
BST2
GAPDH
B
MDA-435
MDA-435R
MDA-231
MDA-231R
BST2
Actin
C
NS mir
BST2 mir2
BST2 mir5
17-AAG(μM) 0 10 25 0 10 25 0 10 25
BST2
Actin
D
NS mir
BST2 mir2
BST2 mir5
17-AAG(μM) 0 10 25 0 10 25 0 10 25
BST2
Actin
E
NS mir
BST2 mir2
BST2 mir5
Absorbance (540nm)
Time (d)
F
NS mir
BST2 mir2
BST2 mir5
Absorbance (540nm)
Time (d)
G
Cells / field
NS mir
BST2 mir2
BST2 mir5
H
Cells / field
NS mir
BST2 mir2
BST2 mir5

APPENDIX: Supporting information

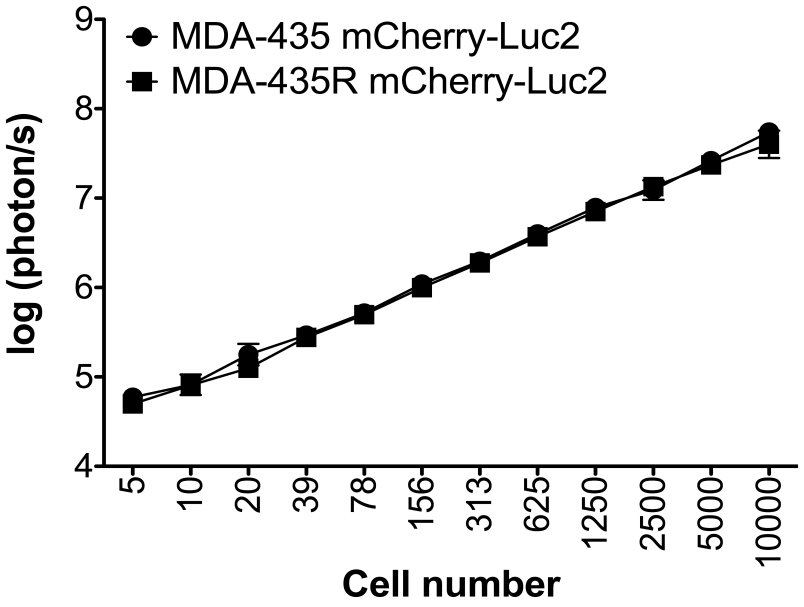


Figure 3.S1. *In vitro* bioluminescence of MDA-435 mCherry-Luc2 and MDA-435R mCherry-Luc2 cells. Cells from each subline were serially diluted from 10000 to 5 cells/well. Luciferin (concentration) was added to each well and incubated for 2 mins. The plate was imaged and datapoints were represented in a log graph of photon/s per cell. Data presented in the graphs are representative of three independent experiments ± s.d.

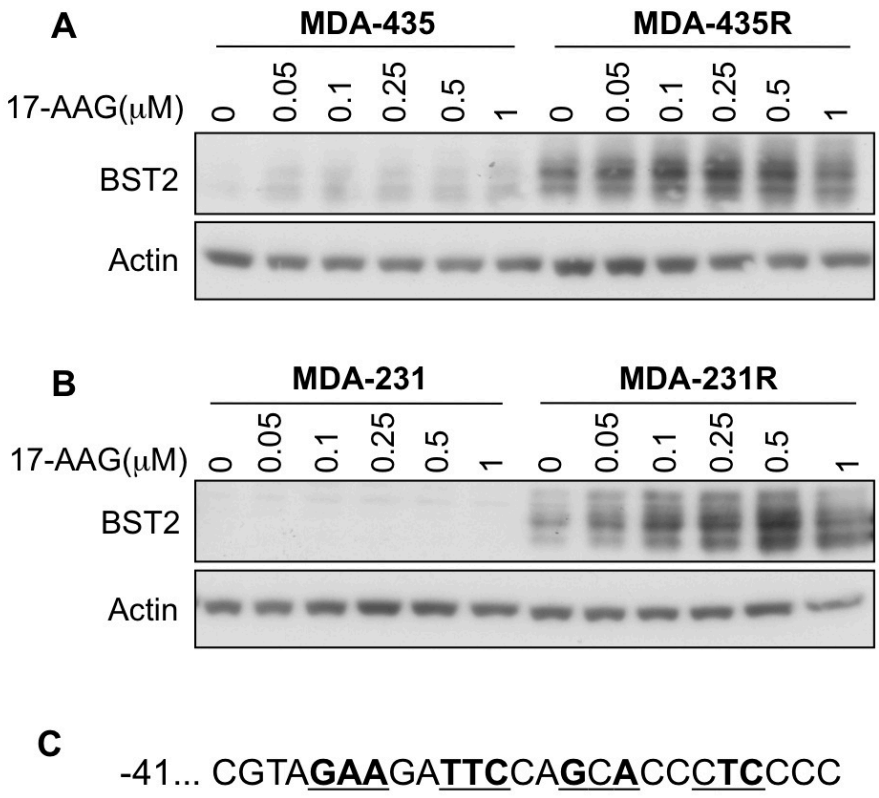


Figure 3.S2. **BST2 expression is induced by 17-AAG treatment.** (A-B) BST2 protein expression in parental and resistant lines of (A) MDA-435 and (B) MDA-231 treated with indicated concentrations of 17-AAG for 24 hrs was analysed by immunoblot with total cell lysate. BST2 protein expression was induced by 17-AAG in a dose dependent manner (A) in the parental and resistant cells of the MDA-435 line and (B) in the resistant cells of the MDA-231 line. (C) Analysis of the promoter region of human BST2 gene revealed the presence of heat shock element (HSE) sites.

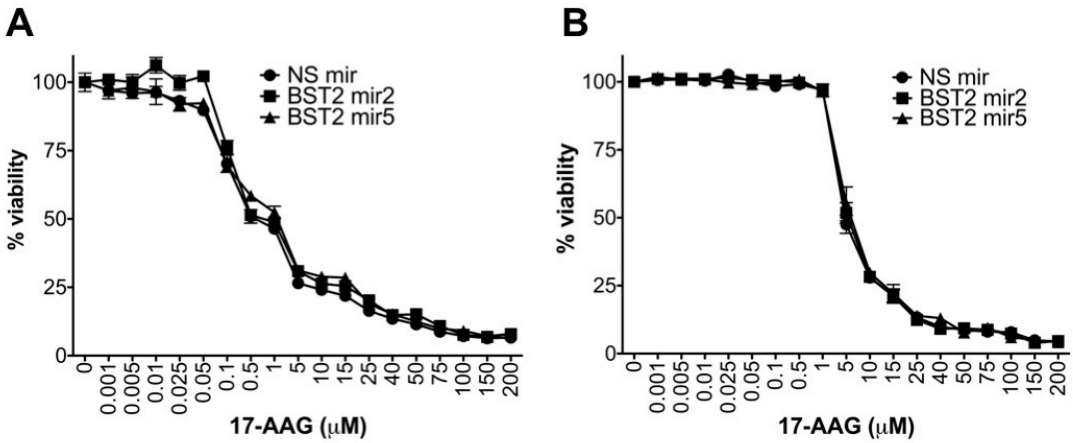


Figure 3.S3. BST2 was not involved in conferring resistance to 17-AAG. Stable BST2 knockdown did not resensitise the (A) MDA-435R or (B) MDA-231R cells to 17-AAG as indicated by 17-AAG-induced growth inhibition assay. Data presented in the graphs are representative of three independent experiments ± s.d., *P<0.05 by student's t-test in comparison with each control.

DECLARATION FOR THESIS CHAPTER 4

In the case of Chapter **4**, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Participated in project hypothesis -Designed and performed experiments -Analysed data -Prepared and wrote the manuscript	65

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Michelle M. Kouspou	Provided technical support, contributed to refinements to manuscript	
Benjamin J. Lang	Provided technical support, contributed to refinements to manuscript	6
Chau H. Nguyen	Provided technical support, contributed to refinements to manuscript	5
A. Gabrielle van der Kraan	Provided technical support, contributed to refinements to manuscript	4
Jessica L. Vieusseux	Provided technical support	
Reece C. C. Lim	Provided technical support	
Matthew T. Gillespie	Contributed to refinements to manuscript	
Ivor J. Benjamin	Provided Hsf1 -/- mice	
Julian M.W. Quinn	Supervision; project co-ordination and development of hypothesis; provided technical support; contributed to writing and refinements to manuscript	
John T. Price	Supervision; project co-ordination and development of hypothesis; contributed to writing and refinements to manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to the work.

Candidate's signature [redacted] **Date 16/12/2013**

Main Supervisor's Signature [redacted] **Date 9/12/2013**

Chapter 4

The Role of Hsf1 in HSP90 Inhibitor-induced Osteoclast Formation

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Molecular Stress-inducing Compounds Increase Osteoclast Formation in a Heat Shock Factor 1 Protein-dependent Manner*

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Background: HSP90 inhibitors increase osteoclast formation and bone loss.
Results: Altered Hsf1 activity impacts the ability of stress-inducing compounds to modulate osteoclast formation.
Conclusion: Hsf1 plays an important role in stress-associated osteoclast formation, potentially via MITF.
Significance: We identified a novel pathway whereby agents inducing stress can enhance osteoclast formation.

Many anticancer therapeutic agents cause bone loss, which increases the risk of fractures that severely reduce quality of life. Thus, in drug development, it is critical to identify and understand such effects. Anticancer therapeutic and HSP90 inhibitor 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) causes bone loss by increasing osteoclast formation, but the mechanism underlying this is not understood. 17-AAG activates heat shock factor 1 (Hsf1), the master transcriptional regulator of heat shock/cell stress responses, which may be involved in this negative action of 17-AAG upon bone. Using mouse bone marrow and RAW264.7 osteoclast differentiation models we found that HSP90 inhibitors that induced a heat shock response also enhanced osteoclast formation, whereas HSP90 inhibitors that did not (including coumermycin A1 and novobiocin) did not affect osteoclast formation. Pharmacological inhibition or shRNAmir knockdown of Hsf1 in RAW264.7 cells as well as the use of *Hsf1* null mouse bone marrow cells demonstrated that 17-AAG-enhanced osteoclast formation was Hsf1-dependent. Moreover, ectopic overexpression of Hsf1 enhanced 17-AAG effects upon osteoclast formation. Consistent with these findings, protein levels of the essential osteoclast transcription factor microphthalmia-associated transcription factor were increased by 17-AAG in an Hsf1-dependent manner. In addition to HSP90 inhibitors, we also identified that other agents that induced cellular stress, such as ethanol, doxorubicin, and methotrexate, also directly increased osteoclast formation, potentially in an Hsf1-dependent manner. These results, therefore, indicate that cellular stress can enhance osteoclast differentiation via Hsf1-dependent mechanisms and may significantly contribute to pathological and therapeutic related bone loss.

Maintaining bone mass and quality is critical for sustained health and quality of life by preventing fracture (1). For this reason, bone undergoes continual remodeling throughout adult life to optimize bone quality and structural integrity. This remodeling process involves cycles of bone resorption and formation, mediated by osteoclasts and osteoblasts, respectively (2, 3). Many factors can negatively impact bone health, including a poor diet, gonadal hormonal insufficiency, pathological insult, as well as a range of therapeutic agents (4–7) that often compound the loss of bone mass seen with aging. Factors that are deleterious to bone generally cause a net increase in the formation of the specialized bone-resorbing cell, the osteoclast, causing sustained bone loss that can result in low bone mass, *i.e.* osteopenia or osteoporosis (8, 9), that is not compensated for by increased bone formation. Such bone loss is associated with decreased bone strength and, thus, an increased fracture risk, particularly in the spine, hip, and wrist, with any resulting fractures ultimately leading to a severely diminished quality of life and increased rate of mortality, particularly in elderly patients (10). Localized rapid bone loss may also cause pain and hypercalcemia (4).

It is increasingly recognized that chemotherapeutic agents have a major negative impact upon bone by increasing bone loss and fracture risk more rapidly and severely than seen in normal age-related bone loss (4, 6). Although both hormonal and non-hormonal cancer therapies promote bone loss by inducing hypogonadism, chemotherapeutics can also directly impact osteoclasts (as well as the bone-forming osteoblasts) to cause loss of bone mass and structural integrity, although the mechanisms that underlie this have still to be fully elucidated (4, 11–13). Because of the effectiveness of a number of cancer treatments providing improved survival rates, especially in older patients who may already have low bone mass, it is of

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increasing importance to determine the effect of therapeutics on bone turnover and bone loss. Moreover, it is important to identify the mechanisms by which anticancer agents may result in bone loss so that preventative measures, such as administration of antiosteolytic treatments, may be designed effectively.

The process of osteoclast formation is fundamental to the resorption of bone during both physiological and pathophysiological bone resorption. Osteoclasts are multinucleated, hematopoietically derived cells (3) that are highly active and relatively short-lived. Thus, their formation is a highly regulated point of control for bone resorption and is dependent upon the action of RANKL,[3] a TNF-related molecule whose production is locally regulated by many osteotropic hormones. RANKL typically acts in concert with M-CSF, a survival and proliferation factor for osteoclast progenitors and macrophages. RANKL, through interaction with its cognate receptor RANK, activates a cascade of critical transcription factors in osteoclast progenitors, notably involving NFκB, AP-1 (cFos/cJun dimer), NFATc1, and MITF. These factors, in turn, activate osteoclastic gene expression and induce cell fusion, resulting in mature, functional, multinucleated osteoclasts (14, 15).

Heat shock protein 90 (HSP90) is a molecular chaperone that is required for the stability and functionality of a diverse range of proteins (16). In particular, its action is critical for the stability and activity of mutated and overexpressed oncogenic proteins that enhance the survival, growth, and invasive potential of cancer cells (16, 17). Consistent with this, HSP90 is highly expressed in many tumor types and has been associated with poor patient outcomes (16–18). Thus, HSP90 has emerged as a major cancer therapeutic target and, as such, a number of HSP90 inhibitors have been developed, many of which have undergone or are currently in clinical trials (19).

We have found previously that the geldanamycin-derived HSP90 inhibitor and anticancer agent 17-AAG increases bone loss in mouse models through the direct stimulation of osteoclast formation (20). Furthermore, although 17-AAG proved to be effective in reducing the tumor burden at extraosseous sites, it actually increased the tumor burden within the bone and caused elevated bone loss even in the absence of tumor cells (20). Increased tumor growth in bone probably reflects the well characterized effects of the release of tumor growth factors from the bone matrix and is, therefore, secondary to the bone destruction caused by the pro-osteoclastic effects of 17-AAG. Consistent with our findings, Yano *et al.* (21) demonstrated that 17-AAG treatment enhanced prostate tumor growth in the bones of mice, which could be abrogated by the administration of inhibitors of osteoclast formation and function. In addition to 17-AAG, we have demonstrated that other structurally unrelated HSP90 inhibitors also enhance osteoclast formation (20, 22). To date, the mechanism by which HSP90 inhibitors stimulate osteoclast formation has not been clearly defined, although Src kinase and the elevated expression of the essential osteoclast transcription factor microphthalmia-associated transcription factor (MITF) may play roles (21, 22). However, HSP90 inhibition itself seems unlikely to be directly critical in 17-AAG actions on osteoclasts because many of the RANKL signaling pathways required for osteoclast formation (*e.g.* NFκB activation) are at least partly HSP90-dependent.

An alternative possibility is that the ability of 17-AAG to activate the transcription factor heat shock factor 1 (Hsf1) may play a central role in its effects on osteoclasts. Hsf1 is a critical regulator of stress responses in mammalian cells and is essential for the response to a broad range of stress stimuli, including the regulation of heat shock proteins (HSPs) (23–25). Fundamental to this response is the fact that Hsf1 associates with HSP90 under normal conditions, maintaining Hsf1 in an inactive monomeric state (26, 27). However, upon 17-AAG binding to the N-terminal ATPase domain of HSP90 or upon cellular stress, Hsf1 dissociates from the HSP90 complex, forming homotrimeric complexes, undergoes phosphorylation and SUMOylation (28), and binds to heat shock element sites within the promoters of target genes (28, 29). This results in a characteristic pattern of gene expression that is observed during stress (*e.g.* elevated levels of HSP70 and other HSPs), aiding cell survival. Thus, we examined whether the Hsf1-mediated stress response induced by HSP90 inhibition is responsible for enhancing osteoclast formation.

In this study, we report that the effects of 17-AAG upon osteoclast formation are indeed Hsf1-dependent and that, consistent with this, other Hsf1-inducing stressors have similar effects. Moreover, within the context of the stress response, we found that Hsf1 plays a major role in enhancing the levels of the critical osteoclast formation factor MITF. Our results implicate, for the first time, the role of Hsf1 in osteoclast formation and the influence of stress-induced MITF expression, which points to a direct effect of cell stress and MITF in inducing bone loss that may be important in many diseases that affect bone.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The HSP90 inhibitors 17-AAG, 17-DMAG, and radicicol were obtained from LC Labs (Woburn, MA), and coumermycin A1 and novobiocin were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). KNK437 was a gift from Kaneka Corp. (Takasago, Japan), and quercetin, methotrexate, and doxorubicin were purchased from Merck Millipore (Kilsyth, VIC, Australia). Anti-HSP70 (HSPA1A) antibody (catalog no. ADI-SPA-812) was purchased from Enzo Life Sciences (San Diego, CA). Anti-HSP105/110 (HSPH1) antibody (catalog no. SC-6241) was purchased from Santa Cruz Biotechnology (Dallas, TX). The pan anti-actin antibody (catalog no. MS-1295-P) was purchased from Thermo Fisher Scientific (Scoresby, VIC, Australia), and the anti-Hsf1 antibody (catalog no. 4356) was obtained from Cell Signaling Technology (Danvers, MA). IgG HRP-conjugated secondary antibodies for immunoblotting were purchased from Thermo Fisher Scientific. Recombinant murine soluble RANKL ($RANKL^{158-316}$-GST fusion protein) was obtained from Oriental Yeast Co. (Tokyo, Japan), and human M-CSF and TGFβ (TGFβ1 isoform) were from R&D Systems (Minneapolis, MN). L-cell conditioned medium (a source of secreted murine

[3] The abbreviations used are: RANKL, receptor activator of nuclear factor κB ligand; MITF, microphthalmia-associated transcription factor; 17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; 17-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin; HSP, heat shock protein; TRAP, tartrate-resistant acid phosphatase; MEM, minimal essential medium; BMM, bone marrow macrophage(s); MNC, mononuclear cell; ANOVA, analysis of variance.

M-CSF) was prepared as described by Yeung *et al.* (30). For tartrate resistant acid phosphatase (TRAP) histochemical staining, fast red violet LB salt (F-1625), naphthol AS-MX phosphate, and dimethylformamide were purchased from Sigma-Aldrich.

Animals—C57Black/6 mice were obtained from Monash Animal Services (Monash University, Clayton, VIC, Australia). The mice were maintained at the Monash Medical Centre Animal Facility (Clayton, VIC, Australia) according to procedures approved by the Monash Medical Centre Animal Ethics Committee B (Clayton, VIC, Australia), authorization no. MMCB-2011/19. The C;129-*Hsf1*^{tm1Ijb}/J (stock no. 010543) (31) were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained in the Animal Resource Laboratories of Monash University (Clayton, VIC, Australia) according to standard husbandry and breeding procedures approved by the Monash Animal Research Platform (MARP) 2 Animal Ethics Committee (Clayton, VIC Australia), authorization no. SOBSB/B/2010/28BC. Mice were maintained on a BALB/cx 129SvEV background, and intercrossed *Hsf1*$^{+/-}$ mice were used to generate *Hsf1*$^{+/+}$, *Hsf1*$^{+/-}$, and *Hsf1*$^{-/-}$ mice. Mouse genotypes were determined by PCR according to the standard protocol for the C;129-*Hsf1*^{tm1Ijb}/J strain provided by The Jackson Laboratory. All mice used in the experiments were age-matched females.

Cell Lines and Culture—RAW264.7 cells were purchased from the ATCC and were maintained in minimal essential medium-α (MEM) (Invitrogen) containing 10% FBS (Thermo Fisher Scientific), penicillin (10000 units/ml), and streptomycin (10000 units/ml) (Invitrogen), and HEPES (Invitrogen). All osteoclast formation assays utilized this medium (MEM/FBS). Primary bone marrow cells for culture were immediately isolated from humanely killed, 6- to 12-week-old mice by flushing the bone marrow cavity of the long bones with PBS in accordance with the MARP Animal Ethics Committee (Monash University, Clayton, VIC, Australia) authorization MARP/2011/048. Primary bone marrow macrophage (BMM) cultures were maintained in L-cell conditioned medium to induce BMM proliferation, as described previously (32) in RPMI 1640 medium (Invitrogen) supplemented with penicillin and streptomycin and 10% heat-inactivated FBS (MEM/HIFBS). All cells were maintained in a 37 °C incubator in a humidified atmosphere containing 5% CO_2.

Hsf1 shRNAmir Knockdown and Hsf1 Ectopic Overexpression—For Hsf1 knockdown, GIPZ lentiviral shRNAmir constructs (V2LMM_226824, V2LMM_82329, V2LMM_82328, V3LMM_415511, and V3LMM_415512) targeted toward mouse *Hsf1* and a GIPZ non-silencing control lentiviral shRNAmir construct (RHS4346) were purchased from Thermo Scientific. The non-silencing control and the targeted mouse GIPZ shRNA constructs were transiently cotransfected with psPAX2 and pMD2.G packaging constructs into HEK293T cells using Lipofectamine LTX according to the instructions of the manufacturer (Invitrogen). The medium was replaced 16 h later, and, after a further 24 h, the lentiviral-conditioned medium was collected and filtered using a 0.45-μm filter. RAW264.7 cells were transduced by the addition of the lentiviral-conditioned medium for a period of 24 h with the addition of 10 mg/ml of Polybrene. Cells were then grown in standard medium, and transduced cells were selected on the basis of GFP expression using FACS (Flowcore Platform, Monash University) with the selection gates being set to normalize GFP fluorescence intensity between the non-silencing and *Hsf1*-silencing shRNAmir-expressing cells. The most efficient knockdowns were achieved by using the V3LMM_415512 and V2LMM_82329 shRNAmirs, which were used for subsequent experiments and are referred to as mir4 and mir5, respectively.

To ectopically overexpress mouse Hsf1 in RAW264.7 cells, a retroviral expression system was employed. The pBABE-Hsf1-IRES-mCherry retroviral construct was generated by excision of mouse Hsf1 from the Hsf1 construct pcDNA3.1(+) mHsf1 (provided by Richard Voellmy, University of Miami, FL) using HindIII endonuclease refilled by T4 DNA polymerase to generate blunt ends, and this was further digested with EcoRI endonuclease. The resulting product was then ligated with pBABE-puro-IRES-mCherry (33) that had been linearized by BamHI digestion, end-filled with T4 DNA polymerase, and then digested with EcoRI endonuclease. The correct orientation of the mHSF1 insert was confirmed by diagnostic endonuclease digestion. HEK293T cells were cotransfected with pCL-Ampho packaging vector (Imgenex, San Diego, CA) and pBABE-Hsf1-IRES-mCherry using Lipofectamine LTX (Invitrogen). Retroviral-conditioned medium generation, RAW264.7 transduction, and selection of mCherry-expressing transduced cells by FACS were performed according to the lentiviral approaches stated previously.

Osteoclast Progenitor Differentiation and Survival Assays—Osteoclasts were generated by culturing RAW264.7 cells for a 6-day period in 96-well plates at a density of 5×10^3 cells/well in MEM/FBS, 20 ng/ml RANKL and in the presence or absence (vehicle control) of HSP90 inhibitors and other stress-inducing agents, as indicated under "Results." The medium and the agents were replaced at day 3, and on day 6, cells were fixed with 4% formaldehyde and histochemically stained for TRAP as described previously (34). TRAP-positive multinucleated cells (MNCs) containing three or more nuclei per cell, quantified using an inverted light microscope, were counted as osteoclasts. To generate osteoclasts from primary murine cells, bone marrow cells were flushed from bisected long bones of C57black6/J, wild-type (*Hsf1*$^{+/+}$), heterozygous (*Hsf1*$^{+/-}$), or knockout (*Hsf1*$^{-/-}$) C;129-*Hsf1*^{tm1Ijb}/J mice with PBS. Cells were centrifuged and then resuspended in MEM/FBS. Bone marrow cells (10^5 cells/well) were stimulated by 20 ng/ml RANKL and 25 ng/ml M-CSF in the presence or absence of the HSP90 inhibitor 17-AAG for 6 days. Cells were then fixed and stained histochemically for TRAP and osteoclast numbers counted. For BMM preparation for Western blotting, bone marrow cells (10^6 cells/ml) were suspended in RPMI/HIFBS supplemented with 30% L-cell-conditioned medium (30, 32), incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 3 days, and then, the non-adherent cell fraction was removed. The resulting adherent proliferating cells were then prepared for analysis. These cells were able to form numerous osteoclasts with RANKL/M-CSF treatment, as described previously (32). For cell survival assays RAW264.7 cells were seeded at 5×10^3 cells/well and treated with a range of HSP90 inhibitor concentrations. After a period of 96 h, cells were fixed in 50%

TCA at 4 °C for 1 h, followed by five washes in distilled water. Cells were stained with sulforhodamine B (Sigma-Aldrich), rinsed, and then cell-bound sulforhodamine B was solubilized in 150 μl of 10 mM Tris-HCl (pH 10.5). The absorbance at 550 nm was measured by spectrophotometry using a Multiskan FC absorbance plate reader (Thermo-Lab Systems, MA).

Immunoblot Analysis—Immunoblot analysis was performed as described previously (20, 22, 35). Briefly, cell lysates were generated using modified radioimmune precipitation assay buffer (50 mmol/liter Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/liter NaCl) containing phosphatase and protease inhibitor mixture (Sigma-Aldrich), sonicated, and then clarified by centrifugation. Protein concentrations were determined using the BCA protein assay according to the instructions of the manufacturer (Thermo Fisher Scientific). Cell lysates were run on 4–12% BisTris gradient SDS-PAGE electrophoresis gels with MES SDS running buffer (Invitrogen) under reducing conditions and transferred to Immobilon-P PVDF membranes (Merck Millipore). Membranes were blocked for 1 h with 3% milk powder (Diploma, Fonterra Food Services, Mount Waverley, Australia) dissolved in PBST (PBS + 0.1% Tween 20). Membranes were then incubated overnight at 4 °C with appropriate primary antibodies. Immunoblot visualization was achieved by incubation with appropriate IgG HRP-conjugated secondary antibodies and an ECL detection system (Supersignal West Pico, Thermo Fisher Scientific) according to the instructions of the manufacturer.

Statistical Analysis—Data were analyzed using Prism 5 software (GraphPad, San Diego, CA), and statistical significance was determined using ANOVA/Dunnett's post hoc test. Quantitative data are presented as mean ± S.E. of three or more pooled experiments, and significance is represented graphically by *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$.

RESULTS

HSP90 Inhibitors Enhance Osteoclast Formation in Association with Induction of the Heat Shock Response—To investigate the role of the HSR and, more specifically, Hsf1, in 17-AAG actions on osteoclastogenesis, we first examined the effects of different HSP90 inhibitors on the maturation of murine RAW264.7 cells, a bipotential osteoclast/macrophage progenitor cell line that responds strongly to RANKL treatment by forming osteoclasts. RAW264.7 cells were treated with a submaximal concentration of RANKL (20 ng/ml) that is sufficient to cause low levels of osteoclast formation over 6 days of incubation. Osteoclast formation was completely dependent on RANKL treatment; *i.e.* in cultures where RANKL was omitted, osteoclast formation or mononuclear $TRAP^+$ cells were never seen, as described previously 22. Unlike primary bone marrow cells or BMM, RAW264.7 cells form osteoclasts without M-CSF treatment; *i.e.* they require only RANKL stimulation.

Treatment of RAW264.7 cells with 17-AAG induced a dose-dependent increase in Hsp70 (Hspa1a) protein expression, consistent with induction of the HSR (Fig. 1*A*), and, consistent with previous findings (20, 21), 17-AAG also increased RANKL-stimulated osteoclast formation in a dose-dependent manner (Fig. 1, *B* and *C*). To confirm that this response was not unique to RAW264.7 cells, examination of the effects of 17-AAG in RANKL- and M-CSF-stimulated primary bone marrow cells derived from C57Black/6 mice was performed. As with that of the RAW264.7 cell line, 17-AAG significantly increased Hsp70 expression (Fig. 1*D*), and this was associated with a marked increase in osteoclast formation (Fig. 1, *E* and *F*). It was noted that the primary bone marrow cultures were more sensitive to 17-AAG with respect to the induction of the HSR and that this correlated with increased osteoclast formation.

In RAW264.7 cells, 17-DMAG, a HSP90 inhibitor that is structurally related to 17-AAG but more potently inhibits HSP90, also enhanced both Hsp70 protein levels (Fig. 2*A*) and RANKL-induced osteoclast formation (Fig. 2*B*) in a dose-dependent manner, although at notably lower concentrations to that of 17-AAG (Fig. 1, *A* and *B*).

To determine the scope of the effect, we examined whether the structurally unrelated HSP90 inhibitor radicicol had similar effects to that of 17-AAG and 17-DMAG. Despite the differing structure of radicicol, it was also found to significantly increase Hsp70 levels (Fig. 2*C*) and significantly increase osteoclast formation (Fig. 1*D*). However, in contrast to these findings, the HSP90 inhibitors coumermycin A1 (Fig. 2*E*) and novobiocin (Fig. 2*G*) did not significantly increase Hsp70 levels, thus failing to induce a robust HSR. Moreover, coumermycin A1 (Fig. 2*F*) and novobiocin (Fig. 2*H*) did not significantly increase osteoclast formation. These two HSP90 inhibitors bind the C-terminal region of HSP90 and, thus, have a different mode of action to that of 17-AAG, 17-DMAG, and radicicol. These results are consistent with the notion that activation of Hsf1 and its ability to induce the HSR induced by N-terminal HSP90 inhibitors enhances RANKL-elicited osteoclast formation.

Pharmacological Inhibition of Hsf1 Reduces 17-AAG Enhancement of RANKL-induced Osteoclast Differentiation—To further investigate whether the HSR was mechanistically important for 17-AAG-enhanced osteoclast formation, we examined whether pharmacological inhibition of the HSR would abrogate the 17-AAG-mediated effect. Quercetin and KNK437 are two compounds that have been shown to inhibit the HSR via impacting Hsf1 functionality (36, 37). Consistent with this, treatment of RAW264.7 cells with KNK437 (Fig. 3*A*) dose-dependently inhibited the induction of Hsp70 protein expression by 17-AAG and significantly abrogated the effects of 17-AAG on RANKL-stimulated osteoclast formation (Fig. 3*B*), indicating a potential influence of Hsf1 on osteoclast formation. Quercetin was also found to inhibit Hsp70 induction by 17-AAG (Fig. 3*C*) and also significantly reduced the effects of 17-AAG upon osteoclast formation (Fig. 3*D*). Of the two compounds, KNK437 was more potent at inhibiting Hsp70 induction and, consistent with this, was more effective at blocking 17-AAG effects upon osteoclast formation (Fig. 3*B*). Interestingly, the enhancement of osteoclast differentiation by TGFβ, a cytokine known to stimulate NFATc1 expression (unlike 17-AAG (22)) and augment RANKL signals (38, 39), was not affected by KNK437-mediated inhibition of Hsf1 (Fig. 3*E*). Thus, this demonstrated a specificity of action of KNK437 upon stress-mediated osteoclast formation and, more importantly, that of Hsf1 in 17-AAG-enhanced osteoclast differentiation.

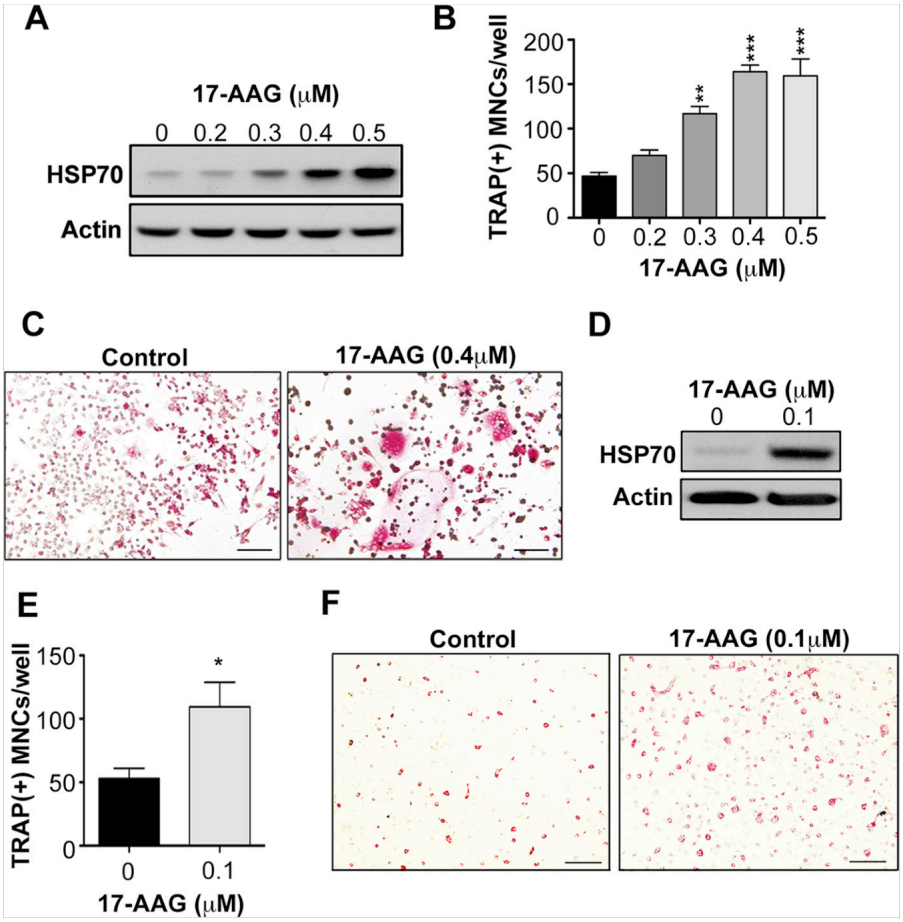

FIGURE 1. **17-AAG enhances RANKL-dependent osteoclastogenesis and induces an HSR.** *A*, murine osteoclast progenitor RAW264.7 cells showed an increase in Hsp70 levels (immunoblot analysis) after treatment with indicated concentrations of 17-AAG for 24 h. *B*, RAW264.7 cells were cultured in 20 ng/ml RANKL and the indicated concentrations of 17-AAG for 6 days, and osteoclasts (TRAP-positive MNCs) were counted. 17-AAG treatment dose-dependently enhanced RANKL-dependent osteoclast formation. *C*, photomicrographs of osteoclasts formed in RANKL-treated RAW264.7 cell cultures showing increased osteoclast formation in 17-AAG-treated (0.4 μM) cells compared with vehicle control (*Veh. Ctl.*). *Red*, TRAP staining. *Scale bars* = 100 μm. *D*, immunoblot analysis of BMMs demonstrated elevated Hsp70 protein levels after treatment with 17-AAG (0.1 μM) after a 24-h treatment period. *E*, as with RAW264.7 cells, mouse bone marrow cells cultured in 20 ng/ml RANKL and M-CSF for 6 days demonstrated a significant increase in TRAP-positive MNCs with 0.1 μM 17-AAG treatment. *F*, photomicrographs of osteoclasts formed in mouse bone marrow cells cultured in 20 ng/ml RANKL and M-CSF for 6 days. Cultures with 17-AAG (0.1 μM) showed an increase in TRAP-positive MNCs when compared with vehicle control. *Red*, TRAP staining. *Scale bars* = 100 μm. *Error bars* represent the mean ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to RANKL (20 ng/ml)-treated vehicle control using ANOVA/Dunnett's post hoc test.

Hsf1 Knockdown Impairs the Effect of 17-AAG on Osteoclastogenesis—To demonstrate a specific involvement of Hsf1 upon 17-AAG actions in osteoclast formation, we reduced Hsf1 expression by RNA interference methods. We utilized an shRNAmir approach on the basis of the design of the primary microRNA-30 transcript allowing for processing via the endogenous RNAi pathways and allowing for more specific silencing than conventional shRNAi. RAW264.7 cells were transduced using lentiviral constructs that expressed either a non-silencing shRNAmir that had no homology to any known mammalian genes or shRNAmirs with specificity for mouse *Hsf1*. Immunoblot analysis of Hsf1 levels confirmed the efficient knockdown of Hsf1 in RAW264.7 cells using two independent shRNAmirs (Fig. 4*A*). Consistent with the knockdown of Hsf1, the 17-AAG-mediated induction of Hsp70 was abrogated significantly (Fig. 4*A*). In non-silencing shRNAmir control RAW264.7 cells, 17-AAG strongly enhanced osteoclast formation, whereas knockdown of Hsf1 by mir4 or mir5 significantly reduced the effects of 17-AAG upon osteoclast formation (Fig. 4*B*), consistent with the effects of the pharmacological inhibition of Hsf1. Of interest, 17-AAG did not increase the steady-state levels of Hsf1, but, consistent with it being a HSP90 client protein, Hsf1 levels were reduced. To ensure that abrogation of 17-AAG-mediated effects upon osteoclast formation because of Hsf1 knockdown was not merely a result of an increased cell death, we tested the sensitivity of the RAW264.7 cells to 17-AAG-mediated cell death. In standard cell survival assays examining increasing 17-AAG concentrations, no differences were observed between the non-silencing control and Hsf1 knockdown cells (Fig. 4*C*), indicating that any effects on osteoclast formation were not due to alterations in cell survival.

Overexpression of Hsf1 Enhances 17-AAG Effects on RANKL-induced Osteoclast Differentiation—To further investigate the influence of Hsf1 in 17-AAG-enhanced osteoclast differentiation, we examined the effect of overexpressing mouse wild-type Hsf1 in the RAW264.7 cell line. To achieve this, we transduced RAW264.7 cells with retroviral vectors expressing mCherry (control) or wild-type Hsf1 (Hsf1WT). Immunoblot analysis

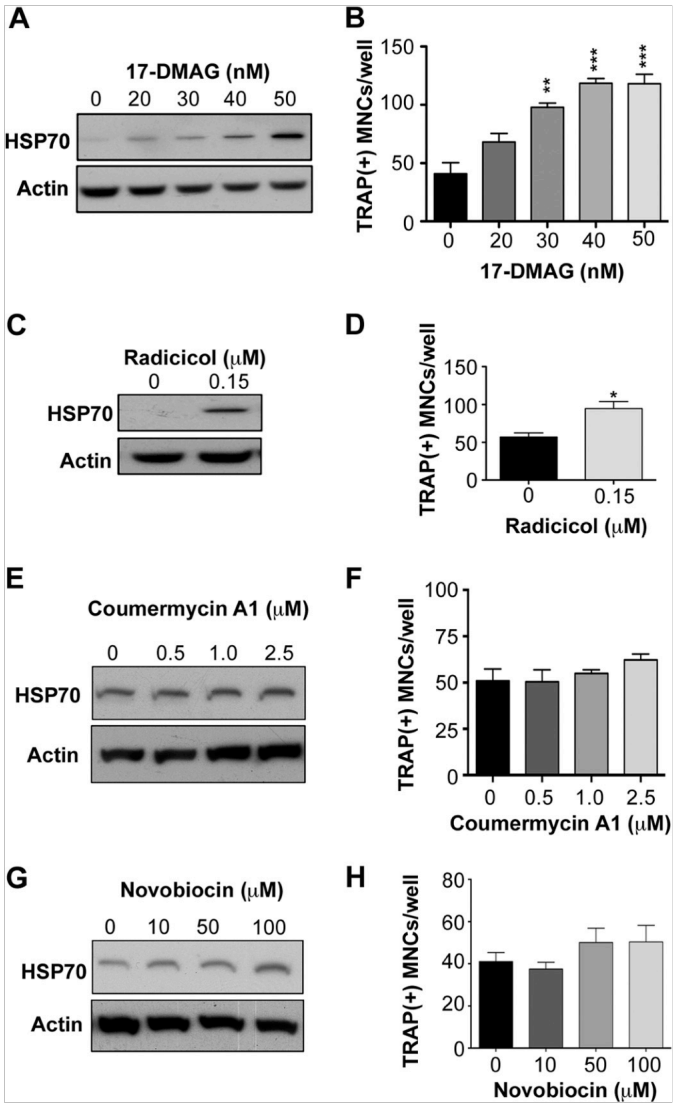

FIGURE 2. **Induction of an HSR by HSP90 inhibitors associates with increased osteoclast formation.** *A*, Hsp70 protein levels in RAW264.7 were dose-dependently increased by 17-DMAG treatment after a 24-h period. *B*, 17-DMAG significantly increased osteoclast formation in RANKL-treated RAW264.7 cells. *C*, the structurally unrelated HSP90 inhibitor radicicol increased Hsp70 levels in RAW264.7 cells after a 24-h treatment period. *D*, radicicol significantly increased osteoclast formation in RAW264.7 cells when compared with vehicle control. The coumermycin A1 (*E* and *F*) and novobiocin (*G* and *H*) HSP90 inhibitors did not induce a heat shock response, indicated by a failure to increase Hsp70 levels in RAW264.7 cells and also failed to increase osteoclast formation. *Error bars* represent the mean ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to RANKL (20 ng/ml)-treated vehicle control using ANOVA/Dunnett's post hoc test.

demonstrated that, although there was a strong overexpression of Hsf1 in the pBABE-Hsf1WT-transduced RAW264.7 cells, Hsf1 was maintained in an inactive state, as demonstrated by the comparative steady-state levels of Hsp70 and Hsp105 between control cells and Hsf1-overexpressing cells (Fig. 5*A*). However, upon treatment with increasing concentrations of 17-AAG, Hsf1-overexpressing cells demonstrated an augmented response, denoted by increased Hsp70 and Hsp105 expression (Fig. 5*A*) in comparison with the vector control (pBABE-mCherry)-transduced cells. Consistent with this finding, RAW264.7 cells overexpressing Hsf1 were more sensitive to the effects of 17-AAG in enhancing osteoclast differentiation (Fig. 5*B*). Notably, however, Hsf1 overexpression did not alter the osteoclastogenic potential of RAW264.7 cells in the absence of 17-AAG. Therefore, elevated Hsf1 levels appeared to sensitize cells to the actions of 17-AAG rather than directly enhance osteoclast differentiation.

17-AAG-enhanced Osteoclast Formation Is Impaired in Primary Bone Marrow Cells Derived from Hsf1$^{-/-}$ Mice—To extend our findings, we examined the role of Hsf1 in 17-AAG-enhanced osteoclast formation in primary cells using bone marrow cells derived from mice that were wild-type (*Hsf1*$^{+/+}$), heterozygous (*Hsf1*$^{+/-}$), and null (*Hsf1*$^{-/-}$) for *Hsf1*. Immunoblot analysis showed that the expression level of Hsf1 in BMM derived from *Hsf*$^{-/-}$ mice was undetectable, whereas its level of expression in *Hsf1*$^{+/-}$ BMM was observed to be significantly lower than those isolated from wild-type (*Hsf1*$^{+/+}$) mice (Fig. 6*A*). Consistent with the steady-state levels of Hsf1 in isolated BMM, the induction of Hsp70 by 17-AAG was absent in the *Hsf1*$^{-/-}$ BMM and reduced significantly in the *Hsf1*$^{+/-}$ BMM when compared with *Hsf1*$^{+/+}$ BMM (Fig. 6*A*). We then examined osteoclast formation in *Hsf1*$^{+/+}$, *Hsf1*$^{+/-}$, and *Hsf1*$^{-/-}$ bone marrow cells (stimulated with 20 ng/ml RANKL and M-CSF) in the presence of 17-AAG. We found that, in *Hsf*$^{-/-}$ cell cultures, 17-AAG failed to significantly elevate osteoclast numbers relative to vehicle control cultures, whereas in *Hsf1*$^{+/+}$ bone marrow cultures, 17-AAG significantly enhanced osteoclast differentiation (Fig. 6, *B* and *C*). Bone marrow cell cultures from *Hsf1*$^{+/-}$ mice also showed a marked impairment of 17-AAG-enhanced osteoclast formation, consistent with the decreased steady-state and activated levels of Hsf1 in these cells (Figs. 6, *B* and *C*).

17-AAG Treatment Enhances MITF Levels in an Hsf1-dependent Manner—MITF has been shown to be a critical regulator of osteoclast formation and function (40–42), although the regulation of MITF protein expression in osteoclasts is not well characterized. We have shown previously that, although 17-AAG has no enhancing effect upon major RANKL-elicited intracellular signaling components (*e.g.* NFκB, c-fos, and NFATc1), we determined that 17-AAG did potently enhance the cellular protein levels of MITF (22). Therefore, we investigated whether 17-AAG enhanced MITF protein levels in a manner that was mediated via Hsf1. We found that increased MITF protein levels caused by 17-AAG treatment of RAW264.7 cells was reduced significantly by pharmacological inhibition of Hsf1 by KNK437 treatment (Fig. 7*A*). Similarly, knockdown of Hsf1 by shRNAmir also inhibited the effect of 17-AAG upon MITF protein induction (Fig. 7*B*). Examination of primary BMM cultures isolated from *Hsf1*$^{-/-}$ mice demonstrated that MITF protein was low or undetectable by immunoblot analysis, either with or without 17-AAG treatment (Fig. 7*C*). Conversely, overexpression of Hsf1 in RAW264.7 cells resulted in the elevation of MITF levels when compared with control cells and was increased further with 17-AAG treatment (Fig. 7*D*). These results indicate that 17-AAG increased RANKL-induced osteoclast differentiation with increased MITF protein levels, which was mediated by the action of Hsf1.

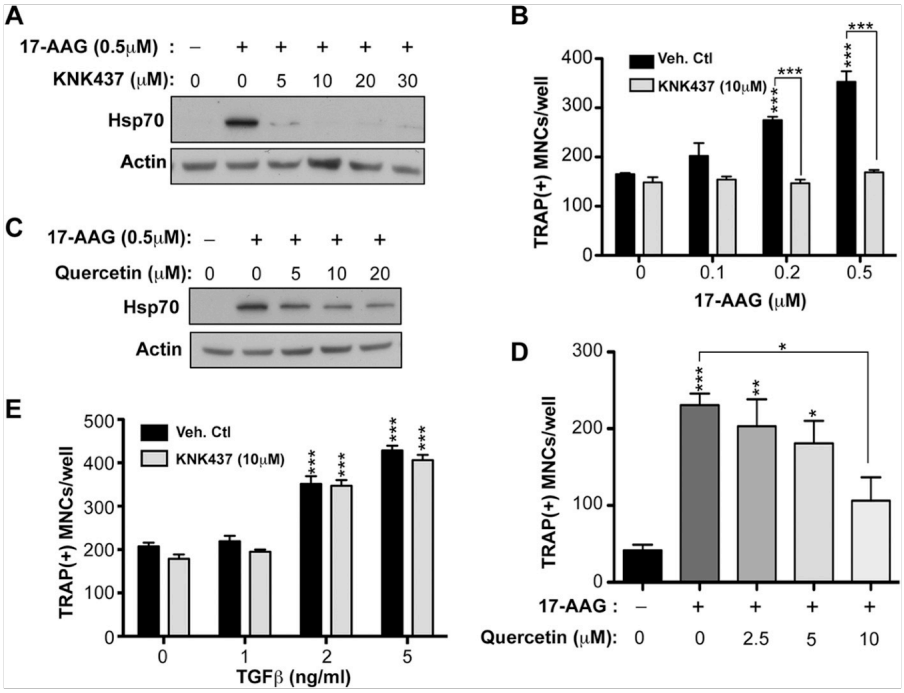

FIGURE 3. **Inhibitors of Hsf1 decrease the effects of 17-AAG, but not TGFβ, on osteoclastogenesis in RANKL-treated RAW264.7 cells.** *A*, immunoblot analysis demonstrated that 24-h cotreatment of RAW264.7 cells with KNK437 ablated 17-AAG-induced Hsp70 protein induction. *B*, KNK437 inhibited 17-AAG-enhanced osteoclast formation in RAW264.7 cells cultured for 6 days in the presence of 20 ng/ml RANKL. *Veh. Ctl.*, vehicle control. Quercetin reduced the effects of 17-AAG treatment on Hsp70 protein expression after 24 h of treatment (*C*) and inhibited the effects of 17-AAG upon enhanced osteoclast numbers in RANKL (20 ng/ml) treated RAW264.7 cells (*D*). *E*, RAW264.7 cells cultured with RANKL showed an increase in osteoclast numbers with TGFβ treatment. However, no effects of KNK437 treatment were observed. *Error bars* represent the mean ± S.E. of four independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to RANKL-treated (20 ng/ml) vehicle control using ANOVA/Dunnett's post hoc test.

Ethanol and Chemotherapeutic Agents Enhance Osteoclast Differentiation Potentially Mediated by Hsf1—Hsf1 is activated to counteract cellular damage and death caused by proteotoxicity of a wide variety of chemical agents. In addition to HSP90 inhibitors, many cytotoxic chemotherapeutic agents also potently activate Hsf1 and the HSR (43–46). Interestingly, some cytotoxic chemotherapeutic agents are also known to promote osteoclast formation (13). We hypothesized that at least some stress-inducing agents, including some currently used cytotoxic chemotherapeutics, may directly enhance osteoclastogenesis via activation of Hsf1 and the HSR (in a manner similar to that of 17-AAG) if the agent was not too directly toxic to RAW264.7 cells. We examined both ethanol, an oxidative stressor that has been shown previously to enhance osteoclastogenesis and induce the HSR (47, 48), and two cytotoxic chemotherapeutic agents, doxorubicin and methotrexate. Immunoblot analysis demonstrated that ethanol (Fig. 8*A*), doxorubicin (Fig. 8*B*), and methotrexate (Fig. 8*C*) all increased Hsp70 protein expression in a dose-dependent manner, consistent with their activation of Hsf1 and the HSR. Consistent with our previous findings regarding HSP90 inhibitors and the HSR, we observed that these three stressors, ethanol, doxorubicin, and methotrexate, all enhanced RANKL-dependent osteoclast formation in a dose-dependent manner in RAW264.7 cells (Figs. 7, *D–F*). As with 17-AAG, pharmacological inhibition of the HSR and Hsf1 by KNK437 in RAW264.7 cultures treated with ethanol (Fig. 7*G*), doxorubicin (Fig. 7*H*), or methotrexate (Fig. 7*I*) inhibited the pro-osteoclastic effects of the agents, although KN437 did not completely ablate the effects of methotrexate. In sum, these results demonstrate that, in addition to 17-AAG, other compounds that can induce Hsf1 activation and the HSR are also able to enhance RANKL-induced osteoclastogenesis, potentially through a mechanism that is at least partly Hsf1-dependent.

DISCUSSION

The ability of 17-AAG to cause bone loss and to increase breast and prostate tumor growth and invasion in bone in murine models indicates that this compound has potentially serious negative effects on bone mass (20, 21). Although 17-AAG itself is not likely to be used clinically, functionally similar, second-generation HSP90 inhibitors are currently undergoing clinical trials and may enter the clinic in the future. Thus, it is imperative to elucidate their effects on bone. 17-AAG and other HSP90 inhibitors have profound stimulatory effects on osteoclast formation (20–22, 49), although contributing influences of other cells to the observed bone loss cannot be ruled out (50). In addition, increases in osteoclast numbers and consequent increased bone resorption potentially increase the risk of metastatic tumor growth in bone because of the release of tumor growth factors from the bone matrix (51).

In this study, we demonstrated that 17-AAG and other stressors act in an Hsf1-dependent manner to increase osteoclast formation from their progenitors and that this may involve an increase in the levels of the transcription factor MITF. We investigated Hsf1 involvement in 17-AAG using a number of approaches, including the use of pharmacological inhibition of Hsf1 and HSR by KNK437. This compound is a potent inhibitor

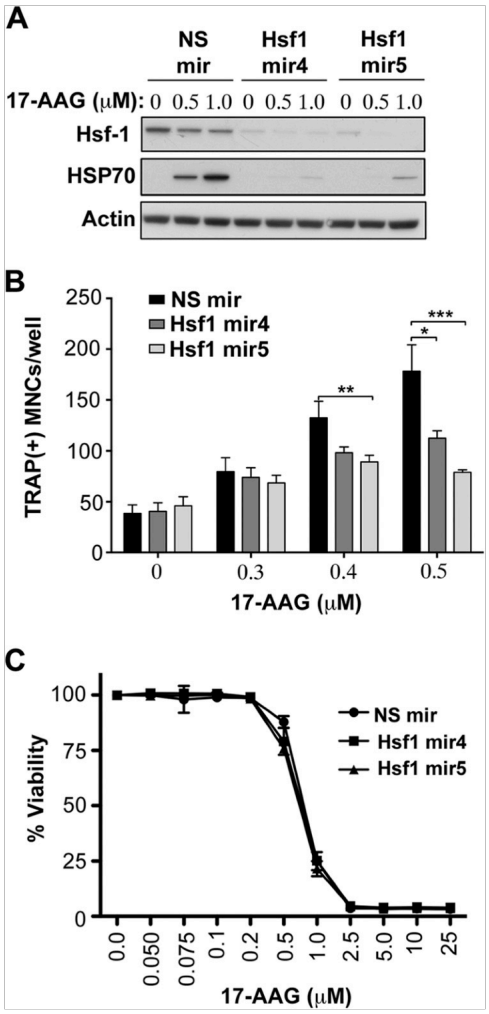

FIGURE 4. **Hsf1 knockdown greatly reduces the effect of 17-AAG upon RANKL-induced osteoclastogenesis.** *A*, lentivirus-transduced RAW264.7 cells with non-silencing (*NS mir*) or *Hsf1*-targeting shRNAmir (*mir4* and *mir5*) constructs were treated with the indicated concentrations of 17-AAG for 24 h. Immunoblot analysis demonstrated decreased Hsf1 protein levels, confirming knockdown. 17-AAG induction of Hsp70 protein was also impaired in the Hsf1 knockdown RAW264.7 cells. *B*, Hsf1 knockdown resulted in a significant reduction of the effects of 17-AAG on osteoclast formation in RAW264.7 cells cultured in 20 ng/ml RANKL. *C*, a dose-response survival assay over 96 h in RAW264.7 cells with indicated concentrations of 17-AAG demonstrated that Hsf1 knockdown had no significant effect upon RAW264.7 cell survival. *Error bars* represent the mean ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to RANKL-treated (20 ng/ml) vehicle control using ANOVA/Dunnett's post hoc test.

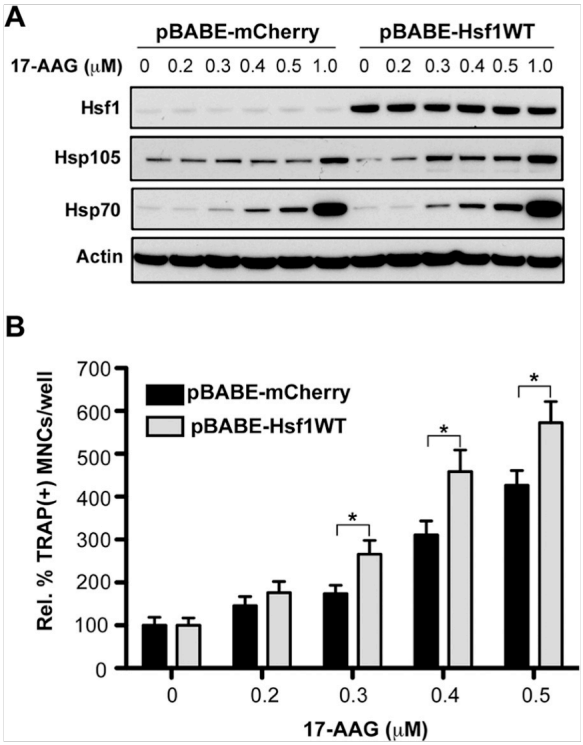

FIGURE 5. **Overexpression of Hsf1 enhances 17-AAG effects on RANKL-induced osteoclastogenesis.** *A*, RAW264.7 cells that had been retrovirus-transduced with pBABE-mCherry control or pBABE-Hsf1WT-mCherry constructs were treated with the indicated concentrations of 17-AAG for 24 h. Immunoblot analysis demonstrated that ectopic expression of Hsf1 increased the levels of Hsp70 and Hsp105 induced by 17-AAG in RAW264.7 cells. *B*, Hsf1 overexpression resulted in a significant increase in TRAP-positive osteoclasts in RAW264.7 cells (cultured in 20 ng/ml RANKL) at increasing concentrations of 17-AAG for 6 days relative to vehicle control. Data are presented as the proportion relative to control (*Rel. %*) ± S.E. from three independent experiments. *, $p < 0.05$ relative to RANKL-treated (20 ng/ml) vehicle control using ANOVA/Dunnett's post hoc test.

of Hsf1-induced expression of HSPs, such as HSP70, but does not affect the basal levels of their constitutively expressed isoforms (36). It was notable that KNK437 administration at 10 μM completely ablated 17-AAG actions on osteoclast formation but did not decrease it below the control baseline levels induced by 20 ng/ml RANKL alone. This concentration of KNK437 also blocked Hsp70 induction. Quercetin, a widely distributed, naturally occurring flavonoid, also reduces HSP induction and has acceptable toxicity in clinical trials (52, 53). However, it should be noted that quercetin also inhibits c-fos and NFκB actions that play a role in osteoclastogenesis, so its effects cannot be assumed to be via Hsf1 alone (54, 55). Quercetin is not used in any currently approved therapies but has been investigated for anticancer and anti-inflammatory actions, so its clinical use to ameliorate pathological bone loss is possible but has not yet been investigated properly.

To more specifically address the role of Hsf1 in 17-AAG osteoclast effects, we targeted *Hsf1* expression by shRNAmirs in RAW264.7 cells. Knockdown of Hsf1 had a similar effect to that of KNK437 in decreasing the effect of 17-AAG on RANKL-induced osteoclast formation as well as inhibiting the induction of HSP70 by 17-AAG. Bone marrow cells from *Hsf1*$^{-/-}$ mice were similarly defective in 17-AAG induction of osteoclast formation. However, the ability of the progenitors to form osteoclasts was not impaired because osteoclast formation in response to RANKL in *Hsf1*$^{-/-}$, *Hsf1*$^{+/-}$, and *Hsf1*$^{+/+}$ were all comparable. Unfortunately, because of fertility problems in these mice (56), we have not been able to undertake a systematic study of the bones or the influence of stressors on their bone parameters. However, with a role for Hsf1 being established, we also sought to identify the sufficiency of Hsf1 induction in mediating 17-AAG actions on osteoclasts. Ectopic overexpression of Hsf1 (28) did not increase osteoclast formation itself but did significantly increase the osteoclastic responsiveness of RAW264.7 cells to 17-AAG. The overexpressed Hsf1 probably remained in an inactive state because we observed no alteration in the steady-state levels of HSP70, indicating that

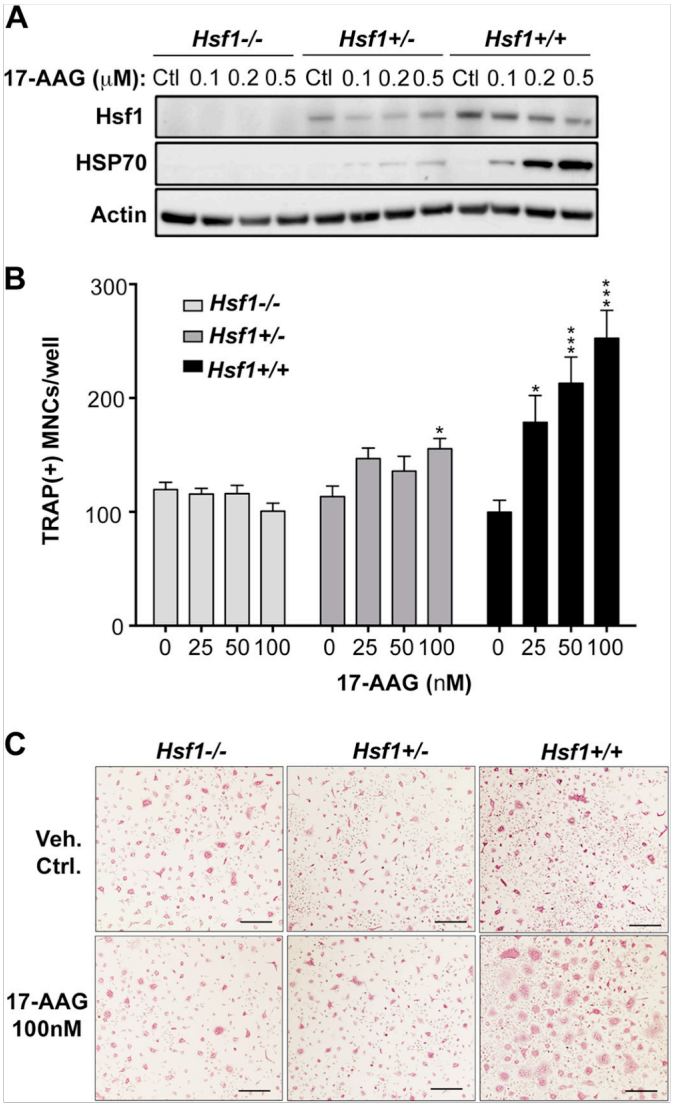

FIGURE 6. **The pro-osteoclastic effects of 17-AAG are impaired in bone marrow cells derived from Hsf1 null mice.** *A*, BMM derived from $Hsf1^{-/-}$, $Hsf1^{+/-}$, and $Hsf1^{+/+}$ mice were treated with the indicated concentrations of 17-AAG for 24 h. Immunoblot analysis showed a complete and partial reduction of Hsf1 protein in $Hsf1^{-/-}$ and $Hsf1^{+/-}$ cells, respectively. Consistent with this, no induction of Hsp70 was observed in $Hsf1^{-/-}$ cells, whereas in $Hsf1^{+/-}$ cells, there was a substantial reduction. *Ctl*, control. *B*, primary bone marrow cells derived from $Hsf1^{-/-}$, $Hsf1^{+/-}$, and $Hsf1^{+/+}$ mice were cultured in 20 ng/ml RANKL, M-CSF, and the indicated concentrations of 17-AAG for 6 days, fixed, and stained histochemically, and then TRAP-positive MNCs were counted. $Hsf1^{-/-}$ cells showed a lack of response to 17-AAG, whereas $Hsf1^{+/-}$ cells demonstrated a marked diminished response to 17-AAG treatment in terms of increased osteoclast formation. *C*, photomicrographs of TRAP-positive (*red*) osteoclast formation in $Hsf1^{-/-}$, $Hsf1^{+/-}$, and $Hsf1^{+/+}$ bone marrow cultures. *Veh. Ctl.*, vehicle control. *Scale bars* = 200 μm. *Error bars* represent the mean ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to RANKL-treated (20 ng/ml) $Hsf1^{+/+}$ control using ANOVA/Dunnett's post hoc test.

Hsf1 expression in itself is insufficient to increase RANKL-induced osteoclast formation but requires activation.

In addition to 17-AAG, we have found other benzoquinone ansamycins, such as herbimycin (20) and 17-DMAG, to also significantly increase RANKL-induced osteoclast formation. However, this action was not limited to this class of compounds because other structurally distinct HSP90 inhibitors, such as radicicol, NVP-AUY922, and CCT018159, also increased osteoclast formation (20, 22). Because these compounds all interact with the N-terminal ATPase site of HSP90, causing inhibition, it could be argued that ATPase site binding may be required for their common actions, and although they greatly stimulate the HSR, it may actually be the inhibition of HSP90 that is mechanistically important for enhanced osteoclast formation (28, 57). However, the HSP90 inhibitor novobiocin and its derivative coumermycin A1, known to inhibit HSP90 by binding the C-terminal of HSP90 and inhibiting its autophosphorylation (thus altering both its chaperone activity and client protein interactions), did not enhance RANKL-induced osteoclast formation (58–60). Moreover, we have found no clear correlation between the potency of the HSP90 inhibitors and their ability to induce osteoclast formation. It should also be noted that these compounds had a minimal effect on the induction of HSR, consistent with previous observations that novobiocin causes a dose-dependent decrease in Hsf1 DNA-binding and transcriptional activities (61). Combined, these results suggest that HSP90 inhibition *per se* may not enhance osteoclast formation and is consistent with a role for the involvement of Hsf1 downstream target involvement.

Although Hsf1 itself has not been suggested previously to play a role in osteoclast formation, several types of Hsf1-dependent cellular stressors have been implicated in pathological bone loss. These include chemotherapeutic agents, such as doxorubicin and methotrexate, that have been shown to cause a decrease in trabecular bone volume in a rat model (13, 62). Similarly, ethanol has been associated with the induction of cellular stress and enhances bone loss *in vivo* through the increase of osteoclast numbers (5). These observations provide circumstantial evidence that Hsf1-dependent cell stress induced by stimuli other than HSP90 inhibitors might indeed enhance osteoclastogenesis, although there is no reason to expect their actions to depend on a single mechanism. However, we confirmed here that ethanol, doxorubicin, and methotrexate cause both enhanced osteoclast formation and a cellular stress response that could be ablated by Hsf1 inhibition by KNK437. Thus, our results demonstrate, for the first time, that compounds capable of activating Hsf1-dependent stress pathways can enhance osteoclastogenesis in a manner similar to that of HSP90 inhibitors. It is important to note, however, that compounds that are simply very toxic to cells or that inhibit signaling essential to RANKL responses may not necessarily drive increased osteoclast formation.

Our findings that MITF levels may be involved in the actions of 17-AAG are particularly interesting. MITF is a transcription factor that is critical for osteoclast formation, as evidenced by the *mi/mi* strain of mice that lack MITF and are devoid of osteoclasts (63). In osteoclast progenitors, MITF levels are also enhanced by RANKL, which triggers a signaling cascade by its interaction with RANK, involving rapid induction of NFκB, p38, AP-1 and NFATc1 activity, and leading to increased MITF levels, typically after 24–48 h. The mechanism linking the elevation of MITF levels to the induction by RANKL treatment is currently controversial, but MITF is essential for many (but not all) gene expression that is required by mature osteoclasts, including TRAP (*acp5*), cathepsin K (*ctsk*), and H^+ ion pump components (40, 64). This requires cooperation

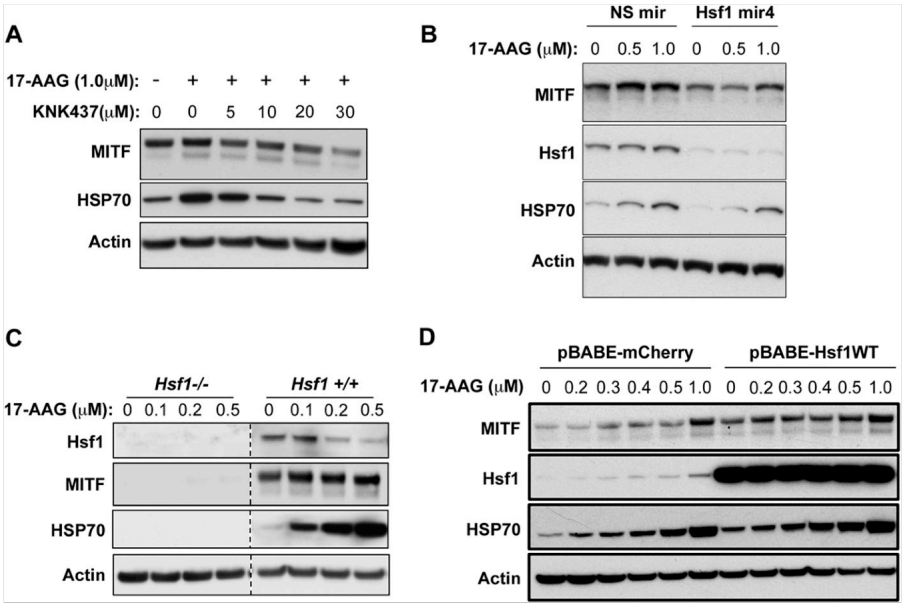

FIGURE 7. **The induction of MITF by 17-AAG osteoclast progenitor cells is dependent upon Hsf1.** *A–D*, protein expression was assessed by immunoblotting of the indicated cell lysates. *A*, Hsf1 inhibition by KNK437 ablated 17-AAG-induced MITF and Hsp70 protein levels after 24 h in RAW264.7 cells. *B*, RAW264.7 cells stably transduced with a lentiviral construct expressing Hsf1 shRNAmir showed a decrease in 17-AAG-induced MITF protein expression after 24 h. *NS*; nonsilencing. *C*, BMM derived from $Hsf1^{-/-}$ and $Hsf1^{+/+}$ mice were treated with M-CSF and the indicated concentrations of 17-AAG for 24 h. $Hsf1^{-/-}$ BMM showed lower MITF protein expression both with and without 17-AAG. *D*, RAW264.7 stably transduced with a retroviral construct expressing Hsf1WT showed an increase in MITF protein expression after 24 h of 17-AAG treatment.

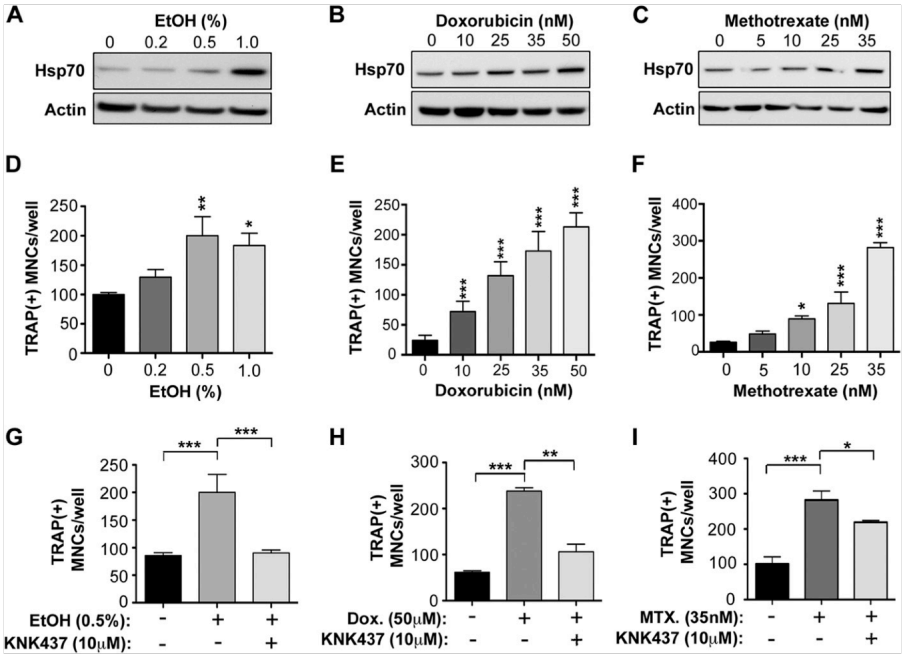

FIGURE 8. **Chemotherapeutic agents and ethanol induce a heat shock response and enhance RANKL-dependent osteoclastogenesis.** Hsp70 protein levels were induced in RAW264.7 cells by EtOH (*A*), doxorubicin (*B*), and methotrexate (*C*) over 24 h, as demonstrated by immunoblot analyses. *D*, RAW264.7 cells treated with RANKL showed a significant increase in osteoclast numbers after 6 days incubation with the indicated concentrations of ethanol. This was also observed when cultures were treated with doxorubicin (*E*) and methotrexate (*F*). *G*, KNK437 treatment inhibited the action of ethanol as well as that of doxorubicin (*Dox.*, *H*) and methotrexate (*MTX*, *I*) on osteoclast formation in RANKL-treated RAW264.7 cells. *Error bars* represent the mean ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to untreated control using ANOVA/Dunnett's post hoc test.

between MITF and the transcription factor PU.1 (the latter is not RANKL-dependent but binds MITF directly) together with AP-1, NFκB, and NFATc1. MITF is a relatively late-activated factor in osteoclast commitment, and it is possible that its induction by 17-AAG results in an increased pool of MITF that may be otherwise rate-limiting. Consistent with the latter, overexpression of MITF or the MITF-E isoform (the latter isoform is a particular target of RANKL) enhances osteoclast formation and action (65, 66). It should be noted that because MITF ablation abolishes osteoclast formation, its inhibition is not informative in addressing MITF mediation of 17-AAG effects.

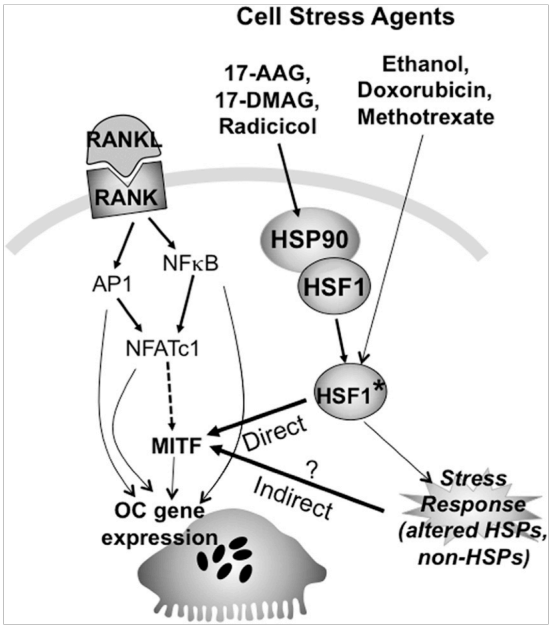

FIGURE 9. **A proposed model of 17-AAG and other cell stress agents upon osteoclast formation.** RANKL binding of RANK induces activation of the NFκB, AP1, and NFATc1 transcription factors. Subsequently, expression of MITF is raised. All four transcription factors are required for osteoclast gene expression. 17-AAG and other HSP90 inhibitors bind to HSP90, inducing a cellular stress response via Hsf1 activation that, in turn, enhances MITF levels either by directly stimulating the *MITF* promoter or indirectly by altering the expression of HSP and/or non-HSP target genes. Other stressor agents (*e.g.* chemotherapeutics) induce a stress response and activate Hsf1, leading to a similar series of events. Elevated MITF levels would, thus, result in an enhanced differentiation response of the osteoclast progenitors to RANKL and greater osteoclast formation. *Hsf1**, activated Hsf1; *OC*, osteoclast.

On the basis of our findings that 17-AAG increases MITF levels and osteoclast formation in a manner sensitive to Hsf1 inhibition, we propose that 17-AAG-induced Hsf1 enhances MITF protein levels and, thereby, amplifies the osteoclastogenic actions of RANKL. This proposed mechanism is summarized in Fig. 9 and incorporates our earlier finding that transcription factors activated early in the RANKL-dependent signaling cascade are not induced by 17-AAG, including NFκB, c-fos (the regulated subunit of AP-1), and NFATc1 (22). The latter findings suggested to us the possibility that a late-acting factor such as MITF would be a more obvious candidate for mediating 17-AAG actions. There is some evidence that MITF can be induced by heat shock elicited by Hsf1 via a direct action on the *MITF* promoter (67). However, for stress-stimulated osteoclast formation, it still has to be determined whether Hsf1 acts directly via *MITF* promoter interaction, by an indirect mechanism such as increased HSP expression that may increase MITF protein stability, or by a combination of both direct and indirect mechanisms. Nevertheless, our findings raise the possibility that any type of cell stress might enhance the levels of this transcription factor, contingent upon other effects the stressor exerts on cells. For example, some recently developed HSP90 inhibitors, such as SNX-2112 and PF-04928473, do not increase osteoclast formation at therapeutically relevant concentrations (49, 68) because, probably, these agents are more potent than 17-AAG at causing degradation of a number of HSP90 clients, such as NFκB, c-fos, NFATc1, and PU.1, which are critical for osteoclast differentiation.

In summary, we have identified a new role for Hsf1 and cell stress in the enhanced formation of osteoclasts that may be highly significant in bone physiology and pathophysiology beyond our focus here on the HSP90 inhibitor 17-AAG. This may result from enhancement of MITF levels, potentially through a direct action of Hsf1 on the *MITF* promoter. 17-AAG actions on osteoclasts may not be solely due to stress or Hsf1 induction, but, nevertheless, inhibition of Hsf1 seems to be a potentially useful approach to reducing osteoclast formation and osteolysis that may be induced by stressor compounds. If stress responses do directly increase the formation of osteoclasts by increasing the responsiveness of osteoclast progenitors to RANKL, we would speculate that other pathological osteolytic stimuli might act, at least in part, by increasing stress via Hsf1 activation and, thus, MITF levels, rather than increasing local net RANKL levels. This raises the possibility that blocking cell stress might reduce excessive pathological osteolysis without necessarily abolishing the bone resorption required for normal bone repair and remodeling.

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Chapter 5

General Discussion

5.1 INTRODUCTION

Despite the key role of HSP90 in the regulation of multiple pathways involved in cancer, HSP90 inhibitors have met with limited success in clinical trials as an anti-cancer treatment. One reason may be due to intrinsic or acquired resistance of cancer cells towards HSP90 inhibitors, which has been shown previously in a number of *in vitro* cancer models. In addition to treatment resistance, prolonged drug pressure can also result in phenotypical changes in tumour cells and the surrounding microenvironment such as the bone tissue. It is therefore critical to understand the mechanism of resistance to HSP90 inhibitors as well as the impact of chronic HSP90 inhibition on tumour cell biology as well as the tumour microenvironment to anticipate potential complications that may arise in the clinic, and to inform the design of better treatment strategies to improve clinical efficacy. To this end, the current study has provided insight into the molecular mechanisms of acquired resistance to a number of structurally diverse HSP90 inhibitors and the alterations in cell biology and metastatic potential of resistant cells. In addition, the current study also describes a novel HSF1-dependent mechanism of bone loss induced by HSP90 inhibition and other stressors.

5.2 SUMMARY OF FINDINGS

5.2.1 Acquired resistance to structurally diverse HSP90 inhibitors arises due increased HDAC activity.

HSP90 inhibitors are unique in that they can simultaneously inhibit multiple pathways that often interact to promote cancer growth and progression. The multi-targeted nature of HSP90 inhibitors was regarded to be more efficient in preventing the development of acquired resistance that is a common problem associated with more selective agents. However, previous studies have shown that chronic HSP90 inhibition in lung cancer and glioblastoma cells can result in the development of resistance towards the benzoquinone ansamycin (BA) family of HSP90 inhibitors that include 17-AAG and GA [1, 2]. The current study extends these findings by demonstrating that prolonged 17-AAG treatment with gradual dose escalation leads to acquired resistance to HSP90 inhibitors in breast cancer and melanoma cell lines. In addition, this study is also the first to demonstrate that cancer cells with acquired resistance to 17-AAG exhibit cross resistance to other classes of HSP90 inhibitors with distinct structures, including second generation HSP90 inhibitors. Although HSP90 inhibitors of the BA family are unlikely to be used clinically due to their high toxicity, functionally similar second generation compounds are currently under intense clinical evaluation and may enter the clinics in the foreseeable future. The cross resistance observed in our resistant cell model is therefore significant in this regard as it indicates an underlying mechanism that is common to different classes of HSP90 inhibitors.

Aberrant expression of HDACs have previously been associated with drug resistance in various cancers [3, 4] and the use of HDAC inhibitors has been shown to be effective in overcoming resistance to chemotherapeutic agents [5-7]. Moreover, HDAC inhibition resulted in the increased depletion of client proteins and apoptosis by 17-AAG in leukemia cell lines [8, 9]. The current study has identified a role for HDAC family members in acquired resistance to HSP90 inhibitors by demonstrating that HDACs 1 and 6 are upregulated at the transcriptional and protein level in MDA-231R cells compared to the parental cells. In addition, co-treatment of MDA-231R cells with HDAC inhibitors and 17-AAG induced more cell death than treatment with 17-AAG alone. Moreover, the inhibition of HDACs also sensitized MDA-231R cells to other HSP90 inhibitors that are structurally distinct from 17-AAG, such as radicicol and CCT018159. Contrary to our expectation, HSP90 was hyperacetylated in MDA-231R cells despite the elevated levels of HDACs 1 and 6, indicating that the underlying mechanism might not be acting through the modification of HSP90. It is possible that histone acetyltransferases (HATs) are upregulated in the resistant cells that resulted in HSP90 hyperacetylation and the increase of HDACs 1 and 6 acts as a compensatory mechanism to maintain acetylation homeostasis. Analysis of HAT expression may be able to confirm this. Furthermore, HDAC5 was significantly downregulated in the resistant cells, which may potentially lead to HSP90 hyperacetylation. Further investigation on the role of HDAC5 on the post-translational modification of HSP90 is warranted to address this possibility. Besides regulating HSP90 acetylation, HDACs are also associated with transcriptional regulation and the deregulation of their expression can lead to decreased response to chemotherapy, potentially through epigenetic silencing of pro-apoptotic and tumour suppressor genes [10, 11]. Indeed, the increased expression of HDACs 1 and 6 is consistent with the decrease in acetylation of histone 3 in resistant cells, suggesting that acquired resistance might arise due to the deregulation of chromatin modification. This was further supported by the resensitisation of the resistant cells to HSP90 inhibitors by a pharmacological inhibitor, SNDX-275 that is highly specific to the class I HDACs which have a predominantly nuclear localization. The expression of genes that are affected by HDACs to confer resistance to HSP90 inhibitors are as yet unclear and further studies are warranted to elucidate the downstream effect of HDAC upregulation in the resistant cells. Notably, HDAC inhibition only partially sensitized MDA-231R cells to HSP90 inhibitors, indicating that additional mediators of resistance are involved. Furthermore, we did not observe any change in the HDAC expression in MDA-435R cells and HDAC inhibition did not improve the sensitivity of these resistant cells to HSP90 inhibitors. Future studies will focus on elucidating the cellular context dependency of HDACs and other molecules that confer resistance. Collectively, our data is the first body of evidence to suggest that HDACs are instrumental in the underlying mechanism of acquired resistance to different classes of HSP90 inhibitors.

5.2.2 Phenotypical changes in cells with acquired resistance to HSP90 inhibitors

The current study is the first to characterize the biological properties commonly associated with metastasis in cancer cells with acquired resistance to HSP90 inhibitors. Our results show that the development of acquired resistance to HSP90 inhibitors is coupled with phenotypical changes that affect the ability of the resistant cells to grow and disseminate. Resistant cells of both models display a lower growth potential *in vitro* and *in vivo* compared to parental cells. Consistent with this, microarray analysis revealed that multiple genes involved in cell cycle progression are downregulated and CSC markers such as key genes in the Wnt signaling pathway is upregulated. Acquired resistance to HSP90 inhibitors as a consequence of chronic 17-AAG treatment might have resulted in the phenotypical switch that favours survival of CSC-like cells that have exited the cell cycle in the face of HSP90 inhibition. Further validation and analysis of CSC markers and associated cell biology in the resistant cells are needed to clarify this.

Our results have also revealed that cancer cells with acquired resistance to HSP90 inhibitors exhibit an enhanced migratory ability *in vitro*, which is consistent with the observations in other chemoresistant models of various therapies [12-15]. Concordantly, GSEA pathway analysis has identified the enrichment of many gene sets involved in cellular migration and cytoskeletal modification in the resistant cells. Furthermore, the increased migratory ability is consistent with EMT features observed in MDA-231R cells, which include a more mesenchymal morphology, upregulation of EMT mediators such as the transcription factor, Snail and increase in mesenchymal markers such as vimentin, ZEB-1 and N-cadherin. Despite the enhanced migration observed *in vitro*, an *in vivo* metastasis model revealed that the formation of secondary tumours in visceral organs, as well as skeletal sites, was impaired in the MDA-435R cells compared to the parental cells. Previous studies have shown that the volume of metastatic tumour mass is highly dependent on the proliferative and survival potential of the disseminated tumour cells (DTCs) once they are established at the secondary site [16-18]. DTCs have been correlated with CSC-like features that include low proliferation rate [19, 20], which is consistent with the upregulation of CSC markers in the resistant cells. Furthermore, tumour cells are subjected to various apoptotic stimuli during the process of metastasis and an increase in sensitivity to these stimuli will affect the establishment of metastatic growth at secondary sites [21]. Consistent with this, GSEA pathway analysis revealed an increase of a number of pathways associated with activation of apoptosis in the resistant cells. Taken together, the impaired growth properties coupled with decreased survival due to the activation of apoptotic pathways are the main limiting steps affecting the metastatic potential of the resistant cells despite the enhanced migratory ability observed *in vitro*.

Interestingly, despite the lower metastatic tumour burden in skeletal sites of mice inoculated with resistant cells, bone lesions genereated were of a similar size to that of mice inoculated with parental cells were observed. This suggests that the resistant cells are more potent at inducing osteolysis compared to the parental cells, possibly due to the upregulation of pro-osteolytic cytokines like

interleukins (IL-8 and IL-25) and chemokines (CXCL16, CXCL28 and CXCL24) as indicated by microarray analysis. This may in turn lead to the production of RANKL, an essential pro-osteoclastic factor by osteoblasts and stromal cells or direct activation of the pre-osteoclastic cells. Furthermore, multiple gene sets that are involved in immune cell activation were also enriched in the MDA-231R cells, which may also lead to the increased activation of myeloid cells that include the osteoclasts. Studies that investigate the expression of cytokines and other osteoclastic factors in the resistant cells should be pursued to further elucidate the underlying mechanism of the pro-osteolytic potential of resistant cells.

5.2.3 Molecular stressors enhance RANKL-dependent osteoclastogenesis in a heat shock dependent manner

Although HSP90 inhibition inhibits tumour growth in soft tissues and perturb signaling molecules involved in osteoclast formation, our group and others have shown that 17-AAG leads to the increase of osteoclastogenesis and osteoclast-dependent bone loss both *in vitro* and *in vivo* [22-24]. The current study extends the findings of these previous reports by showing that the increased RANKL-dependent osteoclast formation is correlated with the activation of the heat shock response by HSP90 inhibitors and other cellular stress inducers, such as ethanol, doxorubicin and methotrexate. Conversely, HSP90 inhibitors that are incapable of inducing heat shock response, such as novobiocin and coumermycin A1 had a minimal effect on osteoclast formation. This is a strong indication that the heat shock response regulated by the transcription factor HSF1 is involved in the enhancement of osteoclast formation. The abrogation of HSF1 activity with pharmacological inhibitors, KNK437 and quercetin was able to inhibit the expression of HSF1-induced HSPs and ablate the enhancing effect of HSP90 inhibitors on osteoclast formation. Knockdown of HSF1 expression using a more target-specific shRNAmir approach shows a similar effect in decreasing stress response and abrogating the enhancing effect of HSP90 inhibitor on osteoclast formation. To further confirm the role of HSF1 in osteoclast formation, a physiologically relevant model using primary bone marrow cells from HSF1-/- mice also failed to respond to HSP90 inhibitors to form osteoclasts. Notably, the abrogation of HSF1 activity and expression does not result in the impairment of osteoclast formation *in vitro*, suggesting that HSF1 is not required for the formation of osteoclast under non-stress conditions. However, HSF1-/- mice have defective fertility and are slightly runted, possibly due to defective bone development. Systematic characterization of the bones of these mice would shed more light on the role of HSF1 in bone metabolism. Furthermore, the role of HSF1 in stress-induced osteoclast formation can be characterized in *vivo* via the treatment of the HSF1-/- mice with HSP90 inhibitors. However, The overexpression of WT HSF1 does not alter osteoclast formation under non-stress conditions but results in increased responsiveness of osteoclast progenitors to HSP90 inhibitors. As discussed in section 1.2.1, HSF1 is bound to HSP90 under normal conditions and is released and undergoes trimerisation to become activated upon HSP90 inhibition. Thus the increased level of WT

HSF1 from overexpression has minimal impact in non-stress conditions but enhances the effect of HSP90 inhibitors on osteoclast formation. Furthermore, increased activation of the HSR by HSF1 overexpression may increase stress levels beyond the capacity of the cells to cope and thus leading to apoptosis [25]. We therefore postulate that this fine balance of the pro-osteoclastic and pro-apoptotic effect of HSF1 may explain the modest impact of the overexpression on osteoclast formation,

Collectively, these data demonstrate that HSF1 alone is not sufficient to increase RANKL-induced osteoclast formation but acts to enhance it during stress. A recent study by our group has revealed that 17-AAG enhances RANKL-dependent osteoclast formation without affecting pro-osteoclastic signaling molecules, such as NFATc1, c-Fos and NFkB expression or activity but significantly increases the protein level and activity of MITF [24]. Results in the current study confirmed the increased protein level of MITF by 17-AAG and the inhibition of HSF1 activity by KNK437 abrogated the induction of MITF by 17-AAG in osteoclast progenitors. Furthermore, MITF protein is largely absent and unresponsive to 17-AAG treatment in the bone marrow cells derived from HSF1-/- mice compared to cells derived from WT littermates. The overexpression of WT HSF1 in osteoclast progenitors also increased MITF protein level in the presence of 17-AAG. Consistent with these data, we also showed that MITF promoter contains HSF1-binding motifs, HSEs, suggesting that MITF expression is indeed HSF1-dependent. Chromatin immunoprecipitation (ChIP) analysis of HSF1 and the MITF promoter should be performed to confirm the role of HSF1 in the regulation of MITF expression. Studies should also investigate the possibility that MITF is dependent on HSF1 at the post-translational level for its stability and activity.

The enhancing effect on osteoclast formation is not limited to BAs, such as GA, 17-DMAG and 17-AAG. In fact, radicicol and other resorcinol-based inhibitors, such as CCT018159 and NVP-AUY922 were also capable of enhancing RANKL-induced osteoclast formation [24]. Furthermore, chemotherapeutic agents such as doxorubicin and methotrexate (MTX) have been implicated in the increase of osteolysis and decrease in bone volume in *in vivo* models [26-28]. In addition, alcohol has also been associated with the induction of cellular stress and increased bone loss *in vivo* [29]. Consistent with these reports, our data showed that doxorubicin, MTX and ethanol were able to induce heat shock response and enhanced RANKL-dependent osteoclast formation in a dose dependent manner. The inhibition of HSF1 with KNK437 abrogated the enhancing effect of these compounds on osteoclastogenesis, which strongly suggests that HSF1 activation induced by stimuli other than HSP90 inhibitors also has a major influence on RANKL-induced osteoclast formation. Further studies using HSF1 knockdown and overexpression models are required to confirm the effect of these compounds on osteoclast formation. It is also important to extend the study in the future to other chemotherapeutic agents to assess their ability to induce cellular stress response and enhance osteolysis. Collectively, the current study presents a strong body of evidence that link cellular stress response to increased osteoclast formation.

5.3 STRATEGIC COMBINATION OF HSP90 INHIBITORS AND HDAC INHIBITORS TO IMPROVE CLINICAL EFFICACY

Single agent activity for HSP90 inhibitors has been modest in the treatment of recurrent, refractory tumors in most trials published to date. This can be partly due to the poor pharmacokinetics and pharmacodynamics of the first generation of HSP90 inhibitors that have been used in these trials. Furthermore, HSP90 facilitates malignant progression indirectly through the chaperoning of multiple proteins without functioning as a classical oncogene in the sense of driving the process itself [30]. This explains that when used as a monotherapy, HSP90 inhibitors have demonstrated mostly cytostatic activity resulting in disease stabilisation but very rarely regression [30]. Therefore targeting HSP90 in combination with other anticancer treatments might be a better strategy to improve overall prognosis of patients. Consistent with this notion, the first significant outcome in clinical trials of 17-AAG was reported in breast cancer patients with HER2 overexpression in combination with the HER2 inhibitor, trastuzumab [31]. Synergistic effects of 17-AAG and the proteasome inhibitor, bortezomib have also been observed in multiple myeloma patients [32]. Unfortunately, minimal objective responses were observed in clinical trials of first generation HSP90 inhibitors in combination with other conventional chemotherapeutic agents [33-35]. The results presented in the current study have extended the current repertoire of HSP90 inhibitor combination treatments by providing a 'proof-of-principle' that concurrent treatment with HDAC inhibitors can potentially combat the development of acquired resistance to HSP90 inhibitors and improve anticancer efficacy. HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) and depsipeptide (Romidepsin) are FDA-approved and have been shown to be clinically effective against haematological cancers [36]. Preclinical studies have shown that the combination of 17-AAG and HDAC inhibitors are highly effective in leukemia cells [8, 9]. Furthermore, the second generation HSP90 inhibitor, NVP-AUY922 has also been shown to act synergistically with SAHA in inhibiting the viability of multiple myeloma cells [37]. It has been reported that HDAC inhibition augments the effect of HSP90 inhibitors through the induction of HSP90 hyperacetylation [8]. The present study however shows that HDAC inhibitors can enhance the effect of HSP90 inhibitors in a HSP90 independent manner, possibly through chromatin remodeling that can result in the upregulation in pro-apoptotic genes and/or the downregulation of survival genes as reported previously [38].

The partial reversal of acquired resistance to HSP90 inhibitors as shown in this study also provides a 'proof-of-principle' that HDAC inhibitors could potentially be used to eradicate tumour cells that develop resistance to HSP90 inhibitors. In fact, HDAC inhibitors have been shown to re-sensitize cisplatin-resistant bladder cancer cells to treatment [39]. Sharma *et al* also showed that resistance to erlotinib in prostate cancer cells is mediated by global changes in the chromatin and the drug resistant subpopulation can be eliminated by HDAC inhibitors [4]. However, the concurrent treatment with

HDAC inhibitor initially might prevent the emergence of cells resistant to HSP90 inhibitors in the first place.

5.4 CHRONIC HSP90 INHIBITION AND CANCER CELL BIOLOGY

Tumours consist of genetically and epigenetically heterogeneous populations of cells due to inherent genetic instability [40]. Lindquist and colleagues have shown that HSP90 allows unstable genetic variants of many proteins to retain "wild-type" biochemical activity to maintain cell viability [41]. However, the capacity of HSP90 as a buffer for genetic aberrations can be overwhelmed in disease states such as cancer due to an increasing load of mutant and/or misfolded oncoproteins, the hostile tumour microenvironment and pharmacological interventions such as HSP90 inhibitors [30]. This can then lead to the unmasking of the underlying genetic variation and the subsequent increase in phenotypic variation, such as the emergence of tumour cell populations with altered cell biology and responsivity to treatment. This warrants caution for sub-lethal levels of HSP90 inhibitor treatment, which may uncover the genotypic diversity of tumour populations and result in malignant progression. Indeed, the current study shows that prolonged 17-AAG treatment leads to profound phenotypical changes in cancer cells compared to the untreated parental cells. While prolonged 17-AAG treatment results in the development of acquired resistance, the metastatic potential of the resistant cells are impaired compared to untreated parent populations. This is in contrast to the observation of increased aggressiveness in cancer cells that have developed resistance to other chemotherapeutic agents [42-44]. We speculate that the impaired metastatic potential of cancer cells treated chronically with HSP90 inhibitor might be due to the selection of CSC-like cells that are survival-driven and dormant. This is supported by a recent study showing that the stable downregulation of heat shock proteins in tumour cells is followed by long-term tumour dormancy *in vivo* [45].

In addition, our study also shows that the resistant cells are highly osteolytic despite the decrease in the formation of metastatic tumours. It is widely accepted that HSP90 inhibitors can lead to increased osteolysis through activation of osteoclast formation [24, 46, 47] but the current study is the first to show that prolonged HSP90 inhibition can also result in the acquisition of a pro-osteolytic phenotype in tumour cells that can enhance the severity of osteolytic bone lesions, possibly through the stimulation of osteoclast formation. Further investigations on the effect of tumour cells resistant to HSP90 inhibitors on bone physiology may reveal the underlying mechanism of enhanced osteolysis in these cancer cells. Taken together, the current study shows that in addition to acquired resistance, prolonged HSP90 inhibition also leads to aberrant changes in cancer cell biology potentially by compromising HSP90 buffering capacity on cancer cell evolution.

5.5 STRESS-INDUCING AGENTS AND THE BONE MICROENVIRONMENT

It is increasingly recognized that cellular stress, such as oxidative stress, hypoxia, acidosis and chemotherapeutic drugs have a major negative impact on the bone by increasing bone loss and fracture risk [48-50]. The current study extends these findings by demonstrating that the HSF1-driven cellular stress response plays a role in osteoclast formation and bone loss potentially through MITF activation. In addition, the presence of tumour cells in the bone results in local acidosis, hypoxia and oxidative stress, which all induce HSF1-dependent stress response [51-53] and can potentially lead to increased osteoclast formation. This can be further magnified by the treatment of tumours by stress-inducing chemotherapeutic agents. Furthermore, results from chapter 3 showed that tumour cells that developed resistance to HSP90 inhibitors are more pro-osteolytic and this can further exacerbate the direct effect of HSP90 inhibitors on osteoclast formation.

The findings from the current study should serve as a caution that compounds intended for cancer therapy should be examined for their ability to increase osteolysis that could lead to impaired bone health. Although chemotherapeutics such as cyclophosphamide, methothrexate and fluorouracil have been reported previously to cause bone loss in cancer patients [54], there have been no bone-related complications reported in patients from clinical studies involving HSP90 inhibitors. This may partly be attributable to the lack of monitoring of bone integrity as a standard procedure in the trials involving HSP90 inhibitors. Furthermore, a low level chronic osteolytic reaction would only be apparent over an extended treatment period and/or in the design of specific clinical trials that examine the effects of treatments upon bone integrity through the use of bone degradation markers in the urine [55]. In order to fully assess potential effects of HSP90 inhibitors on bone, long term monitoring of bone loss should be included as a standard procedure in trials, as reported previously for trials involving other chemotherapeutics [56, 57]. The findings from this study also raise a potential use of HSF1-targeting therapies to prevent or reverse bone loss associated with HSP90 inhibition and/or other chemotherapeutic treatments by reducing excessive pathological osteolysis without abolishing the bone resorption required for normal bone repair and remodeling. In addition, potential combination treatments of HSP90 inhibitors with anti-osteolytic compounds such as bisphosphonate that are clinically available should also be investigated to overcome the potential deleterious effects of HSP90 inhibitors and other stress-inducing agents on bone.

5.6 CONCLUDING REMARKS

The findings from this thesis have extended our current understanding on the impact of HSP90 inhibition on tumour progression and the bone microenvironment through the characterization of the molecular and phenotypical changes in cancer cells with acquired resistance to HSP90 inhibitors as well as establishing a role for HSF1-dependent cellular stress response in osteoclast formation. This thesis has also provided a rationale for novel treatment strategies to prevent or reverse the development of resistance to HSP90 inhibitors as well as to abrogate the pro-osteolytic effect of these compounds.

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