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BAG-1 expression and function in HER2-positive breast cancer

Presented by

Natalia H. Robson

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Declaration

This thesis is the result of original work performed whilst in registered postgraduate candidature under the supervision of Mr Ramsey Cutress and Professor Graham Packham within the Cancer Sciences Unit at the University of Southampton. Unless otherwise stated I carried out this work entirely myself and it forms no part of any other degree or thesis.

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Abstract

Background: In the United Kingdom every year 50,000 women are diagnosed with breast cancer, and 12,000 deaths are attributed to the disease. Human epidermal growth factor receptor 2 (HER2/ neu) is overexpressed in 15- 25% of breast cancer and is a marker of poor prognosis. Treatment with the monoclonal antibody trastuzumab (Herceptin™) can reduce the risk of death by a third and halve the risk of recurrence, but many of those treated do not benefit. The potential for combination treatments is seen in neoadjuvant studies where combination anti-HER2 therapies show greater efficacy than single anti-HER2 therapy.

Methods: BAG-1 is a multifunctional protein frequently overexpressed in breast cancer. BAG-1 interacts with Hsc70/ Hsp70 and the serine/threonine kinase RAF-1 to promote cell survival. This study investigated the potential of targeting BAG-1 in HER2-positive breast cancer. The functional significance of BAG-1 expression in HER2-positive breast cancer cells was investigated using BAG-1 overexpression, and siRNA knockdown. The effect of HER2 inhibition in combination with BAG-1 protein-protein inhibition using Thioflavin S was investigated with cell viability and cell yield assays. Western blots of upstream and downstream proteins were used to explore proposed signalling mechanisms involved. Previous evidence for BAG-1 as a biomarker in breast cancer was assessed against published reporting criteria (REMARK). An immunohistochemical study of BAG-1 expression was performed in a test cohort of ER-positive breast cancers, using the pan-isoform antibody, 3.10G3E2, and a novel BAG-1L isoform-specific antibody.

Results: An increase in endogenous BAG-1L expression occurred following trastuzumab treatment of SKBR3 cells. Additionally overexpression of BAG-1 isoforms by transfection in SKBR3 cells exposed to trastuzumab resulted in an increase in cell number and relative cell viability compared to vector controls. Conversely, the combination of siRNA targeting of BAG-1 and exposure to trastuzumab enhanced reduction of cell viability. The decrease in relative cell viability following trastuzumab treatment was also enhanced following combination with Thioflavin S, a BAG-1:HSC70

interaction inhibitor, in BT-474 cells. This was accompanied by reduced expression of phospho-Akt, suggesting a possible mechanistic link between BAG-1, which interacts directly with RAF-1, and the HER2 signalling pathway.

With a view to determining whether the expression of BAG-1L is elevated in HER2 positive cancers, a pan-isoform anti-BAG-1 antibody (3.10G3E2) and the novel BAG-1L-specific antibody were optimised for immunohistochemical study using tissue microarrays. Further work is required prior to use on tissue from a large cohort of patients diagnosed with HER2-positive breast cancer, however in a second cancer type in which HER2 is known to be overexpressed, oesophageal adenocarcinoma, immunohistochemistry demonstrated an association between advanced tumour regression grade and nuclear BAG-1 expression.

In summary, this work has demonstrated potentiating effects of BAG-1 expression on HER2 signalling pathways, possibly mediated through Akt phosphorylation. The work provides evidence that BAG-1 has the potential to play a role in HER2-positive breast cancer and affect the response to trastuzumab; therefore inhibition of the BAG-1/ RAF-1/Akt pathway may be a potential therapeutic target when used in combination with anti-HER2 therapies.

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List of Abbreviations

ADP Adenosine diphosphate

AQUA Automated quantitative analysis

AR Androgen receptor

ATP Adenosine triphosphate

BrdU Bromodeoxyuridine

BSA Bovine serum albumin

cDNA Complementary deoxyribonucleic acid

CHIP Carboxyl terminus of Hsc70-interacting protein

DCIS Ductal carcinoma in-situ

DMEM Dulbecco's modified Eagles medium

EGFR Epidermal growth factor receptor

ER Oestrogen receptor

ERK Extracellular signal-regulated kinase

FACS Fluorescence-activated cell sorting

GFP Green fluorescent protein

GR Glucocorticoid receptor

GST Glutathione-S-transferase

HGF Hepatocyte growth factor

HER2 Human epidermal growth factor-2

Hsc Heat shock conjugate

Hsp Heat shock protein

IRES Internal ribosome entry sequence

NHR Nuclear hormone receptor

NLS Nuclear localisation sequence

NMR Nuclear magnetic resonance

MAPK Mitogen-activated kinase

MR Mineralocorticoid receptor

mRNA Messenger ribonucleic acid

MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

PARP poly ADP ribose polymerase

PBS Phosphate Buffered Saline

PI3K Phosphophatidylinositol kinase

PR Progesterone receptor

RAR Retinoic acid receptor

RT-PCR Reverse transcriptase polymerase chain reaction

SCC squamous cell carcinoma

SDS-PAGE SDS-Polyacrylamide Gel Electrophoresis

SD Standard deviation

siRNA Small interfering ribonucleic acid

TBS Tris-buffered saline

TMA Tissue microarray

TR Thyroid hormone receptor

ULD Ubiquitin-like domain

UTR Untranslated region

VDR Vitamin D receptor

XTT Sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium) inner salt. N-methyl dibenzopyrazine methyl sulphate functioning as the intermediate electron carrier

Summary of Aims and Objectives

Chapter 1: Introduction

- Review the existing knowledge of BAG-1 structure and function in breast cancer.
- Compare and contrast existing evidence for the role of BAG-1 in proliferation and survival.
- Critically appraise the clinical evidence for BAG-1 in breast cancer using the Reporting Recommendations for Tumour Marker and Prognostic Studies (REMARK) criteria.
- State the hypothesis that a role for BAG-1 exists in HER2-positive breast cancer due to the existence of convergent signalling pathways.

Chapter 2: Materials and Methods

- Novel work: Describe the optimised protocol for immunohistochemical study of BAG-1 in breast cancer using the pan-isoform antibody and a BAG-1L-isoform-specific antibody 3C12 in whole sections and tissue microarray.

- Chapters 3 and 4 use 2 different techniques to explore the functional role of BAG-1 in HER2-positive breast cancer. Chapter 3 uses overexpression studies of BAG-1 using HER2-positive cell lines. The advantage of using overexpression to study BAG-1 is that it is possible to elucidate the roles of individual BAG-1 isoforms. In Chapter 4 knockdown of BAG-1 is achieved in HER2-positive cells using siRNA. siRNA knockdown of BAG-1 provides functional information about the role of BAG-1 at endogenous level, but is not isoform-specific.

Chapter 3: BAG-1 as a potential target in HER2-positive breast cancer

- Overall chapter aim: to provide evidence for the functional role of BAG-1 in HER-positive breast cancer cells by overexpression of individual BAG-1 isoforms.
- Identify endogenous levels and subcellular localisation of BAG-1 in HER2-positive breast cancer cell lines using Western blotting and immunofluorescence.
- Elucidate the effects of overexpression of BAG-1 isoforms on proliferation and relative metabolic activity of HER2-positive breast cancer cells using simple proliferation assays and MTS assays.
- Confirm BAG-1 isoform expression and subcellular localisation by performing consecutive Western blotting and immunofluorescence for biological assays.
- Investigate the effects of overexpression of BAG-1 isoforms on clonogenic potential compared to pcDNA3 and BAG-1 mutants. Confirm BAG-1 expression via Western blotting.
- Establish the optimal concentration of different HER2 family inhibitors for use in biological assays using relative cell viability (MTS) assays.
- Demonstrate BAG-1 dose-response to trastuzumab using Western blotting to further identify the optimal concentration for biological assays.
- Explore the effects of HER2 family inhibitors on the proliferation and relative metabolic activity in HER2-positive breast cancer cells using repeat biological assays using the optimal concentration derived from previous assays.
- Compare the effects of HER2-inhibition on proliferation and relative metabolic activity on cells overexpressing BAG-1 isoforms with those of the topoisomerase-2 inhibitor, doxorubicin, using Western blotting. Confirm the subcellular localisation and relative changes in BAG-1 expression using consecutive immunofluorescence studies.
- Establish if changes in BAG-1 expression and functional effects following HER2 inhibition could be secondary to apoptosis alone using Western blotting of apoptotic markers following exposure of cells to trastuzumab and doxorubicin.
- Elicit if effects seen on BAG-1 expression in HER2-positive breast cancer cells are specific to HER2 family inhibition or occur with other chemotherapeutic agents, such as the anthracycline doxorubicin (Adriamycin).
- Demonstrate concordance between BAG-1 protein and mRNA expression using Western blotting and consecutive qPCR following trastuzumab.

- Confirm the functional effects of overexpression of BAG-1 isoforms are specific to HER2-positive breast cancer cells and HER2 receptor family inhibition by repeating biological assays, Western blotting, and immunofluorescence in ER-positive, HER2-negative MCF-7 cells.

Chapter 4: Crosstalk between BAG-1 and HER2 signalling in HER2-positive breast cancer.

- Overall chapter aim: to provide evidence for the functional role of BAG-1 in HER2-positive breast cancer by knockdown of endogenous levels of all 3 BAG-1 isoforms.
- Demonstrate endogenous expression and localisation of BAG-1 isoforms in different breast cancer cell lines using Western blotting and immunofluorescence.
- Optimise small interfering RNA (siRNA) knockdown of BAG-1 expression in HER2-positive breast cancer cells for subsequent biological assay.
- Establish the effects of trastuzumab on BAG-1 expression in HER2-positive breast cancer cells with and without siRNA knockdown of BAG-1.
- Utilise crystal violet assays to assess the relative density of adherent cells following trastuzumab, alone and in combination with the BAG-1 inhibitor, thioflavin S.
- Investigate the effect of siRNA-induced reduction of BAG-1 expression on proliferation and relative metabolic activity in HER2-positive breast cancer cells.
- Propose a common signalling pathway and mechanism for crosstalk between BAG-1 and HER2 signalling via Western blotting of upstream and downstream signalling proteins following trastuzumab, thioflavin S, or in combination.

Chapter 5: BAG-1 expression in breast, bladder, and oesophageal carcinoma.

- Overall chapter aim: Identify association between clinicopathological parameters and expression of BAG-1 in cancers known to overexpress HER2 for future design of an immunohistochemical study using samples from a treatment-standardised patient cohort and a novel BAG-1L-specific antibody.

- To correlate BAG-1 expression with clinicopathological outcomes with an immunohistochemical study of breast, bladder, and oesophageal adenocarcinoma. Discuss potential for extending study to a treatment-standardised HER2-positive cohort and the existing evidence for HER2 inhibition in bladder and oesophageal cancer.
- Optimise the immunohistochemical protocol for the pan-isoform antibody, 3.10G3E2, and the BAG-1L-specific antibody, 3C12, using both whole sections and tissue microarray.
- Discuss any differences seen between results obtained using immunohistochemistry of whole sections and tissue microarray.
- Trial of automated quantitative analysis (AQUA) following construction of a 'virtual tissue microarray' using a test cohort from the TEAM Trial.
- Demonstrate antibody specificity of both the pan-isoform antibody 3.10G3E2 and the BAG-1L isoform-specific antibody using immunodepletion studies.
- Establish the isotype of the BAG-1L isoform-specific antibody, 3C12, using the Quick Type-M Monoclonal Antibody Isotyping Kit.

Chapter 6: Discussion.

- Discuss advantages of using overexpression models versus siRNA knockdown to investigate BAG-1 expression and function in HER2-positive breast cancer.
- Discuss the following results and associated implications for future work:-
    - Endogenous BAG-1 expression is increased in response to trastuzumab in HER2-positive breast cancer cells.
    - Overexpression of all BAG-1 isoforms protects cell proliferation and relative cell metabolic activity in HER2-positive breast cancer cells exposed to trastuzumab.
    - The functional effect of overexpression of BAG-1 is specific to HER2 inhibitors, but increased expression of endogenous BAG-1 occurs with inhibition of other EGFR family receptors.
    - siRNA targeting of BAG-1 confers a decrease in proliferation in SKBR3 cells exposed to trastuzumab.
    - The combination of trastuzumab and the BAG-1 inhibitor, thioflavin S produce a synergistic decrease in relative cell metabolic activity in HER2-positive breast cancer cells.

  - Altered PI3K/ MAPK signalling protein expression is found in HER2-positive cells following dual inhibition of BAG-1 and HER2.
  - BAG-1 is a potential biomarker in oesophageal adenocarcinoma and is predictive of advanced tumour regression grade.
- Propose future work to support a role for BAG-1 in HER2-positive breast cancer.

Chapter 1: Introduction

Chapter 1: Introduction

Approximately 50,000 women and 350 men were diagnosed with breast cancer in the United Kingdom in 2011. Over 11,600 women and 70 men died of breast cancer in 2012 (http://www.cancerresearchuk.org/cancer-info/cancerstats/types/breast/). The most common type of breast cancer is invasive ductal carcinoma. Ductal cancer of the breast develops from cells that line the lobules of breast or the terminal duct lobular unit. Ductal cancer which has not spread beyond the duct lining is known as ductal carcinoma-in situ (DCIS). Other types of breast cancer are referred to as special type, these include lobular carcinoma. Lobular carcinoma is less common and represents 10% of all breast cancers. Lobular carcinoma is more common in women aged 45-55 and is associated with multifocal tumours. The other breast cancers known as special type are rare and are identified by microscopic features identified by the pathologist. The majority of the special type breast cancers are associated with a favourable prognosis. 87% of females diagnosed with breast cancer will survive for 5 years or more.

The treatments available for breast cancer are surgical resection, chemotherapy, radiotherapy and biological therapies. Surgery includes mastectomy, wide local excision of the cancer, and removal of the axillary lymph nodes with axillary clearance or sentinel node biopsy. Breast conservation such as wide local excision followed by radiotherapy, or mastectomy with breast reconstruction are available to the majority of female patients. Therefore it is important to offer women these aesthetically more acceptable forms of surgery and minimise morbidity whilst preserving optimal oncological outcome. Breast cancers can be classified into those expressing the oestrogen and progesterone receptor (ER-positive, PR-positive), and those which do not (ER-negative PR-negative). Hormone therapy is one of the mainstays of breast cancer targeted therapy and drugs such as Tamoxifen block oestrogen receptor (ER) signalling. Breast cancers are also analysed for amplification of human epidermal growth factor-2 (HER2), a transmembrane tyrosine kinase receptor.

Breast cancers overexpressing the HER2/ neu gene have higher rates of recurrence, shorter disease-free and overall survival, and account for 15-25% of breast tumours diagnosed (Slamon et al., 1987; Slamon et al., 1989). Biological therapies such as the monoclonal antibody trastuzumab (Herceptin™) have made a significant impact on the survival of

patients diagnosed with HER2-positive breast cancer (Mayer et al., 2015). The mechanism of action of trastuzumab is complex. One of the mechanisms better understood is increased degradation of the HER2 receptor (Klapper et al., 2000). A further mechanism is the activation of antibody-dependent cytotoxicity to tumour cells expressing HER2 (Arnould et al., 2006). Trastuzumab also inhibits mitogen-activated kinase (MAPK) and phosphatidylinositol kinase (PI3K) signalling pathways, leading to cell growth arrest and suppressing tumour proliferation (Nahta and Esteva, 2006). However, trastuzumab is currently only licensed for use in combination with chemotherapy and resistance rates in HER2-positive tumours have been reported as high as 74%(Vogel et al., 2002). The diversity of breast cancer has led to a more complex classification system. Breast cancers can be divided into several molecular subtypes associated with different prognoses and response to different therapies. 'Luminal a' cancers are ER-positive and low grade, 'luminal b' cancers are ER-positive and often high grade,' basal-like' cancers are often triple negative for ER, PR and HER2, 'normal type', 'Claudin low' cancers lack tight junction proteins, and HER2-enriched cancers (Prat and Perou, 2011).

The discovery of predictive biomarkers in breast cancer may help to ensure patients can be offered the most acceptable form of treatment whilst maintaining optimal oncological outcome. This could mean radical surgical resection or the adverse effects following chemotherapy could be avoided in certain patients. Predictive biomarkers can also be used to identify a cohort of patients with breast cancer who will benefit most from a certain therapy, or who have a higher chance of recurrence. Assays incorporating a number of predictive biomarkers in breast cancer are now commercially available. Oncotype Dx™ (Genomic Health, Ca) uses reverse transcriptase polymerase chain reaction (RT-PCR) to analyse breast tumours for 5 reference genes and16 genes considered predictive of breast cancer recurrence (Sparano and Paik, 2008). B cell lymphoma-2-associated-athanogene-1 (BAG-1) is one of the 16 genes incorporated into Oncotype Dx™ and is associated with improved prognostic outcomes (Sparano and Paik, 2008).

BAG-1 (Bcl-2-associated athanogene-1) is an evolutionarily conserved protein with homologues ranging from yeast to plants and humans. BAG-1 was originally discovered as a Bcl-2 interacting protein with anti-apoptotic activity in a screen of a mouse embryonic cDNA library in which recombinant human Bcl-2 was used as bait to identify novel binding partners(Takayama et al., 1995). Within the same year, a separate group identified the same protein, which they called RAP46 (Receptor Associated Protein 46kDa) as a glucocorticoid

receptor binding protein in a screen of a human liver expression library (Zeiner et al., 1999). BAG-1 is now known to be a multifunctional protein, which can interact with diverse cellular targets to modulate a plethora of cell signalling cascades regulating cell survival, proliferation, migration, apoptosis, autophagy, proteasomal degradation and transcription under physiological or pathological conditions.

BAG-1 is frequently over-expressed in numerous tumour cell lines (Takayama et al., 1998) and human cancers including invasive breast carcinoma. Overexpression of BAG-1 is protective of breast cancer cell lines following stress induced by heat shock (Townsend et al., 2003), enhances oestrogen receptor (ER) signalling (Cutress et al., 2003), and has been associated with favourable clinicopathological characteristics (Millar et al., 2009). BAG-1 is overexpressed in HER2-positive cell lines, but the function of BAG-1 in HER2-positive breast cancer is still to be investigated (Yang et al., 1999).

One of the proposed mechanisms of action of trastuzumab is inhibition of HER2-driven signalling via both the mitogen-activated protein kinase (MAPK) and PI3K pathways (Nahta and Esteva, 2006). BAG-1 binds the proto-oncogene Raf-1 to phosphorylate the MAPK kinases MEK1 and MEK2, which activate the extracellular signal related kinases ERK1 and ERK2 and promotes cellular proliferation (Sharp et al., 2009b). Thus, it is logical that by investigating how levels of BAG-1 and associated signalling proteins vary in HER2-positive breast cancer cell lines in response to trastuzumab will assist understanding of this common pathway. BAG-1 has been shown to increase breast cancer cell survival via the potentiation of MAPK signalling (Anderson et al., 2010). The mediation of HER2 signalling via MAPK by BAG-1 could be one mechanism by which BAG-1 could promote resistance to trastuzumab.

Investigation of BAG-1 for more than a decade and has revealed a wealth of binding partners and potential pathways of action, however, the exact molecular mechanisms by which it exerts its effects remain unclear. Apoptosis is involved in embryogenesis, defence mechanisms, and tissue homeostasis. The deregulation of apoptosis can lead to the development or progression of cancer (Cory and Adams, 2002). BAG-1 has been implicated in the abrogation of luminal apoptosis in MCF-10A normal mammary epithelial cells (Anderson et al., 2010). Luminal apoptosis is crucial in development of normal mammary architecture. BAG-1 has been shown to reduce luminal apoptosis in MCF-10A cells

producing cells with filled lumens, a phenotype seen in DCIS. This study demonstrates a mechanism by which BAG-1 may orchestrate the disruption of normal apoptotic signalling and play a role in early carcinogenesis.

1.1 BAG-1 structure and localisation.

The BAG-1 gene is located on the human chromosome 9 band 12 and consists of seven exons (Takayama et al., 1995). In humans, BAG-1 is expressed as multiple isoforms via alternate translation initiation from a single mRNA to form three main isoforms with molecular weights of 36 kDa (BAG-1S), 46 kDa (BAG-1M) and 50 kDa (BAG-1L) (Packham et al., 1997). BAG-1S is translated from an AUG codon and BAG-1L from a proximal CUG codon. Both are more abundantly expressed in humans than BAG-1M, also translated from a CUG codon. A fourth isoform of approximately 29kDa has been inconsistently detected in MCF-7 and HeLa cell lines (Yang et al., 1998). The production of the BAG-1S isoform is reliant upon an internal ribosome entry site (IRES) in the 5'-untranslated region (UTR) of BAG-1 mRNA and occurs via a cap-dependent pathway (Coldwell et al., 2001).

Discrete domains common to these isoforms have been identified; an ubiquitin-like domain close to the amino terminus, a BAG domain, and variable number of copies of acidic amino acid repeats. The ubiquitin-like domain (ULD) allows conjugation of substrates targeting them for proteosomal degradation (Takayama et al., 1995), while the BAG domain is an approximately 70 amino acid sequence at the carboxy terminus end, which interacts with the peptide-binding domain of Hsc/Hsp70. The function of the acidic amino acid repeats is not clearly defined, although these sequences play a role in the regulation of glucocorticoid receptor-dependent transcription (Schneikert et al., 1999).

Nuclear magnetic resonance (NMR) spectroscopy experiments reveal the Hsc-70-binding domain of BAG-1 comprises three α-helices. Functional domains in the binding of BAG-1 to the chaperone molecule Hsc-70 were demonstrated by mutagenesis. Surface residues envisaged as integral to chaperone binding were identified by chemical shift analysis and computer modelling. Mutant BAG-1 molecules with Alanine substitutions of these residues in α2 and α3 helices resulted in failure to bind Hsc-70, but Alanine substitution in α1 helices did not inhibit chaperone binding. Alanine substitution of corresponding residues in the bi-lobed ATP-ase region of Hsc-70 shown to bind BAG-1 inhibited BAG-1/Hsc-70 binding (Briknarova et al., 2001).

High-resolution crystal structure analysis permits further definition of BAG-1 domain and Hsc-70 ATP-ase interaction. Highly preserved residues in all known BAG proteins contact Hsc-70 ATP-ase subdomains IA and IIB propagating exposure of a nucleotide-binding cleft and promoting ATP-driven nucleotide exchange. BAG-1 provides a nucleotide-free state for the Hsc-70 ATP-ase, thus establishing a further role for BAG-1 as a nucleotide exchange factor (Sondermann et al., 2002; Sondermann et al., 2001). BAG-1 mediates nucleotide exchange via the BAG domain which causes a conformational switch in the ATP-ase and mediates interaction of chaperone molecules with protein substrates (Brehmer et al., 2001). The BAG domain and the ULD demonstrate structural contiguity between mechanisms for protein folding and proteolytic degradation (Luders et al., 2000).

BAG-1 isoforms have distinct cellular localisation; BAG-1S is predominantly cytoplasmic, as is BAG-1M, but BAG-1M can be transported into the nucleus via interaction with the glucocorticoid receptor (Schneikert et al., 1999). BAG-1L contains a nuclear localisation sequence (NLS) and localises mainly within the nucleus (Brimmell et al., 1999; Schneikert et al., 1999; Takayama et al., 1998). Knee et al. (2001) used deletional mutations and NLS-constructs to show nuclear isoform BAG-1L requires the NH2-terminal binding sequence to enhance androgen receptor transcriptional activity in cells. Deletion of the ubiquitin-like domain in BAG-1L did not affect increased AR activity. Knee et al. (2001) suggested the subcellular location of the BAG-1L isoform accounted for its ability to interact with nuclear AR, however, despite the ability of BAG-1M to translocate to the nucleus, no enhancement of AR activity is found in vivo (Knee et al., 2001). Addition of a NLS to the BAG-1S isoform does not augment ER transcriptional activity, therefore the ability of BAG-1L to stimulate ER is more complex than binding or nuclear localisation alone (Cutress et al., 2003).

Immunohistochemical study of BAG-1 in the murine mammary gland showed an absence of BAG-1 during embryogenesis, but it was present in adult mice. Expression of BAG-1 altered from nucleus to cytoplasm between lactation and later involution implicating BAG-1 in a physiological role in mediating apoptosis in mammary epithelia (Schorr et al., 1999).

1.2.1 Direct binding partners of BAG-1

1.2.1.1 Heat shock protein Hsp70

BAG-1 has multiple molecular binding partners, the most studied being the Hsp70 heat shock protein family. The interaction between BAG-1 and Hsp70 is multifunctional and plays a role in transcription, translocation, and regulation of binding partners in forming multimeric complexes.

Takayama et al. (1998) reported binding of recombinant BAG-1 to a 70kDa protein, later identified via screening of complementary DNA (cDNA) libraries as Hsc/ Hsp70 (Takayama et al., 1998). The specificity of binding between BAG-1 and Hsp70 was confirmed as assays using both endoplasmic Hsc70 (BiP) and the bacterial form of Hsp70 (Dnak) with BAG-1 failed to show interaction (Zeiner et al., 1999). The N-terminal domain of Hsc/Hsp70 binds ATP and subsequent BAG-1 independently of the chaperone molecule carboxy terminal domain (Takayama et al., 1997). Pretreatment with dephosphorylating alkaline phosphatase prior to GST-BAG-1 experiments induces interaction between Hsc/Hsp70 and BAG-1. Interaction between BAG-1 and Hsp70 was quantified by the incorporation of radiolabelled Hsp70. Hsc/Hsp 70 are phosphoproteins and phosphorylation of Hsp70 is crucial in their function, and therefore it is logical that phosphorylation should inhibit binding BAG-1 and subsequent Hsc/Hsp70-BAG-1 complex activity (Zeiner et al., 1997). Isothermal titration microcalorimetry and comparative molecular modelling with the homologous protein GrpE and its interaction with DnaK, have shown that BAG-1 binds Hsc/Hsp70 with a 1:1 monomeric stoichiometry (Stuart et al., 1998).

Biochemical in vitro assays have shown that BAG-1 regulates chaperone activity of Hsc/Hsp70 by mediating refolding of proteins. BAG-1M and BAG-1S have been implicated in inhibition of refolding of heat-denatured β-galactosidase (Gebauer et al., 1997; Luders et al., 2000; Takayama et al., 1997). However, Luders et al. (2000) also reported contrasting findings suggesting BAG-1S stimulated protein refolding. The conflicting evidence might be explained by variation in reagents and the quantities used for each assay (Gassler et al., 2001). The underlying mechanism of the stress response co-ordinated by BAG-1-Hsp70 interaction involves the folding and refolding of proteins. Hohfeld et al. (1998) showed Hsc70/Hsp70-mediated regulation of refolding damaged substrates helps preserve function

in response to cellular stress (Hohfeld, 1998). Mutation of the Hsc70/Hsp70 binding site confers loss of this prosurvival effect in BAG-1S (Townsend et al., 2005).

The diverse functional roles of BAG-1 appear to rely repeatedly on its interaction with Hsc/Hsp70 (Alberti et al., 2003). Hsp70 preserves cell function by binding to incomplete peptides and subsequent interaction with BAG-1, acting as a nucleotide exchange factor, permits adenosine diphosphate (ADP) hydrolysis. The synthesised polypeptide substrate can then undergo folding or proceed along a functional pathway (Hohfeld and Jentsch, 1997). Structural analysis using x-ray crystallography revealed the BAG-1 BAG domain opens the ATPase nucleotide binding cleft of Hsc70 to allow ADP release (Sondermann et al., 2001). The resulting increase in adenosine triphosphatase (ATPase) activity relies upon the additional presence of the heat-shock protein 40 (Hsp40). Hsp40 confers ADP-bound BAG-1 thereby regulating chaperone function (Gassler et al., 2001; Hohfeld and Jentsch, 1997; Sondermann et al., 2001).

BAG-1 is able to simultaneously bind Hsc70 and interact via the ubiquitin-like domain (ULD) with the proteasome (Luders et al., 2000). Elevating the amount of intracellular BAG-1 is associated with increased ATP-dependent interaction between Hsc70 and the proteasome. A proposed further role for BAG-1 is the ability to target protein substrate released by the chaperone to the proteasome for proteolytic degradation (Demand et al., 2001). Protein substrate released by Hsc/Hsp70 are bound by BAG-1 thereby allowing interaction of associated proteins with carboxyl terminus of Hsc70-interacting protein (CHIP) (Ballinger et al., 1999). CHIP can facilitate the ubiquitination of chaperone substrates such as glucocorticoid hormone receptor (GR) and Raf-1 kinase. Immunoprecipitation experiments using COS-7 cells co-transfected with BAG-1S, CHIP and GR demonstrated BAG-1/CHIP-induced GR degradation (Demand et al., 2001). CHIP functions as an E3 ligase in the ubiquitination of Raf-1 kinase in bacterial lysates (Demand et al., 2001). CHIP provides a mechanism by which BAG-1 may promote a switch in the function of Hsc/Hsp70 from protein folding to degradation (Demand et al., 2001; Luders et al., 2000). The degradation pathway may be regulated by the BAG-1/Hsc70/CHIP complex as the addition of a polyubiquitin chain to BAG-1 is facilitated by CHIP, targeting the complex to the proteasome (Alberti et al., 2002).

The interaction between BAG-1 and Hsc/Hsp70 contributes towards cell behaviour by participating in several key functional pathways. BAG-1 exerts its prosurvival effect partially

through its association with Bcl-2. Interaction with Bcl-2 is ATP-dependent, thereby implicating phosphoproteins such as Hsc70 (Takayama et al., 1997). Decreased expression of Bcl-2 in C2C12 myogenic cells causes decreased Hsp70-mediatedprotection against oxidative stress and release of the apoptotic mitochondrial protein, Smac. Selective inhibition of Bcl-2 protein expression using an antisense oligonucleotide can inhibit Hsp70-mediated protection against hydrogen peroxide-induced oxidative stress. Levels of the mitochondrial protein, Smac, are increased, in addition to activated caspases 3 and 9, indicating increased apoptosis (Jiang et al., 2011). However, there is currently no evidence to support the role of a BAG-1/ Hsp70/Bcl-2 complex in regulating mitochondrial activity.

Another cellular process involving both BAG-1 and Hsc70 is stress-signalling. BAG-1 increases proliferation under normal physiological conditions, but on application of stress it functions as a molecular switch to reduce proliferation and increase Hsc/Hsp70 chaperone activity (Song et al., 2001). This switch in functional activity between proliferation via Raf-1 signalling to Hsc/Hsp70–mediated degradation is discussed in section 1.2.2.2.

BAG-1 and Hsc/Hsp70 interaction has also been shown to play a role in the regulation of transcription. In vitro transcription assays have shown the addition of the BAG-1M homolog, HAP46, results in significantly larger RNA molecules than in the absence of BAG-1 (Zeiner et al., 1999). The addition of Hsp70 to the assay did not increase transcription enhanced by BAG-1 (Zeiner et al., 1999). However, electrophoretic mobility shift assays identified the nucleic acid binding ability of BAG-1. Deletional mutants of the amino acid terminal, but not the carboxyl terminal abrogated the DNA-binding ability of BAG-1 (Zeiner et al., 1999). A further shift was observed when DNA fragments were exposed simultaneously to BAG-1 and Hsc70 (Zeiner et al., 1999). Approximately half of Hsc70 bound to GST-HAP46 fusion protein attached to GSH-Sepharose was found to dissociate at 42°C. Controls showed GST-HAP46 (BAG-1M) to be stable at raised temperatures and DNA complexes with Hap46 alone showed no increased mobility shift on exposure to heat (Zeiner et al., 1999). HeLA, DU145 and 3T3 Swiss cells transfected with green fluorescent protein (GFP)-tagged BAG-1 showed preferential nuclear localisation at 42°C using confocal laser-scanning microscopy (Zeiner et al., 1999). These results support the hypothesis that BAG-1 interacts with Hsc/Hsp70 during heat-stressed conditions to preserve transcriptional activity.

BAG-1 and Hsc70/Hsp70 interaction has been reported to regulate growth inhibition demonstrated by assays following blockage of the Hsc70 ATPase binding domain with a

small peptide inhibitor (Sharp et al., 2009b). Peptide assays were used to find overlapping peptides in the binding regions of BAG-1/Hsc70. The BAG-1 C-terminal consists of three alpha helices, helices 2 and 3 interacting with the ATPase region of Hsc70/Hsp70. The resultant small peptides were coupled to Penetratin and incubated with ZR-75-1 or MCF-7 cells. MTS assays were then performed, but this type of assay assesses only the amount of enzymatic metabolic activity of the cells, not growth or proliferation (Figure 1.3). We can, therefore, only conclude from the Sharp et al. (2009) study that inhibition of BAG-1 and Hsp70 binding reduces cellular metabolic activity in breast cancer cells (Sharp et al., 2009b). In the absence of consecutive experiments to show that the results are not due to an increased amount of apoptosis and less metabolising viable cells, interpretation of these results is limited. Small peptides of only 12 of the 120 amino acid C-terminal domain are effective at blocking BAG-1-initiated growth in MCF-7 and ZR-75-1 breast cancer cell lines. Thioflavin S, a small molecular inhibitor, blocks BAG-1/Hsc70 interactions and suppresses metabolic activity of viable ZR-75-1 cells overexpressing BAG-1. BAG-1 deficient mice embryo fibroblasts showed no effect when treated with thioflavin S (Sharp et al., 2009a). To establish whether BAG-1 has a definite role in breast cancer cell proliferation, further experiments incorporating consecutive MTS-type assays, bromodeoxyuridine (BrdU) or Ki-67 staining, in addition to apoptotic assays, need to be performed. The use of small peptide inhibitors, like thioflavin S, does provide encouraging support for therapeutic targeting of BAG-1 signalling mechanisms.

BAG-1 isoforms have been shown to have the ability to enhance transcriptional activity. BAG-1M has been shown under normal physiological conditions to increase cytomegalovirus (CMV) early gene promoter transcriptional activity (Takahashi et al., 2001). Deletional mutations of the BAG-1M Hsc/Hsp70 binding domain and the nucleic acid-binding amino terminus decreased CMV promoter activity (Takahashi et al., 2001). BAG-1S and BAG-1L showed only weakly increased CMV promoter-induced transcription.

BAG-1M has been shown to decrease DNA binding of the glucocorticoid receptor (GR) (Kullmann et al., 1998). Overexpression of RAP46, homologous to BAG-1M, in mouse S49.1 thymoma cells has been shown to inhibit glucocorticoid-induced apoptosis (Kullmann et al., 1998). BAG-1M downregulation by rapamycin results in an increase in glucocorticoid-induced apoptosis, although this effect may be compensated for by overexpression of BAG-1M (Kullmann et al., 1998). The ability of BAG-1M to downregulate GR transcriptional activity has been localised to the first 8 amino acids of the N-terminal region by DNA binding

and gel shift assays (Schmidt et al., 2008). Molecular chaperones assist nuclear hormone receptors in the cytoplasm by promoting folding to allow binding of the appropriate steroid hormone. Hsp70, and other molecular chaperones such as Hsp90 and Hsp70/Hsp90 organising protein (Hop), orchestrate folding GR in the cytoplasm prior to GR being able to bind steroid hormones (Pratt and Toft, 1997). BAG-1S, BAG-1M and BAG-1L have all been found to decrease chaperone folding activity (Bimston et al., 1998; Hohfeld and Jentsch, 1997; Nollen et al., 2001; Zeiner et al., 1997). BAG-1 has been shown to compete with one chaperone, Hsp70-interacting protein (hip), to decrease glucocorticoid binding (Nollen et al., 2001). However, not all studies have shown a BAG-1-induced decrease in binding of steroid hormone (Schneikert et al., 1999). The activity between BAG-1/ Hsp70/ GR occurs within the cytoplasm, but evidence has shown BAG-1 also has a nuclear role in regulating GR transcription (Zeiner et al., 1999). Confocal immunofluorescence has shown that in the presence of steroid hormone BAG-1M is transported from the cytoplasm by activated GR. In the absence of glucocorticoid, BAG-1M is predominantly seen in the cytoplasm (Schneikert et al., 1999). Interestingly, the negative regulatory effect of BAG-1 on GR transcription can be nullified in a cell-free system by increasing the amount of Hsp70, suggesting a concentration-dependent function (Schneikert et al., 2000). The interaction between BAG-1 and nuclear hormone receptors is discussed further in section 1.2.2.3.

1.2.2.2 Raf-1

Raf-1 is a proto-oncogene which is involved in all aspects of growth control including cell proliferation, differentiation and apoptosis (Zebisch and Troppmair, 2006). Gene knock-out studies have revealed that Raf-1 has a general role in tissue formation as genetic ablation of the Raf-1 gene results in loss of viability and gross developmental defects in mice that are most apparent in placenta, lung and skin (Wojnowski et al., 1998). Raf-1 encodes a serine/threonine protein kinase which can be recruited to the plasma membrane and activated directly or indirectly by Ras, a GTPase. Phosphorylated Raf-1 activates ERK1 and ERK2 mitogen activated protein kinases (MAPKs) via phosphorylation of MEK1 and MEK2 MAPKKs which are present in high concentration to amplify signalling.

ERK1 and ERK2 can phosphorylate a large number of proteins, which are often regulatory in nature, and are located both in the cytoplasm and the nucleus. Transcription factors such as c-myc (Alvarez et al., 1991) and p53 (Wang and Shi, 2001) can be phosphorylated by ERK to control cell growth. Upstream proteins of the MAPK cascade including the EGFR and HER2 epidermal growth factor receptors, Raf-1 and MEK are in fact substrates for ERK, thus it is possible that phosphorylation of ERK serves as a feedback mechanism for the upstream components that lead to its activation (Fritsche-Guenther et al., 2011).

The first evidence indicating that BAG-1 physically associates with Raf-1 came from studies by Wang et al. (Wang et al., 1996). Using co-transfections of insect or mammalian cells with recombinant BAG-1 and different domains of Raf-1 they found that the catalytic domain of Raf-1 is sufficient for interactions with BAG-1. The BAG-1/Raf-1 interaction was also shown in a yeast two-hybrid system, suggesting that the cellular machinery required for BAG-1-mediated activation of Raf-1 is conserved throughout evolution. Additionally, Bag-1 was reported to enhance Raf-1 enzymatic activity directly in vitro, which could be related to its function as a chaperone that maintains Raf-1 in an active state rather than as a direct function. An N-terminal deletion mutant of BAG-1 interacted with comparable efficiencies to the full-length protein with in vitro translated 35S-Raf-1 protein, indicating that the ubiquitin-like domain of BAG-1 is not required for its interaction with Raf-1(Wang et al., 1996).

Further in vitro binding assays by Song et al. using truncated recombinant BAG-1 protein precisely mapped the Raf-1 binding region of BAG-1 and showed partial overlapping with the Hsp70 binding region of BAG-1. Binding of Raf-1 to BAG-1 is tighter than that of Hsp70 as

very high concentrations of Hsp70 are required to displace Raf-1 from the overlapping binding region. Furthermore, activation of Raf-1 by BAG-1 is independent of Ras and is repressed by Hsp70. These results suggest that under physiological conditions BAG-1 signalling is via Raf-1 and results in activation of ERK MAPKs promoting cellular proliferation. However, in the event of cellular stress when Hsp70 levels become higher, Hsp70 will displace Raf-1 thereby negatively regulating BAG-1 and inhibiting cell growth (Song et al., 2001).

Work by Anderson et al. (2010) investigated whether the interaction of BAG-1 with Raf-1 is essential for suppressing luminal apoptosis in immortalized BAG1 over-expressing breast epithelial MCF-10A cells grown in 3D cultures. BAG-1 over-expression confers a decrease in luminal apoptosis of acini, a phenotype observed in invasive breast carcinomas and ductal carcinoma in situ. A reduction in activated caspase 3 and M30 expression, both markers of apoptosis, was found in BAG-1 over-expressing cells. Inhibition of MEK using the specific U0126 compound causes loss of preservation against luminal apoptosis in cells over-expressing BAG-1 (Anderson et al., 2010). No significant change in levels of BAD or Bcl-2 was found but, expression of BIM was suppressed leading to loss of apoptotic signalling and phenotype, indicating the importance of BIM in this pathway.

The relationship between Raf-1 and different isoforms of BAG-1 was not investigated in the study by Anderson et al. (2010). However, regulation of transcription factors by Raf-1 signalling is likely to involve BAG-1L and BAG-1M due to their ability to localise in the nucleus, rather than the shorter cytosolic isoform BAG-1S (Yang et al., 1998). Given the strong phenotypic correlations between the MCF-10A 3D model and mammary morphogenesis in vivo (Debnath and Brugge, 2005), these data also suggest that the BAG-1:Raf-1 interaction may be of importance in the early phases of breast cancer development through a disruption in the normal cellular architecture of the mammary gland. Targeting BAG-1: Raf-1 interaction provides an effective novel strategy for therapeutic intervention in breast cancer. Sharp et al. (2009) have identified Thioflavin S as a potent inhibitor of Bag-1 and showed that this compound has the potential to inhibit basal ERK phosphorylation as well as disrupt the interaction between endogenous BAG-1 and Raf-1 in in MCF7 breast cancer cell line (Sharp et al., 2009a). Thioflavin S is a small chemical compound used to stain amyloid in neurofibrils (Wei et al., 2005). Binding assays measuring luciferase activity in MCF-7 cells showed activation between BAG-1 and Hsc/Hsp70 was also decreased by thioflavin S (Sharp et al., 2009a). This study investigates the effect of thioflavin S and

inhibition of BAG-1 interaction in HER2-positive cells alone and in combination with trastuzumab (Chapter 4 Figure 4.8).

1.2.2.3 Nuclear Hormone Receptors

BAG-1 is expressed in hormone responsive tissues such as breast, prostate and thyroid and its overexpression observed in human cancers. BAG-1 isoforms have the ability to bind nuclear hormone receptors (NHR) and regulate transcriptional activity (Figure 1.2). NHRs shown to interact with BAG-1 include oestrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR, discussed in section 1.2.2.1), retinoic acid receptor (RAR), vitamin D3 receptor (VDR), thyroid receptor (TR) and androgen receptor (AR) (Cutress et al., 2003; Knapp et al., 2012; Schneikert et al., 1999; Liu et al., 1998; Witcher et al., 2001; Guzey et al., 2000; Zeiner et al., 1995; Knee et al., 2001). BAG-1 isoforms may bind NHR indirectly via Hsc70/Hsp70 ATPase binding region, or bind directly via the carboxy terminal of the BAG domain (Schneikert et al., 1999).

BAG-1 isoforms have distinct subcellular localisation (Yang et al., 1998; Knee et al., 2001) but both nuclear and cytoplasmic isoforms are capable of interaction with NHR. Cytoplasmic Hsc/Hsp70 and associated chaperones interact with BAG-1 and assist in the folding and preparation of NHR for binding steroid (Pratt et al., 1997). The Hsc/Hsp70-binding domain at the carboxy terminal of BAG-1 has been shown to be essential for BAG-1L activation of AR, implicating cytoplasmic Hsc/Hsp70 in linking nuclear BAG-1L to NHR activity (Knee et al., 2001).

Expression of the nuclear isoform BAG-1L increases AR transcription, shorter BAG-1 isoforms can bind AR, but not affect AR transcription (Knee et al., 2001). Targeting BAG-1M to the nucleus does result in enhanced AR transcription, proving subcellular localisation of BAG-1 isoforms to be important to NHR function (Knee et al., 2001). Deletional mutant analysis revealed that both the amino terminal of BAG-1 and the Hsc/Hsp70-binding carboxy domain are required for regulation of AR activity (Knee et al., 2001).

Overexpression of the nuclear isoform BAG-1L has been associated with regulation of transcriptional activity of a number of other NHR; Vitamin D receptors (Guzey et al., 2000; Witcher et al., 2001), glucocorticoid receptors (Schneikert et al., 1999) and, crucially, ER (Cutress et al., 2003).

Co-immunoprecipitation experiments using ALVA31 and LNCaP prostate cancer cells have demonstrated that BAG-1L interacts with vitamin D receptors(Guzey et al., 2000). BAG-1L increases transactivation of reporter gene plasmids with a vitamin D response element (Guzey et al.., 2000). DNA synthesis is suppressed and action of vitamin D increased by gene transfer-mediated BAG-1L protein expression in PC3 prostate cancer cells (Guzey et al., 2000). Contrasting results were found using U87 glioblastoma cells to study the effects of BAG-1 on VDR regulation. Glutathione S-transferase (GST) binding assays were used to show BAG-1L bound the VDR. Electrophoretic mobility shift assays (EMSA) using U87 cells stably transfected with BAG-1L showed VDR transcription and vitamin D-induced inhibition was reduced. Cell growth rate was increased in U87 cells overexpressing BAG-1L in the presence of vitamin D relative to controls (Witcher et al., 2001). A later study using oral keratinocytes supported the findings of Guzey et al. (2000). The shorter BAG-1S isoform was shown to be the most abundant in H376, TERT-1 and NK oral keratinocyte cells. Confocal immunofluorescence demonstrated subcellular localisation of BAG-1 isoforms was unchanged by exposure to vitamin D. Reporter gene assays measuring luciferase activity showed overexpression of BAG-1L in H376, TERT-1 and NK cells potentiated VDR transactivation and VDR-mediated transcription. These results suggest a role for BAG-1L in regulating VDR activity and decreasing the development of oral carcinomas (Lee et al., 2007).

The 46kDa BAG-1 protein was initially identified by the screening of a human liver lambda gt11 expression library with GR (Zeiner and Gehring 1995). Immunofluorescence and EMSA studies using COS-7 kidney cells have revealed that activated GR can recruit the BAG-1M isoform from the cytoplasm into the nucleus where BAG-1M reduces GR transactivation (Schneikert et al., 1999) GST-pull-down and deletional mutant assays have shown the BAG-1M isoform requires the hinge region to downregulate GR transactivation (Kullmann et al., 1998; Schneikert et al., 1999). Mineralocorticoid receptors (MR) have been shown by the Schneikert et al. (1999) study to not interact with BAG-1M and regulate NHR function. This leads the authors to hypothesise that the mechanism must be different to one involving the cytoplasmic chaperones Hsc/Hsp70 as both GR and MR can recruit chaperones to the nucleus (Diehl and Schimdt 1993; Bruner et al., 1997). However, a later study shows MR may regulate PR function (Knapp et al., 2012). The hormone binding region of the GR which interacts with the carboxy terminal of BAG-1M also is capable of binding Hsc/Hsp70 supporting the role of a Hsc/Hsp70/BAG-1/NHR complex in regulating GR function. The role of Hsc/Hsp70 and GR is discussed in section 1.2.2.1. Deletion of the amino terminal region of BAG-1M still resulted in translocation to the nucleus, but no change in GR activity

occurred (Schneikert et al., 1999). A serine/threonine-rich region present in the amino terminus of BAG-1M and BAG-1L., but not BAG-1S, may explain the isoform-specific ability to interact with nuclear GR (Schneikert et al., 1999). Levels of both BAG-1M and BAG-1L have been shown to be elevated in cancer (Takayama et al., 1998). Steroids, in particular corticosteroids, have been used in the treatment of both primary brain tumours and brain metastases for over half a decade (Ingraham et al., 1952). These studies may therefore support a role for BAG-1 in assisting response to steroid therapy in the treatment of advanced cancer.

PR-dependent reporter gene assays using COS-7 kidney cells have shown BAG-1M inhibits PR function in the presence of progesterone (Knapp et al., 2012). Deletional mutant assays and co-immunoprecipitation studies demonstrated that PR activity is regulated by BAG-1M through the DNA-binding domain (DBD), rather than the hinge region required by the GR (Kullmann et al., 1998). Interestingly, this study by Knapp et al. (2012) showed BAG-1M also interacts with MR, evidence contrasting to previous studies (Schneikert et al., 1999). Further reporter gene assays demonstrated MR transcription was inhibited by BAG-1M expression (Knapp et al., 2012).

GST-pull-down and yeast two-hybrid assays have confirmed interaction between RAR and BAG-1S (Liu et al., 1998). Reporter gene assays using monkey kidney CV-1 cells showed trans-retinoic acid-induced activity of RAR was decreased by co-transfection of a BAG-1S expression plasmid (Liu et al., 1998). Terminal deoxynucleotidyl transferase assays using breast cancer cell lines MCF-7 and ZR-75-1 have shown BAG-1S overexpression causes resistance to trans-retinoic acid-induced apoptosis (Liu et al., 1998). Immunoblotting has been used to show overexpression of BAG-1S in MCF-7 cells also reduces trans-retinoic acid-induced decrease in Bcl-2 expression (Liu et al., 1998). RAR regulation may be a further mechanism by which BAG-1 promotes cancer cell survival and certainly presents a mechanism for resistance to retinoids.

In addition to establishing a role for BAG-1S in RAR, Liu et al. (1998) demonstrated BAG-1S inhibition of thyroid hormone-induced TR activity (Liu et al., 1998). The ability of the cytoplasmic BAG-1S isoform to interact with TR demonstrates how subcellular localisation of BAG-1 isoforms does not always reveal function. The nuclear isoform BAG-1L is an

important determinant of ER function in breast carcinoma, enhancing oestrogen-dependent transcription. Cutress et al. (2003) reported overexpression of BAG-1L sensitised ER to 17-β-oestradiol –dependent transcription in MCF-7 cells. Transcriptional activity of ER was also increased by the BAG-1M isoform but not BAG-1S. BAG-1L was the only BAG-1 isoform co-immunoprecipitated with overexpressed ERα or ERβ. Addition of an NLS to BAG-1S to localise the isoform to the nucleus did not result in upregulation of oestrogen-dependent transcription, therefore the BAG-1L-specific mechanism is not attributed to nuclear localisation alone (Cutress et al., 2003).

Berry at al. (2008) proposed BAG-1 and CHIP (E3-ubiquitin ligase carboxy terminus of Hsc70-interacting protein) regulate nuclear hormone receptor basal turnover. Blockade of BAG-1 interaction with ERα receptor lysines K302 and K303 resulted in decreased ERα turnover and increased activation of the Hsp90 cochaperone p23. ATP-bound Hsp90 is stabilised by p23 and can form complexes with steroid receptor promoting the binding of steroid (Pratt and Toft., 2003).Yeast binding assays have shown that overexpression of p23 increased interaction with oestradiol and ER transcriptional activity (Knoblauch and Garabedian., 1999). However, p23 has been shown to suppress ER activity independently of Hsp90 (Freeman et al., 2000). ER-negative breast cancer C4-12 cells incorporating amino acid substitutions in receptor lysines K302/ K303 showed increased ER degradation in the absence of oestradiol and increased interaction with BAG-1 and CHIP (Berry et al., 2008). Selective down-regulation of ER by the anti-oestrogen ICI 182, 780 (fulvestrant / Faslodex™) in the presence of 17-β-oestradiol is controlled by receptor lysines K302/ K303 which potentiate ER degradation. C4-12 cells carrying receptor lysine mutations exposed to fulvestrant showed a reduction in ER degradation compared to wild type (Berry at al., 2008). Receptor lysines may have a role in improving response to antioestrogens in breast cancer by decreasing BAG-1/ CHIP-regulated ER turnover (Berry et al., 2008). BAG-1 has also been implicated in ER degradation by siRNA experiments using breast cancer cells. siRNA knockdown of BAG-1 in C4-12 cells resulted in decreased ERα turnover (Berry et al., 2008).

Treatment of breast cancer with anti-hormone drugs Tamoxifen or Faslodex induces expression of BAG-1 (Kyllonen et al., 2006). BAG-1 expression responds to a variety of stresses permitting cell survival (Takayama et al., 1995) or persistence of normal function (Song et al., 2001), it is therefore plausible that BAG-1 is involved in the resistance to antihormone drugs. Resistance to hormone therapy in breast cancer is thought to be related to EGFR/ER signalling and the ability to facilitate ‘back-up’ pathways in the presence of

antioestrogen blockade. Microarray studies have attempted to explain resistance by the discovery of antihormone-induced expression of invasive genes including NFkappaB (Gee et al., 2006). NFkappaB signalling may permit resistance to antihormonal effects via a DNA-binding region shown to be involved in regulating transcription at low concentrations of oestrogen (Zhou et al., 2007). High levels of BAG-1 and NFkappaB expression are maintained in microarray profiling of oestrogen-deprived MCF-7 cells (Gee et al., 2006). In addition, CD59, a regulator of the complement cascade, is increased by antioestrogen therapy suggesting a defence mechanism for tumour cells from the immune response (Gee et al., 2006). Knowledge of compensatory pathways activated during antihormonal treatment of breast cancer may provide a basis for investigating the actions of combination therapy and further understanding BAG-1 and NFkappaB signalling.

1.2.2 Indirect binding partners of BAG-1

1.2.2.1 Bcl-2

Bcl-2 (B cell lymphoma 2) was first implicated in apoptosis through its part in the chromosomal translocations associated with non-Hodgkin's B cell lymphomas (Tsujimoto and Croce, 1986). Bcl-2 is an oncogene that contributes to malignancy by inhibiting apoptosis and thereby promoting cell survival and is one of the most studied apoptotic genes in breast cancer. Overexpression of Bcl-2 has been correlated with increased cell survival in some, but not all breast cancers (Frankfurt et al., 1997; Sierra et al., 1996). Bcl-2 prevents caspase-9 activation and apoptosis by inhibiting the release of cytochrome C from the mitochondria, thereby maintaining integrity of the mitochondrial membrane (Adams and Cory, 1998).

Takayama et al. (1995) first established via molecular cloning and Far Western Blotting that an additional pro-survival protein, BAG-1, was involved in some of the apoptotic signalling attributed to Bcl-2 (Takayama et al., 1995). BAG-1 was shown to have a synergistic effect when bound to Bcl-2 in resisting apoptosis following treatment of transfected Jurkat cells with staurosporine. BAG-1 or Bcl-2 alone only mounted partial and weak resistance to cell death following treatment.

Bcl-2 and BAG-1 have been shown to interact in vitro and in yeast two-hybrid binding assays (Wang et al., 1996). One hypothesis is that BAG-1 acts in a manner similar to Ras and targets Raf-1 to intracellular membranes, hence in close proximity to proteins such as Bcl-2, known to be present in mitochondrial membranes ((Yuryev and Wennogle, 1998)). BAG-1, Bcl-2 and Raf-1 have been described as being capable of forming trimolecule complexes (Wang et al., 1996). This would implicate Bcl-2 to be involved in direct interaction with Raf-1 independently of MAPK proteins. However, the stoichiometry is reported as being only approximately 1%, therefore suggesting a minor functional role of Raf-1 (Wang et al., 1996).

BAG-1 is an indirect binding partner of Bcl-2 and can exert prosurvival effects via direct binding partners, such as Hsp/Hsc70 and Raf-1, and can mediate antiapoptotic function independent of Bcl-2 (Figure 1.3). The regulation of luminal apoptosis is important in both

the development of normal breast epithelial architecture and the development of breast tumours (Bissell and Radisky, 2001). Overexpression of BAG-1 in MCF-10A breast epithelial cells suppresses Raf-1-dependent luminal apoptosis in the absence of any change in Bcl-2 expression (Anderson et al., 2010). Isolated Bcl-2 family expression-induced inhibition of apoptosis in 3D cell culture does not result in luminal filling, but activation of the oncogene HER2 in MCF-10A cells does (Debnath et al., 2002; Reginato and Muthuswamy, 2006).

1.3 BAG-1 and cellular function

1.3.1 Apoptosis

The exact mechanism by which BAG-1 isoforms promote cell survival is not fully understood and potentially involves multiple molecular pathways (Figure 1.3). Identifying whether BAG-1 changes levels of apoptosis or promotes increase in cell number or metabolic activity is critical to understanding how BAG-1 utilises functional signalling pathways. BAG-1 was first described as a Bcl-2 interacting protein, protective against apoptosis by regulating the release of caspase-activating enzymes from the mitochondria (Takayama et al., 1995).

BAG-1 is involved in anti-apoptotic pathways independent of Bcl-2 and the importance of Hsc70 and Hsc70 interaction with BAG-1 has become apparent. Hsc70 is a key player in the prosurvival role of BAG-1 and the interaction between the two molecules depends upon the carboxy terminal binding region. Transfection of ZR-75-1 breast cancer cells with BAG-1 mutants lacking the COOH terminal domain showed no difference in cellular metabolic activity compared to cells with endogenous BAG-1 alone (Kudoh et al., 2002). XTT assays performed on ZR-75-1 cells stably expressing BAG-1L or BAG-1S showed prolonged increase in metabolic activity compared to mutants. Caspase activity assays performed on the same transfected cell population demonstrated no increase in caspase activity in the BAG-1 overexpressing cells compared to controls following serum deficiency. Caspases are proteolytic cysteine proteases which form a chain of enzymatic actions resulting in cleavage of protein substrate and programmed cell death (apoptosis). Caspase activity assays detect activated caspase enzymes and therefore relative apoptosis. In this study the release of fluorescent amino-4-trifluoromethyl-coumerin (AFC) from acetyl-aspartyl-glutamyl-valinyl-aspartyl-AFC substrate peptide was measured by a fluorimeter. This study proceeded to investigate the effects of BAG-1 mutants and BAG-1 isoform overexpression using a mouse xenograft model. The volume of tumours formed via injection of ZR-75-1 cells overexpressing BAG-1L and BAG-1S isoforms were larger in volume than vector controls. Tumours formed from cells overexpressing BAG-1 isoform mutants measured significantly less than the vector controls. The XTT assays in this study in conjunction with caspase activity assays do provide evidence for BAG-1 involvement in mediating cell survival and apoptosis. The decrease in tumour volume of tumours formed by a xenograft overexpressing BAG-1mutants supports a role for BAG-1 in tumour growth. The Kudoh et al. (2002) study does, however, have several caveats; Kudoh et al. (2002) expressed the results of the XTT assay via figures incorporating ‘cell number’ on the y-axes (Kudoh et al., 2002). The study

relies upon estimating number of viable cells by comparison to a standard curve, but does not include any evidence of cell counting to provide the data to label the y-axis as such. XTT assay relies upon a colourimetric change in response to metabolic activity of enzymes within the cells. The change in optical density measured at a specific wavelength is relatively proportional to the number of viable cells. The decrease in tumour volume following overexpression of mutant BAG-1 in a mouse xenograft model compared to vector controls may not be due to a change in cell growth or proliferation. In the absence of Ki-67, PCNA or BrdU staining and caspase activity assays, change in tumour weight could be due to a change in the amount of cell death and not due to an increase in proliferation (Kudoh et al., 2002).

ZR-75-1 and MCF-7 breast cancer cells overexpressing BAG-1 have shown inhibition of retinoic acid receptor activity and susceptibility to retinoic acid (Liu et al., 1998). The study used MTT assays to determine the effect of BAG-1 on 'growth inhibition', although an MTT assay is capable only of demonstrating metabolic activity in viable cells. However, the study did incorporate fluorescence-activated cell sorter analysis (FACS), revealing that BAG-1 overexpression resisted retinoic acid-induced apoptosis (Liu et al., 1998). A later study focused on the effects of BAG-1 overexpression in ER-positive MCF-7 and ER-negative Hs578T cells and their response to chemotherapeutic agents. Following treatment with doxorubicin, fluorouracil (5-FU) or docetaxel, cells overexpressing BAG-1L and BAG-1M demonstrated reduced annexin V staining compared to BAG-1S overexpressing MCF-7 cells and controls. Hs578T cells showed no difference between those overexpressing BAG-1 isoforms and controls. MTS assays were carried out on the same population of cells and showed only cells overexpressing BAG-1L in the presence of 17-β oestradiol resisted a decrease in metabolic activity of viable cells compared to shorter BAG-1 isoforms and vector controls. Hs578T cells do not respond to 17-β oestradiol. Western blots were used to show Bcl-2 expression was increased in MCF-7 cells overexpressing BAG-1L and BAG-1M, but not BAG-1S compared to vector controls. Cyclohexamide chase assays showed concordance as MCF-7 cells overexpressing BAG-1M and BAG-1L showed enhanced stability of Bcl-2 (Liu et al., 2009a). These results suggest that the BAG-1M isoform is able to reduce apoptosis on exposure to chemotherapeutic agents, but is unable to improve the response of ER-positive breast cancer cells to oestrogen.

ER positive MCF-7 cell-derived clones and transient transfections have been used to investigate the effect of BAG-1S overexpression. Townsend et al. (2003) reported that clonogenicity of BAG-1S overexpressing cells was maintained despite heat, radiation or

hypoxic stress. Relative cell viability was measured following (tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of MCF-7 cells overexpressing cells BAG-1S by MTT assay. Cells overexpressing the BAG-1S isoform were shown to resist TRAIL-induced apoptosis and maintain cellular metabolic activity (Townsend et al.,2003). Apoptosis was measured using FACS by applying the Nicoletti Method (Nicoletti et al., 1991). The Nicoletti method uses FACS to measure the proportion of cells with a low intact DNA and high fragmented DNA content (sub-G1 content). The percentage of cells with sub-G1 content was significantly lower in MCF-7 cells overexpressing BAG-1S compared to pcDNA3 controls. Interestingly, cell fractionation and immunofluorescence experiments demonstrated cells overexpressing BAG-1S relocalised from the cytoplasm to the nucleus following heat shock. This suggests the mechanism involved in BAG-1 stress-signalling and resisting apoptosis involves nuclear pathways.

Evidence that a depletion in BAG-1 specifically targets tumour cells for apoptosis exists from a transgenic mouse model of non-small cell lung cancer. Heterozygous mice were produced with a null allele for BAG-1 and expressing C-Raf kinase in type II pneumocytes. The mice developed multifocal adenomas in early adulthood. Mice with a two copies of BAG-1 showed a 75% larger lung tumour area than the mice with BAG-1 haploinsufficiency. Apoptosis was measured by immunohistochemical staining of activated caspase-3 and trypan blue staining. Tumours from mice heterozygous for BAG-1 increased amounts of caspase-3 and trypan blue compared to tumour cells from BAG-1 homozygotes (Gotz et al., 2004). The study addressed whether the difference in tumour size could be due to change in proliferation rather than apoptosis by performing Ki-67 and proliferating cellular antigen (PCNA) staining. There was no difference in staining between BAG-1 haploinsufficient tumours and those homozygous for BAG-1, suggesting no change in proliferation. It is accepted that caspase 3 is a central executioner in apoptosis (Porter and Janicke, 1999). Measuring caspase 3 activity does indicate apoptotic signalling, but caspase 3 assays do have limitations. A dose-response experiment is important as the timing of the caspase 3 assay can change the result (Zhivotovsky et al., 2001). Timing is also important to take into account as although caspase 3 activity indicates apoptosis, there is no absolute that the cell will still not undergo necrosis at a later stage. Mechanisms of cell death depend on many factors; availability of caspases, energy, level and duration of toxicity (Zeiss et al., 2003). In addition, the study stained lung adenomas for phospho-ERK, indicative of mitogenic cascade activity, but no difference was seen between heterozygous and homozygous tumours. This study hypothesised that BAG-1 inhibition could be a strategy to prevent tumour cell survival (Gotz et al., 2004).

Translation initiation of BAG-1S occurs by both cap-dependent scanning and by internal ribosome entry. Following heat shock cap-dependent translation is reduced and translation initiation via the internal ribosome entry segment of BAG-1 mRNA (Coldwell et al., 2001). Translation of eukaryotic mRNA commonly occurs via a cap-dependent mechanism. The IRES provides an alternative method of mRNA translation and involves a complex structural element at the 5' untranslated region (UTR/ leader sequence) (Jang et al., 1990).

Western blotting experiments have shown treatment of cervical cancer HeLa cells with the chemotherapeutic agent cisplatin showed a reduction in complexes required for cap-dependent translation. In contrast, HeLa cells treated with vincristine showed a reduction in phosphorylation of sites required for ternary complex formation, but an increase in phosphorylation of initiation factor 4F required for cap-dependent translation (Dobbyn et al., 2008). An increase in initiation factor 4F activity has previously been observed in stress, but the significance not defined (Fraser et al., 1999). Immunoprecipitation experiments using HeLa cells exposed to vincristine showed only the BAG-1S isoform continued to be expressed at higher concentrations of vincristine. There was no new synthesis of BAG-1 isoforms on exposure to cisplatin (Dobbyn et al., 2008). Plasmid constructs previously designed to test IRES function were employed in luciferase assays using HeLa cells exposed to vincristine or cisplatin. Luciferase activity was decreased by 20-30% in cells exposed to vincristine which contained non-IRES or no construct controls. Cells with the IRES construct a showed only a small decrease in luciferase activity on exposure to vincristine. This small decrease was explained by the authors as likely to represent residual cap-dependent translation. Cells treated with cisplatin showed a marked decrease in luciferase activity, including those containing the BAG-1 IRES construct. No difference in the amount of inhibition of translation was seen between vincristine and cisplatin, therefore these experiments show different mechanisms of translation must exist. Immunofluorescence experiments showed relocalisation of polypyrimidine tract binding proteins (PTB), known to be involved in IRES activity, from the nucleus to the cytoplasm after treatment with vincristine, but not cisplatin (Dobbyn et al., 2008). The BAG-1S IRES could provide a pathway by which breast cancer cells are resistant to vincristine chemotherapy and aid design of future targeted therapies.

3-D cell cultures using MCF-10A breast epithelial cells have been used to demonstrate phenotypical change in cell architecture is mediated by Bim-dependent luminal apoptosis (Debnath and Brugge, 2005). Confocal microscopy and immunohistochemical staining of M30, a molecule produced by caspase cleavage of cytokeratin 18, have been used to detect

apoptosis. Overexpression of BAG-1 showed a reduction in the amount of luminal apoptosis in MCF-10A 3-D cell culture and resulted in an increased number of cells with solid lumens compared to vector controls. Western blotting and densitometry demonstrated increased activation of Raf-1 and ERK1/2, and decreased BimEL expression in MCF-10A 3-D cells overexpressing BAG-1. The MEK inhibitor, U1026, showed a reversal in the BAG-1 induced phenotype, with the amount of luminal apoptosis, ERK1/2 and BimEL levels similar to vector controls (Anderson et al., 2010).

Bim is a Bcl-2 family protein shown to promote apoptosis. Bim has 3 distinct isoforms BimS (short isoform), BimL (long isoform), and BimEL (extra -long isoform) generated by alternative splicing (O'Connor et al., 1998). Mcl-1 is also a member of the Bcl-2 family, but inhibits apoptosis. Serum survival factors activate ERK1/2 and cause dissociation of BimEL and Mcl-1. The dissociation of Bim from Mcl-1 is specific for the BimEL isoform and requires ERK1/2 phosphorylation. Mutations in the BH3 binding domain, known to bind Mcl-1, resulted in increased ERK1/2-dependent BimEL degradation (Ewings et al., 2007). Bim/ Mcl-1 dissociation and subsequent BimEL degradation could therefore be a downstream effect of BAG-1 signalling via the mitogen-activated protein kinase (MAPK) pathway.

It may be important to determine the difference between apoptosis and other types of cell death to define the functional role for BAG-1 and the true potential of BAG-1 as a therapeutic target or biomarker. Apoptosis is programmed cell death which is reversible and involves caspases, a series of proteolytic enzymes and distinct changes in cell morphology (Kerr et al., 1972). Necrosis differs from apoptosis as is an energy-independent mechanism and involves recruitment of inflammatory cells, cell membrane rupture, and uncontrolled, irreversible cell death (Ouyang et al., 2012). Hence, determining if BAG-1 can regulate irreversible cell death or mediate the immune system, would necessitate measuring specifically cell necrosis. Trypan blue exclusion assays determine cell membrane rupture and signify late stage apoptosis, but are limited in providing information about the process which led to cell death. The most accurate methods for measuring necrosis, rather than apoptosis, involve light or electron microscopy and observation of changes in morphology associated with necrosis (Fink and Cookson, 2005). However, apoptosis may also involve cell membrane rupture and apoptotic necrosis can occur. Other forms of programmed cell death exist, such as necroptosis and autophagic cell death, further complicating the definition of a specific functional role for BAG-1. The simplest definition of necrosis is a ‘mass of dead cells’ and provides no differentiation between mechanisms leading to that

point. Viewing apoptosis and necrosis as a continuum would mean there would be limited benefit in determining the role of BAG-1 in different mechanisms of cell death.

1.3.2 Proliferation

BAG-1 has a role in regulating breast cancer cell proliferation in response to stress (Song et al., 2001; Townsend et al., 2003), but the evidence for the role of BAG-1 in proliferation in the normal environment is lacking. Hsp70 is a direct regulator of BAG-1 and an indirect regulator of Raf-1 (Song et al., 2001). Inducible BAG-1 and HSP70 expression were produced using tetracycline-controlled transcriptional activation. Tetracycline responsive expression involves the tetracycline transactivator protein (tTa). tTa is produced by fusing a protein found in Escherichia coli bacteria, tetracycline repressor (tetR), with an activator domain V16 found in the Herpes Simplex virus (Gossen and Bujard, 1992). The tTa protein uses DNA- binding operator sequences and a CMV promoter as a tetracycline response element (TRE). The TRE responds to binding of tTa by increasing expression of the gene downstream of the promoter. A 'tet-off' system expression of the TRE-genes is reduced in the presence of the antibiotic tetracycline. A 'tet-on' system works by tTa only being able to bind operator if bound to tetracycline (Gossen and Bujard, 1992). OT cells exhibiting inducible-expression of Hsp70, OT-tet(Hsp70) were transfected with epitope-tagged haemagglutinin A-BAG-1, pcDNA3 control or Ras. Hsp70 expression was induced by tetracycline to levels seen during heat shock without causing cell stress. Western blotting and immunoprecipitation showed reduced BAG-1/Raf-1 and ERK activity with elevated expression of Hsc70 (Song et al., 2001).

Inducible OT-tet(BAG-1), and OT-tet (BAG-1) cells with mutations in the Hsp70 binding domain, OT-tet (BAG-1 C204A) and OT-tet (BAG-1 E208A), were produced to express BAG-1 following removal of tetracycline. OT23 wild type cells were transfected with control plasmid only as vector controls. Cells were grown in the absence of tetracycline then exposed to heat shock at 43° for 1 hour and incubated at 37 for 2 hours before the addition of (^{3}H) thymidine for 2 hours. DNA synthesis was measured by scintillation and trichloroacetic acid (TCA) precipitation. Cells were also treated with the proliferation marker bromodeoxyuridine (BrdU), which was then visualised following staining with anti-BrdU and FITC. OT-tet cells expressing BAG-1 and the those transfected with vector controls showed complete arrest of DNA synthesis and reduced BrdU staining following heat shock compared to those transfected with mutant Hsp70. These results suggest BAG-1 has a role in the

arrest of DNA synthesis following heat shock, which is abrogated by the inability to bind Hsp70 (Song et al., 2001).

BAG-1 may orchestrate cellular signalling leading to regulation of cell growth via the MAP kinase pathway. High levels of Hsp70 suppress BAG-1 binding of Raf-1 leading to inhibition of downstream MAPK enzyme activity (see section 1.2.1.1). This activity of BAG- 1 is Ras-independent as expression of a Ras-depleted mutant does not inhibit BAG-1/Raf-1 signalling. BAG-1 mutants lacking the Hsp70 binding domain have no effect on cellular growth in the presence of high levels of Hsp70 (Song et al., 2001).

Kudoh et al. (2002) reported no difference in cell growth between ER-positive ZR-75-1 breast cancer cells stably transfected with BAG-1 isoforms and those transfected with control plasmid under normal physiological conditions(Kudoh et al., 2002). Assays were referred to as 'cell growth assays', but the method used was a second generation tetrazolium (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium) or XTT) assay, which relies upon measurement of a colourimetric change produced by metabolites of viable cells and is similar to an MTS assay (discussed in section 2.5). The XTT 'Proliferation Assay' was initially described as a method to measure cell growth and drug sensitivity in cancer cell lines (Scudiero et al., 1988), but is an indirect measure of relative cell growth. A direct measure of cell growth would involve counting cells using an automated cell counter or manually using a haemocytometer. The authors showed transfection of BAG-1 carrying mutations in the carboxy terminal did result in a reduced relative number of viable cells compared by XTT assay. Serum-deprivation of ZR-75-1 cells overexpressing BAG-1 isoforms did cause an increase in the relative number of viable cells by XTT assay (Kudoh et al., 2002). In addition, Kudoh et al. produced a mouse xenograft using ZR-75-1 cells transfected with BAG-1 isoforms, BAG-1 with mutant carboxy terminal, or control plasmid. Tumours overexpressing BAG-1 isoforms were significantly greater in volume compared to those transfected with control plasmid. Tumours formed following injection of ZR-75-1 cells expressing mutant BAG-1 were reduced in volume compared to controls (Kudoh et al., 2002). These results implicate BAG-1 may regulate breast tumour growth in vivo, although the evidence in vitro under normal physiological conditions is less convincing.

Overexpression of BAG-1 or in well-differentiated gastric cancer MKN74 cells did not result in an increase in cell number in vitro. Subsequent injection of MKN74 cells transfected with

BAG-1 into intraperitoneal cavities of nude mice did not produce any difference in tumour size compared to plasmid control (Yawata et al., 1998). There is no evidence for a role for BAG-1 in tumour growth or proliferation in gastric cancer. However, there was a 3.3 fold increase in intraperitoneal disseminated tumour weight in mice transfected with BAG-1. Yawata et al. (1998) did show an increase in resistance to apoptosis in cells overexpressing BAG-1. Apoptosis was induced following serum-starvation and assessed using trypan blue exclusion assays and measurement of DNA fragmentation. This provides further evidence for BAG-1 function in promoting tumour cell survival in stress conditions and may contribute to peritoneal metastases dissemination rate (Yawata et al., 1998). Xenograft models investigating BAG-1 expression and function are discussed further in section 1.4.

BAG-1 is expressed in the majority human glioma cell lines. BAG-1 was also found to be expressed in 15 of 19 human glioblastomas using immunohistochemical analysis of primary tumours (Roth et al., 2000). Transgenic expression of BAG-1 in 2 malignant glioma cell lines, LN-18 and LN-229 significantly reduced growth rate and clonogenicity. Co-expression of Bcl-2 suppressed the effects of BAG-1 overexpression. LN-229 cells overexpressing BAG-1 implanted into mice xenografts showed no difference in tumour growth compared to vector controls. However, implantation of LN-229 cells overexpressing Bcl-2 showed increased tumour growth. Serum starvation of glioma cells revealed that Bcl-2 expression could rescue from apoptosis, but BAG-1 could not. Expression of both BAG-1or Bcl-2 alone resulted in reduced proliferation in serum-starved conditions. These results suggest regulation of glioma proliferation is a function of BAG-1 and may be important in the progression of malignant glioma. The results also implicate that co-expression of BAG-1 and Bcl-2 may function in regulating glioma cell survival (Roth et al., 2000).

BAG-1 overexpression has been shown to suppress cell growth in human epidermal HaCaT cells (Hinitt et al., 2010). Stable transfection of HaCaT cells with either of the BAG-1 isoforms via resulted in inhibition of cell growth compared to vector controls. Scratch assays demonstrated decreased epithelial wound repair in HaCaT cells overexpressing BAG-1 isoforms. Cohesion assays were used to investigate the effects of BAG-1 overexpression on cell adhesion. Overexpression of the short isoform BAG-1S produced an increase in cell clumping and hence increased cell-cell adhesion. These results were supported by dissociation assays showing decreased dissociation of HaCaT cells overexpressing BAG-1S compared to vector controls (Hinitt et al., 2010). In addition, overexpression of BAG-1S and BAG-1M showed reduced cell motility and scattering in serum-free medium exposed to

hepatocyte growth factor (HGF) compared to vector controls. The authors also noted that subconfluent cultures of HaCaT cells expressed lower levels of all 3 BAG-1 isoforms. Overexpression of BAG-1S showed a reduced growth rate compared to both HaCaT cells transfected with BAG-1S with point mutations in the Hsc/Hsp70 binding domain and the vector controls. These results suggest that BAG-1S mediates cell growth through the BAG domain and Hsc/Hsp70 chaperone activity. No increase was seen in ERK1/2 activation following overexpression of BAG-1 isoforms (Hinitt et al., 2010). Mutations in helices 1 and 2 of the BAG domain, corresponding with the Raf-1/BRAF binding domain, most effectively suppressed the reduction in cell motility seen with overexpression of BAG-1S wild type.

Hinitt et al. (2010) initially hypothesised that an increase in hepatocyte growth factor receptor (HGFR) or Raf-1 interaction with BAG-1 may implicate BAG-1 in wound healing and proliferation. BAG-1 is overexpressed in squamous cell carcinoma (SCC) (Wood et al., 2011), therefore the authors hoped to explain the oncogenic activity of BAG-1 by showing an increase in cell growth and proliferation. However, these results show BAG-1 overexpression reduces cell growth, via the BAG domain and interaction with the Hsc/Hsp70 chaperone molecules, and cell motility (Hinitt et al., 2010). The results could be explained by cell function being dependent on the ratio of BAG-1 isoforms present or functional expression of BAG-1 changing with tumour progression. An earlier study found immunohistochemical staining of nuclear BAG-1L in oral SCC to be reduced compared to normal oral keratinocytes (Hague et al., 2002). However, levels of total BAG-1L did not differ between oral carcinoma and normal epithelia, suggesting a change in subcellular localisation of BAG-1L may be implicated in carcinogenesis. There was no difference between total BAG-1 between carcinoma and normal tissue, although 8/13 lymph node metastases showed stronger cytoplasmic staining than the corresponding primary tumour. These results support the loss of regulation of BAG-1 being involved in the progression of oral SCC (Hague et al., 2002).

Expression of different BAG-1 isoforms and subcellular localisation could assist identification of breast tumours which are likely to proliferate and behave in an aggressive manner. The initial findings in vitro and in vivo by Kudoh et al. (2002) suggest BAG-1 expression correlates with increased tumour growth rate.

1.4 Tumour xenograft models expressing BAG-1

Tumour xenograft models have been used to investigate the role of BAG-1 in breast cancer in vivo. Injection of ER-positive ZR-75-1 cells overexpressing BAG-1S, BAG-1L, mutant BAG-1, or plasmid control into mammary fat pads of female nu/nu mice resulted in distinct patterns in tumour growth rate (Kudoh et al., 2002). Tumours produced by cells expressing the shorter isoform BAG-1S or the nuclear isoform BAG-1L were increased 1.4-1.6-fold in size compared to those expressing vector controls. Tumour volume and weight was recorded. Tumours formed from the injection of cells expressing mutant BAG-1 lacking the COOH-terminal displayed slow growth rates and reached sizes of less than a third of those formed from control cells. Oestrogen-releasing or control pellets were implanted into oopherectomised female nu/nu mice. The absence of oestrogen caused a reduction in tumour growth for all mice transfected with either BAG-1 isoforms or plasmid controls. Tumours expressing BAG-1 carrying mutant COOH-terminal showed no discernible difference in reduction of growth on exposure to oestrogen compared to those not exposed. Hence, the overexpression of BAG-1 does not overcome the requirement of ER-positive cells for oestrogen. BAG-1L overexpression in ER-positive MCF-7 breast cancer cells potentiates ER transcriptional activity (Cutress et al., 2003). However, the oestrogen-dependent and oestrogen-independent reduction in cell growth with BAG domain mutants suggests a further BAG-1 signalling mechanism independent of ER activity.

Xenografts have been used to investigate the role of BAG-1 in other cancers. Yawata et al. (1998) used gastric carcinoma MKN74 cells transfected into BALB/c nude mice to attempt to elucidate a role for BAG-1 in growth or apoptosis. MKN74 cells transfected with BAG-1 were injected into the intraperitoneal cavity of BALB/nude mice. This study failed to provide evidence for the function of BAG-1 in mediating growth or proliferation in vivo or ex vivo. However, the study did show that disseminated tumour weight was increased 3.3 fold in mice transfected with BAG-1. Measurement of DNA fragmentation and trypan blue exclusion demonstrated BAG-1 expression in MKN74 cells conferred resistance to apoptosis induced by serum-starvation and anoikis. BAG-1 may play a role in gastric tumour survival and dissemination of peritoneal metastases (Yawata et al., 1998).

Overexpression of BAG-1 in malignant glioma cell lines LN-18 and LN-229 reduced growth and clonogenicity (Roth et al., 2000). However, intracranial inoculation of LN-229 cells

overexpressing BAG-1 into nude mice did not result in a change in tumour growth compared to vector controls. LN-229 cells overexpressing Bcl-2 resisted apoptosis induced by serum-starvation, but elevated levels of BAG-1 did not (Roth et al., 2000).

There are several advantages to using xenograft models; they enable examination of tumour invasion, metastasis, and assessment of therapeutic response in vivo, permit individualised therapeutic trial, trial of multiple therapies in single model, shortened time from transfection to biopsy, and application of tissue microarray and analysis prior to human trial (Richmond and Su, 2008). However, xenografts using human cancer cell lines do often not correlate with therapeutic response in patients (Kerbel, 2003). Xenograft models incorporating cells grown in culture will not accurately represent primary tumour cells as subject to different microenvironmental conditions. The microenvironment in solid tumours has been shown to be different to that of normal tissue (Vaupel et al., 1989; Boyer et al., 1992). The pH, blood flow, oxygen and nutrient supply will vary across regions of solid tumour (Vaupel et al., 1989); therefore cells grown in culture will not mimic these heterogeneous conditions. The cell stroma contains vasculature, macrophages, and cancer-associated fibroblasts. The stromal contents secrete chemical messengers, known as cytokines, and growth factors which influence breast tumour development (Sadlonova et al., 2005). Stroma from the human microenvironment can be implanted within the xenograft to attempt to recreate the microenvironment (Yokota et al., 2013).

Models using human cancer cell lines are at risk of erroneous results due to phenotypic drift, although this can be controlled by using specific cell passage numbers. Cells may adapt quickly to overexpression of BAG-1 and any initial functional effect become less reproducible in higher cell passage numbers (see Chapter 2 Discussion). Clonal isolation with subsequent cell passage leads to selection of certain genetic traits and hence a loss of heterogeneity. This partially accounts for the poor translation (<10%) of studies based on cell culture to clinical trial (Hait, 2010; van der Worp et al., 2010).

Xenograft studies often involve inoculation of transfected cells or tumour transplantation into immune deficient mice. Use of athymic or severe combined immune deficient (SCID) mice ensures cells or tissue are not rejected by the host. However, the immune system has a role

in breast tumour development, therefore immune-deficient models will not be wholly representative of clinical response in patients (Kerbel, 2003).

To combat the some of the problems associated with use of human cancer cell lines, human primary breast tumours can be transplanted can be successfully transplanted onto immune-deficient mice (Visonneau et al., 1998). These models do provide a more accurate in vivo representation of human breast cancer, but produce chimeric tumours and disregard any influence an active immune system may have on results (Whittle et al., 2015).

2D cell models are simplistic and a valid method for hypothesis-generating research. However, as described, 2D cell line culture is not a realistic representation of the human tumour environment, and is subject to clonal selection and loss of heterogeneity. Cells cultured in monolayers only have cell-cell contact at their periphery and adopt a flat morphology unnatural to may human cell lines. 3D cell culture is a relatively time-efficient, inexpensive method to examine tumour behaviour in a structurally relevant context when compared to animal models (Debnath and Brugge, 2005). 3D culture using normal breast epithelial MCF-10A cells has been used to show increased BAG-1 expression abrogates luminal apoptosis, leading to an increase in pre-invasive phenotype. Overexpression of BAG-1 in 3D culture of MCF-10A cells was associated with increased activation of Raf-1 and a reduction in BIM. Luminal apoptosis in normal mammary morphogenesis is BIM-dependent; therefore interruption of this normal process by BAG-1 is a mechanism by which BAG-1 may promote tumourigenesis (Anderson et al., 2010).

Orthotopic grafts involve transplanting tissue into the position it would naturally occur. Breast tumours were implanted subcutaneously initially, but transplantation into the murine inguinal mammary fat pad was shown to be more representative of the breast stroma microenvironment (De Rose et al., 2011). Several studies have incorporated HER2-positive patient-derived breast tumour into orthotopic xenografts (Liu et al., 2010; De Rose et al., 2011; Oakes et al., 2012; Zhang et al., 2013; Li et al., 2013). All of these studies used HER2-positive patient-derived xenografts sourced from primary breast tumour, pleural effusion, or ascites. However, few therapeutic studies have used a HER2-positive breast tumour xenograft (Marangoni et al., 2007; Garcia-Garcia et al., 2012). Marangoni et al. (2007) used 200 samples of human breast cancers transplanted into the interscapular fat pad of

female Swiss nude mice to yield 25 transplantable tumours. 2 xenografts derived from a panel of 25 human breast cancer xenografts were found to have HER2 amplification. Only one of the HER2-positive xenografts responded to the anti-HER2 drug trastuzumab (Herceptin™). Interestingly, all 25 transplantable xenografts showed aggressive phenotypes, but a variation in both genomic profile and HER2 expression. The authors concluded a panel of breast tumour xenografts could provide a preclinical tool to guide therapeutic design (Marangoni et al., 2007). One further HER2-positive xenograft was produced from a patient who had shown resistance to trastuzumab and relapsed, but showed a response to the dual EGFR/HER2 inhibitor lapatinib and a synergistic response with inhibition of the PI3K/Akt/mTOR pathway (Garcia-Garcia et al., 2012).

Despite the many problems associated with the use of xenograft models there are numerous successes. Human breast cancer xenografts overexpressing HER2/neu were used to show the ability of trastuzumab (Herceptin) to increase the response to paclitaxel and doxorubicin, leading to clinical trials (Balsega et al., 1998).

1.5 Transgenic models expressing BAG-1

Transgenic murine models of breast cancer are useful as provide a superior representation of the tumour microenvironment shown to contribute towards tumour progression. Target genes in transgenic mice are altered by overexpression, deletion, or mutation to allow study of the tumour response in vivo. Transgenic murine models can also be used for trial of novel therapeutic agents, prior to clinical trial. However, transgenic models cannot offer the genetic complexity of human tumours, as there can be loss or gain of genes from one tumour cell to the next. The lack of genetic heterogeneity within tumours in transgenic models is a probable reason for the limited predictive value of these models in predicting clinical response. The poor translation of transgenic models to clinical trials is highlighted by the last decade of research into the motor neurone disease amyotrophic lateral sclerosis (ALS). 78 single therapies and 12 combined therapies tested using animal models were included in a review (Benetar, 2007). 1 double-blinded clinical trial was successful from a total of 11 involving the trial of novel drugs for patients with ALS (Bhatt and Gordon, 2007; Vincent et al., 2008).

The only transgenic model demonstrating the effect of carrying a null allele for BAG-1 was produced in pneumocytes rather than breast cancer cells. Transgenic mice heterozygous for a null allele for BAG-1 and expressing the oncogene, C-Raf kinase in type II pneumocytes were produced. The mice developed multiple lung adenomas, but the tumours in the mice with BAG-1 haploinsufficiency underwent tumour cell apoptosis (Gotz et al.,2004).

BAG-1 has been implicated in regulating survival of breast cancer cells (see Section 1.3.1) and apoptosis is a crucial process in the development of functional organs. Immunohistochemical studies in murine models have shown a difference in BAG-1 expression and localisation during mammary development and involution (Schorr et al., 1999). Immunohistochemical analysis revealed a change in subcellular localisation within the mammary gland from lactation to involution. During lactation and early stages of involution BAG-1 was observed to be predominantly nuclear. At 10 days involution BAG-1 was found to be cytoplasmic with absent immunopositivity in the nucleus. Schorr et al. (1999) investigated the role of Bcl-2 family members in mammary morphogenesis and the specific role of each during involution. A combination of expression studies, germline loss of function, tissue-specific loss of function, and transgenic gain of function studies was used to examine role of Bcl-2 family members. Numerous effects were seen in organs leading to abnormal

development. Dominant gain of Bcl-2 expression led to decreased apoptosis during mammary involution and increased progression of c-myc-induced mammary tumours (Schorr et al., 1999).

Furth et al., (1999) used a transgenic mouse model of breast cancer progression in which Simian virus 40 large T antigen (TAg) expression is targeted to mammary epithelial cells using the whey acidic protein (WAP) promoter (WAP-Tag) (Furth et al., 1999). All WAP-TAg mice develop multiple breast adenocarcinomas by maturity. In addition, mice carrying a WAP-TAg-Bcl-2 hybrid gene were bred alongside non-transgenic control mice. Mice carrying the WAP-TAg-Bcl-2 hybrid gene developed tumours faster than the WAP-TAg mice. Quantification of apoptotic cells in situ by microscopy and Apotag assay (Oncor, Gaithesburg, MD, USA) showed a decrease in apoptosis. Immunoblots of BAG-1 extracted from the tissue of the correlating WAP-TAg, WAP-TAg-Bcl-2 and control mice showed an upregulation of BAG-1 expression in the WAP-TAg-Bcl-2 model, suggesting BAG-1 is involved in the anti-apoptotic mechanism leading to hastening of tumourigenesis (Furth et al., 1999). WAP-TAg transgenic mice were also used in a later study to show Bcl-2 overexpression reduces apoptosis in the initial proliferative phase in breast carcinogenesis. In addition, overexpression of Bcl-2 was found to reduce overall rate of cell proliferation. The reduction in proliferative rate correlated with the transition to adenocarcinoma (Li et al., 2000). These studies suggest that the activity of Bcl-2 family members, inclusive of BAG-1, mediates a balance between apoptosis and proliferation and contributes towards the progression of breast carcinogenesis.

BAG-1 has been shown to be crucial in the nervous system as plays a role in neuronal differentiation (Kermer et al., 2002) and mice models without BAG-1 are lethal in utero due to massive neuronal apoptosis (Gotz et al., 2005). Alzheimer's disease is characterised by the formation of distinct plaques and neurofibrillary tangles due to the accumulation of amyloid-beta and hyperphosphorylated Tau protein. The 3XTg mouse model of Alzheimer's disease incorporates mutations in the genes for amyloid precursor protein, Tau, and presenilin (Oddo et al., 2003). Immunohistochemical analysis of sections from the brain of 3XTg mice showed high expression of BAG-1 in neurons strongly immunopositive for an antibody against phosphorylated Tau (Elliot et al., 2007). Immunoprecipitation experiments using P19 neuronal cells demonstrated BAG-1 can interact with Tau protein in an Hsc/Hsp70 chaperone-dependent manner. P19 and human embryonic kidney 293 (HEK293) cells were stably transfected with BAG-1- green fluorescent protein (GFP) or GFP alone. Western blot

analysis showed total Tau protein increased with BAG-1 overexpression. The amount of phosphorylated Tau protein was increased by the same ratio as total Tau. An in vitro 20 S proteasome degradation assay showed BAG-1 reduced degradation of Tau more effectively than Hsc70 alone. Western blot analysis of immunoprecipitated Tau from P19 cells overexpressing BAG-1 showed no change in ubiquitin. Depletion of BAG-1 using small interfering RNA (siRNA) against BAG-1 in P19 neuronal cells showed an increase in phosphorylation and decreased Tau. The antibiotic geldamycin causes an upregulation in heat shock protein activity. Tau protein levels were significantly increased in P19 neurons overexpressing BAG-1 compared to vector control following exposure to geldamycin or heat shock. Repeat experiment in P19 cells with depleted BAG-1due to use of siRNA showed a concordant decrease in Tau protein after heat shock or geldamycin. These results support a role for BAG-1 in regulating the function of Hsc70 in neuronal cells between proteasomal degradation and stabilisation of Tau protein (Elliott et al., 2007).

Huntington's disease (HD) is a neurodegenerative disorder involving autosomal dominant inheritance of the Huntingtin gene. Expansion of an amino acid triplet within the brain leads to damage by mechanisms which remain elusive (Walker, 2007). A transgenic model of Huntington disease using mesencephalic progenitor CSM14.1 cells has shown that BAG-1 overexpression prevents neuronal apoptosis following the antibiotic staurosporine (Liman et al., 2005). Staurosporine is an antibiotic used in research as is an effective kinase inhibitor and activator of caspase 3 (Chae et al., 2000). Cell survival of CSM14.1 cells was assessed by crystal violet assay following staurosporine treatment. The increase in cell survival conferred by BAG-1 overexpression was shown to be dependent on the ability of BAG-1 to interact with Hsc/Hsp70 chaperones. CSM14.1 expressing BAG-1 incorporating mutant Hsc/Hsp70 binding domain showed no evidence of neuroprotection compared to control. Fluorogenic assay of caspase 3 activity showed no reduction in neuronal apoptosis in CSM14.1 cells overexpressing BAG-1 compared to control. Hence, the authors conclude BAG-1 may be functioning through regulation of an alternative apoptotic pathway, such as Hsc/Hsp70/ Apaf-1 interaction. Interestingly, BAG-1 was also found to play a role in neuronal differentiation, but this function was independent of Hsc/Hsp70 (Liman et al., 2005). Another study using immunohistochemical analysis of the HD exon 1 transgenic mouse showed Hsc/Hsp70 were consistently both present in Huntingtin aggregates in intranuclear striatum (Jana and Nukina, 2005). This study then used a cellular model created inducible neuronal cell lines expressing truncated N-terminal Huntingtin (tNhtt) normally and with polyglutamine expansion (HD 16Q and HD 150Q respectively). Immunoprecipitation with anti-GFP and immunoblotting experiments showed an association between BAG-1S expression and

polyglutamine-expanded tNhtt, but not with normal tNhtt. Deletion of the Hsc/Hsp70 binding domain of BAG-1 resulted in absence of interaction between BAG-1 and polyglutamine-expanded tNhtt. MTS assays showed overexpression of BAG-1S protected HD 150Q cells from polyglutamine-expanded tNhtt-induced cell death. Cell viability was further improved upon overexpression of Hsc/Hsp70 in addition to BAG-1. These results indicate BAG-1 is involved in neuroprotection from mutant Huntingtin-induced cell death, although does not confirm a role for BAG-1 in normal neuronal cells or those with polyglutamine expansion (Jana and Nukina, 2005).

The evidence produced from studies of BAG-1 using transgenic models in both breast and other organs appears to support hypotheses drawn from cell line work. BAG-1 is multifunctional and can mediate proteasomal degradation or cell survival depending upon interaction and availability of Hsc/Hsp70 chaperones (Takayama et al., 1997; Elliot et al., 2007).

1.6 The expression and significance of BAG-1 in breast cancer: A review of clinical evidence.

1.6.1 The evidence for BAG-1 as a breast cancer biomarker

BAG-1 (Bcl-2-associated athanogene) is one of 16 genes incorporated into the breast cancer multi-marker predictor Oncotype DX™ (Genomic Health, CA). The Oncotype assay relies upon RT-PCR of mRNA extracted from paraffin-embedded tissue and measures 16 tumour-associated genes. Increased levels of BAG-1 expression are associated with a lower risk of tumour recurrence and a more favourable outcome (Sparano and Paik, 2008). The reports incorporating Oncotype DX™ are exciting and prompt the possibility of additional roles for BAG-1. A need for clarification of existing clinical evidence investigating BAG-1 protein expression in breast cancer was identified and addressed in this chapter.

It is interesting that a protein known to enhance tumour cell survival has been associated with better prognostic outcome in breast cancer (Paik et al., 2004). Cancers overexpressing BAG-1 may be dependent on BAG-1 expression, but be a better prognostic group than those which depend on other pathways, for example, triple negative cancers with other mutations. This paradox may also be explained by the multifunctional ability of BAG-1. BAG-1 can switch between regulating cell survival to promoting proteasomal degradation via interaction with Hsc/Hsp70, or alternative binding partners, depending on the presence or absence of stress (Song et al., 2001). BAG-1 can bind Raf-1 or Bcl-2 independently of Hsc/Hsp70, providing two mechanisms by which BAG-1 expression mediates tumour cell survival (Takayama et al., 1997). The pleotropic character of BAG-1 may also explain how BAG-1 overexpression in tumours is associated with an improved prognosis (Sparano and Paik, 2008), yet also to have been reported to confer drug resistance in breast cancer (Liu et al., 2014; Townsend et al., 2003), and decrease response to chemotherapy in squamous cell carcinoma (Wood et al., 2011). The role of BAG-1 may vary in other cancers as downregulation of BAG-1 has been reported in HeLa cervical cancer cells to promote drug resistance (Takahashi et al., 2003).

Interestingly, Bcl-2 expression is also reported by studies incorporating Oncotype Dx™ to be associated with improved clinical outcome (Sparano and Paik, 2008). Bcl-2 is also an anti-apoptotic protein and is overexpressed in human breast cancer (Rochaix et al., 1999) (see Section 1.2.2.1). In vitro work has shown that downregulating Bcl-2 expression enhances chemosensitivity and implicates Bcl-2 as a therapeutic target (Emi et al., 2005). Overexpression of Bcl-2 has been associated with well-differentiated ER-positive breast

cancers (Lipponen et al., 1995; Silvestrini et al., 1994). However, studies have shown Bcl-2 is an independent prognostic marker regardless of hormone receptor status, tumour size, grade, lymph node status and Nottingham Prognostic Index (Callagy et al., 2006; Dawson et al., 2010). The paradoxical nature of anti-apoptotic protein Bcl-2 being a marker of good prognosis may be explained by overexpression of any of the Bcl-2 family members causing an imbalance between pro- and anti-survival signalling.

A multitude of studies report novel tumour markers, but the majority fail to translate into clinical practice. The Reporting Recommendations for Tumour Marker and Prognostic Studies (REMARK) provide reporting recommendations for tumour marker prognostic studies, and were designed to improve the reporting of such studies. These criteria can also be used an analytical tool against which to assess reports of new cancer biomarkers (Altman et al., 2012; Mallett et al., 2010). The REMARK criteria were utilised to assess 12 existing studies of BAG-1expression in human breast cancer as a tumour biomarker (see Table 1.2). The limitations of existing evidence were examined and used to propose foci for future work.

1.6.2 Clinical study design

The majority of the studies associating BAG-1 expression with clinicopathological variables involve retrospective analysis of paraffin-embedded tissue and patient survival data (Cutress et al., 2003; Dumontet et al., 2010; Millar et al., 2009; Nadler et al., 2008; O'Driscoll et al., 2003; Sirvent et al., 2004; Sjostrom et al., 2002; Tang et al., 2004; Tang et al., 1999; Townsend et al., 2002; Turner et al., 2001) (see Table 1.2). A single prospective study conducted more recently concluded both BAG-1 and CD24 could be associated with adverse survival outcomes (Athanassiadou et al., 2009).

The study by Athanassiadou et al. (2009) involved a small prospective immunocytochemical analysis of 70 tumours from patients diagnosed with primary breast cancer (Athanassiadou et al., 2009). Immunocytochemical analysis was performed on cytological imprint smears taken from macroscopically localised primary breast cancer specimens at the time of surgery. Cells were fixed in paraformaldehyde for 10 minutes and stored at -70°C until immunocytochemistry could be performed. This was the only study of the 12 analysed to use cytological imprint smears rather than immunohistochemistry of paraffin-embedded whole sections or microdots. Immunoreactivity for BAG-1 and CD24, a cell surface protein overexpressed in several human cancers including breast carcinoma, was assessed (Kristiansen et al., 2003). Univariate and multivariate analysis demonstrated BAG-1 and CD24 expression positively correlated with increased stage, grade, size of breast tumours, and positive lymph node status. Univariate analysis showed nuclear BAG-1 was associated with shorter patient survival than cytoplasmic and total BAG-1. Overall CD24 and cytoplasmic CD24 correlated with reduced survival times. These findings contrast significantly with those of the Oncotype Dx™ study, which showed BAG-1 to positively correlate with improved survival outcomes and lower recurrence rate (Sparano and Paik, 2008). There are several explanations that could be responsible for the difference between studies. These are as follows;-

Oncotype Dx™ was designed to predict recurrence in ER-positive node-negative patients and the Athanassiadou et al. (2009) study includes ER-negative and node-positive patients.

The use of cytological imprint smears for immunocytochemical analysis by Athanassiaudou et al., (2009) to assess BAG-1 expression rather than RT-PCR of mRNA as used by Oncotype Dx™

The use of a small cohort of 70 patients by Athanassiadou et al. (2009).

The absence of any other apoptotic molecular markers in addition to BAG-1 in the Athanassiadou et al. (2009) study (Athanassiadou et al., 2009).

11 of the 12 studies utilised patient cohorts in excess of 100 patients, but only 2 studies exceeded 500 patients. Nadler et al. (Nadler et al., 2008) used tissue microarrays incorporating breast tissue from 638 patients with linked outcome data. The significance of this study's findings is diluted as the patient treatment history and tumour stage data are incomplete (Nadler et al., 2008). Dumontet et al. (2010) used tumour blocks from the Breast Cancer International Research Group phase III trial comparing adjuvant TAC (doxorubicin, cyclophosphamide, and docataxel) with FAC (doxorubicin, fluorouracil, and cyclophosphamide) regimes in patients with axillary node-positive breast cancer. The group were able to access 91% (1,350) of the tumours for immunohistochemical analysis of 14 molecular markers, including BAG-1. This study found BAG-1 had no prognostic significance, but showed ki67, p53, and microtubule-related proteins were independent prognostic markers in patients receiving adjuvant chemotherapy for node-positive cancer. However, a significant amount of patient data was missing. 164 (12%) patient tumours of the 1,350 had no data on the presence or absence of multifocal tumours. There was an absence of data on the presence or absence of vascular invasion in 74 (5%) tumours and 343 (25%) tumours missed information regarding extranodal disease (Dumontet et al., 2010).

In general, the breast cancer studies investigating the relationship between BAG-1 and prognostic parameters incorporate larger patient cohorts than studies with the same intent in other cancers. This could be explained by breast cancer being more prevalent than, for example, gastric or oral carcinomas, and that patients are often diagnosed early enough that core biopsies and follow-up periods are appropriate for such analysis. However, most studies involve unselected heterogeneous patient cohorts and fail to take into account the many histological and treatment subgroups encountered in the management of breast cancer. Cutress et al. (2003) decreased the number of variables by selecting a specific cohort of patients all of whom were ER (oestrogen receptor) positive, lymph node negative and had received hormone therapy (Cutress et al., 2003).

11 of the 12 studies clearly stated inclusion and exclusion criteria and the assay control used. 11 of the 12 studies applied both univariate and multivariate analysis, although three studies failed to include hazard ratios and all but two omitted confidence intervals (see Table 1.2). The average follow-up for all 12 studies was approximately 7 years (range 3.3 to 12.8 years).

1.6.3 Assay method: mRNA versus protein

11 of the 12 studies used immunohistochemistry to examine BAG-1 protein expression, but 10 of the 12 studies relied upon manually processing larger sections and using a subjective scoring system based on the H-score technique for staining density Athanassiadou et al., 2009; Cutress et al., 2003; Dumontet et al., 2010; O'Driscoll et al., 2003; Sirvent et al., 2004; Sjostrom et al., 2002; Tang et al., 2004; Tang et al., 1999; Townsend et al., 2002; Turner et al., 2001). 2 studies utilised tissue microarrays and automated quantitative analysis (AQUA) (Millar et al., 2009; Nadler et al., 2008).

3 of the 12 studies investigated BAG-1 expression using immunohistochemical staining and RT-PCR. There is some controversy between even the 3 papers that analysed mRNA levels included in this review to whether there is any correlation between BAG-1 mRNA levels and BAG-1 protein expression. Millar et al. (2009) also demonstrated a strong correlation between BAG-1 protein, BAG-1 mRNA and improved survival outcomes (Millar et al., 2009). Townsend et al. (2002) showed no correlation between BAG-1 mRNA levels or cytosolic BAG-1 with improved overall survival (Townsend et al., 2002). O'Driscoll et al. (2003) failed to show any significant patterns between mRNA levels and BAG-1 and concluded BAG-1 was not a potential prognostic indicator (O'Driscoll et al., 2003). The lack of correlation between studies can be partially explained by post-transcriptional modification, an absence of standard treatment regime, and histological type within the cohorts selected.

An absence of any relationship between gene transcripts and protein levels is not surprising as any analysis at RNA level does not take into account post-translational modifications. RT-PCR of mRNA levels does however permit high throughput, sensitive, specific and highly reproducible analysis. Manual scoring techniques can result in false relationships between cancer biomarker expression and patient outcomes due to inaccurate antibody titre (McCabe et al., 2005). Tissue microarrays are a vast improvement on standard immunohistochemical staining techniques as they are efficient, independent of pathologists if automated quantification is used, highly standardised, and involves minimal damage to the tissue blocks. The main concern regarding tissue microarrays is that the small tissue samples or 'microdots' may not be representative of the whole tumours, and do not address tumour heterogeneity (Shergill et al., 2004). Nadler et al. (2008) addressed tumour heterogeneity by incorporating 2 cores of breast tumour taken from different locations from within the cancer into their tissue microarray (Nadler et al., 2008).

Quantification of protein expression for large patient cohorts incorporated into tissue microarrays uses programmes such as AQUA (Automated Quantitative Analysis), which measures in situ protein or antibody concentration. This method allows for accurate antibody titration, non-subjective quantitative analysis and is far superior to manual techniques in terms of efficiency. AQUA does have some faults; the processing of missing data due to tissue 'spots' being damaged during microarray production may still lead to inaccurate analysis (Camp et al., 2004; Camp et al., 2008). Despite the problems with tumour heterogeneity and handling missing data, tissue microarrays allow efficient analysis of proteins, which are further down the cell production line than RNA and more representative of intracellular interactions involved.

1.6.4 BAG-1 expression in normal breast epithelium and breast cancer

Immunohistochemistry and immunoblotting for BAG-1 in normal breast epithelium shows BAG-1 is present in both nuclei and cytosol, but generally not as abundant as in invasive breast cancer tissue (Takayama et al., 1998; Turner et al., 2001). Turner et al. (2001) reported 28% of 88 normal breast epithelia sections with H-scores exceeding 150 for nuclear BAG-1, yet staining for cytosolic being weak in all sections. Sections incorporating invasive breast carcinoma in this study showed dichotomous subgroups with either significantly elevated cytosolic BAG-1, or almost complete absence compared with adjacent epithelial cells. In contrast, Takayama et al. (1998) study showed increased levels of the nuclear isoform BAG-1L were evident in breast cancer cell lines, especially BT-549 compared with normal breast epithelial cells. This paper hypothesised that the higher levels of nuclear BAG-1 in hormone-responsive tumours, such as prostate and breast, could explain a mechanism of up-regulation and increased activity of hormone receptors (Takayama et al., 1998). Cutress et al. (2003) provided evidence in support of this hypothesis using 138 immunostained sections of human breast cancer from patients who had received hormone therapy. Staining for BAG-1L significantly correlated with positive staining for oestrogen (ER) and progesterone receptors (PR), a downstream target of ER transcription (Cutress et al., 2003).

11 of the 12 reports did not use a separate core of non-malignant or normal breast tissue but scored the cells adjacent to the tumour within the sections. This method controls for variability in antibody concentration between sections during manual staining techniques. However, peritumour cells may be subject to the same environmental changes that accompany malignant progression and perhaps different epigenetic influences to normal breast epithelium. Immunohistochemical staining scores were not given for normal breast tissue controls in 11 of the 12 studies.

In general, the studies reported a high percentage of invasive breast carcinomas showed positive staining for BAG-1, half of the studies reporting >70% of tumours showing positive staining (see Table 1.1). However, 3 studies omitted a value for percentage of samples showing positive staining for BAG-1 (Cutress et al., 2003; Millar et al., 2009; Nadler et al., 2008). Half of the immunohistochemistry studies showed a general pattern of higher cytoplasmic than nuclear BAG-1 immunopositivity (Tang et al., 1999; Turner et al., 2001; Townsend et al., 2002; Athanassiadou et al., 2009; Dumontet et al., 2010) and 9 out of the 12 studies did not give a value for mixed staining. A single study showed higher nuclear BAG-1 staining than cytosolic (Cutress et al., 2003). Interestingly, this study also was the

most controlled in terms of selecting a specific patient cohort in terms of tumour stage and treatment received. 83% of the tumours from this group were ER positive, 84% PR positive.

Two studies failed to give any precise subcellular analysis (Sjostrom et al., 2002; Sirvent et al., 2004). Sjostrom et al. (2002) did not distinguish between cytoplasmic and nuclear BAG-1 and failed to show any relationship between BAG-1 and predictive value for patients receiving chemotherapy (Sjostrom et al., 2002). Sirvent et al. (2004) reported BAG-1 immunoreactivity was mixed, with more cytosolic than nuclear BAG-1 in breast carcinomas, but did not quantify this observation (Sirvent et al., 2004) (see Table 1.2).

1.6.5 BAG-1 expression and associated clinicopathological features in breast cancer

Levels of total BAG-1 expression in invasive breast carcinoma positively correlated with patient survival outcomes in 3 studies with varying degree. Turner et al. (2001) reported BAG-1 was associated with improved disease-free survival, overall survival in both node-negative and node-positive patients (Turner et al., 2001). Nadler et al. (2008) found no difference in all prognostic variables between nuclear and cytoplasmic BAG-1, but correlated total BAG-1 with significantly improved survival outcomes in node-positive patients (Nadler et al., 2008). Millar et al. (2009) found high levels of total BAG-1 was associated with low grade tumours, ER and PR positivity, and improved overall survival. Millar et al. (2009) also found increased levels of BAG-1 were present in the luminal a phenotype (Millar et al., 2009). Luminal a tumours are ER-positive, and or PR-positive, and HER2-positive or ki67-high, and account for 40% of all breast cancers (Goldhirsch et al., 2011). The luminal a breast cancer phenotype is associated with favourable response to hormonal therapy, slow tumour progression, and improved survival outcomes (Blows et al., 2010).

3 of the 12 studies supported the hypothesis that increased nuclear BAG-1 was associated with improved prognosis (Turner et al., 2001; Cutress et al., 2003; Tang et al., 2004). High grade tumours showed weak levels of immunoreactivity for nuclear BAG-1 staining in Turner et al. (2001), evidence supported by the later study by Tang et al. (2004), contradicting an earlier paper published by the same group (Kjoniksen et al., 2005; Tang et al., 2004; Turner et al., 2001). The hypothesis that nuclear BAG-1 is a factor for improved prognosis in breast cancer was supported by a study using a select cohort of node-negative, ER-positive patients treated with hormone therapy (Cutress et al., 2003). Cutress et al. (2003) implicate BAG-1L in mediating the response to hormone therapy in ER-positive tumours and show BAG-1L expression to be predictive of tumour response to Tamoxifen (Cutress et al., 2003). The impact of this study could be regarded as more significant as histological and treatment variables were controlled by selecting a specific cohort. In addition, no data was missing in the Cutress et al. (2003) study for histological type, tumour stage, treatment given, or tumour characteristics (Cutress et al., 2003).

Over half of the studies showed high levels of cytoplasmic staining for BAG-1 in invasive breast cancers. However, 9 of the 12 studies showed no correlation between survival and cytosolic BAG-1. The association between high cytosolic BAG-1 and improved patient outcomes reported in Nadler et al. (2008) were supported by a similar expression pattern in Turner et al (2001). Turner at al. (2001) showed that >60% of stage I or II tumours showed high levels of cytoplasmic staining in addition to 64% of 14 DCIS specimens. High levels of

cytoplasmic BAG-1 staining positively correlated with improved patient overall survival, and suggests a role for cytoplasmic BAG-1 in early tumour progression (Turner et al., 2001).

Only 2 of the 12 studies proposed levels of BAG-1, total or especially nuclear BAG-1, were associated with higher tumour stage, increased tumour size and poorer overall survival(Athanassiadou et al., 2009; Tang et al., 1999).Tang et al. (1999) disagreed with this hypothesis in a later study (Tang et al., 2004). The patient, histological, and treatment data are incomplete for the early Tang at al. (1999) study, which could explain a lack of correlation. However, there are no absent data in the prospective Athanassiadou et al. (2009) study, but there is an increased number of lobular breast cancers included, which could account for difference (Athanassiadou et al., 2009). Dumontet et al. (2010) also included mixed and lobular invasive breast carcinomas, accounting for 13% of breast tumours studied (Dumontet et al., 2010).

The controversy surrounding expression patterns of BAG-1 is apparent, but differences introduced by patient cohort heterogeneity in terms of histological type and number, tumour grade, and treatment may account for these. Another consideration for studies relying upon immunohistochemistry is the level chosen above which represents positive staining. Immunoreactivity of 40% of the total tumour was taken to as the cut-off point for nuclear and cytoplasmic BAG-1 positivity by Millar et al., (2009), yet Sirvent et al. (2004) applied a value of 10% immunoreactivity to represent positivity (Millar et al., 2009; Sirvent et al., 2004). The Dumontet et al. (2010) study used the median percentage of immunoreactive tumour to define the cut-off points for both nuclear and cytoplasmic BAG-1. This study used cut-off values of 20% immunoreactivity for nuclear BAG-1 and 60% for cytoplasmic BAG-1 (Dumontet et al., 2010). There is a need for use of a standardised technique for defining cut-off points for BAG-1 positivity and levels of immunointensity (low, intermediate, high). The use of test and validation cohorts to define cut-off points prior to use within the larger study is recommended by the authors of the REMARK criteria and a review of the prognostic ability of panels of biomarkers (Altman et al., 2012). Software programmes such as X-tile™ can process biomarker data and assist designation of cut-off points for sub-sets using a reproducible method (Camp et al., 2004).

1.6.6 BAG-1 expression and associated clinical outcomes in other cancers

BAG-1 has been investigated in a number of different tumours and associated with clinicopathological parameters. An inverse relationship between BAG-1 levels and patient survival and, or tumour grade is apparent for many of the non-breast tumours (Bai et al., 2007; Clemo et al., 2008; Sun et al., 2010; Sun et al., 2011; Hague et al., 2002; Maki et al., 2007; Xie et al., 2004; Zheng et al., 2010).

One study reported a cohort of 22 oral squamous carcinomas showed high levels of BAG-1 in those of higher grade and with positive lymph nodes (Shindoh et al., 2000). However, this study did not use a standard H-score for BAG-1 immunohistochemistry and judged expression level via densitometry of BAG-1 bands produced by Western blotting. This study did not include subcellular analysis of BAG-1 expression. A number of the earlier non-breast studies include patient cohorts of below 100. This is partially due to the majority of these cancers not being as prevalent as breast cancer, thus more difficult to collate large tissue banks for immunohistochemical studies.

Hague et al. (2002) collated a cohort of 64 patients diagnosed with oral squamous cell carcinoma (Hague et al., 2002). This study compared the 64 tumours and 17 specimens of normal oral epithelia for expression of BAG-1. Oral carcinomas showed a reduced level of nuclear BAG-1 than normal oral mucosa. There was no such reduction in cytoplasmic BAG-1, but less of a difference between levels of cytosolic and nuclear BAG-1 in cancerous specimens compared to normal tissue. Hague et al. (2002) did, however, show lymph node metastases demonstrated higher cytoplasmic BAG-1 staining than corresponding primary tumour. Western blot analysis of lysates produced from normal and cancerous oral cell lines showed increased expression of the BAG-1S isoform in oral cancer cell lines. The group used the MCF-7 ER-positive breast cancer cell line as a reference control for Western blotting. These results support a role for BAG-1 in oral carcinogenesis and a change in expression of BAG-1 isoforms with tumour progression (Hague et al., 2002).

Xie et al. (2004) performed an immunohistochemical study of 85 patients diagnosed with squamous carcinomas of the tongue to conclude that increased total BAG-1 correlated with shorter disease-free patient survival (Xie et al., 2004). A larger study incorporating a tissue microarray of 229 oral squamous cell carcinomas reported BAG-1 was associated with perineural invasion (Coutinho-Camillo et al., 2010). Overall survival was reduced in tumours expressing higher levels of Bcl-2-related protein and disease-free survival lower in patients with tumours expressing higher levels of Bcl-x. However, no significant difference in survival

outcomes was found for BAG-1 expression in oral squamous cell carcinoma (Countinho-Camillo et al., 2010).

The expression of BAG-1 has been investigated in gastric carcinoma cell lines and tissue (Zheng et al., 2010). This study used a range of gastric cell lines from undifferentiated (HGC-27) to well-differentiated (MKN28) and gastric tissue to investigate BAG-1 expression. HeLa cervical cell culture and adjacent non-neoplastic mucosa were used as controls. Analysis of BAG-1 expression was performed using Western blotting and RT-PCR of BAG-1 mRNA of lysates from cell culture or gastric tissue. Analysis of BAG-1 isoform expression was obtained by cell fractionation and subsequent Western blotting and RT-PCR. Interestingly, the 50kDa BAG-1 isoform (BAG-1L) was only present in nuclear fractions from undifferentiated HGC-27 gastric cells. The 50kDa and 46kDa isoforms were weakly present in the cytoplasmic fraction of well-differentiated MKN28 cells, poorly differentiated MKN45 cells and KATO-III cells. The 36kDa BAG-1 isoform was strongly present in the cytosolic fraction of MKN28, MKN45, KATO-III, and moderately differentiated AGS cells. Immunohistochemical analysis showed nuclear BAG-1 was decreased from gastritis, intestinal metaplasia, adenoma to carcinoma. Cytoplasmic BAG-1 was weakly positive in gastric carcinoma compared to normal mucosa. Immunohistochemical staining was performed on 355 gastric carcinomas incorporated into a tissue microarray. In situ hybridisation was also performed on the tissue microarray to confirm the levels of BAG-1 mRNA observed following RT-PCR. BAG-1 mRNA levels were shown to be higher in intestinal metaplasia, than in normal mucosa or carcinoma. Levels of BAG-1 mRNA were higher in tumours from female than male patients. BAG-1 mRNA was also found to positively correlate with depth of tumour invasion and vascular invasion. Immunohistochemical staining of nuclear BAG-1 was shown to reduce with metastasis to lymph nodes, lymphatic invasion, and cytoplasmic BAG-1. Increased immunohistochemical staining of cytoplasmic BAG-1 was associated with invasive depth of gastric carcinoma, but not any other clinicopathological parameters. Univariate analysis using Kaplan-Meier technique showed cumulative survival was increased with nuclear BAG-1 expression than without. The opposite was true for cytoplasmic BAG-1 expression, showing a reduction in cumulative survival with increased expression. Multivariate analysis failed to validate BAG-1 as an independent prognostic factor (Zheng et al., 2010).

Overexpression of BAG-1 in colorectal carcinoma cell lines was initially reported by Takayama et al. (1998). An immunohistochemical of BAG-1 expression in tumours from 86 patients diagnosed with colorectal carcinoma demonstrated BAG-1 to be an independent prognostic indicator of mortality (Kikuchi et al., 2002). Nuclear BAG-1 immunopositivity was

higher in cancers with distant metastases. The study by Kikuchi et al. (2002) showed nuclear BAG-1 to have predictive value for metastasis and poor prognosis. Bai et al. (2007) showed that increased total BAG-1 protein, analysed by immunohistochemical staining, correlated with poorly differentiated tumours, nodal metastasis and decreased survival rate (Bai et al., 2007). This group did not perform subcellular analysis and recruited a relatively small cohort of 80 patients with colorectal carcinoma.

Further evidence is provided by BAG-1 overexpression being identified in medium to large colorectal adenomas and carcinoma (Clemo et al., 2008). Clemo et al. (2008) demonstrated high immunohistochemical staining of BAG-1 in carcinomas compared to normal colonic tissue. In addition, Clemo et al. (2008) showed increasing levels of BAG-1 from normal colonic epithelium, small (< 5mm) adenomas, medium (>5mm<10mm) adenomas, large (>10mm) adenomas, to carcinoma. Clemo et al. (2008) used luciferase reporter assays to show knockdown of BAG-1 by siRNA in HCT116 colorectal cancer cell lines inhibits NF-KappaB and induces apoptosis. Western blot analysis confirmed BAG-1 was reduced following siRNA transfection compared to negative siRNA control (Clemo et al., 2008).

BAG-1 has a role in colorectal cancer survival and progression, a theory supported by 3 additional, more recent studies (Alper et al., 2008; Sun et al.). Alper et al. (2008) investigated the expression of BAG-1 in the adenoma-carcinoma sequence in colorectal cancer (Alper et al., 2008). Samples from 12 hyperplastic polyps, 18 tubular and 19 villous adenomas were analysed by immunohistochemistry. Samples were analysed for BAG-1 and 2 additional apoptotic markers, Bak and survivin, Increased levels of cytoplasmic BAG-1 were found in colonic tumour cells compared with adenoma and normal colonic epithelium (Alper et al., 2008). Sun et al. (2010) used samples from 128 patients diagnosed with colorectal carcinoma and 20 samples of normal colonic tissue to investigate BAG-1 expression (Sun et al., 2010). RT-PCR and immunohistochemical analysis of BAG-1 showed BAG-1 positively correlated with increasing tumour grade, poor prognosis, distant metastasis and Duke's stage. There was no correlation between clinicopathological parameters and Bcl-2 expression in colon carcinoma (Sun et al., 2010). The same group persisted with investigation of the significance of BAG-1 expression in colorectal cancer (Sun et al., 2011). They collated samples from 320 patients diagnosed with colorectal cancer and 30 normal colorectal tissue samples. RT-PCR and immunohistochemical analysis confirmed the findings from their first study. BAG-1 was shown to be associated with increased tumour grade, distant metastases, Duke's stage and prognosis (Sun et al., 2011).

Expression patterns of BAG-1 have been studied in endometrial cancers, although studies so far have failed to reach significance in association with patient survival outcomes. BAG-1 was shown to be highly expressed in poorly differentiated endometrial carcinoma compared to normal endometrium (Moriyama et al., 2004). A later study showed cytosolic BAG-1 increased from normal endometrium and endometrial hyperplasia to carcinoma. Nuclear BAG-1 was found to be more abundant in normal endometrium than cancer (Song et al., 2008).

Cutress et al. (2003) defined a relationship between high BAG-1L expression and oestrogen-dependent ER upregulation, resulting in an improved response to hormone therapy. An interesting yet contrasting relationship is observed in between BAG-1 expression and prostate cancer, a hormone-dependent carcinoma. Expression of BAG-1L has been shown to increase AR transcription (Knee et al., 2001; see section 1.2.2.3) In a large cohort of 263 tumours high expression of BAG-1L was associated with hormone refractory prostate cancer (Maki et al., 2007). BAG-1 expression was investigated in prostate cancer cell lines, xenografts, and patient samples using RT-PCR and immunohistochemistry. BAG-1 gene amplification was found in both of the xenografts and in 6 of the 81 hormone-refractory prostate cancers. No gene amplification was found in any of the 130 untreated prostate carcinomas. The relationship between nuclear BAG-1 expression and refractory prostate cancer was also shown in a smaller cohort of 92 prostate tumours (Krajewska et al., 2006). These results confirm a role for BAG-1 in hormone-responsive tumours, suggesting that nuclear BAG-1 regulates hormone receptor activity and hence response to hormone therapy.

The expression of BAG-1 has been investigated in non-small cell lung carcinoma (NSCLC) (Rorke et al., 2001). Tumour samples were collected from 85 patients diagnosed with NSCLC. Subcellular analysis of BAG-1 isoforms was performed using immunohistochemistry. Cancers expressing high levels of BAG-1 were associated with reduced risk of death. Cytosolic BAG-1 was found to be a positive indicator of improved prognosis in lung carcinoma (Rorke et al., 2001). The regulation of ER by BAG-1 may partially explain the link between clinicopathological parameters and BAG-1 expression in carcinoma of the lung. Liu et al. (2008) showed increased median survival time was associated with lower levels of BAG-1 RNA (Liu et al., 2008). Lung cancer is increasing in prevalence amongst females compared to males, thereby implicating female sex hormones and the ER in increasing susceptibility to carcinogens and promoting tumour progression (Chakraborty et al., 2010).

BAG-1 has been shown by a more recent study to be an independent prognostic factor in patients following radical surgical resection of the pancreatic head (van der Zee et al., 2013).

Immunohistochemical analysis of 217 tumours from patients diagnosed with pancreatic carcinoma showed nuclear BAG-1 is associated with a favourable survival outcome following resection of the pancreatic head, but not the periampullary region.

To attempt to explain further the discrepancy between BAG-1 expression in human cancers and clinicopathological characteristics, published reviews of BAG-1 discuss the role of BAG-1 in hormone-driven tumours, and those which are hormone-independent (Cutress et al., 2002; Sharp et al., 2004). The larger cohort studies of BAG-1 expression in colorectal, gastric and oral squamous cell carcinomas show a negative correlation with favourable prognostic parameters (Bai et al., 2007; Clemo et al., 2008; Sun et al., 2010; Sun et al., 2011; Zheng et al., 2010; Hague et al., 2002). High cytosolic BAG-1 in non-breast tumours has been associated with lymph node metastasis or reduced cumulative survival (Hague et al., 2002; Zheng et al., 2010). Contrasting results exist from studies of BAG-1 in breast cancer (see Section 1.6.5). However, the study by Cutress et al. (2003) meets the majority of the REMARK criteria and was performed on specific cohort of ER-positive, node-negative patients. The study by Cutress et al. (2003) showed increased nuclear BAG-1 in breast carcinoma was associated with an upregulation of ER, improved prognosis, and response to hormone therapy (Cutress et al., 2003). However, the role of BAG-1 in tumourigenesis cannot be clearly defined by the hormone dependency of the tumour, as contrasting results are seen for endometrial and prostate cancer, both of which are hormonally-driven (Moriyama et al., 2004; Song et al., 2008; Maki et al., 2007; Krajewska et al., 2006).

BAG-1 expression and function varies between tumour types. This supports the hypothesis that pathways mediated by BAG-1 in carcinogenesis are diverse and do not always involve regulation of hormone receptor activity, but may be a result of an imbalance in apoptotic proteins, thereby enhancing tumour cell survival.

1.6.7 Conclusion from review of clinical evidence

The benefit of incorporation of molecular biomarkers and traditional prognostic parameters is now well-recognised in breast cancer (Parisi et al., 2010). There is increasing evidence in support of BAG-1 as a cancer biomarker both from work on BAG-1 protein expression and BAG-1 RNA. The development of high-throughput breast cancer assays, such as Oncotype DX™, utilise the evidence that increased BAG-1 RNA is associated with low risk of recurrence and a favourable prognosis. However, RT-PCR relies upon mRNA extraction and not protein expression and there remains a lack of correlation between patterns in BAG-1 protein expression and BAG-1 mRNA.

The clinicopathological outcomes related to BAG-1 remain inconsistent in the majority of cancers studied. One explanation is the ability of BAG isoforms to adapt their functional role to changes in conditions or availability of chaperones. BAG-1M has been shown to switch function depending on the concentration present (Knapp et al., 2014). In vitro studies have shown low levels of BAG-1M stimulate steroid receptor activity, but at high concentrations BAG-1M inhibited AR, GR and MR function. The loss of regulation of BAG-1 could easily lead to an imbalance of protein folding or alternative functional outcomes, which could promote carcinogenesis.

Further studies should revisit BAG-1 protein expression in breast cancer, but in specific patient cohorts with standardised treatment regimes. The cohorts could be further divided according to the molecular classification of breast cancers to potentially identify expression patterns specific to luminal a, luminal b and basal type breast carcinomas.

Some of the studies incorporated experiments using breast cancer cell lines in addition to RNA or protein analysis of human breast tumours. Cell line work can be flawed as heterogeneity has been proven within the most established breast cancer cell lines and a tendency exists for cell lines to phenotypically drift (Burdall et al., 2003). Yet, cell line work may provide support to hypotheses generated from studies using paraffin-embedded human carcinomas or vice versa. Biological assays using ER+/- and HER2 +/- breast cancer cell lines can be used to investigate expression patterns of BAG-1 in response to chemotherapy, or the effect of siRNA knockdown of BAG-1 on cell proliferation and viability. Cell lines permit investigation into intracellular pathways involved, aid identification of other relevant targets for protein or mRNA assays. Tissue microarrays can then be used for large cohorts to investigate expression of any signalling proteins which may further validate BAG-1 as a cancer biomarker and possible therapeutic target.

1.7 BAG-1 in HER2 positive breast cancer

HER2 is a tyrosine kinase receptor and part of the same family of receptors as EGFR (epidermal growth factor receptor). The action of HER2 is unique within the EGFR family of receptors as HER2 does not have its own ligand, but relies upon dimerisation with other receptors. Overexpression of the HER2/Neu receptor has been shown to be associated with a higher risk of metastatic disease and shorter patient survival. Approximately 20% of breast cancers have HER2 gene amplification, leading to HER2 overexpression. The presence of HER2 overexpression in breast cancer has been used to identify tumours responsive to the monoclonal antibody therapy Trastuzumab (Herceptin™).

Previous studies have suggested BAG-1 may function in mediating transcription factors and through the MAPK signalling pathway (Figure 1.2) and via interaction with Raf-1(Sharp et al., 2009a; Wang et al., 1996). Many studies have demonstrated crosstalk and a compensatory effect of altered MAPK signalling on the phosphatidylinositol-3-kinase (PI3-K) pathway (Siddiqa et al., 2008). The mechanism of action of trastuzumab is yet to be wholly clarified, but is known to involve the interruption of HER2 receptor dimerisation and attenuation of receptor signalling along these common signalling pathways. An immunological response courtesy of natural killer cells has also been implicated in contributing towards the action of trastuzumab. Natural killer cells have been shown to induce antibody-dependent cellular cytotoxity (ADCC) in via binding with the Fc gamma receptor. Trastuzumab incorporates an Fc domain which can bind this Fc gamma receptor and trigger ADCC resulting in lysis of cancer cells bound to the drug. Additional proposed mechanisms of action are inhibition of shedding of the HER2 extracellular domain, inhibition of angiogenesis, and induction of G1 cell cycle arrest (Valabrega et al., 2007).

The problem of resistance to HER2-targeting therapies like trastuzumab is unsurprising considering the variety of possible modes of action. Resistance is a significant problem and has been reported to be apparent in as many as 70% of patients treated with trastuzumab alone (Montemurro et al., 2005) and half of those receiving trastuzumab in combination with adjuvant anthracyline or taxane-based chemotherapy (Montemurro et al., 2004). Resistance to HER2-targeted therapy provokes recognition of the need for novel molecular markers of HER2 activity and resistance. BAG-1 shares common signalling pathways and has been shown to be overexpressed in HER2 positive breast cancer cells.

BAG-1 binds RAF-1, as previously discussed, and utilises the MAPK signalling pathway. BAG-1 and the HER2 share common binding proteins and signalling pathways (Figure

1.5).Studies linking clinicopathological parameters to BAG-1 expression in breast cancer have not focused on HER2 positive cancers, although some studies have included HER2 expression in statistical analysis of associated biomarkers. Turner et al. failed to show any significance using a relatively small cohort of breast cancer patients between HER2 positivity, 10-year survival parameters, or the expression of BAG-1 (Turner et al., 2001). A larger immunohistochemical study by Nadler et al., using a tissue microarray incorporating 638 breast cancer patients, looked at BAG-1 and HER2 expression showed that HER2 expression was an independent prognostic factor associated with poorer survival outcomes. This group also showed that ER and PR positivity was associated with improved 10 year survival parameters in patients with higher BAG-1 expression levels, but failed to show any relationship between survival, HER2 and BAG-1 expression (Nadler et al., 2008).

A more recent immunohistochemical study of 292 breast cancer patients, including 51 (18%) HER2 positive cancers, showed a strong negative relationship between HER2 positivity and high BAG-1 expression. Only 5% of the HER2 positive cancers showed high levels of BAG-1 expression. A correlation between HER2 positivity and increased breast cancer-specific death was shown on Cox univariate analysis and an association between HER2 positivity and distant metastases on multivariate analysis (Millar et al., 2009).

Treatment data regarding type and length of course of therapy for the HER2 positive patients were unfortunately not considered prior to association with survival outcomes. This could prove significant considering patients receiving Trastuzumab following adjuvant anthracycline or taxane-based chemotherapy have been shown to have a 52% reduction in developing recurrence (Piccart-Gebhart et al., 2005). These clinical studies are lacking in their ability to demonstrate the significance of the expression of BAG-1 in breast cancer, as previously discussed. To progress from previous studies, and investigate if there is any value in knowing BAG-1 expression levels in HER2 positive patients, future work needs to focus on a treatment-standardised cohort of HER2 positive patients. The aim of this study is to elucidate whether there are any significant patterns in BAG-1 expression in HER2 positive breast cancers and to suggest potential mechanisms of function. The project will also further evaluate ER+ breast cancers using immunohistochemistry and attempt to suggest BAG-1 signalling pathways which may contrast with HER2+ breast tumours.

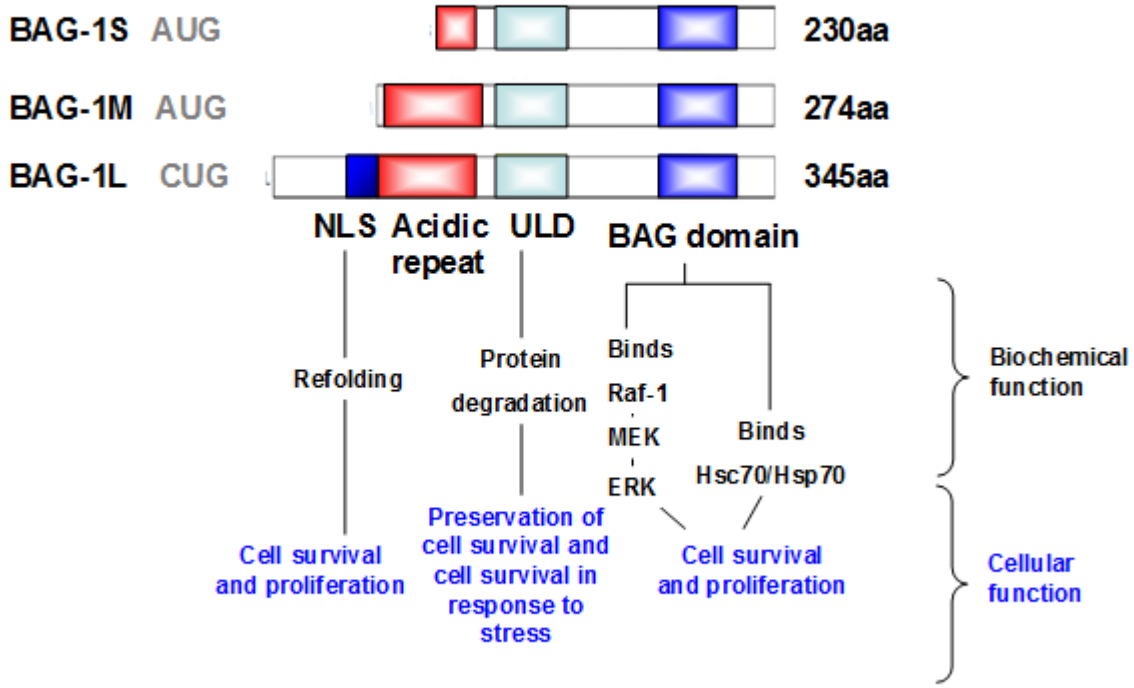

Figure 1.1 Domain structures of BAG-1 isoforms and associated functions.

The structures of the 3 major human BAG-1 isoforms are shown with associated functional domains. Structural domains located at the amino terminus are the nuclear localisation sequence (NLS) and a variable number of copies of amino acid repeats. The function of the amino acid repeats is unclear, although they have been shown to play a role in the regulation of glucocorticoid receptor transcription (Schneikert et al., 1999). The first exon contains the alternate translation initiation sites for BAG-1L (CUG), BAG-1M (AUG), and BAG-1S (AUG) (Yang et al., 1998). The ubiquitin-like domain (ULD) targets substrates for proteasomal degradation (Demand et al., 2001). The BAG domain is located at the carboxy terminus and contains a region capable of binding the molecular chaperones heat shock conjugate/ protein 70 or Raf-1, depending on the presence or absence of cellular stress (Hohfeld and Jentsch, 1997; Townsend et al., 2003).

aa: amino acid; **BAG**; Bcl-2-associated athanogene; **ERK**: extra-cellular signal-related kinase; **Hsc/Hsp70**: heat shock conjugate/ protein 70; **MEK**: mitogen-activated protein kinase kinase.

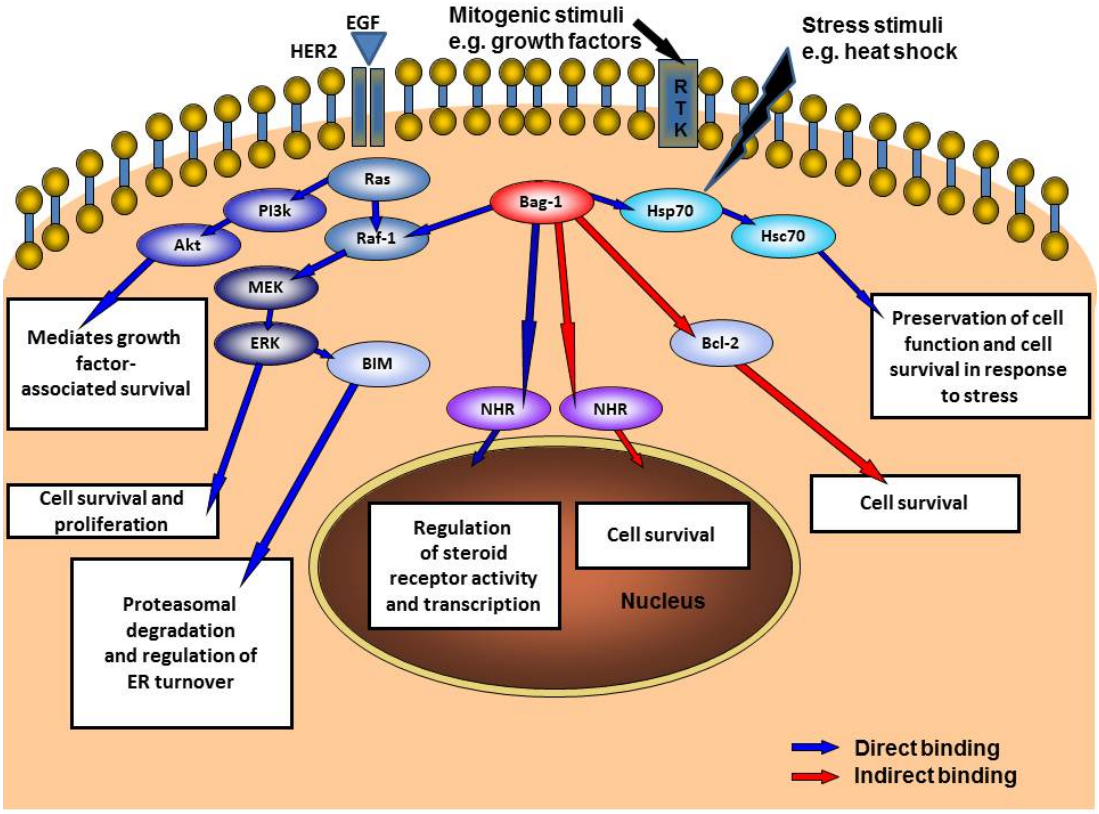

Figure 1.2 BAG-1 binding partners, signalling pathways and functions.

BAG-1 directly binds Raf-1, Hsc/Hsp70, and nuclear hormone receptors (NHR) (Schneikert et al., 1999; Wang et al., 1996; Zeiner et al., 1997). Bcl-2 is an indirect binding partner of BAG-1(Takayama et al., 1995). Raf-1 and Hsc/Hsp70 compete to bind BAG-1 via the BAG domain. BAG-1 preferentially binds Raf-1 in normal physiological conditions and BAG-1 stimulates Raf-1 kinase activity to cause cellular proliferation. In the presence of cellular stress levels of Hsc/Hsp70 are elevated and the chaperone molecules can displace Raf-1(Song et al., 2001). The change in binding partner with environmental conditions acts as a functional switch between cell proliferation and proteasomal degradation. BAG-1 can simultaneously bind Hsc/Hsp70 and the proteasome (Luders et al., 2000). This interaction allows BAG-1 to target Hsc70-bound protein substrate for degradation (Demand et al., 2001). The phosphatidylinositol-4, 5-bisphosphate kinase (PI3K) pathway is altered in 70% of breast cancers and often involves HER2 amplification. There is no evidence of direct interaction between BAG-1 and PI3K proteins. However, changes in PI3K activity can affect mitogen-activated protein kinase (MAPK) signalling, which is downstream of BAG-1 and Raf-1. Inhibition of PI3K can lead to ERK-dependent up-regulation of BIM and subsequent apoptosis (Ebi et al., 2013).

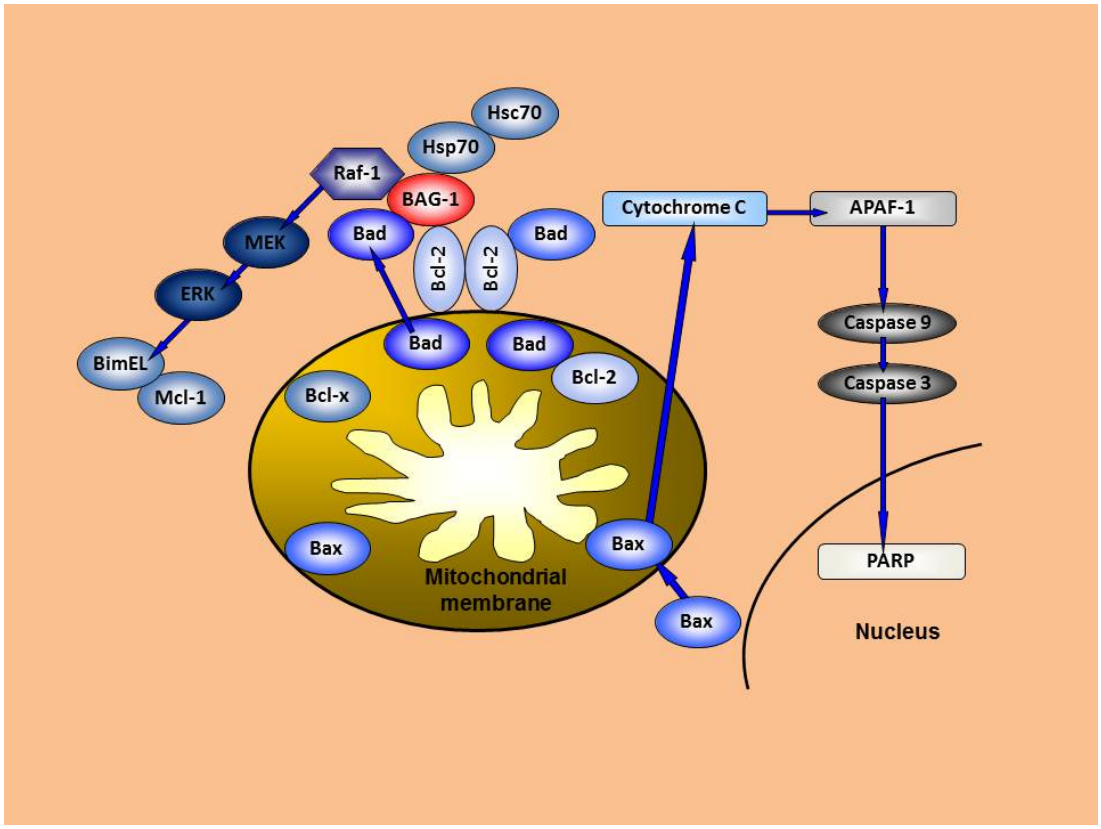

Figure 1.3 BAG-1 binding partners mediating apoptosis

BAG-1 indirectly binds Bcl-2 via Raf-1 to suppress apoptosis (Wang et al., 1996). BAG-1 can also regulate survival via direct interaction with chaperone molecules Hsc/Hsp70 (Takayama et al., 1997). BAG-1 overexpression in MCF-10A breast epithelial cells has been shown to increase activation of Raf-1 and ERK1/2 and cause decreased BimEL expression (Anderson et al., 2010). Bim is a pro-apoptotic protein member of the Bcl-2 family with 3 distinct isoforms, BimS, BimL, and BimEL. The dissociation of Bim from Mcl-1 is specific for the BimEL isoform and requires ERK phosphorylation. BimEL degradation and subsequent reduction of apoptosis occurs following dissociation of BimEL from Mcl-1(Ewings et al., 2007). Bcl-2 family proteins regulate the release of caspase activating enzymes from mitochondrion. Caspase-3 activation results in cleavage of poly (ADP-ribose) polymerase (PARP) and apoptosis. Cleaved PARP can be measured as a marker of apoptosis (see Chapter 3 Section 3.2.9). BAG-1 has been shown to promote Akt-mediated phosphorylation of the pro-apoptotic protein BAD (Gotz et al., 2005).

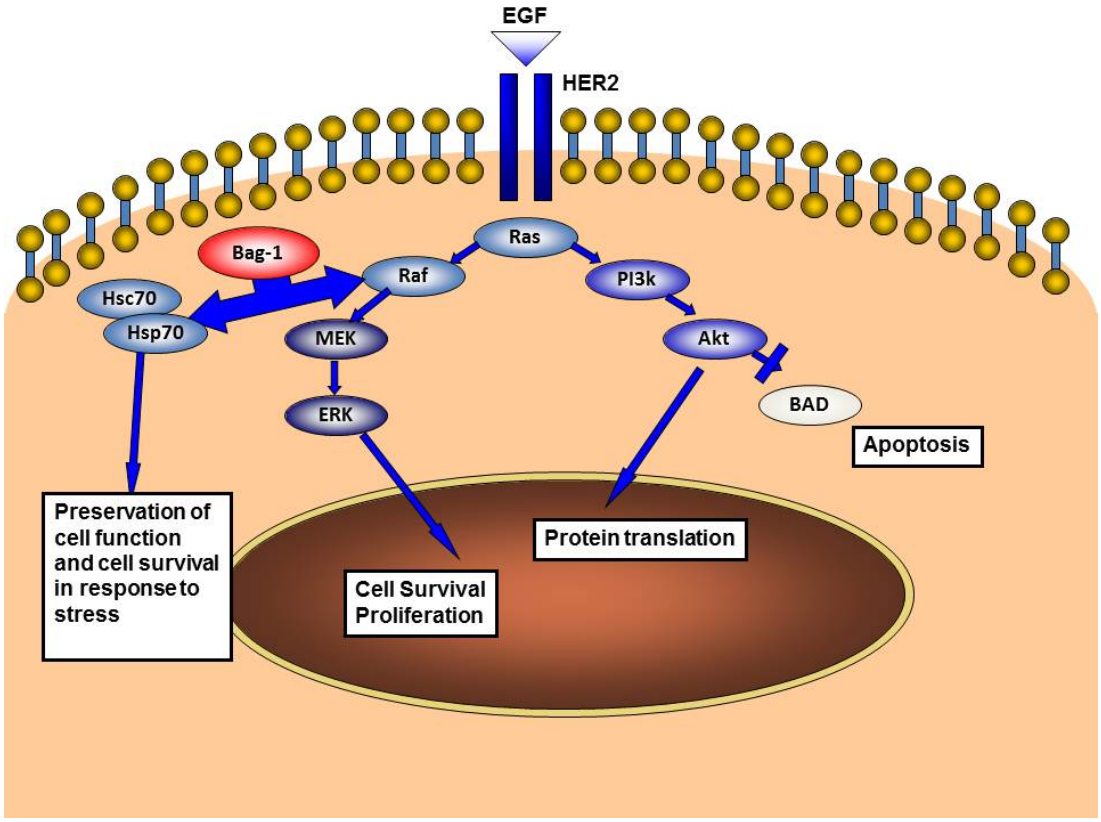

Figure 1.4 HER2 signalling pathways and BAG-1

Inhibition of HER2 signalling via the PI3K and MAPK pathways are proposed mechanisms of action of the drug trastuzumab (Herceptin™)(Nahta and Esteva, 2006). BAG-1 competitively binds Raf-1 and Hsc/Hsp70. In response to stress BAG-1 binds Hsp70 and curtails signalling via downstream ERK kinases (Townsend et al., 2003). BAG-1 has also been shown to increase Akt-mediated phosphorylation of pro-apoptotic protein BAD (Gotz et al., 2005).

	Subcellular BAG-1 staining (% positive)			Total BAG-1 staining (%)			
Study	**Nuclear**	**Cytoplasmic**	**Mixed**	**Weak**	**Moderate**	**Strong**	**% Positive**
Tang et al., 1999	18.2	57.1	1.4	23.6	35.7	17.9	NG
Turner et al., 2001	23.0	65.0	NG	NG	NG	NG	NG
Sjostrom et al., 2002	N/A	N/A	N/A	NG	NG	NG	60
Townsend et al., 2002	47.0	84.0	NG	NG	NG	NG	92.0
O'Driscoll et al., 2003	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cutress at al., 2003	66.0	27.0	NG	NG	NG	NG	NG
Tang et al., 2004	0.5	85.5	NG	13.5	25.0	61.0	86.0
Nadler et al., 2008	NG	NG	NG	NG	22.0	78.0	NG
Millar et al., 2009	54.0	63.0	NG	NG	NG	NG	NG
Sirvent et al., 2004	Generally mixed. More cytosolic than nuclear. No data given.			NG	NG	NG	80.6
Athanassiadou et al., 2008	27.1	48.6	8.6	NG	NG	NG	70.0
Dumontet et al., 2010	79.2	77.0	NG	NG	NG	NG	NG

Table 1.1 Review of immunohistochemical evidence of level of BAG-1 expression in breast cancer. (NG- data not given; N/A- not applicable).

Author		Millar	Nadler	Sirvent	Tang[1]	Cutress	O'Driscoll	Townsend	Turner	Athan.	Tang[2]	Siostrom	Dumontet
Introduction													
	State marker	√	√	√	√	√	√	√	√	√	√	√	√
	Objectives	√	√	√	√	√	√	√	√	√	√	√	√
	Hypotheses	√	√	√	√	√	√	√	√	√	√	√	√
Methods													
Patients													
	Disease stage	√	I		I	√	√	I	I	√	I	√	√
	Co-morbidities												
	Inclusion/ exclusion criteria	√			√	√	√			√			
	Treatment received	√	I		√	√	I	I	√	√	√	√	√
	Treatment randomised?												
Specimen													
	Type	√	√	√	√	√	√	√	√	√	√	√	√
	Controls	√			√	√	√	√	√	√			
Assay													
	Assay method	√	√	√	√	√	√	√	√	√	√	√	√
	Controls	√			√	√	√	√	√	√	√		
	Blinded?								√				
Study													
	Prospective (P)/	R	R	R	R	R	R	R	R	P	R	R	R
	Follow-up in years (median)	5.3	10	4.5	3.7	5.6	6	12.8	12.1	3.3	8	3	4.6
	Define clinical end-points	√			√	√	√		√	√	√	√	√
	Sample size (N)/ Power	√ (292)	√ (638)	√ (186)	√ (185)	√ (138)	√ (106)	√ (160)	√(122)	√ (70)	√(140)	√(126)	√(1350)
Statistics													
	Methods	√	√		√	√	√	√	√	√	√	√	√
Results													
Data													
	Patient flow (Consort)												
	Prognostic variables	√	√		√	√	√	√	√	√	√		
	Demographic characteristics	√	I		√	√	√	√	√	√	√	√	√
	Missing data	NO	YES	YES	YES	NO	YES	YES	YES	NO	YES	YES	YES
Analysis													
	Prognostic variables	√	√	√	√	√	√	√	√	√	√	√	√
	Univariate analysis	√	√	√	√	√	√		√	√	√	√	√
	Multivariate analyses (+HR)	√	√	√	√	√	No HR		√	√	√	NO HR	√
	Estimated effects (+CI)				√							NO CI	
	Further investigations	√								√			
Discussion													
	Interpret with hypotheses	√	√	√	√	√	√	√	√	√	√	√	√
	Future research/ clinical value	√	√	√	√	√	√	√	√	√	√	√	√

Table 1.2 Analysis of BAG-1 and patient outcome studies using the REMARK profile for reporting prognostic tumour marker studies (McShane et al. Br J Cancer 2005; 93: 387-391). I:Incomplete data; R: Retrospective; P; Prospective; HR: Hazard ratios; CI: Confidence intervals. 1: Tang et al., 2004; 2: Tang et al., 1999.

Author	Experimental Methods	Results	Apoptosis / Proliferation
Liu et al. 1998			
	i.Transient transfection and stable breast cancer cell lines (ZR-75-1 or MCF-7s) overexpressing BAG-1 treated with retinoic acid and growth inhibition measured by MTT assay. FACS.	Dominant negative effects of BAG-1 mutants on Inhibition of RAR activity and resistance to retinoic acid. FACS confirms apoptosis	Apoptosis
Song et al. 2001			
	BAG-1 proteins expressed by removal of tetracycline in wild type OT-tet (BAG-1) cells and OT-tet (BAG-1 C204A or E208A)mutants Cells treated with BrdU, then BrdU detected with anti-BrdU and FITC. Same population of cells incubated with (^{3}H)Thymidine heat shocked, prior to DNA synthesis being measured by TCA precipitation and scintilation counting.	Heat shock induced arrest of DNA synthesis is dependent on BAG-1-Hsp 70 interaction. Thymidine incorporation and BrdU staining was decreased in wild type and OT-tet compared to mutants deficient in the Hsp70 binding region.	Demonstrates a role for BAG-1 in proliferation and mitogenesis but only following heat shock.
Kudoh et al. 2002			
	i.ZR-75-1 cells transfected with BAG-1 mutants lacking the COOH-terminus of BAG domain. Immunoblot analysis of BAG-1 expression in cells expressing mutants compared to endogenous BAG-1 and BAG-1L	No difference in rate of cell accumulation in controls compared to those expressing BAG-1. Cells overexpressing BAG-1 mutants accumulated at a slower rate. XTT assay	Cell number on y axis of figure for XTT assay- is metabolic activity. No apoptosis assay done (caspase etc) to show proliferation not apoptosis or vice versa. BrdU staining would clarify.
	ii. Stable transfection with BAG-1 or control expression plasmids followed by XTT growth assays in serum-deficient media.	BAG-1S and BAG-1L show prolonged survival and continue to grow compared to mutants.	Apoptosis
	iii. Stable transfection with BAG-1 or control expression plasmids followed by caspase activity assays	Caspase not elevated by serum-deficiency in cells overexpressing BAG-1.	Apoptosis
	iv. Anchorage-independent cell culture followed by XTT growth Assays	Cells overexpressing BAG-1 mutants declined in growth after detachment compared to those overexpressing wild-type BAG-1.	Anchorage-dependance
	v. Tumour xenograft study	Tumours expressing BAG-1 mutants weighed significantly less than controls or wild-type.	Xenograft tumour growth
Townsend et al. 2003			
	i. MCF-7 cell-derived clones stably expressing BAG-1S or transient transfection of BAG-1 isoform or mutant constructs exposed to different levels of stress (heat, radiation, hypoxia) followed by long-term growth assays (colony count).	BAG-1S-overexpressing cells were protected from growth inhibition by stressful conditions.	Clonogenicity
	ii. TRAIL-induced apoptosis followed by MTT cell viability assay.	Cells overexpressing BAG-1S reduced TRAIL-induced apoptosis.	Apoptosis
	iii.Coimmunoprecipitation and immunoblot analysis of BAG-1 mutants and their association with chaperones with or without heat shock.	Mutants inhibiting BAG-1-HSP-70 interaction failed to reduce growth inhibition following heat shock.	Clonogenicity
Gotz et al. 2004			
	Mouse model heterozygous for BAG-1 carrrying null allele and expressing C-Raf kinase in type II pneumocytes. (Transgenic model)	Absence of BAG-1 expression produces tumour cell apotosis	Apoptosis
Dobbyn et al. 2008			
	Luciferase assays following vincristine in HeLa cells transfected with BAG-1 constructs with or without BAG-1 IRES.	Cells with BAG-1 IRES showed only slight decrese in luciferase activity (BAG-1 synthesis)	Apoptosis

Sharp et al. 2009			
	Small peptides from the BAG-1 C-terminus were coupled to Penetratin and incubated with ZR-75-1 or MCF-7 cells before MTS assays (Cell Titer Aqueous One Solution Reagent™)	Small peptides from helices 2 and 3 of the BAG-1 domain inhibited metabolic activity in both ZR-75-1 and MCF-7 cells.	'Growth inhibition' reported but MTS assay alone so demonstrates metabolic activity only not cell gowth, proliferation, or cell number.
Liu et al. 2009			
	MCF-7 and Hs578T cells transfected with BAG-1 constructs and treated with Doxorubicin, docetaxel and 5-FU. MTS assays performed in addition to FACS and Annexin V staining Cyclohexamide chase assay.	BAG-1L and 1M showed resistance to apoptosis compared with controls and BAG-1S. Only BAG 1L showed increase in cell metabolic activity in presence of oestrogen compared to control. BAG-1L / 1M showed increased Bcl-2 stability and expression with cyclohexamide.	Apoptosis
Anderson et al. 2009			
	MCF-10A cells were infected with a retroviral vector transfected with BAG-1 construct. Cell population incubated with or without MEK inhibitor U0126. Confocal imaging and 3D culture performed following immunofluorescence to display phenotype. Immunoblots of BIMEL, activated caspase-3 and MEK/ERK signalling cascade kinases.	BAG-1 overexpressing MCF-10A cells activate the MEK/ERK pathway via RAF-1 leading to BIMEL phosphorylation and decreased luminal apoptosis. The effect is reversed following addition of U0126, a MEK inhibitor.	Apoptosis

Table 1.3 Review of published experimental evidence for the role of BAG-1 in apoptosis and proliferation

Chapter 2: Materials and Methods

Chapter 2: Materials and Methods

2.1 Solutions and Buffers

2.1.1 General Reagents

Phosphate Buffered Saline (PBS)

125mM sodium chloride (NaCl)

16mM Na_2HPO_4

10mM $NaH_2\ PO_4$

Hydrochloric acid (HCl) to correct to pH 7.2

Tris (Tris[hydroxymethyl]laminomethane) – Buffered Saline – TBS)

10mM Tris-HCl pH 8.0

150mM NaCl

TBS-Tween 0.05% (TBS-T)

400ml TBS

200µL Tween

Citrate Buffer (10mM Citric Acid)

1.92g anhydrous citric acid

Distilled water (dH_2O) 1L

Correct with sodium hydroxide (NaOH) to pH6.0

Tris-EDTA (TE Buffer) (10mM Tris Base, 1mM EDTA)

0.37g Ethylenediaminetetra-actetic acid (EDTA)

0.55g Tris base

dH_2O to 1L

Correct to pH9.0 with NaOH

10x PBS

Dissolve the following in 800ml dH_20:

80g NaCl

2g potassium chloride (KCl)

14.4g Na_2HPO_4

2.4g KH_2PO_4

Adjust pH with NaOH until pH 7.4

Add dH_2O until 1L

4% Paraformaldehyde

For 10ml: 8.5ml dH_2O

0.4g paraformaldehyde (PFA)

20 µL 10N NaOH

Solution vortexed for 5 minutes until PFA dissolved and then the following was added:

1ml of 10x PBS

50µL of 1M $MgCl_2$

200µL HCl to adjust the pH 7.2-7.4.

Adjust to 10ml with dH_2O

2.1.2 Protein Analysis Reagents

Protein Running Buffer

25mM Tris Base

200mM Glycine

0.1% sodium dodecyl sulphate (SDS)

Transfer Buffer

200ml Protein Running Buffer

500ml Ethanol

Distilled H_2O up to 2L

RIPA Buffer (Lysis Buffer)

900mg NaCL

790mg Tris Base (Sigma-Aldrich, UK)

75ml distilled H_2O

HCl to correct to pH 8.0

10ml 10% NP40 (IPEGAL Nonidet P-40)

2.5ml 10% Sodium deoxycholate

1ml 100mM EDTA

Stripping Buffer

2% SDS

100 mM β-mercaptoethanol

50mM Tris, pH 6.8

2.1.3 Cell Culture Media

The antibiotics penicillin and streptomycin were used in the 1% DMEM but omitted from the other media as they were used initially to assist reproducibility when attempting to produce stable clones overexpressing specific BAG-1 isoforms (data not shown). However, this study did not succeed in producing stable clones overexpressing BAG-1 isoforms, but instead used clones transiently overexpressing BAG-1 isoforms. The use of antibiotics can prophylactically protect against contamination and aid reproducibility of cell culture experiments. However, use of antibiotics can alter the microenvironment and lead to misleading results (Kuhlmann, 1995). Therefore, for all experiments included in this study antibiotics were omitted from cell culture unless otherwise stated.

1% Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, UK)

500ml DMEM

5ml (v/v) foetal bovine serum (FBS)

2mM L-glutamine

100µg/ml penicillin G

100µg/ml streptomycin

Serum-free medium

500ml DMEM

2mM L-glutamine

Complete DMEM

500ml DMEM

10% FBS

2mM L-glutamine

Freezing Medium

5ml DMEM

4ml FBS

1ml dimethyl sulfoxide (DMSO)

Plastic flasks and plates were supplied by Greiner Bio-One™ Ltd, unless otherwise stated.

2.2 Cell Culture

Lonza™, UK, supplied all cell culture media and trypsin, unless otherwise stated.

2.2.1 Mammalian Cell Lines

All cell lines used were maintained in complete medium (DMEM supplemented with 10% FBS and 2nM glutamine). Cell lines used are described in Table 2.1.

2.2.1 Cryopreservation of Cell Lines and Thawing

Cells were cryopreserved using 1ml aliquots of freezing media following trypsinisation and resuspension. Labelled aliquots were stored in cryotubes and stored at -80°C overnight prior to transfer to liquid nitrogen for long-term storage. Aliquots were thawed in the palm and transferred to 15ml falcon tubes. 5ml complete DMEM was added and the falcon tube centrifuged at 1150 revolutions per minute (rpm) for 3 minutes to remove DMSO. Pellets were then resuspended in 1ml complete medium before transferring to T25 plastic culture flasks (Griener™, UK) and incubated at 37°C and 5% CO_2.

2.2.2 Cell Passage

Cells were grown as adherent monolayers in sterile culture flasks (Greiner™, UK) in a humidified atmosphere (5% CO_2) at 37°C. Cell passage was performed at approximately 70% confluence. Medium was pre-warmed in a water bath to 37°C before use. Existing medium was aspirated away prior to rinsing with 5mls PBS (cell culture-tested PBS supplied by Lonza™). The PBS, any cellular debris, and traces of serum were then also aspirated away as serum contains trypsin inhibitors. 5ml trypsin/EDTA (0.05% trypsin, 5mM EDTA, Lonza™) was then added to flasks before incubating for 1-5 minutes, depending on cell line.

Cells were split using a 1:3 ratio once 70% confluent, unless stated otherwise. Cells were checked for detachment at 1 minute intervals under a microscope. Once detached, an equal volume (5ml) of complete medium was added to the flasks to inactivate the trypsin. A 50µl sample of suspended cells was placed in an Eppendorf tube and cells counted using a haemocytometer and trypan blue. An 100µL pipette was used to gently agitate the cell suspension and add 50µL of trypan blue. The haemocytometer was cleaned with 70% alcohol prior to use and a new coverslip used. A Gilson pipette was used to gently draw up the cell suspension and rested on the edge of the chambers of the haemocytometer. Capillary action was allowed to fill the chambers and care was taken to not flood the haemocytometer. Healthy cells unstained by trypan blue were counted within 16 corner squares. Cells within the squares, and any on the right hand or bottom gridline, were counted. Cells were counted in each set of 16 corner squares and the number of cells calculated using the total of all 4 sets divided by 4 and multiplied by 2 $x10^4$. The cell suspension was then centrifuged at 1150rpm for 3 minutes to remove remaining trypsin. Cells were transferred to new pre-labelled flasks and diluted to the desired concentration with complete medium, or, if surplus cells, spun ready for cryopreservation, or disposed of.

Cell line	Supplier/ Gift from	Reference	Tissue of origin (all human)	Comments
MCF-7	Professor G. Packham	(Soule et al., 1973)	Breast (originally derived from pleural effusion)	ER-positive, HER2-negative
SKBR3	Professor G. Packham	(Trempe, 1976) (DeFazio-Eli et al., 2011)	Breast (pleural effusion)	ER-negative, HER2-positive, HER1-positive
BT-474	ATCC	(Lasfargues et al., 1978; Liu et al., 2009b)	Breast	ER-positive, HER2-positive HER1-positive
T24	Dr Simon Crabb	(Bubenik et al., 1973; Kato et al., 1978)	Bladder	Express HER2, although weakly compared to BT-474 breast cancer cells (Nagasawa et al., 2006)
UMUC3	Dr Simon Crabb	(Grossman et al., 1986)	Bladder	Express HER2, although weakly compared to BT-474 breast cancer cells (Nagasawa et al., 2006)

Table 2.1 Human cell lines.

All cell lines were obtained from Professor G. Packham, unless stated otherwise, and were originally from either the American Type Tissue Collection (ATCC) or the European Collection of Cell Cultures (ECACC).

2.2.3 Testing for Mycoplasma contamination

MycoAlert™ Mycoplasma Detection Kit (Lonza) was used to test for contamination with mycoplasma on a monthly basis as part of a laboratory group rota. All new cell lines were placed in 'quarantine' until tested. Water baths, pipettes, and shared reagents, were all tested in addition to cultured cells and media. The detection kit is a biochemical test capable of identifying contamination with the 6 most common mycoplasma contaminants and the majority of the 180 mycoplasma species. The 2 kit reagents were simply added to the culture supernatant. Any viable mycoplasma found in the sample supernatant are lysed and the enzymes react with MycoAlert™ Substrate causing the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). Luciferase enzyme activity in the MycoAlert™ Reagent is enhanced by the presence of ATP. Measurement of luminescence using a Varioskan Flash plate reader (Thermo Electron Corporation) before and after the addition of the MycoAlert™ Substrate produced a ratio which confirmed the presence or absence of mycoplasma. The test procedure took approximately 20 minutes. The kit is expensive (approximately £10/ test) and mycoplasma can be detected via PCR, but testing via PCR can take a whole day and can require a second PCR reaction, therefore, for efficiency, the kit was used.

2.3 Transfection of Cell Lines

Cells were routinely incubated at 37°C and 5% CO_2 until approximately 70% confluent prior to harvesting. 200,000 cells were plated per well of a 6 well plate. 6 centrifuge tubes (1 tube per well of 6 well plate) were labelled and assigned to the following contents (Table 2.3). The serum-free medium was added to the centrifuge tube first, then the Fugene™ reagent, taking care not to touch the plastic of the centrifuge tube. The tubes were then gently flicked to mix the contents and left for 5 minutes at room temperature. The DNA was added to each

labelled centrifuge according to Table 2.3 and left to stand at room temperature for 30 minutes. Medium was aspirated from the plated cells in the 6-well plate and replaced with fresh, pre-warmed complete medium. The contents of each centrifuge tube was then uniformly added to corresponding pre-labelled wells of the 6-well plate and the plate incubated for 48 hours at 37°C and 5% CO_2. To attempt to ensure uniform distribution between the 6 wells, the total volume of each centrifuge tube was divided by 6.5 and this amount added to each well. Dividing the total volume by 6.5 rather than 6 allowed for any loss due to contents remaining in the centrifuge tube.

To assess transfection efficiency prior to use of the above protocol in further experiments co-transfection with a plasmid incorporating enhanced green fluorescent protein (pEGFP, Clontech™) was used (Cormack et al., 1996). The percentage of cells transfected was measured 48 hours after transfection with pEGFP and plasmids containing BAG-1 constructs (Table 2.2). 100ng of pEGP was transfected with plasmids containing BAG-1 constructs prior to adding the Fugene HD™ reagent (Table 2.2). Cells were harvested at 48 hours and trypsinised with 0.5% trypsin (Lonza) and analysed using flow cytometry to determine percentage of cells expressing pEGFP. Analysis was kindly carried out by Dr Emmanouil Papadakis using fluorescence-activated cell sorting (FACS, Becton and Dickinson). Background and autofluorescence were determined using non-transfected cells and the mock transfection with Fugene HD™ reagent alone. Results were determined from 3 independent experiments and transfection efficiency using the protocol described above was above 70% (data not shown).

BAG-1 Expression Construct	Source (Townsend et al., 2003)
BAG-1S	Generated by inserting EheI fragment containing human BAG-1S open reading frame in to the EcoRV site of pcDNA33 (Invitrogen, UK)
BAG-1M	Generated by replacing the HindIII to XcmI fragment of plasmid pcDNA33-BAG-1S using PCR and optimised BAG-1M translation start site (using primers p46/ M (CCCAAGCTTGAATTCCGCCATGGCTCAGCGCGGGGGGGCGC) and 3'NESTED (CGCCATTTCCTCC-CCGGTCACCTC) (Oswel, Southampton, UK)
BAG-1L	Generated using an optimised BAG-1L start site using primers p50/L-r (CCAAGCTTGAATTCCGCCATGGCTCAGCGGGGGGGCGC) and 3'NESTED in plasmids pcDNA33-BAG-1S,
BAG-1S mutants incorporating mutations in the Hsp70 binding domain (used in colony forming efficiency assay see Section 2.10)	BAG-1S deletion mutants BAG-1SH3A and BAG-1SH3B were kindly provided by Dr Emmanouil Papdakis produced using PCR and the following primers: (5′-ACCCAGGGCGAAGAGATGAATCGG and 5′-TCAAGCTTCAGCTTGCAAATC) (Townsend et al., 2003)

Table 2.2 Expression plasmids used in the production of cells transiently overexpressing BAG-1 isoforms or BAG-1S mutants. BAG-1S mutants incorporated mutations of the Hsp70 binding domain.

Centrifuge tube	DNA (concentration)	Ratio of DNA: Fugene HD™ reagent	Volume DNA (µl)	Fugene HD™ reagent (µl)	Serum-free media (µl)
1	cDNA (4.45µg/µL)	3:1	0.45	6.00	93.50
2	P29 (0.87µg/µL)	3:1	2.30	6.00	91.70
3	BAG-1S (0.36µg/µL)	3:1	5.60	6.00	88.40
4	BAG-1M (0.37µg/µL)	3:1	5.40	6.00	88.60
5	BAG-1L (0.5µg/µL)	3:1	4.00	6.00	90.00
6	Untreated (no DNA)	N/A	0.00	6.00	94.00

Table 2.3 Quantities of DNA, reagents, and media for transfection of MCF-7, SKBR3 and BT-474 breast cancer cell lines.

BAG-1 expression constructs used are listed in Table 2.2. Manufacturer's recommended ratio of Fugene HD™ reagent: DNA is 3.0:1 for MCF-7 cells. Promega™ protocols (http://www.promega.com/techserv/tools/FugeneHdTool) do not exist for SKBR3 or BT-474 cells, but the DNA: Fugene HD™ reagent ratio for cell lines not optimised is 3:1.

N/A: not applicable

2.3.2 Production of Cell Lines Transiently Overexpressing BAG-1

SKBR3 cells were transfected with BAG-1 constructs as described in section 2.3. SKBR3 cells were seeded from single colonies and maintained under G418 (Geneticin) selection. G418 is an aminoglycoside antibiotic used to achieve cell selection by blocking polypeptide synthesis. Cells undergoing mitosis are more susceptible to G418 selection than those which are not. Colonies were grown over several weeks, with serial Western blots performed to assess isoform expression. Initial experiments attempted to produce stable clones overexpressing BAG-1 isoforms using G418 selection, but failed to succeed (data not shown). Therefore, cells transiently overexpressing BAG-1 were produced and selection using the antibiotic G418 was not required**.** The protocol for transfection with BAG-1 DNA was followed (see 2.3.1), Cells were harvested at 48 hours from 6-well plates and transferred into 6 pre-labelled T75 culture flasks and incubated at 37°C and 5% CO_2. Once approximately 70% confluent, cells were tested for BAG-1 isoform expression using Western blots (section 2.9.3) and used for biological assays as described (section 2.5 and 2.6).

2.4 siRNA knockdown of BAG-1 in Breast Cancer Cell Lines

2.4.1 siRNA (Ambion) knockdown without siGLO® Green (Thermo Scientific) optimisation

To study the functional role of BAG-1 in HER2-positive breast cancer cells knockdown of BAG-1 expression using siRNA was performed (Chapter 4). BAG-1 siRNA is not isoform-specific, but does provide a tool for use in biological assays studying the functional effects of the reduction of BAG-1 in breast cancer cells. siRNA targeting BAG-1 was obtained from Ambion™ (oligonucleotide sequences: GGUUGUUGAAGAGGUCAUAtt and GGGAAAAUCUCUGAAGGAAtt). Non-targeting negative control siRNA was provided by Dharmacon (siGENOME™ SMARTpool).

Cells were harvested for use at 70% confluency and 200,000 cells per well plated in a 6-well plate. 10 microfuge tubes were labelled and filled according to the following table (Table 2.4). The medium was changed 24 hours following plating of cells and replaced with 2ml fresh complete medium per well. Tubes 1 and 2, were combined and flicked gently to mix contents and allowed to stand for 20 minutes at room temperature. The contents were then added to corresponding pre-labelled wells of the 6-well plate containing cells and fresh medium.

Treated cells were then incubated at 37°C and 5% CO_2 for 48 hours. Cells were harvested at 48 hours and either transferred into T75 flasks for further experiments, or collected for cell lysates. Lysates were used for Western blotting to confirm siRNA knockdown of BAG-1 compared to mock and negative controls, in addition to determining optimal concentration of siRNA for effective BAG-1 knockdown.

	Tube 1			Tube 2	
	Microfuge tube	siRNA	Optimem™	Dharmafect™ 1 (Dharmacon)	Optimem™
Mock	1	0	100	4	96
5nM stock	2	1	99	4	96
10nM stock	3	2	98	4	96
25nM stock	4	5	95	4	96
Negative siRNA	5	1	99	4	96

Table 2.4 Quantities of siRNA, reagent and media for siRNA knockdown of BAG-1 in breast cancer cell lines. All quantities listed are in µL.

2.4.2 siRNA knockdown with siGLO® Green optimisation of siRNA concentration

To determine the optimal transfection protocol serial Western blots were performed varying cell number (data not shown) and siRNA concentration (Figure 4.2). siGLO® Transfection Indicators are fluorescent oligonucleotides that facilitate optimal conditions for transfection. These indicators were used to ascertain transfection efficiency during siRNA experiments. Cells were harvested at 70% confluency and 200,000 cells plated per well in a 6-well plate. siRNA was diluted to 1:25 to 2µM stock in x1 siRNA dilution buffer. Tubes were prepared as Table 2.5.

Tube 1 was added to tube 2 and incubated for 20 minutes at RT after briefly inverting repeatedly to mix (Table 2.5). The 475µl total was added to1.6mls of warmed medium and gently mixed by inversion before being pipetted into corresponding wells. Samples were incubated at 37°C and 5% CO_2 for 12 hours overnight.

After 12 hours of incubation fluorescence was visualised using Olympus Excellence Pro™ software and images captured using an Olympus IX81 Inverted Fluorescence Microscope. Fluorescence attributed to siGLO™ corresponding with cellular distribution supports adequate transfection. Background fluorescence may indicate inadequate transfection or fluorescent cell debris may suggest reagent toxicity. Cells were harvested from plates by scraping and pellets snap frozen and stored at -20°C for lysate preparation. Western blots were performed to confirm BAG-1 expression.

Tube	Well no. Reagent(s)	1 NT	2 Lipid	3 siGLO	4 siRNA (10nM)	5 siRNA (50nM)	6 siRNA (100nM)
1	siGLO® (50µM)	0	0	5.94	10	50	100
	/siRNA(100µM)	237.5	130	231.56	227.5	187.5	17.5
2	Dharmafect ™1/	0	9.5	9.5	9.5	9.5	9.5
	medium	237.5	228	228	228	228	228
	Total (µl)	475	475	475	475	475	475

Table 2.5 Quantities of reagents required for preparation of tubes 1 and 2 for siGLO optimisation of siRNA concentration.

2.5 MTS Assays

MTS assays determine relative cell viability and have been used in this study to assess the functional effects of overexpressing individual BAG-1 isoforms (Chapter 3), reducing total BAG-1 expression using siRNA knockdown (Chapter 4), with and without HER2 inhibitors or doxorubicin (Chapters 3 and 4). MTS assays have been used in previous studies to provide evidence for the role of BAG-1 in tumour proliferation (Song et al., 2001; Townsend et al., 2003) (Section 1.3.2). MTS assays do not directly measure proliferation, therefore this study included simultaneous simple cell counting assays (section 2.7) and the results compared. The assay depends upon a colourimetric reaction produced by metabolically active cells and the CellTiter 96A® Aqueous One Solution Reagent which contains 3-(4, 5-dimethylthiazol2-yl)-5-(3-carboxymethoxyphenyl)-2-(-4-sulfophenyl-2H-tetrazolium) (MTS). The MTS is converted to the coloured compound formazan by living, metabolically active cells and therefore, the amount of formazan, or change in photoabsorbance due to colour change, is proportional to the number of living cells. Consecutive measurements at a series of defined time-points can therefore analyse relative cellular activity.

2.5.1 MTS assays performed as functional biological assays following overexpression or siRNA knockdown of BAG-1.

Cells were plated in 96-well plates at a density of 10,000 cells per well and incubated at 37°C and 5% CO_2 for 24 hours in complete medium. The medium from the plates was then aspirated away and discarded. Fresh complete medium or medium containing a chemotherapeutic drug at a concentration defined previously by previous cell viability assays was added to each well (Figures 3.5A, 3.7, 3.14A, and 3.18). At defined time-points (0 hours to 6 days), media was aspirated away and replaced with 5µL CellTiter 96® Aqueous One Solution Reagent (Promega, UK). The 96-well plate was then incubated for a further 90

minutes at 37°C and 5% CO_2. The colourimetric reaction was analysed by a Varioskan Flash plate reader (Thermo Electron Corporation) by measuring changes in absorbance at 490nm. Triplicate wells containing complete medium alone and drug solvent (Table 2.6) were analysed to obtain a value for background from the coloured medium which could then be subtracted following plate reader analysis.

2.5.2 MTS assays performed as dose-response experiments for assessment of IC_{50} following treatment of cells with trastuzumab (Herceptin™), doxorubicin, lapatinib and erlotinib

Cells were seeded in complete medium in 96 well plates at a density of 5,000 cells per well. Plates were then incubated for 24 hours prior to treatment at 37°C and 5% CO_2. Cells were then treated with a range of drug concentrations around a value derived from a review of previous literature. MTS reagent (Cell Titer 96A® Aqueous One Solution Reagent) was added to wells following the removal of medium at time points from 0 hours to 6 days. Changes in photoabsorbance were read using a Varioskan Flash plate reader (490nm). Untreated cells were plated in triplicate and MTS reagent added at each corresponding time-point to provide a control. Triplicate wells with complete medium alone or with drug solvent (Table 2.6) were also included in the assay to provide a value for background to be subtracted from final readings. Mean values were calculated from the readings for triplicate wells. All experiments were repeated twice and the mean values plotted on a survival curve using GraphPad Prism 4 and the IC_{50} value calculated. All drugs were a kind donation from the Oncology Pharmacy at Southampton General Hospital. The supplier and how each drug was solubilised are shown in Table 2.5.

Drug	Supplier	Solubilisation method as per supplier's protocol
Herceptin	Roche™	Pale yellow powder dissolved in sterile water by pharmacists at Southampton General Hospital and kept at 4°C until use. 440mg of lyophilised drug is reconstituted with 20ml of sterile water, containing 1.1% benzyl alcohol to give a stock solution of 21mg/ml (Genentech™, Roche). The stock solution was then diluted to desired concentration with warmed complete medium immediately before use. Untreated cells were incubated with sterile water as a vehicle control.
Lapatinib	GSK™	Yellow solid tablet crushed by pharmacists prior to donation to laboratory. Dissolved in the laboratory in dimethyl sulfoxide (DMSO, Sigma™) as a 1000-fold stock solution and stored at 4°C in aliquots after brief use of vortex and sonication. Cells were incubated with DMSO as a vehicle control.
Erlotinib	Roche™	Supplied as lyophilised powder. Reconstituted into a 10mM stock (10mg powder with 2.54ml DMSO) with DMSO and stored in aliquots at -20°C. Cells were incubated with DMSO as a vehicle control.
Doxorubicin	Medac™	Red solution in 5ml vials of 2mg/ml doxorubicin hydrochloride provided by pharmacy and divided into aliquots for storage at 4°C. Dilutions were prepared using complete medium. Aliquots were used within one month of receipt of vial from pharmacy. Untreated cells were incubated with PBS as a control.
Thioflavin S	Professor G.K Packham	Compound stored as yellow powder and soluble in distilled water/ DMSO. (Originally from Sigma-Aldrich, Poole, Dorset (Sharp et al., 2009a)

Table 2.6 Drugs used in the study.

All drugs were donated by the Oncology Pharmacy at Southampton General Hospital and were collected on the day of use/ solubilisation and stored as per supplier's protocol. Herceptin was replaced after a maximum of 3 months storage at 4°C. An effort was made to use fresh Herceptin whenever available due to an observed decrease in cell death and inhibition of proliferation with Herceptin that had been stored for over 3 months (data not shown).

2.6 Cell yield assays

Simple cell counting assays were used as a direct measure of cell yield following BAG-1 overexpression (Chapter 3), siRNA knockdown of BAG-1 (Chapter 4), and treatment with HER2 inhibitors or doxorubicin. Cells were seeded at a density of100,000 cells/ml in 6-well plates. At defined time points, 100µL of trypsinised cells was extracted from each well and placed in a CASYCup® with 10mls of CASYton® ready-to-use isotonic saline. The cell density of viable cells was determined using the CASY® Technology Cell Counter (Roche) according to manufacturer's instructions. All readings were performed in triplicate and a mean average determined from results.

2.7 SDS-PAGE and Western Blotting

2.7.1 Preparation of Cell Lysates

To establish endogenous expression in different cell lines cells were routinely harvested once approximately 70% confluent. Otherwise, cells were harvested for lysates at the time point determined by each experiment. Cells were detached from culture plates using a cell scraper and transferred with the conditioned medium into 15ml falcon tubes. Plates were rinsed with PBS to collect any remaining cells. The cells and conditioned medium were then centrifuged at 10,000rpm (11,180g) for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 1ml of PBS prior to transfer into a 1.5ml eppendorf tube. The resuspended pellet was then centrifuged at 13000rpm for a further 10 minutes.

1ml of cold Radioimmunoprecipitation Assay (RIPA) buffer and protease inhibitors (Pierce™, Thermo Scientific) were added to the pellet once the supernatant had been carefully aspirated away. Use of a broad spectrum protease inhibitor cocktail prevents proteolytic degradation ensuring proteins of interest remain intact. The Pierce™ EDTA-free protease inhibitor cocktail contained serine, cysteine, aspartic acid, and aminopeptidases commonly present in cell lysates. The cocktail does not contain EDTA as EDTA is a metalloproteinase inhibitor. RIPA buffer is suited to lysis and protein extraction of mammalian cells in preparation for SDS-PAGE (denaturing polyacrylamide gel electrophoresis). RIPA buffer is named after the assay method for which it was developed (radio-immunoprecipitation assay). The buffer solution incorporates two ionic detergents and one non-ionic detergent in Tris buffer: 25mM Tris•HCl, pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS).The eppendorfs were then briefly vortexed and then set down on ice for 20 minutes. The eppendorfs were then re-centrifuged for 10 minutes at 13,000rpm at 4°C and the supernatant aspirated into microfuge tubes before being transferred for storage at -20°C.

2.7.2 Bovine Serum Albumin (BSA) Protein (Bradford) Assay

The Bradford Assay was developed to measure the quantity of protein in a solution. The assay is used to ensure equal amounts of protein from cell lysates are loaded on to gels for Western blotting (section 2.7.3.2). The assay is based on a colourimetric change produced by Coomassie Brilliant Blue dye. Under acidic conditions the red unbound form of the dye interacts with protein to form a blue bound form, which is stable and amenable to measurement of photoabsorbance at a wavelength of 595nm.

A 96-well plate was labelled for 7 BSA standards and each sample in duplicate. BSA standards were made using 1mg/ml BSA according to Table 2.7. 20µL of the BSA standards and samples were pipetted into duplicate wells. The amount of protein in each sample was calculated from the standard curve calculated from photoabsorbances of BSA standards. BioRad protein assay reagent (BioRad Laboratories, USA) was diluted 1:5 with dH_2O and 180µL pipetted into each of the wells containing standards or samples. The 96-well plate was then allowed to sit for 5 minutes at room temperature. Absorbances for the standards and the samples were measured at a wavelength of 595nm using a Varioskan Flash™ plate reader.

2.7.3 Western Blotting

2.7.3.1 Preparation of gels

7.5-12% Tris-HCl acrylamide denaturing gels were made with a 4% acrylamide stack 24 hours in advance of use (Tables 2.8A and 2.8B). Gels were wrapped in damp paper towel and clingfilm to keep moist, and stored at 4°C. Gels were made with 10 or 15 comb loading wells.

Protein (µg)	0	1	2	3	4	5	6
BSA (µL)	0	3	6	9	12	15	18
Lysis Buffer(µL)	1	1	1	1	1	1	1
dH_2O (µL)	57	54	51	48	45	42	39
Total volume per well (µL)	20	20	20	20	20	20	20

Table 2.7 Quantities for standards for BSA protein assay.

Resolving Gel %	7.5	10	12
30% polyacrylamide (ml)	3.75	5.0	6.0
1.5M Tris (pH8.8) (ml)	3.5	3.5	3.5
10% ammonium persulfate (µl)	75	75	75
10% SDS	150	150	150
TEMED (µl)	10	10	10
dH_2O (ml)	7.28	6.03	5.03
Total volume (ml)	15	15	15

Table 2.8A Formulae for preparation of resolving Tris-HCl acrylamide gels (Bio-Rad™, http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6201.pdf).

Stacking Gel %	4
30% polyacrylamide (ml)	1.98
0.5M Tris-SDS (pH6.8) (ml)	3.78
10% ammonium persulfate (µl)	75
10% SDS	150
TEMED (µl)	15
dH_2O (ml)	9
Total volume (ml)	15

Table 2.8B Formula for preparation of stacking Tris-HCl acrylamide gels (Bio-Rad™).

2.7.3.2 Loading and resolution of gels with subsequent transfer to nitrocellulose blotting membranes.

Gels were placed in a BioRad Mini-PROTEAN 3 Cell and approximately 500ml protein running buffer added. Using values calculated from BSA protein assay, 20-40µg protein was loaded from each sample in equal amounts. Protein samples were vortexed briefly with 10µL of 3x SDS Sample Buffer Red and 0.1M dithiothreitol (DTT; Cell Signalling Technology), in addition to distilled water to create an equal sample volumes of 15µL. The samples and protein marker (New England Biolabs, MA) were heated for 5 minutes at 95°C. 15µL of protein marker was loaded in the first loading well and samples were loaded into adjacent wells.

Gels were run in protein running buffer for 40 minutes at 200V. The gels were then sandwiched between 10 x 8 cm filter paper and 10 x 7 cm nitrocellulose membrane (Whatman plc, UK), surrounded by sponge layers (BioRad Laboratories) and placed in a BioRad Mini-Trans Blot Cell. The cell was placed in a plastic tray and surrounded by ice and gels transferred over 75 minutes at 100V in 500ml of transfer buffer.

2.7.3.3 Blocking of membranes and staining with primary and secondary antibodies.

Nitrocellulose membranes were blocked with 5% milk (Marvel) made in TBS-Tween (0.1%) for 1 hour at room temperature on automatic tilt-table or rollers. The membrane was stained with primary antibody (Table 2.9) overnight at 4°C also on automatic tilt-tables or rollers. The following morning, membranes were washed 3 x 5 minutes in 10ml TBS-Tween (0.1%) and stained with correlating horseradish peroxidise secondary antibodies for 1 hour at room temperature on rollers or tilt-table.

2.7.3.4 Developing and imaging of membranes

The membranes were washed a further 3 x 5 minutes in TBS-Tween (0.1%) prior to adding 1ml of Supersignal West Pico Chemiluminescent substrate reagent (Thermo Fisher Scientific Inc, IL). A BioRad Fluor-S MultiImager installed with BioRad Quantity One Software was then used to detect chemiluminescence.

2.7.3.5 Stripping of membranes

Antibodies were removed from membranes using 5ml of stripping buffer for 20 minutes on automatic rollers or tilt-tables. The membranes were then washed 3 x 5 minutes with deionised water (10ml) and blocked again in 5% milk (Marvel) in TBS-Tween (0.1%) for 1 hour at room temperature.

2.7.3.6 Densitometry and statistical analysis

Images captured using the BioRad Fluor-S MultiImager were saved as TIFF files and densitometry analysis was carried out using Image J density analysis software (http://rsb.info.nih.gov/ij/). Experiments were repeated in triplicate to allow calculation of standard deviation (SD) using Prism software. Error bars shown represent SD. A minority of experiments were only repeated in duplicate and do not include error bars.

Name/ Antibody to	Supplier/ Gift from	Reference	Type	Antigen (human unless specified)	Concentration
TB2	Professor G. Packham	Brimmell et al. 2002	Ra Po	GST-Bag-1S	1:1000
3.10G3E2	Professor G. Packham	Brimmell et al.2002	Mo Mo	GST-Bag-1S	1: 1000 (unless otherwise stated)
HER2	Abcam	Zhang YJ et al. 2013	Ra Po	ErbB2 C-terminus peptide	1:400
EGFR	Abcam	BADe LK et al. 2011	Ra Mo	EGFR Tyr1068 phospho-peptide	1:1000
PARP	Abcam	Zhou et al. 2006	Ra Po	PARP N-terminal peptide	1:200
Akt	Abcam	Stan D et al. 2011	Ra Po	C-terminal peptide	1:1000
p-Akt (Ser473)	Abcam	Jang S et al. 2012	Ra Mo	Ser473 phospho-specific peptide	1:1000
p-Akt (Thr308)	Abcam	Kokobo T et al. 2009	Ra Po	Thr308 phospho-specific peptide	1:1000
ERK1/2	Abcam	Hellmann et al. 2010	Ra Po	ERK peptide conjugated to immunogenic carrier protein	1:200
p-ERK1/2	Abcam	-	Ra Po	Phopsho-specific ERK peptide	1:200
BAD	Santa Cruz	He Y. et al 2010	Mo Mo	Recombinant BAD	1:400
p-BAD	Cell Signalling	Wang et al. 2008	Ra Po	Phopsho-specific (Ser112) peptide	1:800
Raf-1	Santa Cruz, Biotech	-	Ra Po	C-terminal peptide	1:1000
3C12	Professor G. Packham	-	Mo Mo	BAG-1L	1:800 or 1:1000 as stated

Table 2.9 Primary antibodies used for Western Blotting

Ra Po: Rabbit Polyclonal, Mo Mo: Mouse Monoclonal

2.8 Immunocytochemistry of fixed cells.

Immunofluorescence was used to ascertain the subcellular localisation of endogenous BAG-1 in HER2-positive breast cancer cells (Figures 3.2A, 3.11, 3.13, 3.17 and 4.1) with and without exposure to HER2 family inhibitors.

2.8.1 Fixation of cells

19mm coverslips (Thermo Fisher Scientific) were immersed in 100% laboratory grade ethanol and then placed using forceps into the wells of sterile 12-well plates. The wells and coverslips were then rinsed thoroughly 3 times with sterile PBS. 50,000 cells per well were seeded and incubated in complete medium overnight. The following morning, media was aspirated away and coverslips gently washed a further 3 times with sterile PBS. Cells were then fixed in 4% paraformaldehyde in PBS for 15 minutes and washed with TBS-Triton (0.1%).

2.8.2 Immunocytochemistry

Cells were incubated for 30 minutes in 3% BSA diluted in PBS-Triton (0.1%). A clean staining tray was then lined with strips of clean Parafilm M (Camlab, UK) and small pools of 200µL 3.10G3E2 BAG-1 (1:1000 diluted in PBS) antibody pipetted at 5 cm intervals. The coverslips were then carefully elevated using a sterile needle and forceps from the wells and overturned face-down onto the antibody pools. The trays were then covered and incubated at 4°C overnight. In the morning, coverslips were gently elevated from the parafilm and replaced back into their respective wells face-up. The cells were then washed 3 x 5 minutes with PBS-Triton (0.1%).

The coverslips were again gently elevated and overturned (face-down) onto 50 µL of 1:1000 Alexa Fluor® (Invitrogen) secondary antibody and incubated at room temperature in darkness for 1 hour. The coverslips were again returned to their respective wells and washed 3 x 5 minutes in PBS-Triton (0.1%).

2.8.3 Mounting coverslips

25 x 75 x 1mm slides were pre-labelled accordingly, coverslips then elevated from wells and overturned onto 2 drops of Vectashield Hardset containing DAPI (Vector Labs, UK)

2.8.4 Imaging

Intracellular fluorescence was imaged using Olympus Excellence Pro™ software and captured using an Olympus IX81 Inverted Fluorescence Microscope.

2.9 RNAase extraction and qualitative real time PCR

2.9.1 RNA extraction (Qiagen Mini-prep kit)

Qiagen Mini-Prep Kit was used for RNA extraction from samples. RNAse-free DNAse stock solution was made up according to manufacturer's protocol. 10µL of RNAse-free DNAse was added to the buffer RDD using filter tips to pipette into a centrifuge tube. The centrifuge tube was then vortexed for 30 seconds and placed on ice. 350µL of RLT buffer was added to samples in labelled microfuge tubes and vortexed vigorously for 1 minute. 350µL of 70% ethanol was then added to each sample (700µL total).

Samples were then placed in RNAeasy spin columns and placed in collection tubes (2ml tubes). The samples were then centrifuged at 10,000rpm (11,180g) for 30 seconds at RT. The supernatant was then discarded and 350µL of RW1 buffer added before further centrifugation at 10,000rpm (11,180g) for 30 seconds at RT. 80µL of RNAse-free DNAse stock solution was then added to samples, whilst ensuring no contact was made between the disc and the pipette. A further 350µL RW1 buffer was then added and the samples centrifuged at 10,000rpm (11,180g) for 30 seconds. 500µL RPE buffer was then added before centrifugation for 30 seconds at 10,000rpm (11,180g) and repeated centrifugation for 2 minutes.

The spin column was then placed in the new collection tube (2ml) and centrifuged for 1 minute at RT. The aim of this step is a 'dry spin' to help remove residual ethanol after the RPE wash. Samples are then centrifuged for 1 minute at 10,000rpm (11,180g) at RT and 'nano-drop' analysis performed using 2µL of sample to assess the quantity of RNA extracted.

2.9.2 Generation of cDNA (complementary DNA) by reverse transcription

Reverse transcription was set up using a 'no template' (RNA replaced with dH_20) and a 'reverse transcriptase' control (an untreated sample). The amount of RNA required for 1µg was calculated using the 1000/ x, where x was the value given from the nano drop analysis (Table 2.10).

Primer annealing was performed using a Gene Amp® PCR System 9700 (PE Applied Biosystems) using the PCR cycler program 'xw>70' of the DNA Thermal Cycler 480 (Applied Biosystems). Tubes were mixed by agitating and spinning down the contents using brief centrifugation. RT-PCR mastermix was prepared whilst primer annealing took place (Table 2.11)

Tubes were removed at the end of the xw>70 program from the cycler and immediately placed on ice. 10µL of mastermix was added to each sample microfuge tube and mixed by agitation and brief centrifugation. The tubes were then placed in the PCR cycler and the programme 'xw>cDNA' selected. On completion of the programme 75µL dH_2O was added per sample and samples stored at -80°C.

Sample/ reagent	Quantity
RNA (1µg final)	x µL
Oligo dT primer (100µg/µl of RNA)	1µL
dH_20 (nuclease free)	y µL
Total	15 µL

Table 2.10 Quantities of samples and reagents for making cDNA using reverse transcription. The amount of RNA required for 1µg was calculated using the 1000/ x, where x was the value given from the nano drop analysis. 'y' represents the amount of nuclease-free distilled water added required to make total up to 15µL.

Sample/ reagent	Quantity per sample (reaction)
5x M-MLV buffer	5µL
dNTP(10mm) mix	1.25µL
RNAsin	0.625µL
M-MLV RT enzyme	1µL
dH_20 (nuclease-free)	2.125µL
Total	10µL

Table 2.11 Quantities for PCR mastermix. All quantities are for one sample or reaction. A slight excess was made up for multiple samples e.g. for 5 samples quantities were multiplied by 5.2.

2.9.3 Qualitative Real Time PCR

Taqman® assay on demand probe sets (Applied Biosystems, UK) were used to amplify BAG-1 and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase, H) genes. Quantitative real time PCR using Taqman® probes detect fluorescence relating to DNA amplification of specific primer targets (Table 2.12). Taqman probes contain a reporter dye and a quencher. When the probe is intact the quencher is in close proximity to the fluorogenic dye which prevents fluorescence. During PCR, the probe anneals downstream of the primer binding site and, when amplification takes place by Taq DNA polymerase, the probe is cleaved. This results in release of quencher activity and subsequent fluorescence which is detected by the qRTPCR system. Higher starting concentrations of the nucleotide sequence of interest require fewer PCR cycles for a detectable increase in fluorescence above the baseline level of 0.1. A crossing threshold (CT) value was produced for each sample which corresponds to the number of cycles of PCR when the baseline was crossed. CT values were produced for each sample, including no cDNA controls.

PCR Primer	Primer Sequence
GAPDH Forward	5'-CATCATCCCTGCCTCTACTG-3'
GAPDH Reverse	5'-TTGGCAGGTTTTTCTAGACG-3'
BAG-1 (pre-designed Taqman® probe.	Assay ID: HS00185390_BAG-1 Part number: 4331182

Table 2.12 PCR primer sequences.

Taqman® mastermix contains PCR reagents and was used for detection of amplified primer targets. 5µl of cDNA, reverse transcription control or RNase free water was added per well (MicroAmp™ Optical 96-Well Reaction Plate, Applied Biosystems , UK) in duplicate or triplicate to each primer set.

Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, UK) was used to perform the qRTPCR reaction. The thermal cycle was: 2 minutes at 50^{o}C, 10 minutes at 95^{o}C, then 40 cycles of: 15 seconds at 95^{o}C and 1 minute at 60^{o}C.

2.9.4 Statistical analysis

The comparative ΔΔCT method (Equation 1) was used for statistical analysis of qRTPCR results.

Equation 1: ΔΔCT= power (2, ΔCT) where ΔCT is the change in mean crossing threshold.

BAG-1 CT values were normalised to average GAPDH to correct for variation in start-point cDNA concentration. Subsequent analysis for each sample relative to control samples was performed. Data were paired and normal distribution was assumed so a paired Student's t-test was performed (Excel, Windows version 7) to identify statistical significance of changes in BAG-1 expression between treatments. Normal distribution can be tested for using a Shapiro-Wilk test, although this was not performed in this study(Henderson, 2006).

2.10 Crystal violet assays

Clonogenic assays were used to determine if overexpression of BAG-1 isoforms could alter the clonogenicity of HER2-positive breast cancer cells. A cell colony is defined as a cluster of at least 50 cells. The crystal violet assay is a method for quantification of cell colonies adhering to multi-well plates and has been shown to correlate with manual counting of cell colonies (Ochel, 2005). Crystal violet stains the DNA within cells and the amount of dye taken up by the monolayer can be quantified in a spectrophotometer or plate reader after solubilisation with acetic acid. The results in chapter 3 (Figure 3.4) were achieved using manual counting of cell colonies using 12-well plates.

To determine the effect of overexpression of BAG-1 on the clonogenic potential of SKBR3 cells clonogenic assays were conducted comparing cells overexpressing BAG-1 isoforms and BAG-1S mutants to pcDNA3 control**.** SKBR3 cells were transfected with BAG-1 isoforms, BAG-1S mutants or pcDNA3 control (constructs listed in Table 2.2). BAG-1S mutants incorporated mutations in the Hsp70 binding domain shown to be important in cell survival (Townsend et al., 2003). A mock transfection was included using the Fugene HD™ reagent alone. Following 48 hours of incubation cells were trypsinised and seeded into 12-well plates or lysed with RIPA buffer for Western blots. Cells seeded in the 12-well plates underwent serial dilution with complete medium until single colonies could be visualised (approximately 50,000 cells per well) and placed under G418 (neomycin) selection (500µg/ml). Following a 14 day incubation period under G418 selection, the cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes. Cells were fixed in 4% PFA-PBS to preserve cell morphology. Plates were then washed three times with distilled water. Cells were then stained with 70µL per well of 0.1% crystal violet for 20 minutes at RT. The plates were then rinsed extensively with distilled water and left to dry overnight. Colonies were counted manually in each well of the 12 well-plates. All wells were in triplicate and the experiment

repeated three times. Data were derived from the mean colony number of triplicate wells (+/- SD).

Crystal violet assays can be also be used to quantify the relative density of cells adhering to multi-well plates and has been reported to be a rapid and reproducible method of assessing cell survival (Franken et al., 2006). Crystal violet stains the DNA within cells and the amount of dye absorbed by the monolayer is quantified by spectrophotometer. BT-474 cells were used as seeding with 2,000 to 10,000 SKBR3 cells in 96-well plates resulted in either few viable cells or an uninterpretable dense monolayer. The SKBR3 cells seeded in 96-well plates showed little difference in crystal violet staining between conditions, despite obvious differences observed when cells were seeded in 6-well plates (data not shown). 7,000 BT-474 cells were seeded in 96 well plates. Plates were incubated overnight at 37°C and 5% CO_2 until approximately 70% confluent. Cells were washed with PBS and fixed in 4% PFA-PBS. Cells were then stained with 70µL per well of 0.1% crystal violet for 30 minutes at RT. 100µL 50% acetic acid in PBS was then added to each well and left for 30 minutes at RT to solubilise the crystal violet stain. The plate was agitated every 10 minutes for 30 seconds to dislodge detached from fixed cells. Additional agitation at 30 minutes was required until to ensure uniform appearance with no dense areas of colouration. Changes in photoabsorbance were read using a Varioskan™ Flash plate reader (570nm) Results were calculated as a percentage of the photoabsorbance recorded for untreated cells. All wells were in triplicate and results calculated from 3 independent experiments (+/-SD).

2.11 Antibody isotype assay

Antibodies consist of one or more copies of a Y—shaped unit incorporating 4 polypeptide chains. Each Y-shaped unit contains 2 copies of a heavy polypeptide chain and 2 copies of a light chain. The light chains can be classed as kappa (κ) or lambda (λ). The heavy chains determine the class of antibody. There are 5 antibody isotypes in mammals: IgG, IgM, IgA, IgD, and IgE (gamma (γ), mu (μ), alpha (α), delta (δ) and epsilon (ε), respectively). It is important to determine the isotype of an antibody as they differ in terms of function, biological properties, and location (http://www.abcam.com/protocols/antibody-structure-and-isotypes).

A Quick Type-M Mouse Monoclonal Antibody Isotyping Test Kit™ (Micratech, LLC) was used to determine the isotype of a novel monoclonal BAG-1L-specific antibody. The monoclonal antibody sample was diluted to a concentration of 1.0μg/ml in 1% BSA / PBS. 150μL of this diluted sample was then added to the development tube. An isotyping strip was then removed from the desiccant vial and the tube recapped.

150μL of the sample was then pipetted into the development tube and incubated at RT for 30 seconds. The tube was vortexed for 5 seconds to ensure the coloured microparticle solution was adequately suspended. Results were interpreted at 5 minutes, once positive flow control bands had been seen. Blue bands at 5-10 minutes of the class or subclass window were recorded in addition to any blue bands in the kappa or lambda window on the reverse of the strip. The red band was removed from the base of the test strip after photographing the results to ensure no further band development and the strip to be kept as a permanent record.

2.12 Immunohistochemistry

2.12.1 Immunohistochemistry for BAG-1 using whole sections

Sections were cut from paraffin-embedded blocks and kept at 4°C prior to use. Care was taken to not allow slides to dry at any time during the procedure. Superfrost Plus™ adhesive slides (Thermo Scientific) were used as are coated to increase cell and tissue adherence.

2.12.1.1 Deparaffinisation and rehydration

Slides were placed in 24-slide racks and de-waxed in xylene (Sigma-Aldrich, UK) for 5 minutes x2 in separate baths. The slides were then processed through 4 baths containing 88%, 88%, 80% and 70% respectively of laboratory grade ethanol for 2 minutes each. The slides were then placed in a Perspex tub with gently running cold tap water for a further 2 minutes

2.12.1.2 Antigen retrieval

Antigen unmasking was achieved using 10mM citrate buffer pH 6.0. The slides were placed in full racks and submerged in approximately 330ml buffer in plastic containers with perforated lids. The microwave turntable was balanced by always using 3 containers complete with full racks of slides. The sections were heated using medium power for 25 minutes and then placed under running tap water until cool.

2.12.1.3 Blocking steps

All whole sections were blocked with 3% hydrogen peroxide solution for 10 minutes at room temperature. The sections were then washed 3 x 5 minutes in TBS and then blocked with TBS/ foetal calf serum 5% for 1 hour.

2.12.1.4 Immunohistochemistry

200µL 3.10G3E2 BAG-1 antibody (1:1000) was applied to each slide and incubated at 4°C overnight in a covered staining tray. The sections were then washed 3 x 5 minutes in TBS. Slides were drained and biotinylated second stage antibody (rabbit anti-mouse 1:400, Abcam) was applied and incubated for 30 minutes. Slides were then drained and washed three times before application of 200µL of streptavidin-biotin peroxidase complexes (Vectastain™, ABC Kit, Vector Labs) and further incubation for 30 minutes. During this step 200µL DAB (3, 3-diaminobenzidine**)** chromogen was mixed with 1ml of DAB Substrate at least 10 minutes prior to use. The DAB solution (Vector Laboratories) was then applied in volumes of 200µL per slide and slides incubated in darkness for 5 minutes at room temperature. The slides were then immersed in running tap water before being counterstained via 20 seconds in Mayer's haematoxylin. Sections were then washed for a further 2 minutes in running tap water.

2.12.1.5 Dehydration, clearing and mounting of whole sections

Slides were placed in 24-slide racks and de-hydrated by processing through 4 baths containing 70%, 80%, 88% and 88% respectively of laboratory grade ethanol for 2 minutes each. The slides were then cleared in xylene (Sigma-Aldrich, UK) for x2 5 minutes in

separate baths. The slides were then placed in a Perspex tub with gently running cold tap water for 2 minutes mounting with DPX (Leica, UK)

2.12.2 Immunohistochemistry demonstrating BAG-1 using breast cancer tissue microarray

Sections were cut from paraffin-embedded tissue microarray blocks and prepared on slides (Thermo Scientific Superfrost™ Plus) a maximum of 24 hours prior to use. Following preparation the slides were kept at 4°C overnight and at all times prior to use.

2.12.2.1 Deparaffinisation and rehydration

Slides were placed in 24-slide racks and de-waxed in xylene (Sigma-Aldrich, UK) for 5 minutes x3 in separate baths. The slides were then processed through 4 baths containing 100%, 100%, 95% and 95% respectively of laboratory grade ethanol for 2 minutes each. The slides were then placed in a Perspex tub with gently running cold tap water for a further 2 minutes 2.1).

2.12.2.2 Antigen retrieval

A pressure cooker (Nordic Ware®) was filled with 1L of citrate buffer (pH6). The rubber seal was positioned inside the cooker and placed inside the microwave without the lid at medium power for 12-14 minutes (until the buffer begins to boil). The dewaxed and rehydrated slides were then placed into the pressure cooker and the lid secured along with the red stopper. The microwave was then re-started at medium power and observed until the yellow stopper

began to rise. Once the yellow stopper had risen a stopwatch was started for 5 minutes and at the end of this period the pressure cooker removed from the microwave. The red stopper was removed with caution and whilst wearing a protective face mask. Once the yellow stopper had fallen the lid was removed and the contents left to cool for 20 minutes at room temperature. The cooker was then placed in a sink under running cold water for 2 minutes.

2.12.2.3 Blocking steps

Endogenous peroxidise was blocked for all sections using 3% H_2O_2 solution for 10 minutes. The sections were then washed in water for 2 minutes in a water bath with magnetic stirrer. Slides were then cleared of excess solution and a DAKO Pen used to ring around sections. Several different blocking solutions were trialled prior to staining, summarised in the Table 2.13. Following blocking steps sections were not rinsed in preparation for staining. Final TMA sections were blocked in foetal bovine serum, although a minority of TMA cores showed superior results with normal horse serum (Chapter 5).

2.12.2.4 Immunohistochemistry

200µL anti- BAG-1 antibody (3.10G3E2) was added to each slide and incubated at 4°C between 4 hours to overnight (12 hours). Sections were then washed in TBS-Tween (0.05%) for 2 x 5 minutes on a mixer. Excess TBS was wiped gently from around sections and 200µL Envision secondary antibody (3-4 drops) added before a further 30 minute incubation period at room temperature. Sections were covered with an opaque plastic staining tray lid during all periods of incubation.

Sections were then washed in TBS-Tween (0.05%) for a further 2 x 5 minutes on a mixer. During this step, 20µL DAB Chromogen and 1µL DAB substrate were mixed at least 10 minutes prior to use. Excess TBS-Tween (0.05%) was then removed from sections and 200µL of DAB solution applied to each section. Sections were then covered using the opaque staining tray lid and incubated for 10 minutes in darkness a room temperature. Sections were then washed for 2 minutes in water in a mixer, counterstained in haematoxylin (TAAB, UK) for 20 seconds, re-washed in running tap water for approximately 2 minutes (until water running clear), and immersed in Scott's Tap Water Substitute (TAAB, UK) for 5 seconds.

2.12.2.5 Dehydration, clearing and mounting of sections

Slides were placed in 24-slide racks and de-hydrated by processing through 4 baths containing 95%, 95%, 100% and 100% respectively of laboratory grade ethanol for 2 minutes each. The slides were then cleared in xylene (Sigma-Aldrich, UK) for x2 5 minutes in separate baths. The slides were then mounted with DPX (Leica, UK).

2.12.2.6 Imaging

TMA cores were imaged using the Ariol® imaging system and software (Applied Imaging Corporation).

Blocking Solution	Concentration (TBS-T 0.05%)	Incubation Time
Dako Protein-Free Block	1:1	20 minutes at room temperature
Casein	1:10	30 minutes at room temperature
Normal Goat Serum	1:20	1 hour at room temperature
Normal Horse Serum	1:20	1 hour at room temperature
Foetal Bovine Serum	1:10	1 hour at room temperature

Table 2.13 Blocking solutions used for immunohistochemical study of BAG-1.

2.13 Immunodepletion using pan-isoform 3.10G3E2 antibody or isoform-specific 3C12

Immunodepletion assays can be used to remove a protein of interest from tissue and confirm the specificity of an antibody. Tissue is incubated with the antibody targeting the protein of interest, in this case BAG-1/ BAG-1L, and glutathione S-transferase (GST)-tagged protein. Purification of the protein is achieved by incubation with glutathione sepharose beads (Glutathione Sepharose 4B™, GE Healthcare, Sigma Aldrich) and centrifugation to separate the supernatant.

2.13.1 Deparaffinisation and rehydration

Slides were de-waxed in xylene (Sigma-Aldrich, UK) and rehydrated through graded ethanol. The slides were then placed in gently running cold tap water for a further 2 minutes.

2.13.2 Antigen Retrieval

Antigen unmasking was achieved using 10mM citrate buffer pH 6.0. The sections were heated using medium power for 25 minutes and then placed under running tap water until cool.

2.13.3 Blocking

All whole sections were blocked with 3% hydrogen peroxide solution for 10 minutes at room temperature. The sections were then washed 3 x 5 minutes in TBS and then blocked with TBS/ foetal calf serum 5% for 1 hour.

2.13.4 Purification of glutathione S-transferase proteins using Glutathione Sepharose 4B™ (GE Healthcare, Sigma Aldrich)

40µL of 50% Gluthathione Sepharose 4B™ slurry were added, as per manufacturer's protocol, to 100µg BAG-1S-GST protein and 100µL BAG-1 antibody (1:800) in 1% PBS into a centrifuge tube. Samples were then incubated with intermittent gentle agitation via pipette at room temperature for 30 minutes. Supernatant was then separated and using centrifugation at 500 rpm for 5 minutes. The supernatant was then stored on ice until applied to previously prepared paraffin-embedded sections.

2.13.5 Immunohistochemistry

200 µL of BAG-1 3.10G3E2 antibody (1:800) or BAG-1S-GST + 3.10G3E2 + GSH beads or BAG-1-GST + GSH beads or BAG-1S-GST alone were applied to multiple sections from one tumour (Table 2.14) Immunohistochemical staining was performed as per the protocol in section 2.13.1.4 .

2.13.6 Dehydration, clearing and mounting of whole sections

Slides were de-hydrated through graded ethanol and cleared in xylene (Sigma-Aldrich, UK). The slides were then placed in gently running cold tap water for 2 minutes mounting with DPX (Leica, UK).

Slide	Antibody/ Reagents
1	200µL BAG-1 antibody (1:800)
2	200µL supernatant from BAG-1 antibody (1:800) + BAG-1S-GST (100µg/ ml) + GSH beads
3	200µL supernatant from BAG-1S-GST (100µg/ ml) + GSH beads
4	200µL BAG-1S-GST (100µg/ ml)
5	TBS control

Table 2.14 Antibody and reagents applied to slides 1-5 for immunodepletion of primary antibody. Experiment performed in duplicate for pan-isoform anti-BAG-1 antibody 3.10G3E2 and isoform-specific antibody 3C12.

2.14 XTile™ analysis of immunohistochemical scoring of tissue microarrays and clinicopathological variables

Scores from immunohistochemical studies were compiled using Excel (Microsoft™) database. The maximum combined score for both nuclear and cytoplasmic BAG-1 'Tquickmax' was copied into an XTile™ (Yale, USA) file along with corresponding disease-free and overall survival for each tumour.

Optimal cut-off points for significance were designated using the XTile™ Kaplan-Meier function and the high/mid/low grouping criteria. These scoring cut-offs were then applied to the original Excel data to organise the cohort into distinct groups of low, medium and high staining for BAG-1. The data were processed for statistical significance using SPSS® software.

Chapter 3: BAG-1 as a potential target in HER2-positive breast cancer

Chapter 3: BAG-1 as a potential target in HER2-positive breast cancer.

3.1 Introduction

The overall aim of this chapter is to provide evidence for the functional role of BAG-1 in HER2-positive breast cancer cells by overexpression of individual BAG-1 isoforms. Previous studies have established functional roles in breast cancer for BAG-1 isoforms using overexpression. BAG-1 overexpression has been shown to promote survival (Wang et al., 1998), proliferation (Townsend et al., 2003) and cochaperone activity (Hohfeld et al., 1998; Townsend et al., 2003). Many studies have explored the interaction of BAG-1 isoforms in breast cancer with the oestrogen receptor (ER) (Cutress et al., 2003), but no studies have focused specifically on the effect of BAG-1 isoform expression in HER2-positive breast cancer cells. The hypothesis tested in this chapter is that overexpression of BAG-1 isoforms in HER2-positive breast cancer cells affects the response of breast cancer cells to trastuzumab (Herceptin™).

The HER2 receptor is a transmembrane tyrosine kinase receptor and a member of the EGF receptor family. EGFR/ ErbB (HER1), HER2/ ErbB2, HER3/ ErbB3, and HER4/ErbB4 constitute the EGRF family. All of the EGFR family, except HER2, use an extracellular domain to bind specific ligands. All preferentially undergo dimerisation with HER2 upon binding of signalling proteins, e.g. growth factors and participate in crosstalk engaging a number of signalling pathways, of which Raf-1 is constituent (Hynes et al., 2000). BAG-1 binds to and activates Raf-1 to be able to implicate BAG-1 specifically in HER2 signalling. The convergence of BAG-1 and HER2 signalling pathways by Raf-1 suggests a mechanism by which modulation of Raf-1 by BAG-1 may mediate response to HER2 inhibition. This chapter compares the effects of BAG-1 isoform overexpression on the therapeutic potential of HER2-specific inhibition, to those of inhibition of other EGFR family receptors and doxorubicin. The mechanistic pathways behind the response of BAG-1 to trastuzumab treatment are almost certainly complex, as trastuzumab has been shown to have several mechanisms of action in breast cancer cells (Valabrega et al., 2007). The mechanisms of action of trastuzumab are discussed in the introduction to Chapter 1, but briefly consist of inhibition of MAPK/PI3K signalling(Nahta and Esteva, 2006), antibody dependent cytotoxicity to tumour cells (Arnould et al., 2006), and increased degradation of the HER2 receptor (Klapper et al., 2000). Lapatinib is a small molecule tyrosine kinase inhibitor inhibits receptor signal processes by binding to the ATP-binding pocket of the EGFR/HER2 protein kinase domain, preventing self-phosphorylation and subsequent activation of the downstream

signaling mechanism (Nelson and Dolder, 2006). Lapatinib has been show to induce apoptosis in SKBR3 trastuzumab-resistant cells (Nahta et al., 2007). To determine whether the elevation of BAG-1 in response to trastuzumab was specific to HER2 another EGFR family inhibitor, erlotinib, was used. Erlotinib specifically inhibits EGFR (HER1/ErbB1) and has no direct effect on HER2, although it is known that significant cross-talk occurs between members of the EGFR family (Shou et al., 2004).

The chapter begins by establishing the endogenous levels and subcellular localisation of BAG-1 in HER2-positive SKBR3 cells by Western blotting and immunofluorescence studies respectively (Figure 3.2). The SKBR3 cell line was initially derived from the pleural effusion of a female breast cancer patient and is ER-negative and HER2-positive (Trempe, 1976) (Chapter 2 Table 2.1). The basal rate of cell death has been reported in SKBR3 cells as less than 10% (Li et al., 2005). SKBR3 cells have been used to study the effects of HER2 family inhibitors on breast cancer cells resistant to HER2 inhibition with trastuzumab (Nahta et al., 2007). This study starts with ER-negative SKBR3 cells as this is independent to ER signalling and potential crosstalk between ER and HER2 reported by studies investigating resistance to anti-HER2 therapy (Arpino et al., 2008; Ocana et al., 2006). To compare the effect of BAG-1 overexpression and HER2-inhibition in the presence of ER signalling the ER-positive, HER2-positive breast cancer cell line BT-474 was used (Table 2.1). Co-inhibition of ER, HER2, and EGFR activity in breast cancer cells has been shown to be effective in reducing proliferation and oestrogen-dependent gene expression in antioestrogen-resistant breast cancer(Chu et al., 2005).

To investigate the isoform-specific role for BAG-1 in proliferation and cell survival it was crucial to produce HER2-positive cells overexpressing each BAG-1 isoform. To ensure overexpression was present throughout timecourse experiments and allow serial experiments on the same cell stock, attempts were made to produce stable clones. Transfected SKBR3 cells confirmed by Western blot to be overexpressing BAG-1 isoforms were seeded from single colonies and maintained under G418 (Geneticin) selection. Initial immunoblots showed promise and stocks were harvested and frozen for subsequent experiments. For subsequent use these stocks were recovered and allowed to recover in culture prior to use in biological assays. Unfortunately Western blots performed at the point of commencing assays showed BAG-1 isoforms were no longer overexpressed and hence not valid for use in the proliferation assays. Both ELISA screening and further immunoblots from colonies in repeated attempts to produce stable clones failed to show isoform-specific expression. Cell density , time of harvesting, G418 concentration and selection time were all varied in turn to exclude the possibility that the expression plasmid was lost in over-confluent

cells, or that the cell selection was inefficient and allowing cells not expressing the plasmid to persist. However, these measures were unsuccessful.

Initial Western blots had confirmed overexpression of specific BAG-1 isoforms in SKBR3 cells at 48 hours instigated experimental design incorporating SKBR3 cells transiently overexpressing the desired BAG-1 isoform. Thus, all cell yield and cell viability assays were performed at this point with correlating Western blots to confirm expression at each time point of every assay. Recognising that time points beyond 72 hours had resulted in loss of specific BAG-1 isoform expression in initial experiments, necessitated finding effective concentrations of all drugs used within 48 hours of treatment.

The chapter uses simple cell counting assays to study the effect of BAG-1 isoform overexpression on proliferation in HER2-positive cells. MTS assays are also used to explore the effects of increasing BAG-1 isoform expression on cell viability in HER2-positive cells. To explore the effect of BAG-1 isoform expression on clonogenicity in HER2-positive cells crystal violet dissolution assays were performed. Immunofluorescence experiments are used to demonstrate subcellular localisation of BAG-1 with and without HER2 family inhibition. To establish that functional effects of BAG-1 expression are not secondary to apoptosis alone, Western blots performed following trastuzumab and doxorubicin include expression of cleaved poly ADP ribose polymerase (PARP). Cleavage of PARP and generation of an 89kD fragment can be used as a marker of apoptosis (Soldani and Scovassi, 2002). Results differ between studies investigating concordance between BAG-1 protein and mRNA expression (Millar et al., 2009; O'Driscoll et al., 2003; Townsend et al., 2002). This chapter uses Western blotting and qPCR to investigate the relationship between BAG-1 protein and mRNA expression in HER2-positive breast cancer cells with and without exposure to trastuzumab.

An alternative method of studying the function of BAG-1 in HER2-positive cells rather than using alternative cell lines or HER2 inhibitors would be to use siRNA to knockdown HER2. Treatment of SKBR3 cells and MCF-7 cells overexpressing HER2 with HER2 siRNA has been shown to cause an increase in late G_1/S-phase growth arrest (Choudhury et al., 2004).

3.2 Results

3.2.1 The effect of overexpression of BAG-1 isoforms on cell yield and relative cell viability of SKBR3 cells.

Immunocytochemistry was performed on SKBR3 cells transiently transfected with BAG-1 isoforms to confirm the subcellular localisation of BAG-1 isoforms (Figure 3.1). BAG-1L was shown to be predominantly localised to the cell nucleus, whilst BAG-1M and BAG 1-S were mostly cytoplasmic. The pcDNA3 control showed minimal BAG-1 staining using indirect immunofluorescence. To determine BAG-1 isoform expression Western blotting was performed consecutively from lysates taken from the stock of transfected SKBR3 cells used to perform cell yield and MTS assays (Figure 3.2).

To investigate the effects of BAG-1 overexpression on proliferation and relative metabolic activity of HER2-positive breast cancer, two types of biological assay were performed. SKBR3 cells were transfected with BAG-1 isoforms or pcDNA3 control. Western blots confirmed transient overexpression of BAG-1 isoforms compared to endogenous BAG-1 in untreated cells or pcDNA3 control (Figure 3.2). MTS assays established an increase in metabolic activity in viable cells transiently overexpressing the isoforms BAG-1L and BAG-1M compared to those overexpressing the short isoform BAG-1S or pcDNA3 (Figure 3.3A). A simple counting method was also employed for the same cell population using a Casy® Technology Cell Counter (Schärfe System/ Roche). This straight forward cell yield assay supported the results from the MTS assay, demonstrating a relative increase in cell yield with both overexpression of BAG-1L or BAG-1M compared to pcDNA3. Experiments were performed in triplicate and results were concordant with those from MTS assays (Figure 3.3B).

3.2.2 The effect of overexpression of BAG-1 isoforms on the clonogenic potential of SKBR3 cells.

To determine the effect of overexpression of BAG-1 on the clonogenic potential of SKBR3 cells clonogenic assays were conducted comparing cells overexpressing BAG-1 isoforms and BAG-1S mutants to pcDNA3 control**.** SKBR3 cells were transfected with isoform-specific BAG-1 constructs, BAG-1S mutants incorporating mutations in the Hsp70 binding domain, or pcDNA3 control (constructs listed in Table 2.2) and incubated for 48 hours. A mock

transfection was included using the Fugene HD™ reagent alone. Cells were then trypsinised and either seeded into 12-well plates or lysed with RIPA for Western blots. Cells seeded in the 12-well plates underwent serial dilution with complete DMEM until single colonies were identified (at approximately 50,000 cells/ well). Colonies were then placed under G418 selection. After an incubation period of 2 weeks the cells were fixed with 4% PFA for half an hour, then rinsed with PBS and allowed to dry overnight. Colonies were then stained with 0.1% crystal violet and manually counted. Each well was repeated in triplicate and the data were derived from the mean number of colonies from triplicate wells from 3 repeat experiments. Consecutive Western blots were performed from lysates from the transfected stock to confirm BAG-1 isoform transient overexpression compared to pcDNA3 control at 48 hours (Figure 3.5).

Cells transiently overexpressing the short cytosolic isoform BAG-1S demonstrated reduced clonogenicity compared to the longer BAG-1 isoforms BAG-1M, BAG-1L, the pcDNA3 control and the BAG-1S mutant BAG-1SH2 (Figure 3.4). BAG-1SH3A and BAG-1SH3AB showed elevated clonogenicity compared to BAG-1S. SKBR3 cells overexpressing the BAG-1S isoform showed significantly reduced clonogenicity compared to pcDNA3 ($p=0.039$, $p=0.461$, 0.382 for BAG-1S, 1M and 1L respectively using the one-way ANOVA test and Bonferroni method in GraphPad Prism™). SKBR3 cells overexpressing BAG-1SH3A and BAG-1SH3AB mutants (see Section 2.1 and Table 2.2) showed a significant increase in clonogenicity compared to pcDNA3 control ($p=0.028$, $p=0.046$ respectively).

3.2.3 Isoform specific expression of BAG-1 isoforms in SKBR3 cells used in clonogenic assays.

To confirm the isoform specific overexpression of BAG-1 isoforms in the clonogenic assays Western blotting was performed in parallel. Western blotting was conducted using lysates from the stock of cells used for the clonogenic assays. Blots confirmed low levels of endogenous expression of BAG-1 isoforms in both mock transfection (results not shown) and pcDNA3 controls (Figure 3.2B).Overexpression of each of the BAG-1 isoforms and mutants was confirmed using lysates taken from the pools of transfected cells submitted to G418 selection for the clonogenic assays (Figure 3.5). BAG-1S and the mutant BAG-1SH2 were expressed at higher levels than other BAG-1 isoforms.

3.2.4 The response of HER2-postive cells to trastuzumab treatment.

To deduce the concentration of trastuzumab at which reduces viable cell yield to 50% (IC50) a MTS cell viability assay was performed (Figure 3.6A). At approximately 24 to 48 hours 50% decrease in cell viability was observed with a concentration of 50µM trastuzumab (Figure 3.5A). This trastuzumab concentration was then employed for all subsequent experiments. At 48 hours of treatment with 500µM trastuzumab over 70% of cells were detached or appeared apoptotic, therefore this concentration would not provide enough viable cells for further experiments. A similar IC50 for trastuzumab was derived using BT-474 cells from dose response experiments performed by Dr Emmanouil Papadakis (data not shown). Western blotting was performed to determine if BAG-1 isoform expression was altered at any of the concentrations used in the dose response experiment (Fig 3.6B). Results showed a previously unreported increase in the expression of BAG-1S ($p=0.014$, at 50µM trastuzumab using one-way ANOVA and Bonferroni multiple comparisons analysis in GraphPad Prism™. p-values are to 3 decimal places) and BAG-1M ($p=0.038$) following trastuzumab compared to untreated controls. There was a decrease in BAG-1S and BAG-1M expression between 500µM and 1mM with little change in expression of BAG-1L. This could be due to a functional switch of BAG-1 isoforms reported previously during stress (Song et al., 2001) or a change in mode of cell death. This experiment did not investigate caspase or cleaved PARP expression which could indicate apoptosis, rather than growth inhibition. Pan-isoform expression was most significantly increased following 500µM trastuzumab ($p=0.003$).

The experiment was repeated using an alternative HER2-positive cell line, BT-474. Western blotting showed no obvious increase in BAG-1 expression in BT-474 cells treated with trastuzumab compared to untreated controls (Figure 3.6C). There was a decrease in expression of the HER2 downstream signalling protein phospho-Akt (p-Akt473) following trastuzumab. The experiment using BT-474 cells was repeated and results shown in Chapter 4 (Figure 4.7) demonstrating an increase in BAG-1L after trastuzumab and a similar decrease in p-Akt (473). The difference between experiments and lack of variation in expression of BAG-1 in the experiment may be explained by saturation of bands, suggested by dense bands correlating with β-actin expression (Figure 3.6C). The expression of HER2 signalling proteins following trastuzumab or the BAG-1 inhibitor thioflavin S in BT-474 cells is discussed in Chapter 4, where the effects of a combination of the drugs are reported.

3.2.5 The effect of trastuzumab on the cell yield and relative cell viability on SKBR3 cells overexpressing BAG-1 isoforms.

This experiment focused upon investigating the functional effect of an increase in BAG-1 expression seen in SKBR3 HER2-positive cells exposed to trastuzumab. Initial experiments had not shown a similar increase in BAG-1 expression in BT-474 cells following trastuzumab, so all cell yield and relative cell viability assays were performed using SKBR3 cells. SKBR3 cells transiently overexpressing BAG-1 isoforms were treated with 50µM trastuzumab and an MTS assay performed to assess relative cell viability Figure 3.7B). Cells overexpressing BAG-1L, BAG-1M and BAG-1S showed an increase in relative cell viability compared to those transfected with pcDNA3 control following treatment with 50µM trastuzumab at 48 hours. This experiment showed overexpression of all BAG-1 isoforms individually reduced any decrease in cell viability due to trastuzumab compared to the pcDNA3 control. Results correlated with a simple direct cell yield assay using the CASY® Cell Counter to count the number of viable cells (Figure 3.7A). Readings were repeated in triplicate from 3 individual 100µl samples from triplicate wells for each isoform. There was a small increase in cell yield in cells transfected with pcDNA3 exposed to trastuzumab compared to untreated cells. This was unexpected as the cell yield of SKBR3 cells transfected with pcDNA3 and exposed to trastuzumab should be less than, or at least similar to, the parental control. The results seen are not simply due to continuing proliferation despite trastuzumab as were normalised to the increase in cell yield of untreated parental SKBR3 cells. This may suggest an underlying mechanism of resistance has been triggered in SKBR3 cells as HER2 resistance has been reported in up to 70% of HER2-positive breast cancers(Pohlmann et al., 2009).

This experiment does not include a direct measure of proliferation. To be able to ascertain if BAG-1 also increases cell proliferation, and not just cell yield and relative cell viability, would require incorporation of bromodeoxyuridine (BrdU). BrdU incorporation has been used to show BAG-1 expression caused arrest of DNA synthesis following heat stress in OT-Tet (BAG-1) cells (Song et al., 2001) (discussed in Chapter 1 section 1.3.2). To assess the impact of ER signalling on the assay results the experiments could have been repeated in ER-positive HER2-positive BT-474 cells. Crosstalk between ER and HER2 signalling exists, so this would be an important consideration for planning future work (Arpino et al., 2008).

3.2.6 The effect of doxorubicin (250nM-2μM) on the relative cell viability of SKBR3 cells over 48 hours.

To establish the changes in BAG-1 expression and proliferation seen in SKBR3 cells were specific to treatment with anti-HER2 therapy, rather than a result of non-specific cell killing or growth inhibition, the chemotherapeutic agent doxorubicin (Adriamycin) was used. Doxorubicin is an anthracycline used in many different types of cancers. One of the main mechanisms of action of doxorubicin is blockage of the enzyme topoisomerase-2, needed by cancer cells to continue to proliferate and grow (Marchand et al., 2010). The optimal concentration of doxorubicin for treatment of SKBR3 cells was established using an MTS assay to deduce the IC50. At approximately 24 to 48 hours 50% relative cell viability was observed with a concentration of 1μM doxorubicin (Figure 3.8). This concentration of doxorubicin was then employed for all subsequent experiments.

3.2.7 The effect of doxorubicin on the proliferation (A) and the relative cell viability (B) on SKBR3 cells overexpressing BAG-1 isoforms.

To investigate if effects on BAG-1 expression and proliferation of SKBR3 cells were specific to anti-HER2 therapy rather than non-specific killing, biological assays were repeated using 1 μM doxorubicin. SKBR3 cells overexpressing BAG-1 isoforms or transfected with pcDNA3 control were seeded in 96-well plates and treated after 24 hours of incubation with 1 μM doxorubicin. A simple proliferation assay was performed by counting cells at 8, 24, 36 and 48 hours of exposure to doxorubicin using a CASY® Cell Counter (Figure 3.9A). The assay was performed using triplicate wells and undertaken three times. Results showed less of decrease in cell yield at 48 hours in cells overexpressing BAG-1L and BAG-1M, but no difference in those overexpressing BAG-1S, which showed results similar to pcDNA3 vector controls ($p= 0.016$, $p= 0.023$, $p=0.728$ for BAG-1L, 1M, and 1S, respectively using ANOVA and Bonferroni analysis in GraphPad Prism™).

An MTS assay conducted in parallel using the same pools of SKBR3 cells transiently overexpressing BAG-1 isoforms demonstrated a difference between cells overexpressing BAG-1 isoforms compared to pcDNA3 after 48 hours of exposure to doxorubicin (Figure 3.9B). There were comparably more viable cells in cultures overexpressing BAG-1 following doxorubicin, most marked at 36 hours treatment and in those cells overexpressing BAG-1S. This contrasted with the simple cell yield assay performed (Figure 3.9A) suggesting BAG-1 overexpression may slightly decrease the effect of doxorubicin. At 36 hours a protective

effect was demonstrated in cells overexpressing BAG-1S, compared to other BAG-1 isoforms and the pcDNA3 control. At 48 hours overexpression of all BAG-1 isoforms showed an increase in relative cell viability compared to the pcDNA3 control (p= 0.019, p=0.010, p=0.023 for BAG-1S, BAG-1M, and BAG-1L respectively using ANOVA followed by the Bonferroni analysis in GraphPad Prism™). Overexpression of BAG-1 isoforms did not allow SKBR3 cells to continue to grow in the presence of doxorubicin. However, there was a significant decrease in the effect of doxorubicin on relative cell viability (metabolic activity) compared to pcDNA3 controls. The experiment does not differentiate between whether BAG-1isoform expresssion enhances metabolic activity of cells or increases cell survival.

3.2.8 PARP and BAG-1 expression in SKBR3 cells following treatment with 50μM trastuzumab or 1μM doxorubicin.

PARP is a family of proteins involved mainly in DNA repair and programmed cell death (Lloyd et al., 2002). The expression of cleaved PARP can indicate apoptosis (Smulson et al., 1999)**.** PARP-1 is a target of apoptotic caspases and the 89kDa fragment can be detected during the late stages of apoptosis (Soldani et al., 2001). The PARP fragments are no longer capable of performing the function of DNA repair and it is hypothesised the larger fragment interacts with other proteins important in apoptosis such as p53(Malanga et al., 1998).

Western blotting of BAG-1 isoform and PARP expression following 1μM doxorubicin was performed using the stock of cells used for both cell yield and MTS assays (Figures 3.9 and 3.10). Increased expression of cleaved PARP and the presence of an 89kDa protein corresponding with the large PARP fragment were found following 48 hours doxorubicin treatment. However, uncleaved PARP (116kDa) was also increased with exposure to doxorubicin and is most likely a response to DNA damage. The ratio of uncleaved to cleaved PARP remained unchanged with both trastuzumab and doxorubicin compared to untreated cells, indicating no increase in apoptosis. Exposure to trastuzumab appears to cause a decrease in uncleaved PARP and cleaved PARP expression at 24 and 48 hours compared to untreated cells. BAG-1L is increased at the same time points compared to untreated cells and those treated with doxorubicin. These results do not indicate changes in apoptosis, but do demonstrate elevation of BAG-1L expression following trastuzumab but not doxorubicin. A further assay detecting caspase-3 activity would indicate if early apoptosis was present.

The expression of BAG-1S was found to decrease after 8 hours in SKBR3 cells treated with trastuzumab or doxorubicin and in those left untreated. This contrasted with previous figures, where BAG-1S is the most abundant isoform (Figures 3.5 and 3.6). The explanation for this

may be that cells were harvested for Western blots at a lower confluency than the desired 70%.

3.2.9 qPCR analysis of BAG-1 mRNA expression in BT-474 cells following 48 hours of treatment with 50μM trastuzumab.

To determine whether there is correlation between levels of increased BAG-1 protein expression associated with treatment with trastuzumab and BAG-1 mRNA, qPCR was performed. Quantitative real time PCR using Taqman® probes detects fluorescence relating to DNA amplification of specific primer targets. Taqman probes contain a reporter dye and a quencher. When the probe is intact the quencher is in close proximity to the fluorogenic dye which prevents fluorescence. The probe anneals downstream of the primer binding site and, when amplification takes place by Taq DNA polymerase, the probe is cleaved. Subsequent release of quencher activity produces fluorescence which is detected by the qRTPCR system. Higher starting concentrations of the nucleotide sequence of interest require fewer PCR cycles for a detectable increase in fluorescence above the baseline level of 0.1. A crossing threshold (CT) value for each sample is produced which corresponds to the number of cycles of PCR when the baseline is crossed. CT values were produced for each sample, including no cDNA controls. BAG-1 CT values were normalised to average GAPDH to correct for variation in start-point cDNA concentration. BAG-1 mRNA expression was seen to increase with treatment of BT-474 cells with trastuzumab (Figure 3.11), corresponding with BAG-1 protein expression demonstrated by previous Western blots from the same stock of treated cells.

BT-474 cells were used instead of SKBR3 cells after failing to produce interpretable results using the qRTPCR system on numerous occasions using SKBR3 cells. Future work will endeavor to repeat this experiment in SKBR3 cells.

3.2.10 Immunocytochemistry showing levels of endogenous BAG-1 in SKBR3 cells following treatment with 50μM trastuzumab or 1μM doxorubicin.

To further demonstrate the response of BAG-1 to trastuzumab and doxorubicin in SKBR3 cells immunocytochemistry was performed. Immunofluorescence due to endogenous expression of BAG-1 was observed to increase following 8 hours treatment with 50μM trastuzumab. BAG-1 expression following trastuzumab was seen to increase in SKBR3 cells in both the cytosol and nucleus (Figure 3.12). Low levels of immunofluorescence were

detected in both untreated SKBR3 cells and those treated with 1µM doxorubicin confirming the results from Western blotting (Figure 3.10).

3.2.11 Western blots showing BAG-1 expression in MCF-7 cells following treatment with 50µM Trastuzumab or 1µM Doxorubicin.

To assess if the increased expression of BAG-1 seen with exposure to trastuzumab was specific to HER2-positive cells, experiments were repeated in HER2-negative, ER-positive MCF-7 cells (Figure 3.13)**.** A dose response experiment had previously been performed by Dr Emmanouil Papadakis for MCF-7 cells (results not shown) to determine the IC50 for trastuzumab (25-50µM). Previous published studies in using trastuzumab in MCF-7 cells also show 50µM trastuzumab is a valid concentration for use in biological assays (Emlet et al., 2007). Western blotting of MCF-7 breast cancer cells showed no difference in BAG-1 expression between untreated cells and those treated with trastuzumab or doxorubicin.

3.2.12 Immunocytochemistry showing endogenous BAG-1 localisation in MCF-7 cells following treatment with 50µM trastuzumab or 1µM doxorubicin.

To observe if there was any alteration in subcellular localisation of BAG-1 in MCF-7 cells or visible response to trastuzumab immunocytochemistry was performed. There was no increase in indirect immunofluorescence due to endogenous BAG-1 expression in MCF-7 cells with either trastuzumab or doxorubicin (Figure 3.14).

3.2.13 The effect of lapatinib (500nM-100µM) on the relative cell viability of SKBR3 cells over 48 hours.

To investigate if the BAG-1 response to trastuzumab seen in HER2-positive cells was specific to HER2 signalling or could be elicited by HER1 (EGFR), the dual EGFR/HER2 tyrosine kinase inhibitor lapatinib was employed. Lapatinib crosses the blood brain barrier and has been shown to increase progression-free survival in combination with capecitabine in patients with central nervous system metastases from breast cancer (Geyer et al., 2006). To determine the optimal concentration of lapatinib for use in subsequent experiments a dose response was performed using SKBR3 cells. An MTS cell viability assay performed

over 48 hours showed approximately 50% response of SKBR3 cells with 1 μM lapatinib between 24 and 48 hours (Figure 3.15). Data were derived from the mean of 3 independent assays. Lapatinib was used at this concentration for all subsequent assays.

3.2.14 The effect of lapatinib on the proliferation and the relative cell viability on SKBR3 cells overexpressing BAG-1 isoforms.

SKBR3 cells were treated with 1μM lapatinib for up to 48 hours. No difference in proliferation or relative cell viability was seen between SKBR3 cells overexpressing BAG-1 isoforms and those transfected with pcDNA3 following 1 μM lapatinib at 36 hours (Figure 3.16). At 48 hours there was an increased number of viable cells counted in cultures overexpressing BAG-1S relative to those containing pcDNA3.

3.2.15 BAG-1 expression in SKBR3 cells following treatment with 1μM lapatinib.

To determine BAG-1 expression in HER2-positive cells following lapatinib Western blotting was performed. Western blotting demonstrated total BAG-1 and BAG-1L expression in SKBR3 cells was increased with lapatinib compared to untreated cells at both 8 and 24 hours (Figure 3.17). However, there was no obvious difference in total BAG-1, or BAG-1L expression at 48 hours, although levels of BAG-1M were increased in the cells treated with lapatinib.

3.2.16 Immunocytochemistry showing endogenous BAG-1 localisation in BT-474 cells following treatment with 1μM lapatinib.

Immunocytochemistry was performed to determine the effect of lapatinib on endogenous levels of BAG-1. Endogenous BAG-1 expression was shown via indirect immunofluorescence to elevate in ER-positive HER2-positive BT-474 cells following lapatinib. BAG-1 was seen to increase, but be excluded from nuclei at 36 and 48 hours incubation with lapatinib. This experiment was attempted on numerous occasions using SKBR3 cells to allow continuity with former assays, but failed to show an adequate number of viable cells fluorescing, even on revision of the treatment dose of lapatinib (500nM). The optimal concentration ascertained from the MTS cell viability assay (Figure 3.15) caused

approximately 90% cell death by the point of cell fixation. Cells fixed and showing any immunofluorescence were distorted and appeared to be undergoing apoptosis. The experiment was repeated successfully using BT-474 cells, which are also HER2-positive, but ER-positive. BAG-1 levels did increase with lapatinib treatment in BT-474 cells, but a different pattern of subcellular localisation was observed (Figure 3.18). At 36 hours and beyond BAG-1 had localised to the cytoplasm and there was clear nuclear-sparing. This had not been observed in SKBR3 cells treated with trastuzumab and could be due to the interaction of BAG-1 with ER rather than HER2.

3.2.17 The effect of erlotinib (250nM-2μM) on the relative cell viability of SKBR3 cells over 48 hours.

To determine whether BAG-1 overexpression alters the response of SKBR3 cells to an alternative EGFR family inhibitor, erlotinib, was used. Erlotinib solely inhibits EGFR (HER1/ErbB1) and has no direct effect on HER2. Erlotinib is not currently a standard treatment used in breast cancer, although previous studies have suggested erlotinib may inhibit triple negative breast cancer (Ueno and Zhang, 2011). The optimal concentration of erlotinib for subsequent assays was derived from 3 repeat MTS assays. An approximately 50% response was seen a concentration of 1μM erlotinib over 24 and 36 hours incubation (Figure 3.19).

3.2.18 The effect of erlotinib on the proliferation and the relative cell viability on SKBR3 cells overexpressing BAG-1 isoforms.

To investigate if the response of SKBR3 cells overexpressing BAG-1 following trastuzumab is specific to HER2 inhibition, cell yield and MTS assays were repeated with the HER1 (EGFR) inhibitor erlotinib**.** 3 independent cell yield assays and MTS cell viability assays from the same stock of SKBR3 cells showed no difference between cell cultures overexpressing BAG-1 isoforms and pcDNA3 control following erlotinib (Figure 3.20).

3.3 Discussion.

Chapter 1 reviews the literature incorporating analysis of previous studies investigating the role of BAG-1 in survival and proliferation. This review revealed there was incomplete experimental evidence to conclude BAG-1 is involved in proliferation of breast cancer cells. This chapter sought to clarify if there is an isoform-specific role for BAG-1 in proliferation of HER2 positive breast cancer – a specific subset of breast cancers. Previous studies had used MTS-type assays (MTS, MTT or XTT assays) to investigate the role of BAG-1 in cell growth and proliferation (Liu et al., 1998; Kudoh et al., 2002; Townsend et al., 2003; Sharp et al., 2009), but these are cell viability assays, determining the amount of cellular metabolic activity present alone.

To strengthen the evidence for the role of BAG-1 in proliferation of breast cancer cells it was important to establish if the differences observed between BAG-1 isoforms truly indicated a difference in cell yield, rather than cell metabolic activity or apoptosis. MTS assays were performed alongside a simple cell counting method using a CASY® Cell Counter. Expression of cleaved PARP was also performed to indicate if changes seen in cell yield or viability could be due to apoptosis. This study did not incorporate any additional markers of apoptosis or proliferation as the focus shifted towards immunohistochemical studies attempting to establish BAG-1 as a biomarker in HER2-positive breast cancers (Chapter 5). However, future work will incorporate staining for markers of DNA synthesis, such as BrdU or Ki-67. Fluorescence-activated cell sorting (FACS) and measurement of annexin V staining could also assist clarification regarding quantification of apoptosis alongside immunoblots for caspase expression.

The study also did not establish if alterations in other modes of cell death were responsible for differences seen in cells overexpressing BAG-1 in response to trastuzumab. Apoptosis (Type 1 cell death) is active programmed cell death important in embryological development. Autophagic (Type 2) cell death is defined by autophagic vacuolisation of the cytoplasm and can be identified by transmission electron microscopy. Necrosis (Type 3 cell death) can be defined as pathological failure of homeostasis (Kroemer and Levine, 2008).To measure necrotic and autophagic cell death future work would need to include measurement of morphological characteristics by time-lapse microscopy and molecular markers of autophagy such as LC3 (Krysko et al., 2008; Tanida and Waguri, 2010). However, establishing which mode of cell death may be less important as the process of cell death can be considered as a continuum, rather than distinct stages (Raffray and Cohen, 1997).

Previous studies have demonstrated the value of target protein overexpression in characterising BAG-1 isoforms. Functional structural domains have been identified within BAG-1 which display discrete interactions with BAG-1 isoforms. Perhaps, the most clinically significant study in breast cancer showed that overexpression of the nuclear isoform BAG-1L upregulates ERα transcription and is associated with improved response to Tamoxifen in ER positive, node negative patients (Cutress et al, 2003).

The results do support the hypothesis of the chapter that overexpression of BAG-1 isoforms preserves cell yield and relative metabolic activity on exposure to trastuzumab in HER2-positive SKBR3 cells. Overexpression of both BAG-1L and BAG-1M result in an increase in metabolic activity and number of viable SKBR3 cells compared to the short isoform, BAG-1S and controls in the absence of trastuzumab (Figure 3.3B). In the presence of trastuzumab overexpression of all BAG-1 isoforms resulted in higher metabolic activity and compared to those transfected with pcDNA3 control (Figure 3.7B). An increase in BAG-1 expression has been shown to alter response to chemotherapy in MCF-7 and Hs578T cells (Liu et al., 2009a). Liu et al. (2009) which reported MCF-7 cells overexpressing either BAG-1L or BAG-1M showed resistance to apoptosis induced by doxorubicin, docetaxel, or 5-FU. Apoptosis was determined by FACS and Annexin V staining. Only cells overexpressing BAG-1L maintained metabolic activity of viable cells in MTS assays following treatment with chemotherapeutic drugs (Liu et al., 2009a).

This chapter confirmed subcellular localisation of BAG-1 in cells overexpressing BAG-1 isoforms, demonstrating BAG-1M and 1S can be found in the cytosol, but BAG-1L to be predominantly localised to the nucleus (Figure 3.1), This chapter's results using BT-474 cells suggest that the mechanism behind altered cell yield in response to lapatinib may involve nuclear BAG-1 (Figure 3.18). However, the exclusion of nuclear BAG-1 with 48 hours of lapatinib in BT-474 cells was not observed following trastuzumab in SKBR3 cells (Figure 3.12). These results are interesting, but are limited as the effects seen in BT-474 cells may be due to ER rather than HER2 signalling and would explain why similar results were not found in ER-negative SKBR3 cells. Unfortunately immunofluorescence experiments using SKBR3 cell cultures and lapatinib failed to be interpretable as cells were distorted or not viable at the concentrations of lapatinib determined by the dose response assays.

SKBR3 cells were transfected with constructs of BAG-1 isoforms and immunoblots confirmed the HER2 positive cells were overexpressing specific isoforms (Figure 3.2). Colonies of cells overexpressing BAG-1 isoforms were counted at defined time points to

determine the influence of BAG-1 on clonogenicity (Figure 3.4). Cells overexpressing the short isoform BAG-1S demonstrated reduced clonogenicity compared with larger isoforms (Figure 3.4). The specific function and effect of BAG-1S on clonogenicity have previously been studied in MCF-7 breast cancer cells (Townsend et al., 2003). Townsend et al. (2003) performed long-term growth assays of MCF-7 cell-derived clones and MCF-7 cells transiently overexpressing BAG-1S or BAG-1 mutants with a disrupted Hsp70 binding region. The cells overexpressing BAG-1S did not show any reduction in number of colonies following heat shock, whilst BAG-1 mutants demonstrated reduced clonogenicity. The same study showed BAG-1S overexpressing cells showed resistance to TRAIL-induced apoptosis compared to BAG-1 mutants (Townsend et al., 2003). The results published by Townsend et al. (2003) do suggest expression of BAG-1S regulates clonogenicity although contrast with this study, which shows a reduction in colony formation. This may be due to the differences in the role of BAG-1S in different breast cancer cell lines or that the preservation of colony number in cultures overexpressing BAG-1S is specific to heat-induced stress.

The overexpression of all BAG-1 isoforms protects cellular yield (Figure 3.3A) and relative cell viability (Figure 3.3B) on exposure to trastuzumab, but the expression of the control pcDNA3 does not. This may be a consequence of complex signalling, as the pathways and mechanisms involved in the action of trastuzumab are multiple(Vu and Claret, 2012). One explanation is that the inhibition of HER2 signalling following blockade of receptor dimerisation is predominantly along the MEK/ERK pathway. BAG-1 has been shown to function via binding Raf-1 and potentiating the MEK/ERK pathway (Anderson et al., 2010). Overexpression of BAG-1 in MCF-10A cells has been shown to result in decreased luminal apoptosis, a phenotype similar to that of DCIS, and enhanced activation of Raf-1. Use of the MEK U0126 inhibitor reverses the BAG-1-associated phenotype (Anderson et al., 2010). Hence, increased BAG-1 expression, Raf-1 activation and potentiation of the MEK/ERK pathway may abrogate the effect of HER2 inhibitors along this pathway. Blockade of HER2 signalling via the PI3K pathway leads to a compensatory increase in MEK/ERK activity in SKBR3 and BT-474 cells (Serra et al., 2011). Decreasing Raf-1/MEK/ERK signalling by inhibition of BAG-1 may provide a mechanism for combating HER2 resistance.

Chapter 4 will further investigate the MEK/ERK and the PI3k-Akt pathways using siRNA knockdown of BAG-1 expression and the small molecule inhibitor, thioflavin S (see section 1.2.2.2). One hypothesis is that BAG-1 enhances the compensatory signalling induced by HER2 inhibition. An alternative explanation is that trastuzumab mediates signalling pathways involving preferential competitive binding of Raf-1 to Ras, whilst BAG-1 may function independently of Ras (Song et al., 2001) it is plausible this results in an increase in BAG-1-

mediated enhanced MEK/ERK activity. Previous research has shown BAG-1 interacts with a variety of nuclear hormone receptors (NHR – see section 1.2.2.3). Therefore a compensatory shift from PI3K activity to increased Raf-1/ERK/MEK phosphorylation following trastuzumab could alter the availability of Hsc/Hsp70 chaperone molecules. The availability of Hsc/Hsp70 chaperones has been shown to alter the function of BAG-1interaction with NHR between regulating DNA synthesis and degradation (Schneikert et al., 2000; Schneikert et al., 1999).

Previous studies investigating the isoform-specific roles of BAG-1 isoforms in breast cancer have implicated BAG-1S in promoting cell survival in response to stress (Townsend et al., 2003). This study has shown overexpression of BAG-1S is associated with reduced clonogenicity in SKBR3 cells. Isoform-specific roles have been identified for BAG-1S previously in different cancers. Overexpression of BAG-1S in HaCaT human epidermal cell cultures has been shown to increase cell clumping in cohesion assays compared to vector controls(Hinitt et al., 2010). Overexpression of BAG-1M and BAG-1S was found in the same study to reduce colony scattering and hence implicated BAG-1 in regulating cell motility. Overexpression of all BAG-1 isoforms in HaCaT cells was associated with decreased cell yield and inhibited wound closure in scratch assays. These results and our study support a role for BAG-1S in mediating communal activity within cell cultures and the tissue remodelling events that occur during cancer invasion. Mutations in helix 1 of the BAG domain of BAG-1S allow Hsc/Hsp70 binding but were shown to reduce the growth inhibitory effect of BAG-1S overexpression. This indicates the Hsc/Hsp70 chaperones interaction with BAG-1 is important for mediating growth in HaCaT cell. However, the Raf-1 binding domain was implicated in BAG-1S-mediated cell motility by mutational analysis in the same study. Interestingly, no changes were seen in ERK/MEK activity suggesting Raf-1 acts via an alternative functional pathway (Hinitt et al., 2010).The part BAG-1 isoforms play in motility and migration has not yet been investigated in breast cancer cells.

SKBR3 cells demonstrated an increase in both BAG-1S and BAG-1M following trastuzumab on immunoblotting (Figure 3.6), which corresponds with results showing increased cytoplasmic BAG-1 using indirect immunofluorescence (Figure 3.12). A later Western blot also using SKBR3 cells exposed to trastuzumab demonstrated increased BAG-1L expression (Figure 3.10). The difference between BAG-1 isoform expression on Western blots in Figures 3.6 and 3.10 may be explained by different levels of endogenous BAG-1 isoforms in untreated controls. Failed attempts at producing stable clones overexpressing individual BAG-1 isoforms for this study demonstrated how difficult it was to obtain

reproducible isoform expression (results not shown). However, the Western blots in Figures 3.6 and 3.10 are representative of 3 independent experiments for each. Immunoblots and corresponding immunofluorescence demonstrated upregulation of BAG-1 isoform expression unsurprisingly did not occur with trastuzumab in HER2-negative ER-positive MCF-7 cells (Figures 3.13 and 3.14). Therefore, the lack of response in HER2-negative MCF-7 cells suggests the response to trastuzumab in HER2-positive cells is an on target effect.

To investigate if results obtained using the HER2 inhibitor trastuzumab were specific for HER2 inhibition or occurred with inhibition of other members of the EGFR family experiments were repeated using lapatinib and erlotinib. Duplicate experiments using the dual EGRR (HER1)/ HER2 inhibitor lapatinib in the place of trastuzumab showed elevated BAG-1L expression on immunoblots at 8 and 24 hours, but no difference at 48 hours (Figure 3.16). The reason BAG-1L did not increase at 48 hours exposure to lapatinib may be secondary to elevated endogenous levels of the isoform present in untreated controls. Immunocytochemistry in BT-474 cell cultures correlated with these findings and interestingly showed increased total expression, but nuclear exclusion of BAG-1 at 48 hours, not present at 24 hours (Figure 3.18). The HER1 inhibitor erlotinib was used in repeat assays in the place of lapatinib or trastuzumab. However, no change in cell yield or cell metabolic activity was seen between cells overexpressing BAG-1 isoforms and pcDNA3 following exposure to erlotinib, suggesting results seen were specific to HER2 inhibition (Figure 3.20). SKBR3 and BT-474 cells have been shown to express high levels of both HER1 and HER2 (DeFazio-Eli et al., 2011; Liu et al., 2009b) (Chapter 2 Table 2.1)

The findings of previous studies investigating the pattern of BAG-1 mRNA expression in comparison to BAG-1 protein expression in breast cancer cells are inconsistent. No similarity was found in the pattern of BAG-1 mRNA expression in breast cancers with BAG-1 protein expression in a study by Townsend *et al.* 2002 (discussed in Chapter 1). However, this chapter suggests that BAG-1 gene expression is induced by trastuzumab or trastuzumab increases mRNA stability (Figure 3.11). Differences between studies may be subsequent to previous studies using breast cancer tissue rather than cell culture and this study not representing the complete tumour environment.

In summary, this chapter has shown BAG-1 expression increases with trastuzumab treatment in HER2 positive cells. This elevation in BAG-1 expression is absent in HER2 negative, ER positive MCF-7 cells. The increase of BAG-1 expression following HER inhibition is not specific to SKBR3 cells, but occurs in other HER2 positive cell lines, such as

BT-474 cells (Figure 3.18). BAG-1 expression increases following treatment with other HER2 inhibitors, such as lapatinib (Figure 3.17). SKBR3 cells exposed to lapatinib and trastuzumab showed increased BAG-1 expression was associated with increased cell yield and viability compared to pcDNA3 (Figures 3.7 and 3.17 respectively). SKBR3 cells overexpressing BAG-1 isoforms showed similar levels of cell viability and cell yield on exposure to the EGFR (HER1)-specific inhibitor, erlotinib (Figure 3.20). This suggests the BAG-1 functional response to trastuzumab is specific to HER2, rather than occurring with other EGFR family members. However, it is important to recognise many studies have identified one of the mechanisms of resistance to hormonal therapy is the ability of the EGFR family to participate in cross-signalling between themselves and ER (Nicholson RI et al., 1999.; Shou et al.,2004). It is interesting that a pattern of exclusion of nuclear BAG-1 following lapatinib was seen in ER-positive HER2-positive BT-474 cells (Figure 3.18), but not ER-negative HER2-positive SKBR3 cells following trastuzumab (Figure 3.12)

This chapter supports a role for BAG-1 in the response of HER2-positive breast cancer to trastuzumab. These results have shown overexpression of all BAG-1 isoforms appears to alleviate the effects of trastuzumab and enhanced cell yields and metabolic activity are above that of the controls. The results from this chapter suggest BAG-1S expression may prevent the decrease in cell yield seen with exposure to lapatinib (Figure 3.16). Cell yield was not decreased in SKBR3 cells transfected with pcDNA3 exposed to trastuzumab, but was decreased by lapatinib compared to controls. This suggests that inhibition of pcDNA3 transfected cell cultures occurs through the HER1 inhibitor and could explain why overexpression of BAG-1M and BAG-1L do not increase relative cell yield.

Further work incorporating a number of HER2 family-positive cell lines is required to establish the effects of BAG-1 overexpression in HER2-positive breast cancer. The next chapter attempts to implicate potential functional signalling mechanisms involved using BAG-1-targeting siRNA and the small molecule inhibitor, thioflavin S (NSC1948) in HER2-positive cell cultures.

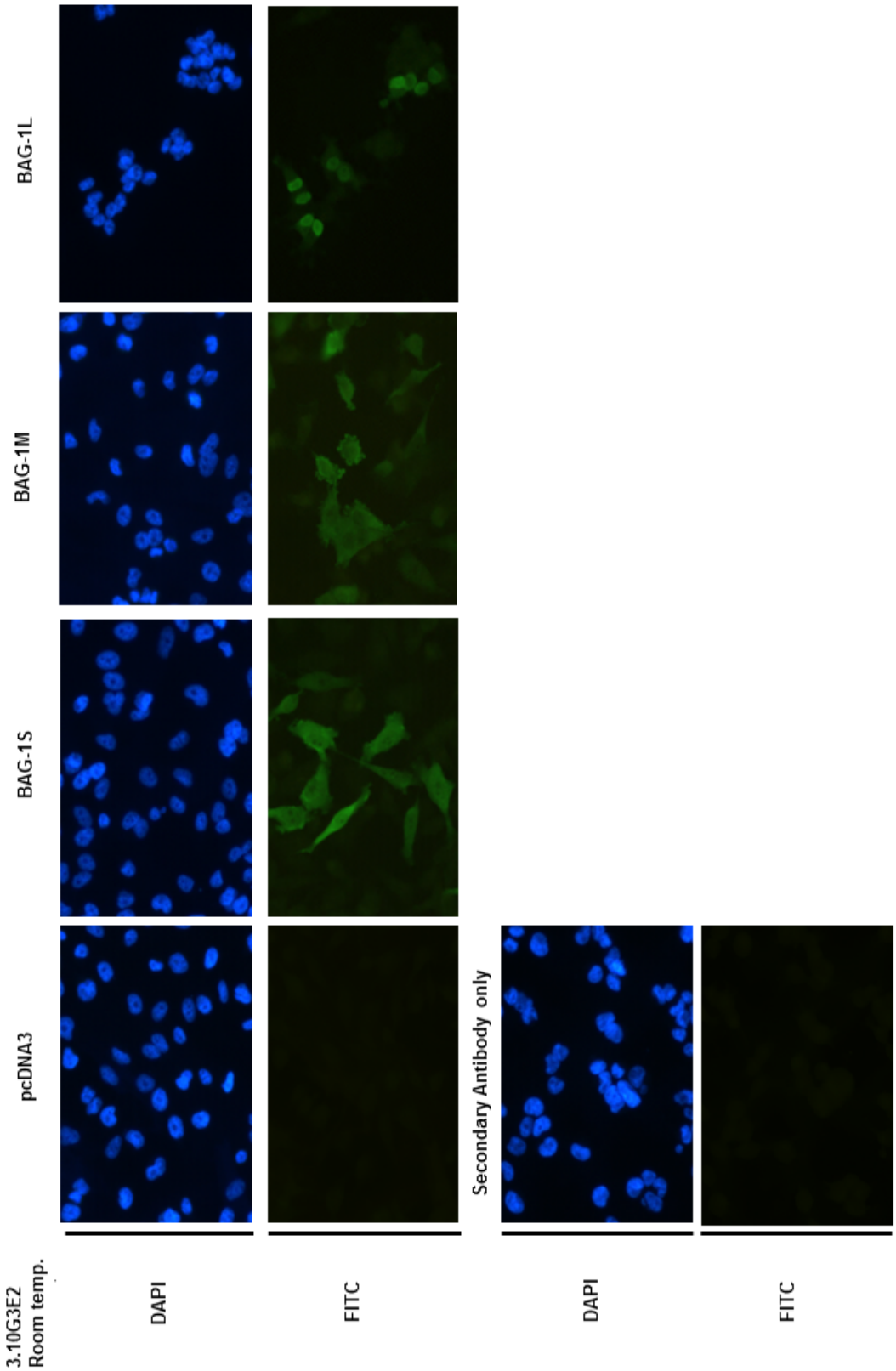

Figure 3.1 Subcellular localisation of BAG-1 isoforms in SKBR3 cells
Immunocytochemistry was performed consecutively from the stock of cells used for cell yield and cell viability assays (Figure 3.3) To demonstrate subcellular localisation of transfected BAG-1 isoforms the 3.10G3E2 primary antibody (1:1000) was used and Alexa-Fluor® 488 rabbit-mouse secondary (Cell Signalling). Nuclei were visualised by staining with DAPI incorporated into VECTASHIELD® Hardset mounting media (Vector Laboratories). Consecutive Western blotting was performed to confirm BAG-1 isoform expression (Figure 3.2).

Figure 3.2 Overexpression of BAG-1 isoforms in SKBR3 cells

Consecutive Western blots were performed using the same stock of SKBR3 cells transfected with isoform-specific constructs used for immunocytochemistry (Figure 3.1),cell yield, and cell viability assays (Figure 3.3). Cells were lysed with RIPA buffer at approximately 70% confluency. Nitrocellulose membranes were probed for BAG-1 (3.10G3E2) and β–actin was used as a loading control.

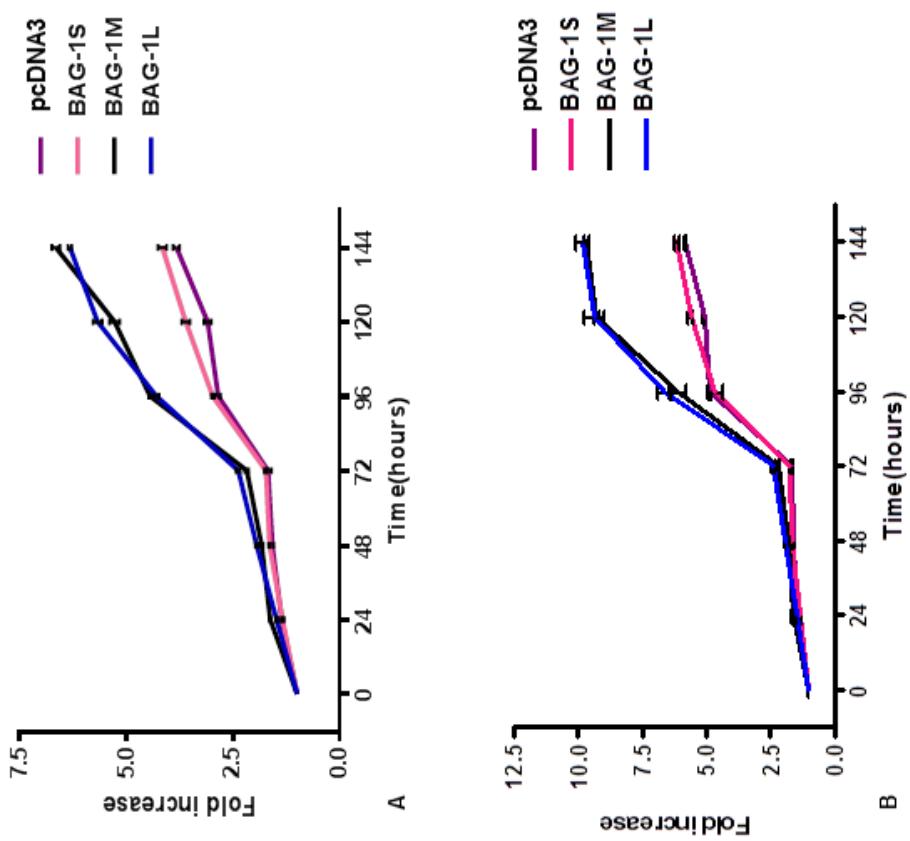

Figure 3.3 The effect of overexpression of BAG-1 isoforms on cell yield and relative cell viability of SKBR3 cells.

SKBR3 cells were transfected with isoform-specific BAG-1 constructs. At 24 hour intervals over 6 days cells were counted using a Casy Counter™ (A) or relative cellular metabolic activity calculated using Cell Titer 96® AQueous One Solution reagent (B). The relative number of metabolically active cells was derived by measuring photoabsorbance following a colourimetric reaction accompanying production of the metabolite formazan. All data shown are the means of wells treated in triplicate (+/-SD).

Figure 3.4 The effect of overexpression of BAG-1 isoforms on the clonogenic potential of SKBR3 cells.

SKBR3 cells were seeded in 6 well plates at a concentration of 300,000 cells /well. Following 24 hours incubation according to standard cell culture protocol, cells were transfected with isoform-specific BAG-1 constructs, BAG-1 mutants, pcDNA or pEGFP (data for pEGFP not shown, see section 2.3). A mock transfection was included using Fugene HD transfection reagent alone. Following 48 hours of incubation cells were trypsinised and seeded into 12-well plates or lysed with RIPA buffer for western blots. Cells seeded in the 12-well plates underwent serial dilution with DMEM until single colonies were visualised (~50,00cells / well) and placed under G418 selection (500µg/ml). Following a 14 day incubation period under G418 selection, the cells were fixed with 4% paraformaldehyde for 30 minutes. Cells were then rinsed with PBS and stained with 0.1% crystal violet for 20 minutes before being left to dry overnight. Colonies were counted in each well of the 12-well plates and data derived from the mean colony number of the triplicate wells (+/-SD).

Figure 3.5 Expression of BAG-1 isoforms in SKBR3 cells from stocks used for clonogenic assays (figure 4.3).

SKBR3 cells transfected with isoform-specific BAG-1 constructs, BAG-1 mutants, or pcDNA were lysed for western blotting using RIPA lysis buffer at approximately 70% confluency. Untreated cells, cells from a mock transfection, and pcDNA were lysed as controls at correlating time points. Expression of BAG-1S mutants, BAG-1SH2, BAG-1SH3A and BAG-1SH3AB was also checked and correlated to clonogenic assay results. Nitrocellulose membranes were probed for BAG-1 (3.10G3E2) and β–actin was used as a loading control.

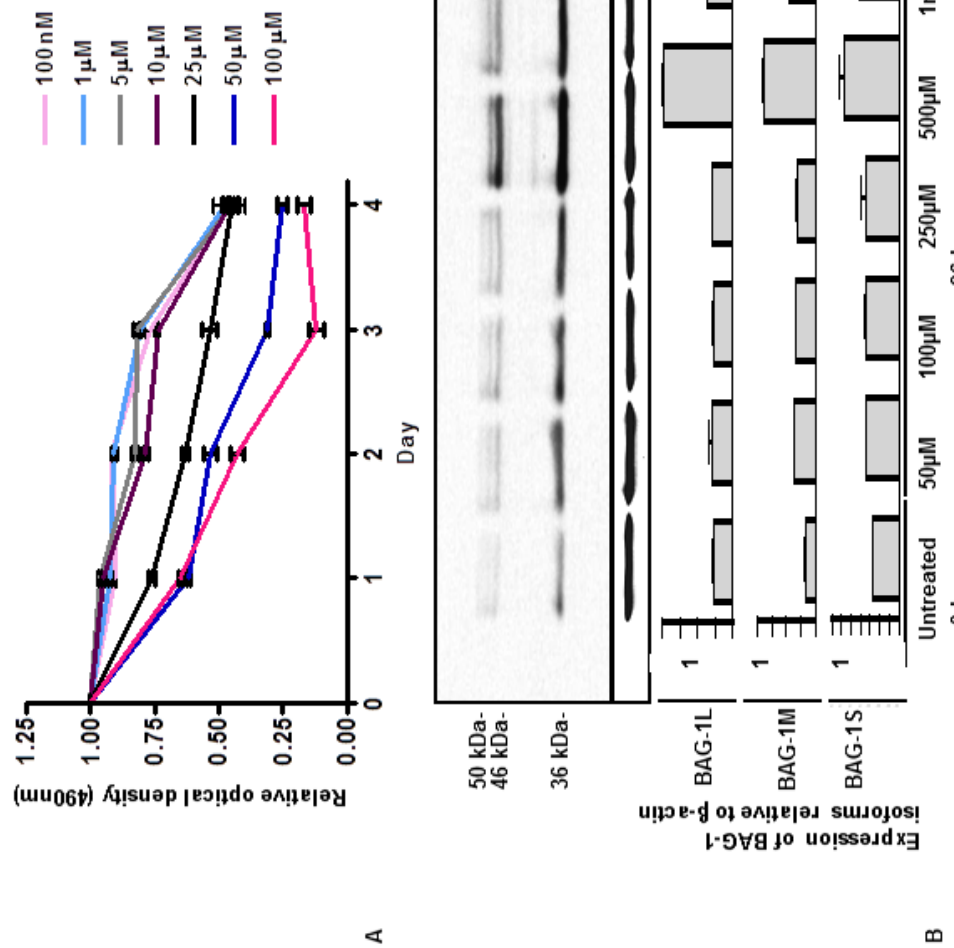

Figure 3.6 The response of SKBR3 cells to concentrations of Trastuzumab from 100nM- 100µM over 96 hours.

A Dose response curve; SKBR3 cells were seeded in 96 well plates at a concentration of 5,000 cells/ well. Following incubation for 24 hours cells were treated in triplicate with Trastuzumab at concentrations from 100nM-100µM. The number of metabolically active cells was derived using CellTiter 96® Aqueous One Solution reagent. Data were derived from 3 repeated experiments using the mean relative photoabsorbance of triplicate wells at 490nm (+/-SD).
B Western blot demonstrating dose-response; SKBR3 cells were seeded in 6-well plates and grown in DMEM under standard conditions until 70% confluent. Cells were treated with 50µM Trastuzumab for 96 hours and observed under the microscope. Cells were harvested and lysed with RIPA buffer and BAG-1 expression determined by Western blot using the anti- BAG-1 antibody 3.10G3E2.

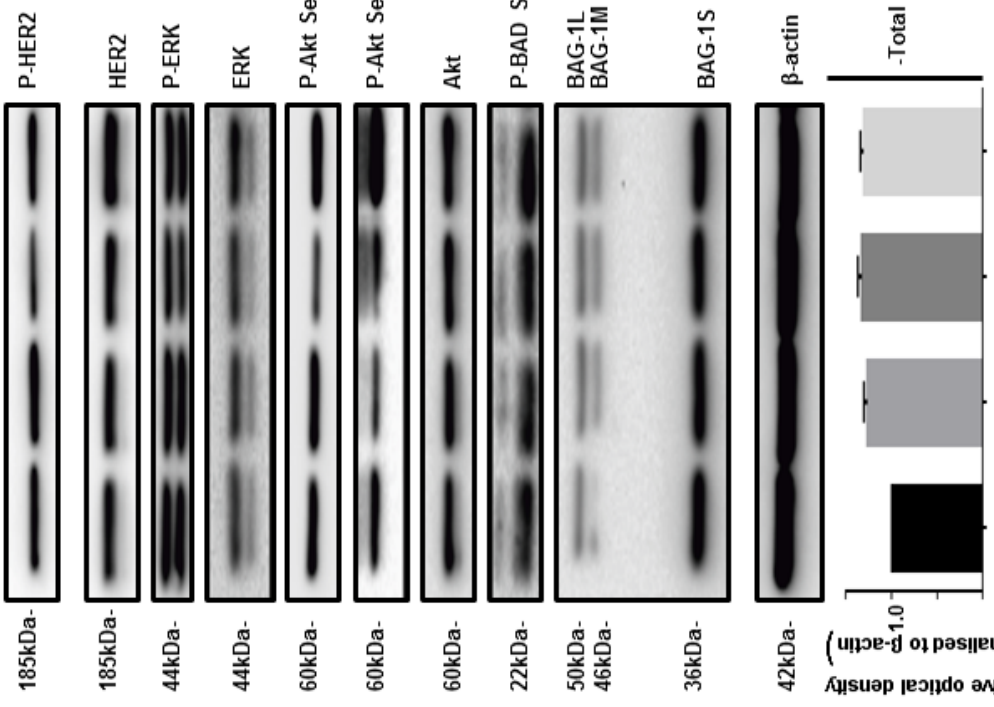

Figure 3.6C Expression of BAG-1 and HER2 signalling molecules following trastuzumab (50μM) or thioflavin S (50μM).
BT-474 cells were treated with thioflavin S (50μM), trastuzumab (50μM), or a combination of the two and the expression of downstream signalling molecules determined by Western blotting. IC50 using BT-474 cells was derived from previous dose-response experiments performed by Dr Emmanouil Papdakis (data not shown). Boxes show non-consecutive lanes from the same Western blot. Data are representative of 3 independent experiments performed by author although some of the blots above are kindly provided by Dr Emmanouil Papadakis.

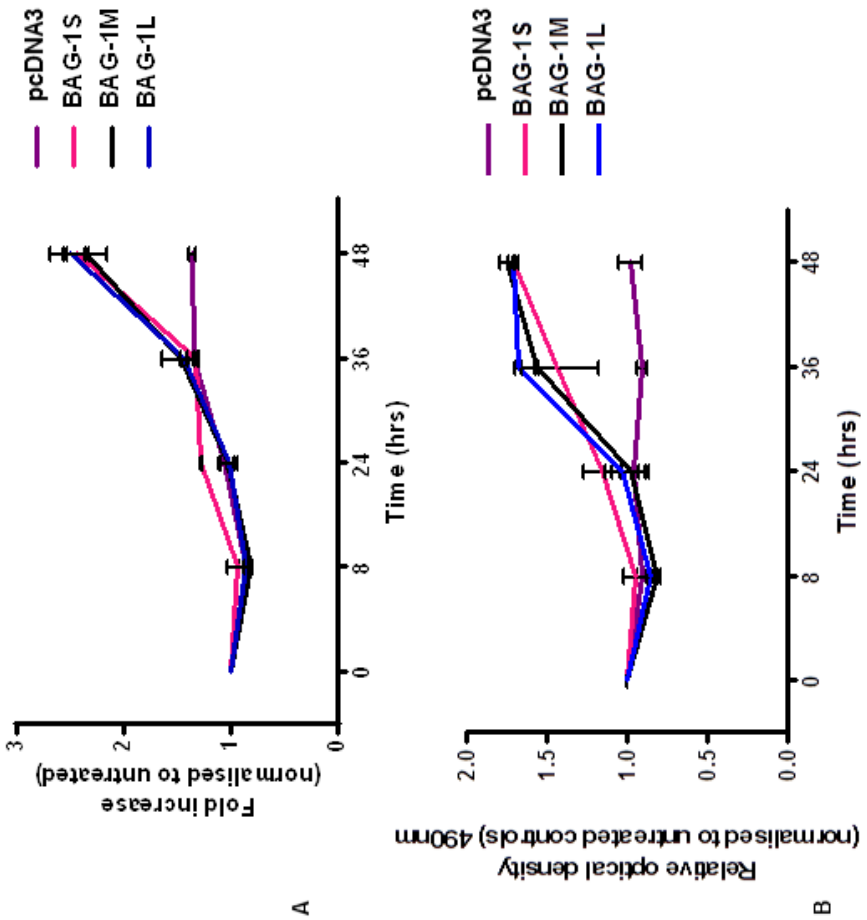

Figure 3.7 The effect of Trastuzumab on the cell yield (A) and the relative cell viability (B) on SKBR3 cells overexpressing BAG-1 isoforms.

SKBR3 cells were transfected with isoform-specific BAG-1 constructs. Following 48 hours incubation cells were harvested and seeded in 96 well plates at a concentration of 5,000 cells/ well. Following a further 24 hours of incubation in DMEM, cells were treated with 50μM Trastuzumab. At 8, 24, 36 and 48 hours cells were counted using a Casy Counter™ (A) or relative cellular metabolic activity calculated using Cell Titer 96® AQueous One Solution reagent (B). The data shown are derived from 3 repeat experiments using the mean relative photoabsorbance (490nm) of wells treated in triplicate (+/-SD).

Figure 3.8 The effect of doxorubicin (250nM-2µM) on the relative cell viability of SKBR3 cells over 48 hours.

SKBR3 cells were seeded in 96 well plates at a concentration of 5,000 cells/ well. Following incubation for 24 hours cells were treated in triplicate with doxorubicin at concentrations from 100nM-100µM. The number of metabolically active cells was derived using CellTiter 96® Aqueous One Solution reagent. Data was derived from 3 repeated experiments using the mean relative photoabsorbance of triplicate wells at 490nm (+/-SD).

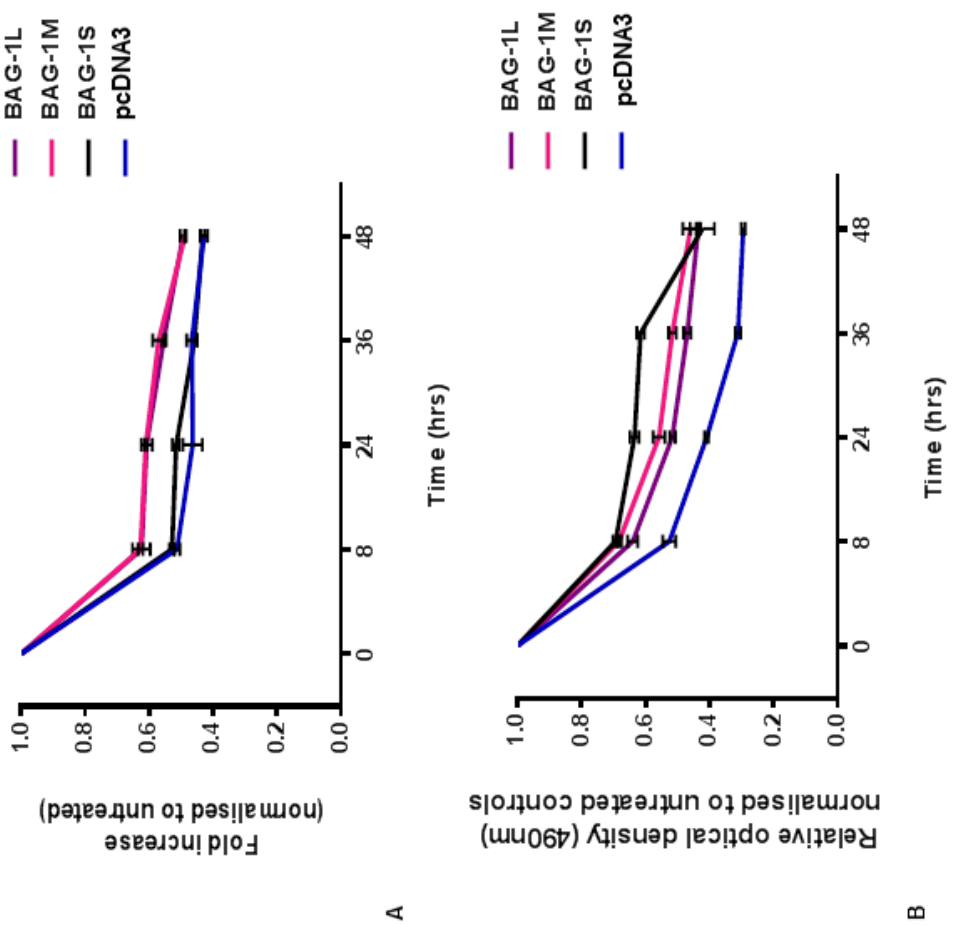

Figure 3.9 The effect of doxorubicin on the cell yield (A) and the relative cell viability (B) on SKBR3 cells overexpressing BAG-1 isoforms.

SKBR3 cells were transfected with isoform-specific BAG-1 constructs. Following 48 hours incubation cells were harvested and seeded in 96 well plates at a concentration of 5,000 cells/ well. Following a further 24 hours of incubation in DMEM, cells were treated with 1μM doxorubicin. At 8, 24, 36 and 48 hours cells were counted using a Casy Counter™ (A) or relative cellular metabolic activity (cell viability) calculated using Cell Titer 96® AQueous One Solution reagent (B). The data shown are derived from 3 repeat experiments using the mean relative photoabsorbance (490nm) of wells treated in triplicate (+/-SD).

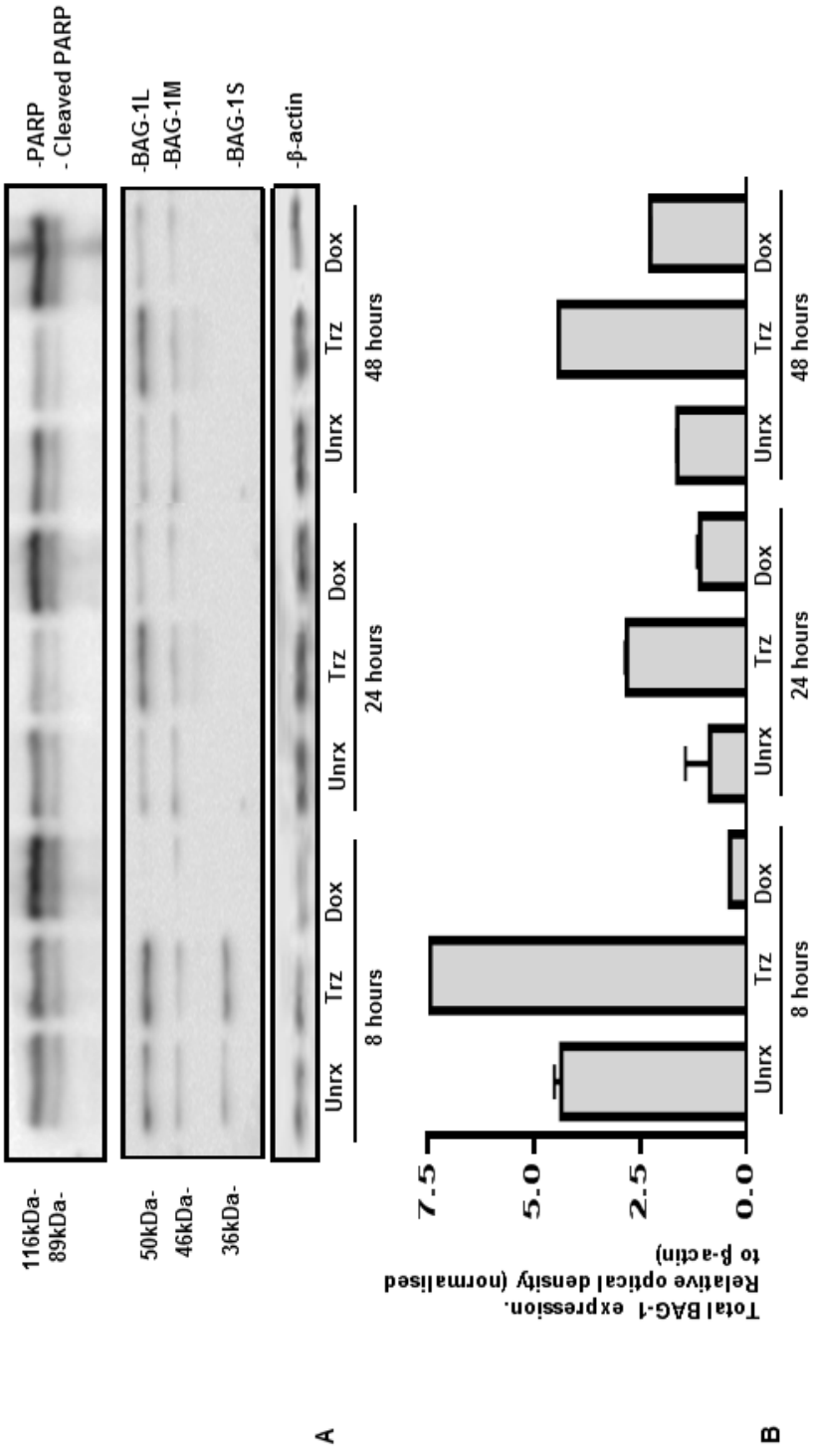

Figure 3.10 PARP and BAG-1 expression in SKBR3 cells following 8-48 hours treatment with 50μM trastuzumab or 1μM doxorubicin.

SKBR3 cells were lysed for Western blotting (A) using RIPA lysis buffer following 8, 24 and 48 hours of treatment with 50μM trastuzumab or 1μM doxorubicin. Untreated cells were lysed as controls at correlating time points. Nitrocellulose membranes were probed for BAG-1 (3.10G3E2) and PARP (Cell Signalling). β–actin was used as a loading control. B: Densitometric analysis of total BAG-1 expression. Data were derived from 3 individual experiments (mean +/- SD).

Figure 3.11 qPCR analysis of BAG-1 mRNA expression in BT-474 cells following 48 hours of treatment with 50μM trastuzumab.

Qiagen Mini-Prep Kit was used for RNA extraction from BT-474 cells following 48 hours exposure to 50μM trastuzumab. Reverse transcription was set up using a 'no template' (RNA replaced with dH_20) and a 'reverse transcriptase' control (an untreated sample). Primer annealing was performed using a Gene Amp® PCR System 9700 (PE Applied Biosystems).Taqman® assay on demand probe sets (Applied Biosystems, UK) were used to amplify BAG-1(Assay ID: HS00185390_BAG-1Part number: 4331182) and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase, H) genes. The comparative ΔΔCT method (ΔΔCT= power (2, ΔCT) where ΔCT is the change in mean crossing threshold) was used for statistical analysis of qRTPCR results. BAG-1 CT values were normalised to average GAPDH to correct for variation in start-point cDNA concentration. Subsequent analysis for each sample relative to control samples was performed. Data was paired and normal distribution was assumed so a paired students t-test was performed

Data shown is from 3 independent experiments (p=0.0262).

Figure 3.12 Immunocytochemistry showing endogenous BAG-1 localisation in SKBR3 cells following 8-48 hours treatment with 50μM trastuzumab or 1μM doxorubicin.

SKBR3 cells were seeded on sterile coverslips at a concentration of 100,000 cells / well. Endogenous BAG-1 expression was detected at each time point following fixation in 4% PFA using the 3.10G3E2 BAG-1 antibody followed by Alexa-Fluor® 488 rabbit anti-mouse secondary (Cell Signalling). Nuclei were visualised with DAPI incorporated into VECTASHIELD® Hardset mounting media (Vector Laboratories).

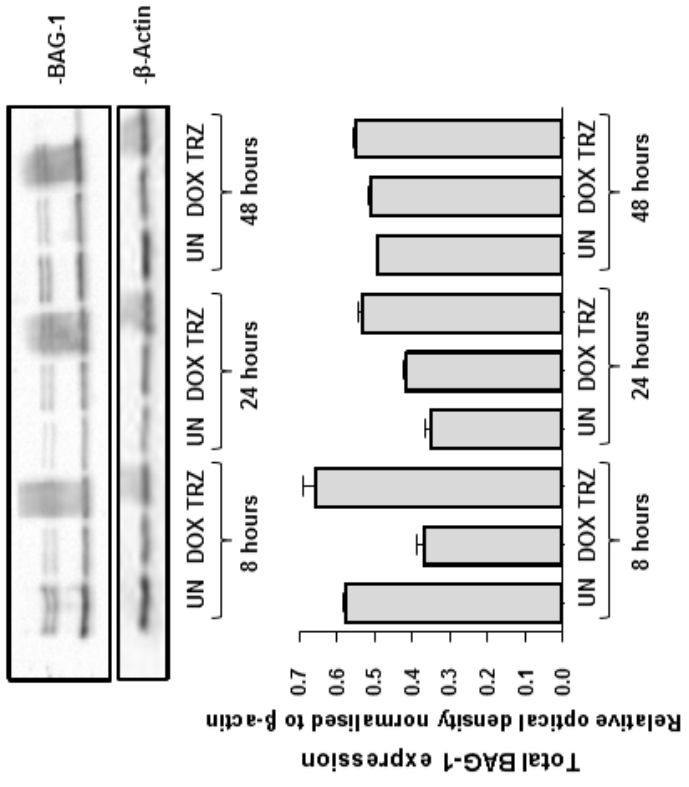

Figure 3.13 Western blots showing BAG-1 expression in MCF-7 cells following 8-48 hours treatment with 50µM Trastuzumab or 1µM Doxorubicin.

MCF-7 cells were lysed for western blotting using RIPA lysis buffer following 8, 24 and 48 hours of treatment with 50µM Trastuzumab or 1µM Doxorubicin. Untreated cells were lysed as controls at correlating time points. Nitrocellulose membranes were probed for BAG-1 (3.10G3E2). β–actin was used as a loading control. Data were derived from 3 individual experiments (mean +/- SD).

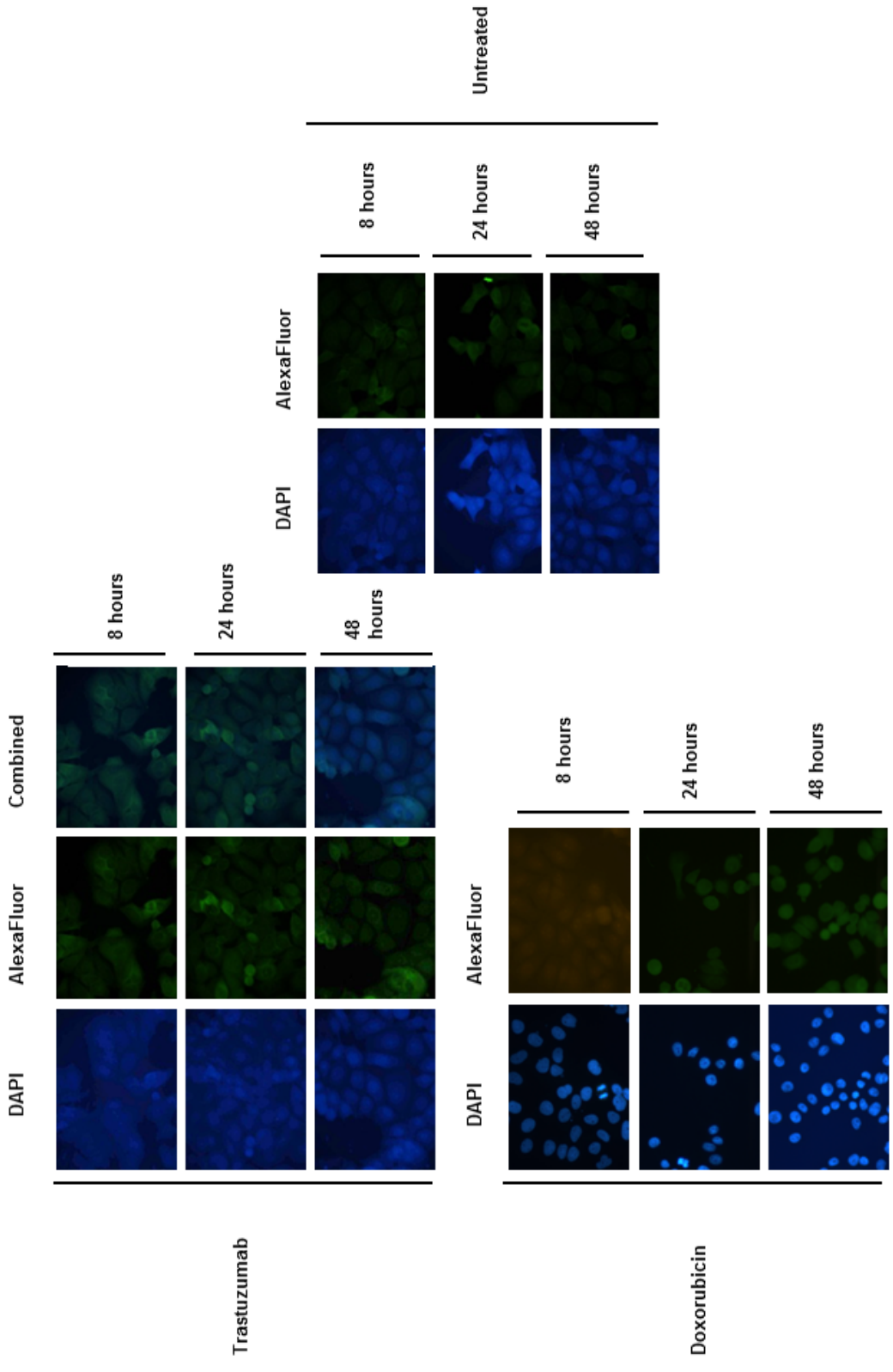

Figure 3.14 Immunocytochemistry showing endogenous BAG-1 localisation in MCF-7 cells following 8-48 hours treatment with 50μM trastuzumab or 1μM doxorubicin.

MCF-7 cells were seeded on sterile coverslips at a concentration of 100,000 cells / well. Endogenous BAG-1 expression was detected at each time point following fixation in 4% PFA using the 3.10G3E2 anti-BAG-1 antibody followed by Alexa-Fluor® 488 rabbit anti-mouse secondary (Cell Signalling). Nuclei were visualised by staining with DAPI incorporated into VECTASHIELD® Hardset mounting media (Vector Laboratories).

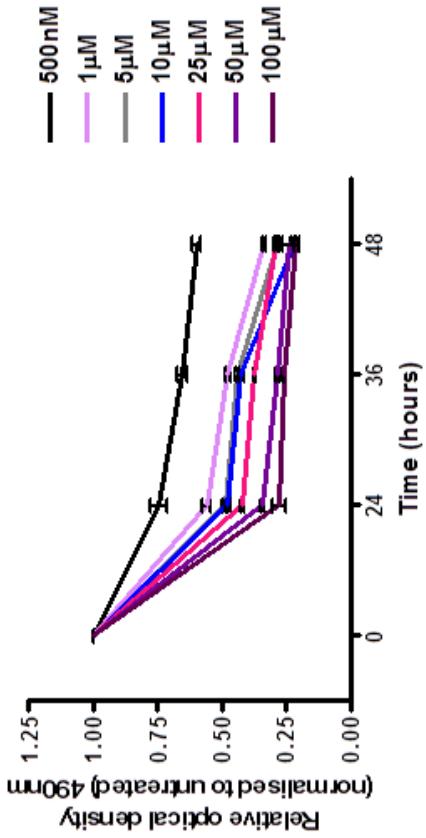

Figure 3.15 The effect of lapatinib (500nM-100µM) on the relative cell viability of SKBR3 cells over 48 hours.

SKBR3 cells were seeded in 96 well plates at a concentration of 5,000 cells/ well. Following incubation for 24 hours, cells were treated in triplicate with lapatinib at concentrations from 500nM-100µM. The number of metabolically active cells was derived using CellTiter 96® Aqueous One Solution reagent. Data were derived from 3 repeated experiments using the mean relative photoabsorbance of triplicate wells at 490nm (+/-SD).

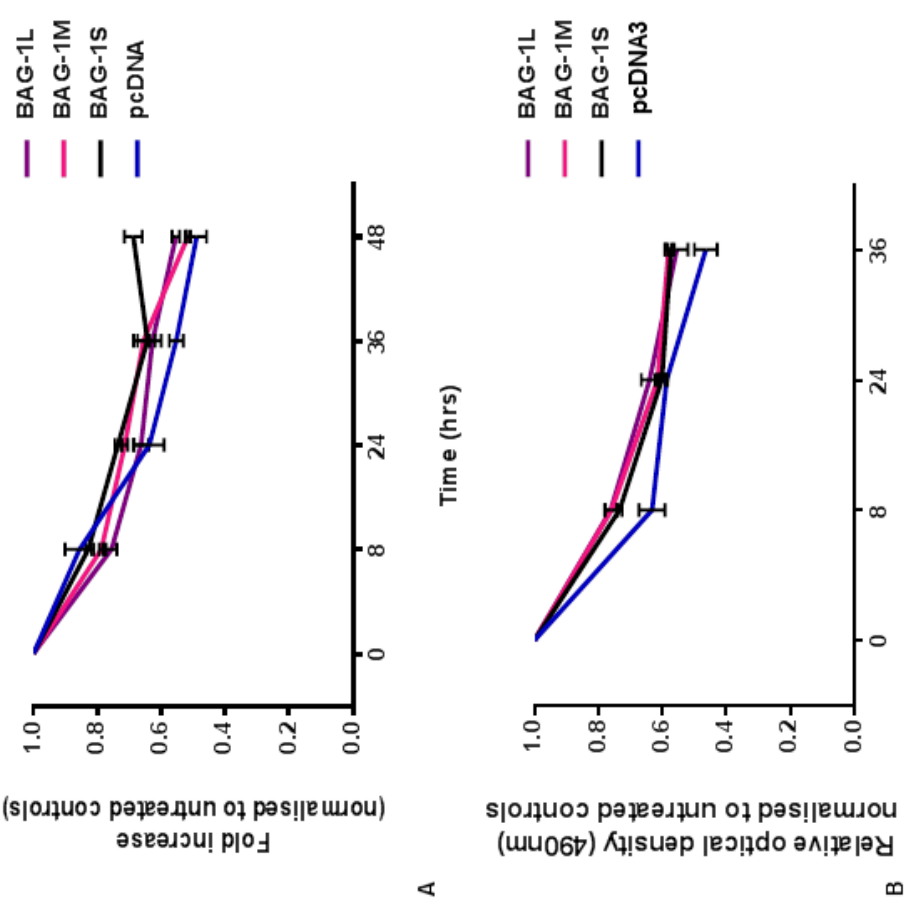

Figure 3.16 The effect of lapatinib on the cell yield (A) and the relative cell viability (B) on SKBR3 cells overexpressing BAG-1 isoforms.

SKBR3 cells were transfected with isoform-specific BAG-1 constructs. Following 48 hours incubation cells were harvested and seeded in 96 well plates at a concentration of 5,000 cells/ well. Following a further 24 hours of incubation in DMEM, cells were treated with 1μM lapatinib. At 8, 24, 36 and 48 hours cells were counted using a Casy Counter™ (A) or relative cellular metabolic activity calculated using Cell Titer 96® AQueous One Solution reagent (B). The data shown are derived from 3 repeat experiments using the mean relative photoabsorbance (490nm) of wells treated in triplicate (+/-SD).

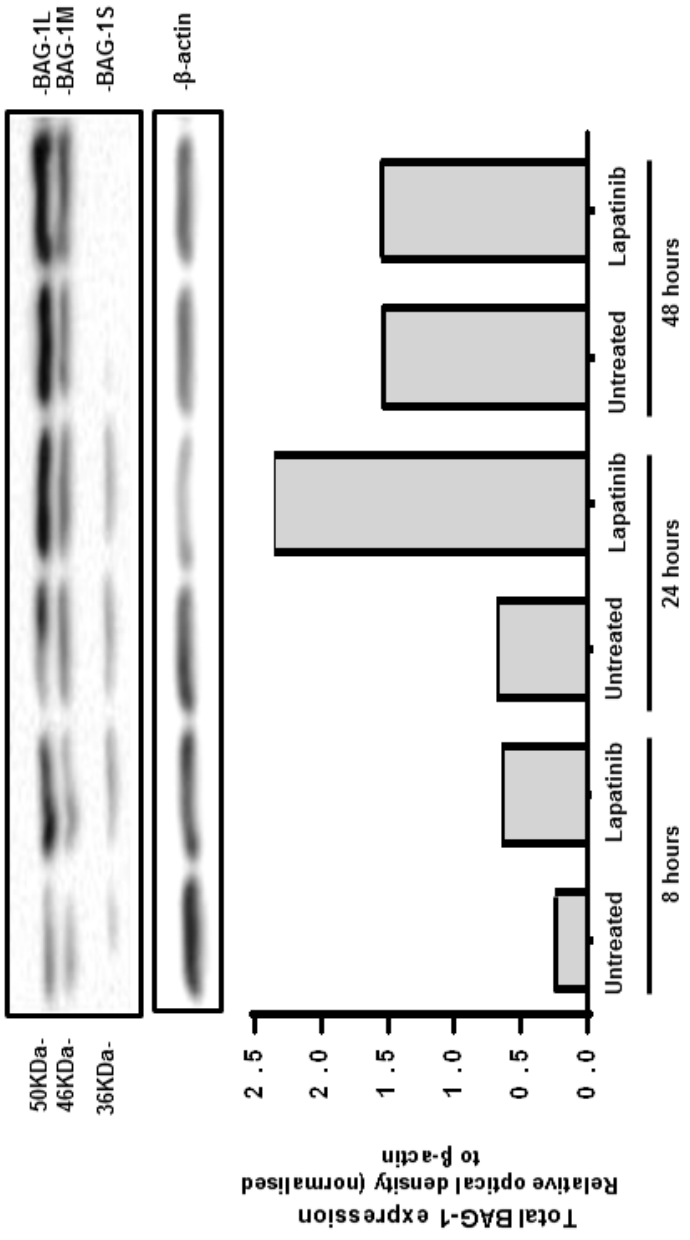

Figure 3.17 BAG-1 expression in SKBR3 cells following 8-48 hours treatment with 1μM lapatinib.

SKBR3 cells were lysed for western blotting using RIPA lysis buffer following 8, 24 and 48 hours of treatment with 1μM lapatinib. Untreated cells were lysed as controls at corresponding time points. Nitrocellulose membranes were probed for BAG-1 (3.10G3E2). β–actin was used as a loading control. Data were derived from the mean of 2 independent experiments.

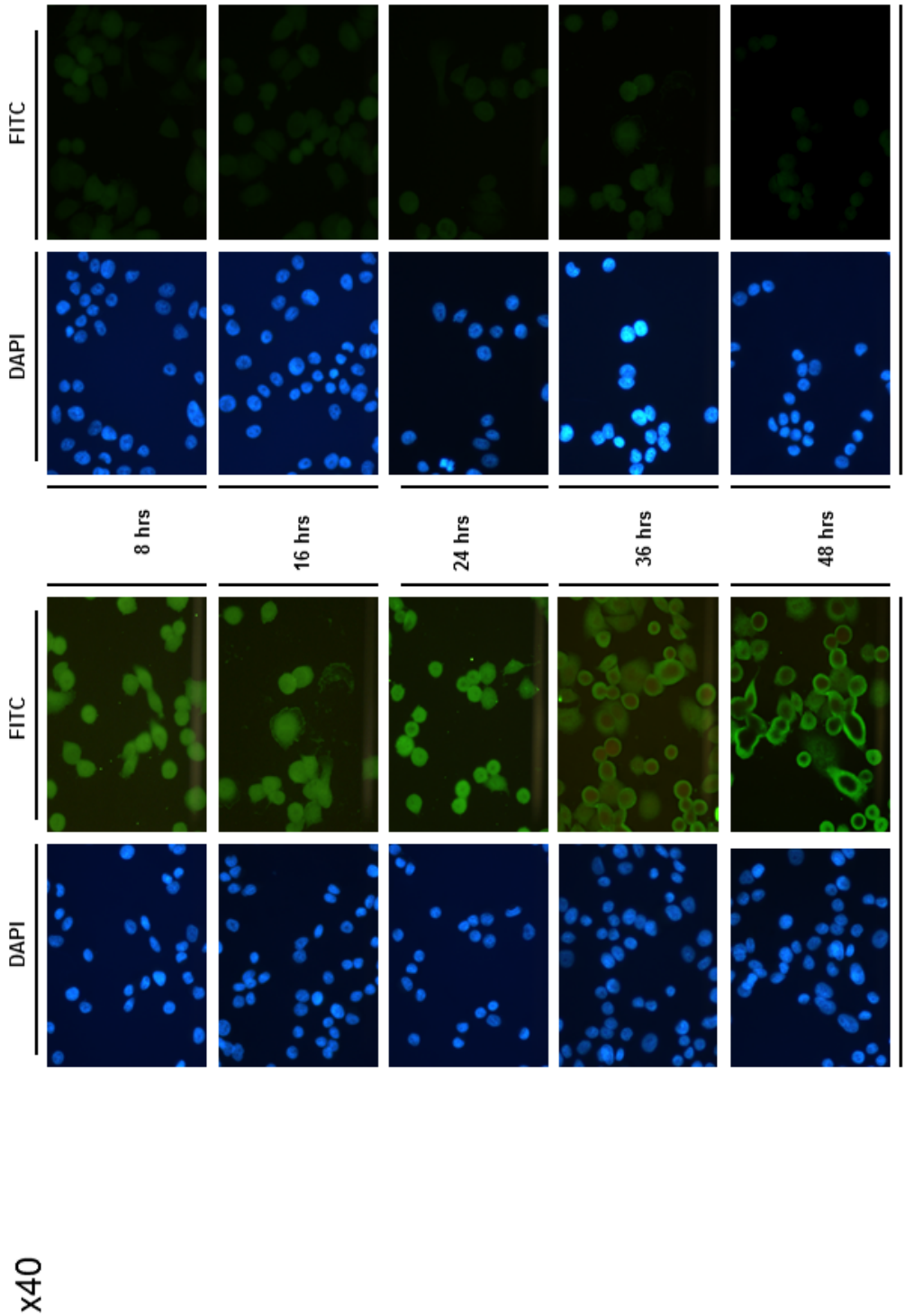

Figure 3.18 Immunocytochemistry showing endogenous BAG-1 localisation in BT-474 cells following 8-48 hours treatment with 1μM Lapatinib.

BT-474 cells were seeded on sterile coverslips at a concentration of 100,000 cells / well. Following 24 hours incubation in standard culture medium, cells were treated with 1μM Lapatinib for 8-24 hours. Endogenous BAG-1 expression was detected at each time point following fixation in 4% PFA using the 3.10G3E2 BAG-1 antibody followed by Alexa-Fluor® 488 rabbit anti-mouse secondary (Cell Signalling). Nuclei were visualised by staining with DAPI incorporated into VECTASHIELD® Hardset mounting media (Vector Laboratories).

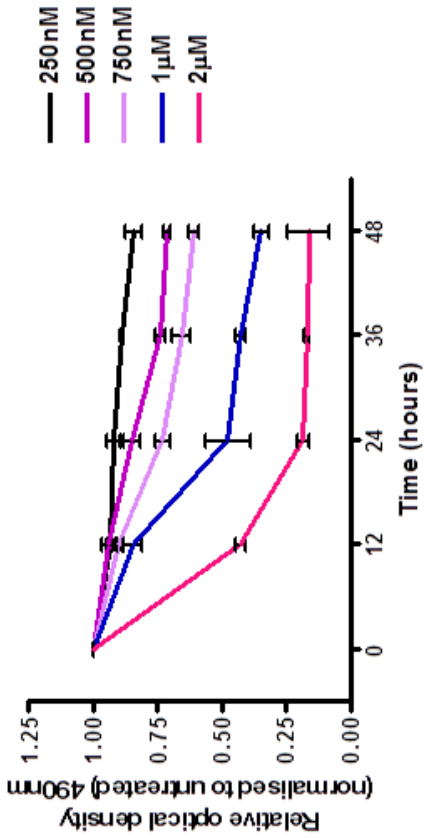

Figure 3.19 The effect of erlotinib (250nM-2µM) on the relative cell viability of SKBR3 cells over 48 hours.

SKBR3 cells were seeded in 96 well plates at a concentration of 5,000 cells/ well. Following incubation for 24 hours cells were treated in triplicate with lapatinib at concentrations from 250nM-1µM. The number of metabolically active cells was derived using CellTiter 96® Aqueous One Solution reagent. Data were derived from 3 repeated experiments using the mean relative photoabsorbance of triplicate wells at 490nm (+/-SD).

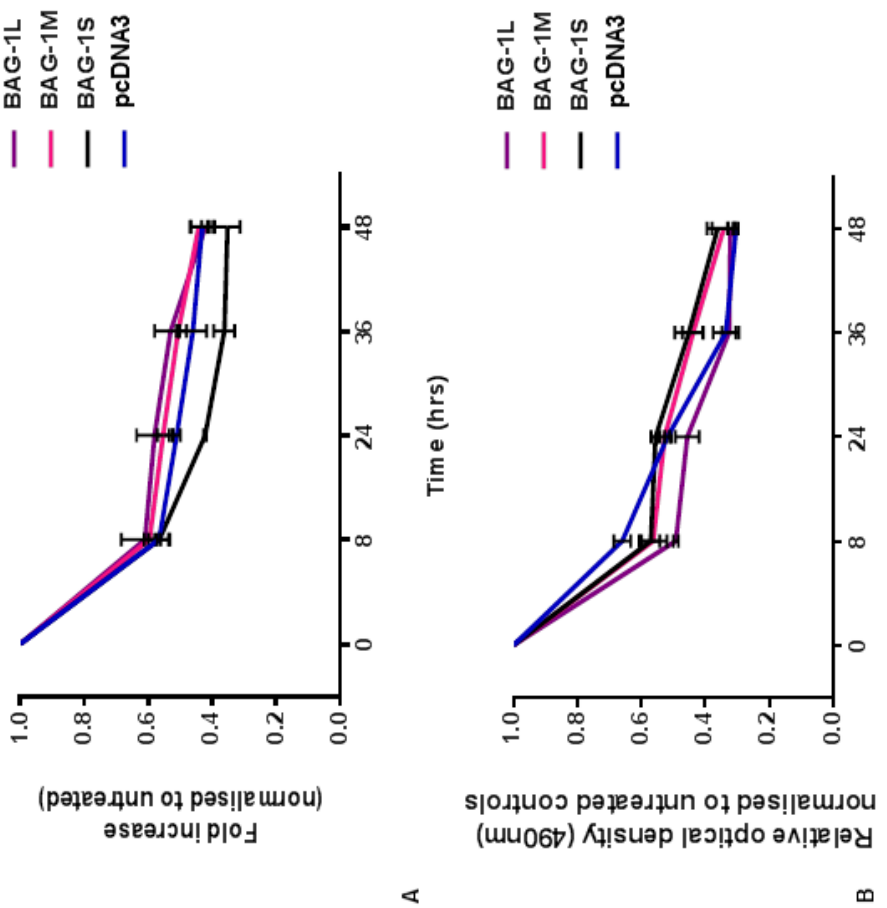

Figure 3.20 The effect of erlotinib on cell yield (A) and relative cell viability (B) on SKBR3 cells overexpressing BAG-1 isoforms.

SKBR3 cells were transfected with isoform-specific BAG-1 constructs. Following 48 hours incubation cells were harvested and seeded in 96 well plates at a concentration of 5,000 cells/ well. Following a further 24 hours of incubation in DMEM, cells were treated with 1μM erlotinib. At 8, 24, 36 and 48 hours cells were counted using a Casy Counter™(A) or relative cellular metabolic activity calculated using Cell Titer 96® AQueous One Solution reagent (B). The data shown derived from 3 repeat experiments using the mean relative photoabsorbance (490nm) of wells treated in triplicate (+/-SD).

Chapter 4: Crosstalk between BAG-1 and HER2 signalling in HER-positive breast cancer.

Chapter 4: Crosstalk between BAG-1 and HER2 signalling in HER2-positive breast cancer.

4.1 Introduction

Chapter 3 investigated the effects on cell yield and viability of overexpression of individual BAG-1 isoforms in HER2-positive breast cancer cells (Section 3.2.1). The aim of this chapter was to study the function of endogenous BAG-1 in HER2 positive breast cancer. To achieve this we utilised siRNA targeting BAG-1 and studied the functional effects via cell viability assays, using MTS reagent, and cell yield via simple cell counting techniques. A further aim of this chapter was to investigate the response of HER2 positive cells to HER2 inhibition by trastuzumab (Herceptin™) following knockdown of endogenous BAG-1 with siRNA. Repeat biological assays were performed in the presence of trastuzumab with and without knockdown of BAG-1 to elucidate effects on cell yield and cell viability. The main objective of this chapter was to ascertain if reduction of BAG-1 using siRNA sensitises HER2-positive cells to trastuzumab. A further objective was to identify potential signalling mechanisms involved, thereby testing the hypothesis that BAG-1 may potentiate MAPK signalling and abrogate the response to trastuzumab. An additional objective was to assess if use of the BAG-1 inhibitor, thioflavin S, in biological assays mediated the response of HER2-positive BT-474 cells to trastuzumab, and corresponded with results using siRNA knockdown of BAG-1. Thioflavin S (NSC71948) was identified as a small-molecule chemical inhibitor of binding between the C-terminus of the BAG domain and Raf-1. In addition, thioflavin S interferes with BAG-1-mediated protein-protein interactions between Hsc70/Hsp70 known to be functionally important in BAG-1-regulated survival pathways (Sharp et al., 2009).

The expression of BAG-1 in normal breast cell lines compared to breast cancer cell lines has been published by Tang et al. (1999) (Tang et al., 1999). Western blotting was used to show the most abundant BAG-1 isoform in all cell lines was BAG-1S. The only detectable isoform in HS574, HS578, and HS787 normal breast cells was BAG-1S. BAG-1S expression was higher in the majority of breast cancer cell lines compared to normal breast cell lines. BT-474 cells showed high levels of BAG-1L and weak expression of BAG-1M, but BAG-1S was still the most abundant isoform. MCF-7 cells and SKBR3 cells were shown to weakly express BAG-1L in addition to high levels of BAG-1S.

Small interfering RNA (siRNA) are a group of double-stranded RNA molecules consisting of 20-25 nucleotides and first discovered in plants in by Hamilton and Baulcombe in 1999 (Hamilton and Baulcombe, 1999). siRNA plays an important part in RNA inhibition (RNAi) pathway and has proved a useful tool for molecular biologists to reduce the expression of specific genes. Any gene in which the sequence is known can be targeted by siRNA. The siRNA is a result of a dicer enzyme that converts double stranded RNA or small hairpin RNA into siRNA. These useful RNA molecules can then be transfected into cells to knockdown a specific gene of interest.

Knockdown of BAG-1 using siRNA has been used in breast cancer research previously. BAG-1 siRNA has been used to demonstrate knockdown of MAPK1 overcame resistance to trastuzumab in SKBR3 cells (Boyer et al., 2013). This study suggests BAG-1 may act via the MAPK pathway to sensitise SKBR3 cells to trastuzumab.

RNA interference has been used to target other genes involved in breast cancer development, metastasis and response to chemoradiotherapy. An example is the targeting of the p53 negative regulator Hdmx, overexpressed in 5% of primary breast tumours. Reduction of Hdmx in MCF-7 breast cancer cells by siRNA resulted in p53-dependent decreased clonogenic potential (Danovi et al., 2004). A further example is the targeting of CXC chemokine receptor-4 (CXCR4**),** shown to be overexpressed in the invasive breast cancer cells MDA-MB-231 (Muller et al., 2001). Transfection of MDA-MB-231 cells with tetracycline-inducible siRNA targeting CXCR4 results in decreased tumour migration in vitro (Chen et al., 2003). Direct inoculation with naked CXCR4-targeting siRNA into a SCID mouse model slowed tumour development (Liang et al., 2005).Studies using RNA interference have been performed specifically looking at HER2-positive breast cancer. RNA targeting polio-like kinase-1 (PLK-1) bound with a Her2-ScFv-protamine peptide fusion protein (F5-P) reduces growth in HER2-positive SKBR3 and BT-474 breast cancer cells in vitro. Injection of PLK-1 siRNA into mouse xenograft models of HER2-positive breast cancer resulted in decreased growth, metastasis and prolonged survival (Yao et al., 2012).

Excitingly, siRNA therapy has reached clinical trials in breast cancer. RNA interference identified loss of PTEN activates the PI3K pathway and is implicated in resistance to anti-HER2 therapy in breast cancer (Berns et al., 2007). Protein kinase N3 (PKN3) is a protein

downstream of PI3K and siRNA reduction of PKN3 in breast cancer cells inhibits breast tumour growth and metastasis (Unsal-Kacmaz et al., 2012). A phase 1 clinical trial using siRNA targeting PKN3 in patients diagnosed with breast cancer is in the stages of completion (http://www.clinicaltrials.gov/ct2/show/NCT00938574?term=atu027&rank = 1).

Knockdown of BAG-1 using siRNA has also been utilised in several other cancers. Clemo et al. (2008) showed a potential role for BAG-1 in colorectal cancer using siRNA knockdown of BAG-1 in HCT116 cells. Reducing BAG-1 expression via siRNA knockdown resulted in an inhibition of NF-κB activity and increased apoptosis, thus implicating BAG-1 as a target for adjuvant therapy design (Clemo et al., 2008). Wang et al. used siRNA knockdown of BAG-1 expression in H1299 non-small cell lung cancer cells to demonstrate BAG-1 function in upregulating p73-dependent transcription. Use of both chemotherapeutic agents and radiotherapy have been associated with an up-regulation of p73-mediated apoptosis, therefore these pathways could be further potentiated by BAG-1 (Wang et al., 2009). More recently, Yoshino et al. have proposed a role for BAG-1 in squamous oesophageal cancer using siRNA knockdown. Decreasing expression of BAG-1 reduced radiosensitivity of TE12 cells, suggesting a contrasting proapoptotic role for BAG-1 in oesophageal cancer (Yoshino et al., 2010).

BAG-1 increases proliferation in breast cancer cells (Kudoh et al., 2002, Townsend et al., 2003, Sharp et al., 2009 see Chapter 1, Table 1.1), but this function has not been validated by siRNA knockdown of BAG-1 in HER2-positive breast cancer. There are at several proposed mechanisms of action contributing to the response of HER2 positive cells to trastuzumab (Herceptin™), which have been previously discussed in Chapter 1 (Pohlmann et al., 2009). Briefly, the main mechanisms of action of trastuzumab are interruption of MAPK/PI3K signalling (Nahta and Esteva, 2006), antibody-dependent cytotoxicity to tumour cells (Arnould et al., 2006), and increased degradation of the HER2 receptor (Klapper et al., 2000). This chapter investigates if siRNA knockdown of BAG-1 has any gross effect on HER2-positive cell response to trastuzumab treatment.

4.2 Results

4.2.1 Expression and localisation of endogenous BAG-1 proteins in breast cancer cells.

SKBR3, BT-474 and MCF-7 cells were harvested at 70% confluence after 48 hours incubation and used for Western blots and immunofluorescence. Images were obtained using fluorescence microscopy to demonstrate the subcellular localisation of endogenous BAG-1 (Figure 4.1). Immunofluorescence showed predominantly cytoplasmic BAG-1 in SKBR3 cells compared with the BT-474 and MCF-7 cells, which showed both nuclear and cytoplasmic BAG-1 (Figure 4.1). Western blots similar ratios of BAG-1 isoforms in all 3 cell lines. All cell lines revealed a small amount of endogenous BAG-1M compared to the other isoforms. BAG-1S was the most abundant BAG-1 isoform expressed in all cell lines, as originally reported by Tang et al. (Tang et al., 1999). BAG-1 has 3 distinct isoforms produced from a single strand of mRNA. Consequently, siRNA does not provide direct information about isoform-specific function. However, characteristic localisation patterns of BAG-1 isoforms are demonstrated by overexpression of isoform-specific BAG-1 constructs in chapter 3 confirming BAG-1S and BAG-1M are present predominantly in the cytoplasm and BAG-1L in the nucleus (Figure 3.1).

4.2.2 Optimisation of siRNA knockdown of BAG-1 using siRNA concentrations of 25nM-100nM.

To optimise siRNA knockdown of BAG-1, BAG-1 expression following siRNA knockdown in SKBR3 cells was determined using Western blotting (siRNA targeting BAG-1 oligonucleotides was supplied by Ambion™, see Chapter 2). Blots incorporated lysates from untreated HER2 positive cells and from those transfected with non-targeting negative controls (siGENOME™ SMARTpool, Dharmacon) (Figure 4.2). Image J optical densitometric analysis of Western blot bands was used to deduce mean percentage knockdown from the 2 independent experiments. Transfection of SKBR3 cells with BAG-1 siRNA at an optimal siRNA concentration of 25nM produced a mean average of 67% knockdown compared to the negative control and 59% compared to the untreated control. An siRNA concentration of 50nM was associated with increased cell death and detachment visible under the microscope compared to more dilute concentrations. This may account for the loss of effect seen at the highest concentration of 100nM.

Acceptable levels of siRNA knockdown are often reported at 90% or above, however, evidence has shown that achieving truly representative knockdown of gene expression is a balance between keeping transfected cells healthy and high levels of knockdown. Studies have shown that if 90% siRNA knockdown is obtained, then the amount of apoptosis and unhealthy cells is higher than if 60-65% knockdown is achieved (Ambion Technotes Volume 12). During optimisation of our working protocol we encountered problems with cell toxicity to the concentration and exposure time to Dharmafect™ transfection reagent. This necessitated 6 hourly microscopic observations of cells and termination of the experiment if more than 10% cell death was observed after transfection with siRNA. Transfection medium was changed to complete medium after 24 hours and an MTT assay performed to establish >80% viable cells, as per manufacturer's protocol (ThermoScientific). This study achieved a knockdown of BAG-1 of 67% of the negative siRNA control and we can rationalise this as optimal as at this level of knockdown there is a larger proportion of functionally normal healthy cells, therefore more representative of in vivo conditions.

Important factors in creating the optimal representative gene knockdown are the viability of the transfected cells, choosing the correct transfection reagent, and exposure time. This study adhered closely to manufacturer's protocol (Ambion) and included initial optimisation steps such as finding the optimal siRNA concentration and density of cells seeded. Cells seeded were at the earliest passage available (ranging from passage 6 to 28), and kept in warm medium at all times.

At siRNA concentrations of 50nM or more the cells became more detached and there was visible increased cell death under the microscope prior to harvesting. An attempt to ensure a healthy monolayer of attached cells was harvested at 70% confluency, but due to the proportion of detached cells at the higher siRNA concentrations, these were included in preparation of the pellet for lysates. The Western blots performed at 100nM siRNA showed decreased knockdown (Figure 4.2A) which may be explained by the proportion of detached and pre-apoptotic cells used.

4.2.3 BAG-1 expression in SKBR3 cells following siRNA knockdown of BAG-1 and treatment with Trastuzumab (50µM).

To investigate the effects of BAG-1 in HER2-positive breast cancer cells endogenous BAG-1 was knocked down using siRNA. Experiments were repeated with and without trastuzumab

to ascertain if siRNA knockdown of BAG-1 altered the response of SKBR3 cells to trastuzumab. The concentration of trastuzumab used was derived from the dose response experiment reported in Chapter 3 (Figure 3.6A)

Western blotting and subsequent optical densitometric analysis confirmed transfection of 25nM BAG-1 siRNA over 48 hours resulted in adequate knockdown (Figure 4.3). 200,000 cells were seeded in T75 flasks and incubated for a further 48 hours with or without 50µM trastuzumab. The medium for untreated controls was replaced with fresh complete medium, which was assumed appropriate as the solvent used for trastuzumab was distilled water (see Chapter 2, Table 2.5). Lysates were produced from cells expressing endogenous BAG-1 alone with and without trastuzumab, and from cells with BAG-1 knocked down via siRNA with and without trastuzumab treatment. All lysates were run in parallel lanes on a single Western blot.

Probing for BAG-1 using the 3.10G3E2 anti-BAG-1 antibody showed increased BAG-1L expression in those cells exposed to trastuzumab without siRNA knockdown (mean increase of 34.9%) compared to untreated cells (Figure 4.3). Following siRNA knockdown, the BAG-1L levels were reduced to 60.3% of the siRNA negative control without trastuzumab. Exposure to trastuzumab with siRNA knockdown of BAG-1L expression was reduced to 37.2% of the negative siRNA control at 48 hours. Hence, these results showed BAG-1L levels were elevated in those treated with siRNA and trastuzumab compared to siRNA alone. BAG-1 isoform expression was seen to unexpectedly increase between 0 hours and 48 hours in untreated cells. Results were normalised to untreated control at 48 hours.

Nitrocellulose membranes were probed for signalling proteins up and downstream of BAG-1. HER2 expression was initially investigated in SKBR3 cells, previously reported to be HER2-positive (see Table 2.1). HER2 levels were not changed by either trastuzumab or siRNA knockdown of BAG-1. Trastuzumab is known to interrupt PI3K signalling (Nahta and Esteva, 2006). Akt (Protein kinase B) is a cytosolic protein kinase and is downstream of the PI3K pathway (Song et al., 2005). Activation of Akt can phosphorylate the proapoptotic protein BAD at serine 136 and indirectly interact with mTOR, which is integral to growth regulatory pathways. Dysregulation of the PI3K/ Akt/ mTOR pathway has been associated with the development of breast cancer (Lauring et al., 2013). Total Akt expression was not altered by siRNA knockdown of BAG-1 or treatment with trastuzumab. The activated form of Akt (phosphorylated at serine 473) was shown to be reduced by siRNA knockdown of BAG-1 and trastuzumab. Trastuzumab alone in the absence of BAG-1 knockdown showed a change in band pattern, suggesting a shift in alternative phosphorylation sites other than

serine 473. Total ERK1/2 was shown to be weakly elevated with siRNA knockdown and trastuzumab compared to untreated cells. There was no obvious difference in ERK1/2 expression with either of trastuzumab or siRNA alone. Unfortunately expression of activated ERK1/2 (phosphorylated ERK1/2) could not be obtained for this experiment, but later experiments using BT-474 cells did show a reduction in activated ERK1/2 with trastuzumab (Figure 4.7).

4.2.4 The effect of siRNA knockdown of BAG-1 expression on the metabolic activity of SKBR3 cells treated with 50μM trastuzumab.

Previous studies have implicated BAG-1 in the proliferation and survival of cancer cells as discussed in chapter 1. In this chapter siRNA was used as a tool to decrease levels of BAG-1 and analyse the effects in untreated HER2-positive cells and those subjected to HER2 inhibition with trastuzumab. MTS assays were performed to assess the effects of decreasing BAG-1 expression via siRNA knockdown on relative metabolic activity with and without trastuzumab.

Western blotting and subsequent optical densitometric analysis confirmed transfection of 25nM BAG-1 siRNA over 48 hours resulted in adequate knockdown. MTS assays were then performed on reseeded SKBR3 cells following siRNA knockdown of BAG-1 and on cells transfected with negative control in the absence of trastuzumab. MTS assays were carried out on out on cells proceeding siRNA BAG-1 knockdown or transfection of negative control treated with 50μM trastuzumab. Each 96-well plate contained untreated controls for cells with BAG-1 knocked down and negative controls. Each condition was performed in triplicate and data were derived from 3 independent experiments using the mean photoabsorbance of the triplicate samples (+/-SD).

MTS assays showed a decrease in cell viability with both siRNA depletion of BAG-1 and with trastuzumab. Hence no significant difference was seen in cell viability following siRNA reduction of BAG-1 or after treatment of HER2-positive cells with trastuzumab.

4.2.5 The effect of siRNA knockdown of BAG-1 expression on cell yield of SKBR3 cells treated with 50μM trastuzumab.

To attempt to correlate and support the findings of MTS assays (Figure 4.5) simple cell yield assays were performed with or without siRNA knockdown and trastuzumab. All data were normalised to the value derived for the mock transfection at zero hours in the absence of

trastuzumab. SKBR3 cells transfected with the control negative siRNA continued to show the cell yield was comparable with the mock control. There was no difference between SKBR3 cells transfected with BAG-1 siRNA and those transfected with negative siRNA in the presence of trastuzumab.

4.2.6 Crystal violet assays using BT-474 cells treated with 50μM trastuzumab, Thioflavin S, or both for 96hours.

The ability of a single cell to form colonies is one method of assessing tumour cell survival. Dilution of cell suspensions and identification of single cell colonies followed by manual counting of cell colonies over a time period was used in Chapter 3 to assess clonogenicity of cells overexpressing BAG-1 isoforms (Figure 3.4). To assess the effect of drugs over a time period and the size of colonies formed it is necessary to measure the colony diameter, count cells per colony, or measure the photoabsorbance of colonies stained with crystal violet (Figure 4.6). The crystal violet assay is a method for quantification of the relative density of cells adhering to multi-well plates and has been reported to be a rapid and reproducible method of assessing cell survival. Crystal violet stains the DNA within cells and the amount of dye absorbed by the monolayer is quantified by spectrophotometer. BT-474 cells were used as seeding with 2,000 to 10,000 SKBR3 cells in 96-well plates resulted in either few viable cells or an uninterpretable dense monolayer. The SKBR3 cells seeded in 96-well plates showed little difference in crystal violet staining between conditions, despite obvious differences observed when cells were seeded in 6-well plates (data not shown). The experiment was subsequently repeated by Dr Emmanouil Papdakis twice, in addition to performing corresponding Western blots using BT-474 cells treated with trastuzumab or thioflavin S (Figure 4.7). Dr Emmanouil Papadakis had derived the optimal concentration for thioflavin S treatment of BT-474 cells using MTS dose response experiments (data not shown).The combination of trastuzumab and the small molecule inhibitor, thioflavin S, showed an additive effect than either drug alone (Figure 4.6). CalcuSyn™ software was used to determine that the combination of the inhibitors resulted in synergistic rather than additive killing of BT-474 cells (Results not shown. Titration and further work demonstrating synergy have since been performed by Dr Emmanouil Papadakis, CRUK Centre, Southampton).

4.2.7 Effect of trastuzumab and thioflavin S treatment on HER2 pathway signalling molecules.

To indicate potential signalling mechanisms and support the hypothesis of an additive role in the function of BAG-1 in HER2-positive breast cancer cells further Western blots were probed for up and downstream proteins following HER2 inhibition. BT-474 (Figure 4.7A) and MCF-7 (Figure 4.7B) cells were treated with thioflavin S (50µM), trastuzumab (50µM), or a combination of the two, and the expression of downstream signalling molecules determined by Western blotting. Dose-response experiments to determine the IC50 for treatment of MCF-7 and BT-474 cells with thioflavin S were performed by Dr Emmanouil Papadakis (data not shown).

Expression of BAG-1L was increased in response to trastuzumab in HER2 positive BT-474 cells but not in HER2 negative ER positive MCF-7 cells. Expression of other BAG-1 isoforms was not significantly upregulated on exposure to trastuzumab during this experiment. Treatment with thioflavin S alone increased BAG-1L expression moderately in BT-474 cells and BAG-1S expression in MCF-7 cells. An increase in BAG-1S expression was observed following treatment with a combination of trastuzumab and thioflavin S. Expression of HER2 was independently inhibited by trastuzumab and thioflavin S in BT-474 cells and further reduced in combination. This is inconsistent with the unchanged expression of HER2 following trastuzumab treatment of SKBR3 cells (Figure 4.3), Downstream Raf-1 expression correlated with the expression pattern observed for HER2; levels decreasing following independent treatment with trastuzumab or thioflavin S, with an enhanced inhibitory effect seen in combination.

The protein kinase Akt plays a role in cell survival and apoptosis via PI3 kinase signalling pathways (Franke et al., 1995). There was an increase in total Akt expression with trastuzumab and thioflavin S, alone and in combination. Activation of Akt is dependent on phosphorylation at the Thr308 site by PDK1 and phosphorylation of the carboxy terminus at Ser473 (Bayascas and Alessi, 2005). Phospho-Akt (Thr308) expression was seen to be unchanged with trastuzumab treatment in BT-474 cells relative to β-actin (phospho-Akt Thr308 blots were performed by Dr Emmanouil Papdakis). No increase was seen in expression of phospho-Akt (Ser473) with trastuzumab. Expression of phospho-Akt (Ser473) was diminished compared to untreated control with the addition of thioflavin S to trastuzumab in BT-474 but not MCF-7 cells.

BAD is a member of the Bcl-2 family that promotes cell death by displacing Bax from binding to Bcl-2 and Bcl-x (Schorr et al., 1999). BAD is phosphorylated by Akt at serine 136 to promote cell survival (Datta et al., 1997). Phospho-BAD (ser136) expression levels were unaltered by exposure to trastuzumab or thioflavin S alone, but reduced by combination therapy, which may indicate reduced anti-apoptotic signalling (Western blots for phospho-BAD performed by Dr Emmanouil Papadakis).

Expression of the MAPK signalling proteins ERK1/2 showed no change in levels after trastuzumab or with a combination of trastuzumab and thioflavin S. Phopsho-ERK1/2 expression showed an increase with thioflavin S in BT-474 cells, but no change in expression in combination with trastuzumab. No obvious changes were seen in phosphor-ERK1/2 expression with thioflavin S in MCF-7 cells, although appeared to decrease with trastuzumab and in combination with thioflavin S. Enhanced phospho-ERK1/2 expression following thioflavin S might be expected if Hsp/Hsc70 binding were compromised by thioflavin S and may result in an increase in BAG-1 and Raf-1 activity. The abrogation of this increase in phospho-ERK1/2 expression by the combination of trastuzumab and thioflavin S may indicate upstream signalling is important for BAG-1 to facilitate Raf-1 and ERK1/2 interaction.

4.3 Discussion

Use of siRNA is not without challenges. The RNAi pathway is complex and has many junctions with other signalling pathways, thus is capable of producing non-specific effects. Incomplete complementary genes may still produce a response to siRNA and result in off-target effects. This has been partially addressed in this study by the use of a negative control siRNA, in addition to mock transfection controls.

In vivo, epithelial cells may mistake the double-stranded siRNA for viral material and attempt to mount an immune response (Robbins et al., 2008; Robbins et al., 2009). The risk of an immune reaction is elevated if larger amounts of siRNA are used, thus has been combated by the introduction of microRNA, which can achieve similar gene knockdown at smaller concentrations. The optimal concentration for the BAG-1 siRNA in this in vitro study was high at 25nM. Many published protocols use as little as 1nM siRNA, therefore the chances of off-target effects may be considerable in this study and translation of results into in vivo models may be limited.

There is strong evidence in literature for the role of BAG-1 in breast cancer cell survival (Townsend et al., 2003), therefore reduction in BAG-1 expression via siRNA-based combination therapy could improve therapeutic response. BAG-1 isoforms are derived from a single strand of mRNA, therefore siRNA is not isoform-specific. This chapter could therefore not attempt to add to characterisation of BAG-1 isoforms, but investigate the effects of generic BAG-1 knockdown in SKBR3 and BT-474 breast cancer cells.

4.3.1 siRNA knockdown of BAG-1 in HER2-positive SKBR3 cells causes a decrease in relative cell viability.

This study has demonstrated reduction in BAG-1 expression via siRNA knockdown in HER2 positive cells results in a reduction in relative metabolic activity measured by MTS assay (Figure 4.6). Levels of metabolic activity measured by MTS were slightly higher in cells with BAG-1 knocked down but not exposed to trastuzumab. However, there was no obvious difference between the relative metabolic activity of cells with BAG-1 knocked down and trastuzumab and those treated with trastuzumab alone. A number of studies have proposed a role for BAG-1 in proliferation of breast cancer cells (Kudoh et al., 2002; Townsend et al., 2004; Sharp et al., 2009). However, on review of these studies, MTS assays, or similar, have been performed in most in isolation, or with correlating immunoblots, or only following

cellular stress. An MTS assay, or MTT assay, provides a relative measure of metabolic activity in viable cells, but does not quantify cell number or amount of cellular proliferation.

4.3.2 Targeting of BAG-1 using siRNA reduces cell yield in SKBR3 cells and inhibition of BAG-1 with thioflavin S potentiates response to trastuzumab in BT-474 breast cancer cells.

No previous studies have shown convincingly BAG-1 expression results in increased proliferation in breast cancer cells, although many have implicated BAG-1 in this role (Table 1.3). This study begins to address the effects of BAG-1 on cell yield and viability in HER2-positive SKBR3 and BT-474 breast cancer cells. Western blotting confirmed an optimal concentration of 25nM siRNA targeting BAG-1 produces adequate reduction in BAG-1 expression at over 48 hours on Western blotting (Figure 4.2).

The aim of this chapter was to add further evidence to the hypothesis suggested in chapter 3 that overexpression of BAG-1 protects HER2-positive breast cancer cells and results in a relative increase in cell yield and viability (Figures 3.5 and 3.7). Targeting of BAG-1 by siRNA allowed repeat biological assays to assess if reduction in BAG-1 sensitises SKBR3 cells to trastuzumab. To report upon how siRNA knockdown BAG-1 affects cell yield and viability this study used a simple cell counting method (Figure 4.5), in addition to MTS assays (Figure 4.4).

The increase in BAG-1L expression observed in chapter 3 with trastuzumab was evident again, but was abrogated by the addition of BAG-1 siRNA (Figure 4.3). SKBR3 cells showed decreased relative metabolic activity with exposure to BAG-1 siRNA and trastuzumab alone and with a combination. There was evidence of additive reduction in cell viability with the combination of trastuzumab and thioflavin S, compared to untreated BT-474 cells (Figure 4.6). Parallel cell yield assays showed similar reduction with siRNA knockdown of BAG-1 in untreated cells, yet no additional effect of siRNA knockdown of BAG-1 in cells treated with trastuzumab (Figure 4.5). An extension of the duration of the experiment may have produced more convincing results, although beyond 48 hours inadequate cells could be harvested for Western blotting, rendering it impossible to confirm expression of BAG-1 in cells used for these assays. Cell death appeared to be most marked in cells treated with trastuzumab and siRNA, as the cell cultures showed a high number of floating, distorted cells and cultures were less confluent at time points attempted beyond 48 hours. This suggests that mediation by BAG-1 of apoptotic pathways may be more dominant than those regulating cell growth in these experiments. Cell death by apoptosis was not investigated during this experiment, but

could be investigated using caspase activity assays or FACS analysis of Annexin V staining (discussed in Chapter 3). Western blots could also include downstream signalling proteins such as BimEL and Mcl-1. The pro-apoptotic protein Bim has been shown to be phosphorylated by ERK1/2 causing dissociation from Mcl-1 and an increase in BimEL degradation (Ewings et al., 2007). Knockdown of MAPK1 (ERK2) has been shown to reverse trastuzumab resistance in SKBR3 cells (Boyer et al., 2013). This study hypothesises the mechanism of action of BAG-1 sensitisation to trastuzumab may be via potentiation of ERK1/2 signalling, therefore assessing the expression of functional proteins downstream may prove helpful.

4.3.3 Thioflavin S in combination with trastuzumab shows an additive reduction in relative cell viability compared to either drug alone.

Crystal violet assays using BT-474 cells showed a reduction in viable cells with both trastuzumab and thioflavin S alone (Figure 4.6). In combination, a greater decrease in cell viability was seen compared to untreated cells suggesting an additive between the two drugs. The experiment was repeated twice using triplicate samples and a similar effect was seen. An accurate calculation of drug synergy was not possible for this experiment as only one concentration of each drug was used. To calculate true synergy using a programme such as CalcuSyn™ a dose-response titration must be performed for each drug and in combination (This calculation has been performed by Dr Emmanouil Papadakis using 3 independent experiments and has confirmed synergy between trastuzumab and thioflavin S. Data not shown).

4.3.4 Altered expression of Akt, phospho-Akt and phospho-ERK following inhibition of BAG-1: Hsc /Hsp70 interaction by thioflavin S and, or trastuzumab suggests potential signalling pathways involved in sensitisation of BT-474 cells to trastuzumab.

The PI3K pathway has a well-established role in breast cancer and many drugs used in breast cancer target this signalling pathway(Baselga, 2011). Suggestions of crosstalk between BAG-1 and HER2 signalling is provided by the use of the small molecule inhibitor, thioflavin S (NSC1948) identified by Sharp *et al.* (2009). Western blotting of HER2-positive BT-474 cells following exposure to trastuzumab and thioflavin S, alone and in combination, showed a reduction in phospho-Akt expression (Figure 4.7). Total Akt expression was also

increased in BT-474 cells exposed to thioflavin S alone and in combination with trastuzumab. This suggests the sensitisation of HER-2-positive cells by BAG-1 may occur via the PI3K pathway. However, further cell yield assays using thioflavin S in a number of HER2-positive cell lines are required to qualify this hypothesis.

Bands are cut due to differences in the loading order between blots. This is not ideal as reduces ability to accurately compare bands. However, all bands shown are representative of results from 4 independent experiments. This study would have also benefitted from further cell yield and viability assays incorporating siRNA knockdown of BAG-1 in HER2-negative MCF-7 cells. This would have provided a control to confirm that the possible potentiation of the response to trastuzumab of SKBR3 cells by BAG-1 involves mediation of HER2 activity.

The diminished HER2 signalling and correlating Raf-1 expression demonstrated following treatment of BT-474 cells with trastuzumab is strengthened further by the addition of thioflavin S. Thioflavin S (NSC71948) was identified as a small-molecule chemical inhibitor of binding between the C-terminus of the BAG domain and Raf-1. In addition, thioflavin S interferes with BAG-1-mediated protein-protein interactions between Hsc70/Hsp70 known to be functionally important in BAG-1-regulated survival pathways (Sharp et al., 2009). The additive effect of thioflavin S and trastuzumab suggests a role for BAG-1 in the mediating the response of HER2-positive cells to anti-HER2 therapy. Reduction of by MAPK1 (ERK2) by siRNA knockdown has been shown to reverse resistance to trastuzumab in SKBR3 cells (Boyer et al., 2013). The results in this chapter show a decrease in ERK1/2 with thioflavin S alone and in combination with trastuzumab in BT-474 cells. There was an increase in phospho-ERK 1/2 with thioflavin S alone, suggesting ERK1/2 activation is mediated by BAG-1. The results from Western blotting (Figure 4.7) and the crystal violet viability assay (Figure 4.6) suggest BAG-1 siRNA knockdown may alleviate BAG-1 potentiation of ERK1/2 signalling and sensitise BT-474 cells to trastuzumab. However, this study cannot prove that the results seen are due to thioflavin S alone, and not due to ‘off-target’ effects of the compound. The study would have benefited from repeat cell yield assays using SKBR3 cells transfected with BAG-1 Hsc/Hsp70 binding mutants. This would help determine if it is the interaction between BAG-1 and Hsc/Hsp70 which mediates the response of HER2-positive breast cancer cells to trastuzumab. BAG-1 mutants were not used in these experiments, but cell viability assays using crystal violet staining were performed using a small molecule compound, Thioflavin S, known to disrupt the interaction between BAG-1 and Hsc/Hsp70 (Figure 4.6)(Sharp et al., 2009a) .

Akt phosphorylates BAD at Ser136 as part of one of many cell signalling pathways enhancing cell survival (Datta et al., 1997). Our experiments showed expression of phospho-BAD reduced on exposure to trastuzumab and thioflavin S, but not with either drug alone (Figure 4.7). This suggests BAD-mediated prosurvival signalling is receptive to inhibition of Bag-1 binding Raf-1 but in the presence of concomitant disruption of HER2 signalling by trastuzumab. Future experiments using combination therapy should demonstrate a corresponding increase in total BAD expression to confirm reduction in antiapoptotic signalling and phospho-BAD. Future work will include cell yield and viability assays using thioflavin S in a panel of HER2-positive and negative cell lines. In addition, markers of apoptosis should be investigated in HER2-positive cells treated with thioflavin S, alone and in combination with trastuzumab. This could be done by using caspase activity assays or FACS analysis of Annexin V.

Use of siRNA targeting of BAG-1 in these HER2-positive cell lines and subsequent treatment with trastuzumab are preliminary steps in dissecting apart these signalling pathways to identify potential targets, or if BAG-1 may itself act as a biomarker in HER2-targeted therapy. This chapter has not shown BAG-1 siRNA convincingly alters the response of SKBR3 cells to trastuzumab. However, the combination of the BAG-1 inhibitor thioflavin S and trastuzumab in BT-474 cells shows an additive reduction in cell viability and altered expression of PI3K/ MAPK pathway proteins. Future work involves further development of compounds to disrupt the BAG-1/HSC70 interaction in vivo and concurrent in vitro experiments to define response to anti-HER2 drugs in numerous HER2-positive breast cancer cell lines. Future studies should determine the bioavailability and penetration of the plasma membrane of breast tumour cells by thioflavin S. This would help ascertain to whether the BAG-1/Hsp/Hsc70 interaction is an effective target to facilitate cell death in response to trastuzumab.

Figure 4.1 Expression and subcellular localisation of endogenous BAG-1 in breast cancer cell lines.
SKBR3, BT-474 and MCF-7 cells were harvested at 70% confluency and used for consecutive immunofluorescence study and Western blots of endogenous BAG-1 expression. BAG-1 was detected by using 3.10G3E2 anti-BAG-1 antibody (1:1000) and Alexa-Fluor® 488 rabbit-mouse secondary (Cell Signalling). Nuclei were stained using VECTASHIELD®Hardset mounting media with DAPI (Vector Laboratories). Nitrocellulose membranes were probed for BAG-1 (3.10G3E2) and β-actin was used as a loading control.

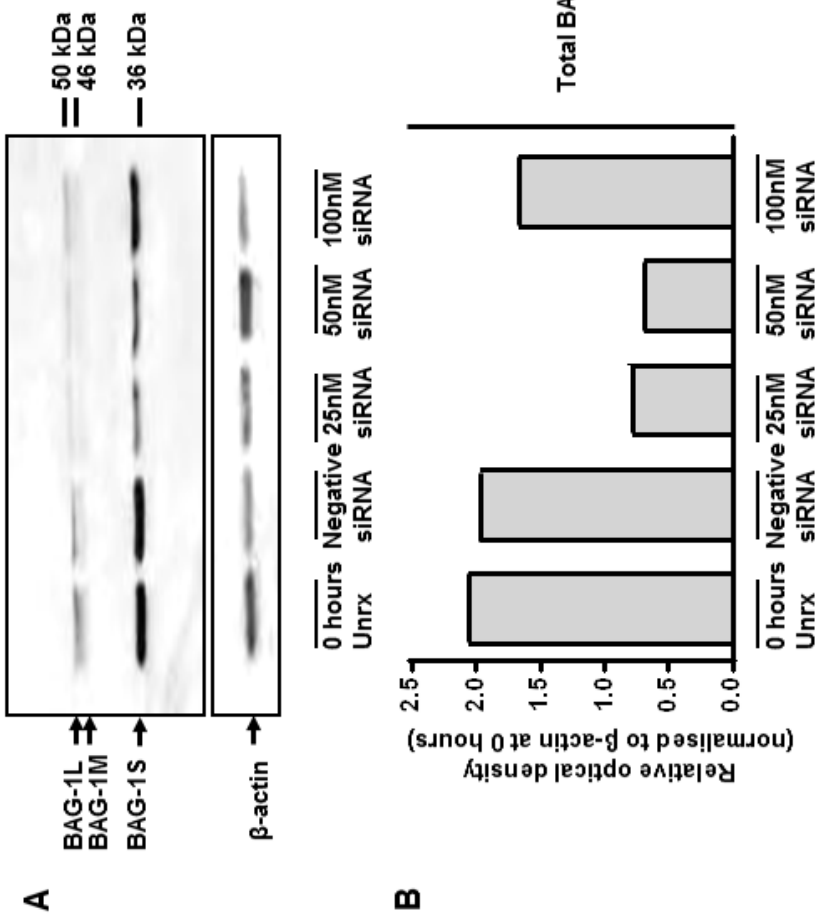

Figure 4.2 Optimisation of siRNA knockdown of BAG-1 using siRNA concentrations of 25nM-100nM.

SKBR3 cells were seeded and grown in DMEM under standard conditions until 70% confluent. Cells were incubated in Opti-MEM (Invitrogen) and exposed to siRNA oligonucleotide and Dharmafect (Dharmacon) reagent at siRNA concentrations of 25, 50 and 100nM for 48 hours. Controls for this experiment were the Negative siRNA (Ambion) and untreated SKBR3 cells (mock- no siRNA). Cells were harvested at 48 hours, lysed with RIPA buffer and the down-regulation of BAG-1 determined by Western blot (A). Data were derived from the means of 2 independent experiments using standard densitometric analysis (B).

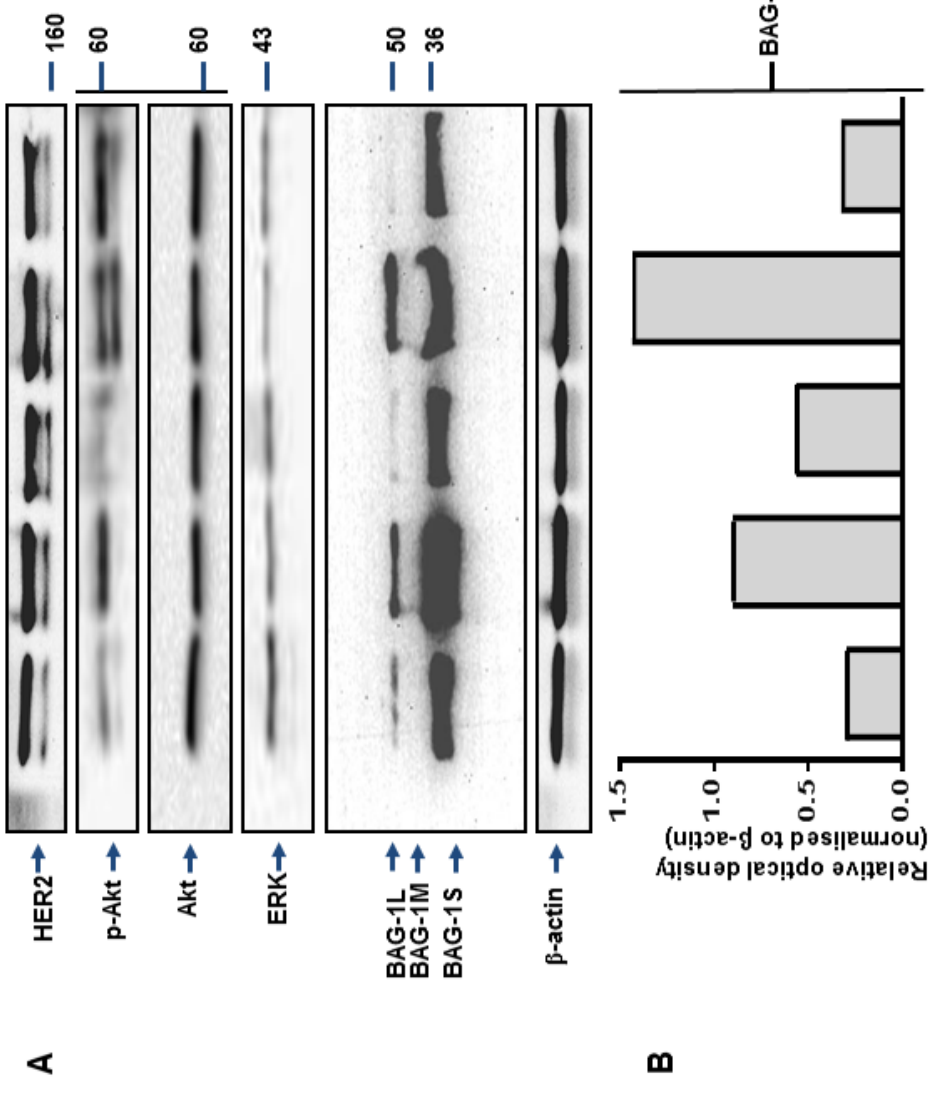

Figure 4.3 Western blot showing BAG-1 expression in SKBR3 cells following siRNA knockdown of BAG-1 and treatment with trastuzumab (50μM).

200,000 SKBR3 cells were seeded in T75 flasks and grown in DMEM under standard conditions. The cells were then incubated in Opti-MEM (Invitrogen) and exposed to 25nMsiRNA oligonucleotide (Ambion) and Dharmafect™(Dharmacon) reagent for 48 hours. Controls for this experiment were the negative siRNA (siGENOME ™Dharmacon) and untreated SKBR3 cells. Cells were harvested at 48 hours, lysed with RIPA buffer and the down-regulation of BAG-1 determined by Western blot (A). Data were derived from 2 independent experiments (B).

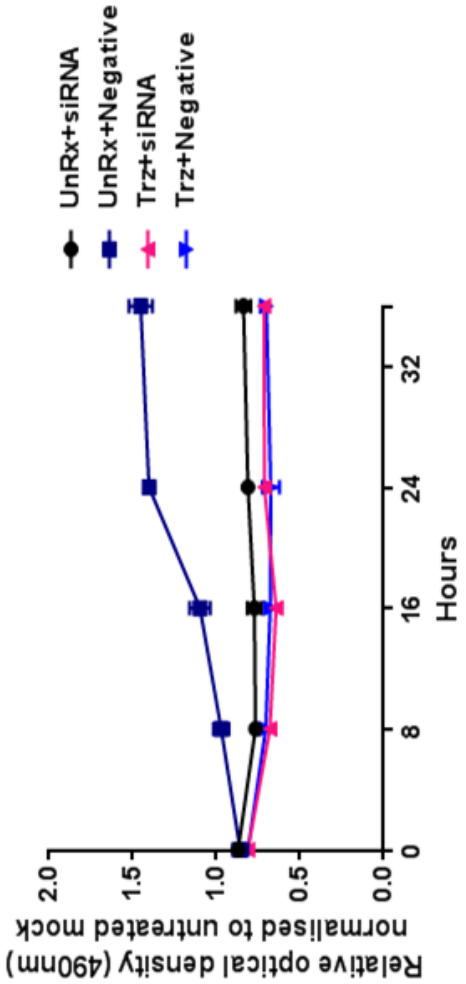

Figure 4.4 The effect siRNA knockdown of BAG-1 expression on the viability or relative metabolic activity of SKBR3 cells treated with 50µM trastuzumab.

SKBR3 cells were seeded and grown in DMEM under standard conditions until 70% confluent. The cells were then incubated in Opti-MEM (Invitrogen) and exposed to siRNA oligonucleotide (Ambion) and Dharmafect™ (Dharmacon) reagent at an siRNA concentration of 25nM for 48 hours according to Dharmacon protocol. Cells were observed at regular intervals for toxicity and an MTS assay performed on a sample to ensure >80% cell viability. Western blotting was performed on a sample 200,000 cells to confirm siRNA knockdown of BAG-1. Cells were then trypsinised and re-seeded in 96-well plates in triplicate. Following 24 hours of incubation, cells were treated with 50 µM Trastuzumab or fresh standard culture medium alone. Plates were read at 0, 8, 16, 24 and 36 hours following a 90 minute incubation with CellTiter 96® Aqueous One Solution reagent. Data were derived from three independent experiments using the mean photoabsorbance (490nm) of the triplicate samples (+/-SD).

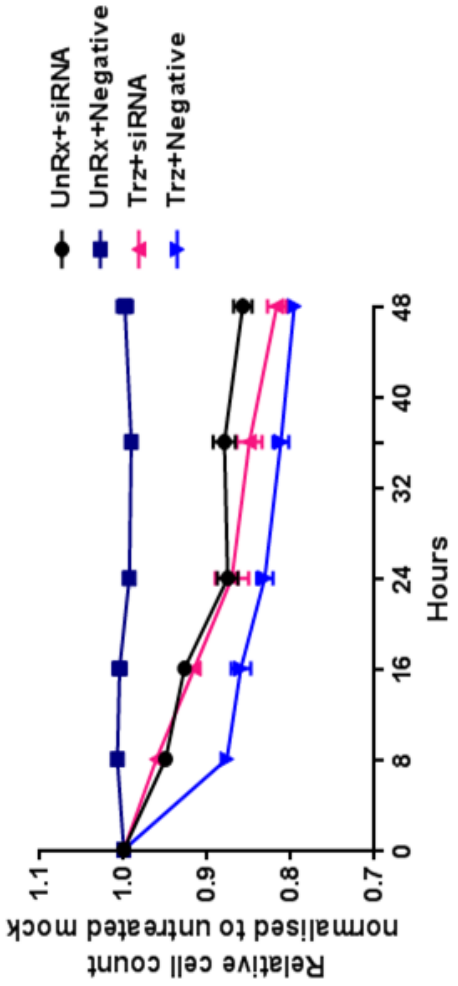

Figure 4.5 The effect of siRNA knockdown of BAG-1 expression on cell yield of SKBR3 cells treated with 50µM Trastuzumab.

SKBR3 cells were seeded and grown in DMEM under standard conditions until 70% confluent. The cells were then incubated in Opti-MEM (Invitrogen) and exposed to siRNA oligonucleotide and Dharmafect™ (Dharmacon) reagent at an siRNA concentration of 25nM for 48 hours according to Dharmacon protocol. Cells were observed at regular intervals for toxicity and an MTS assay performed on a sample to ensure >80% cell viability. Western blotting was performed on a sample 200,000 cells to confirm siRNA knockdown of BAG-1. Cells were then trypsinised and re-seeded in 96-well plates in triplicate. Following 24 hours of incubation, cells were treated with 50 µM Trastuzumab or fresh standard culture medium alone. Cells were counted at 0, 8, 16, 24, 36 and 48 hours from treatment. Data were derived from the mean of triplicate samples (+/-SD).

Figure 4.6 Crystal violet assays using BT-474 cells treated with 50µM trastuzumab, thioflavin S, or both for 96hours.

7,000 BT-474 cells were seeded in 96 well plates. Plates were incubated overnight at 37°C and 5% CO_2 until approximately 70% confluent. Cells were washed with PBS and fixed in 4% PFA-PBS. Cells were then stained with 70µL per well of 0.1% crystal violet for 30 minutes at RT. 100µL 50% acetic acid in PBS was then added to each well and left for 30 minutes at RT to solubilise the crystal violet stain. The plate was agitated every 10 minutes for 30 seconds to dislodge detached from fixed cells. Additional agitation at 30 minutes was required until to ensure uniform appearance with no dense areas of colouration. Changes in photoabsorbance were read using a Varioskan™ Flash plate reader (570nm) Results were calculated as a percentage of the photoabsorbance recorded for untreated cells. All wells were in triplicate and results derived from 3 independent experiments (+/- SD).

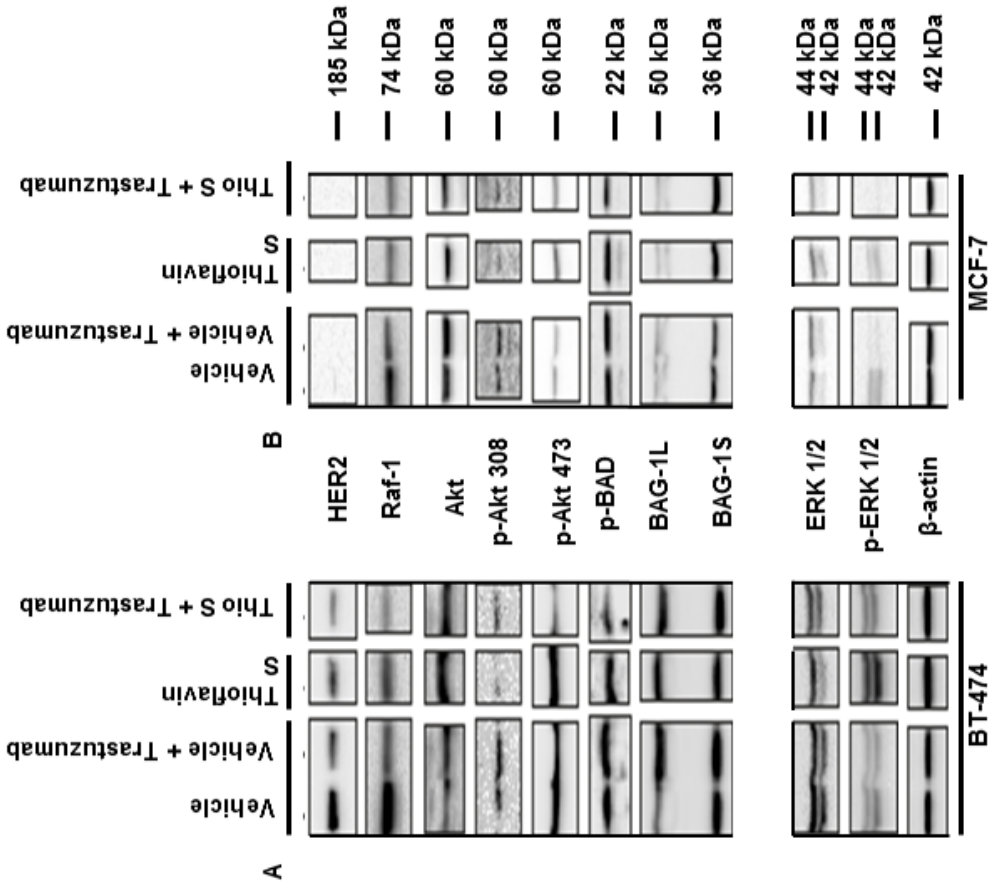

Figure 4.7 Effect of trastuzumab and thioflavin S drug treatment on HER2 pathway signalling molecules.

BT-474 (A) and MCF-7 (B) cells were treated with thioflavin (thio) S (50μM), trastuzumab (50μM), or a combination of the two and the expression of downstream signalling molecules determined by Western blotting. Boxes show non-consecutive lanes from the same Western blot. Data is representative of 4 independent experiments performed by author although some of the blots above are kindly provided by Dr Emmanouil Papadakis.

Chapter 5: BAG-1 expression in breast, bladder, and oesophageal cancer

Chapter 5: BAG-1 in breast, oesophageal and bladder adenocarcinoma.

The initial aim of this chapter was to provide immunohistochemical evidence for the use of BAG-1 as a biomarker in HER2-positive breast cancer. The aim was to validate the pan-isoform anti-BAG-1 antibody 3.10G3E2 using a pre-validated large cohort of samples taken from patients diagnosed with ER-positive breast cancer. A further aim was to then test a novel BAG-1L isoform-specific antibody using the standardised patient cohort and compare results to those using the 3.10G3E2 BAG-1 antibody. The intention was to then analyse data for correlation of clinicopathological parameters with HER2 status. A future aim was to then use results to design further immunohistochemical investigation of a treatment-standardised cohort of HER2-positive breast cancers currently being collected at Southampton General Hospital for use by the Cancer Sciences Unit. The chapter describes steps taken to optimise both antibodies for use with breast cancer whole sections and tissue microarray. The pan-isoform BAG-1 antibody required further optimisation prior to use on the large breast cancer cohort, therefore alternative cancers known to overexpress HER2 were investigated. The chapter is divided into 3 sections according to the cancer studied and results presented and discussed within each section.

5.1. BAG-1 in breast cancer

5.1.1 Introduction

Identification of predictive biomarkers in cancer provides another dimension to the tailoring of therapy to each patient. The inclusion of BAG-1 in the commercial tumour array, Oncotype Dx™, and the high-throughput assay, PAM50, provides substantial support to the role of BAG-1 in the development and progression of breast cancer (Kelly et al., 2012).

The existing immunohistochemical biomarker studies of BAG-1 in breast cancer were analysed using the REMARK criteria in Chapter 1 and correlation with clinical parameters described. In summary, increased BAG-1 expression correlated with favourable survival outcomes and a trend towards improved prognosis (Cutress et al., 2003, Millar et al., 2009, Nadler et al., 2008, O'Driscoll et al., 2003). There is mixed evidence to support the role of both nuclear and cytoplasmic BAG-1 and previous studies fail to meet many of the REMARK criteria. However, high nuclear BAG-1 immunoreactivity in ER+ positive cancers treated with hormonal therapy is an independent predictor of survival. In addition, predictive

immunohistochemical analysis of ER, PgR, HER2 and Ki67 (IHC4) was improved by measurement of nuclear BAG-1 expression (Afentakis et al., 2013).

BAG-1 expression has been studied archival tissue blocks from the Tamoxifen and Anastrazole arm of the Arimidex or Tamoxifen, Alone or in Combination (ATAC) Trial (Howell et al., 2005). BAG-1 expression has been shown to positively correlate with expression of hormone receptors ER and PR, in patients with hormone receptor-positive localised breast cancer receiving hormonal therapy (Afentakis et al., 2013) . BAG-1 was shown to negatively correlate with HER2 expression and tumour grade, although the HER2-positive cohort was relatively small (103 of the 963 patients). BAG-1 expression has previously been reported to positively correlate with HER2 RNA expression in node-negative breast cancer, although the significance of the relationship was weak (p=0.048)(Petry et al., 2010). The contrasting results may be explained by differences between BAG-1 protein and RNA expression, or by ER positivity of the patient cohort used by the Afentakis et al. (2013) study compared with the Petry et al. (2010) study (Afentakis et al., 2013; Petry et al., 2010). Therefore, there is a current lack of strong evidence to support a relationship between BAG-1 and HER2 expression. This chapter uses a previously described ER-positive cohort to optimise the protocol for immunohistochemical study of BAG-1 using tissue microarray. The chapter also attempts to correlate HER2 and BAG-1 expression in other cancers known to overexpress HER2. The Afentakis et al. (2013) study also showed strong correlation between nuclear and cytoplasmic BAG-1 expression in breast cancer, suggesting there is no additional predictive value gained from analysing the expression of both. Afentakis et al. (2013) hypothesise that a BAG-1L-specific antibody may offer superior prognostic information. One of the aims of this chapter was to optimise the protocol for immunohistochemical study of BAG-1L expression using TMA and a novel BAG-1L-specific antibody developed in-house, 3C12.

Tissue Microarrays have allowed tissue traditional analysis of standard paraffin blocks to become high-throughput, whilst using a minimal amount of tissue. Tissue microarrays may be used for immunohistochemistry, in situ hybridisation, fluorescence in situ hybridisation (FISH), in situ PCR, RNA, DNA or protein expression analysis, TUNEL assay for apoptosis, and morphological characterisation of tissue. The first multi-tumour blocks were constructed in 1986 by H. Battifora, and the first tumour microarray produced by Kononen at al. in 1998 (Battifora, 1986; Kononen et al., 1998). Tissue microarrays are relatively cost effective as use less reagent and are time efficient. They allow for increased reproducibility and decrease variables between samples as many samples may be processed at once, under exact conditions, in the same run. A limited amount of suitable tissue from large patient cohorts and a call for standardisation of immunohistochemical detection of biomarkers has

led to a surge in TMA studies. Automated analysis systems such as AQUA™ eradicate any bias introduced by human scorers, although these systems are still in their infancy (McCabe et al., 2005).

Only a few previous studies have utilised tissue microarrays in attempts to validate BAG-1 as a biomarker, but have included heterogeneous patient cohorts and have omitted information regarding standardisation of techniques, or pre-validation steps (Chapter 1)(Nadler et al., 2008; Millar et al., 2009). BAG-1 warrants further investigation in large controlled patient cohorts using TMA. This chapter attempts to reinvestigate the expression of BAG-1 in breast cancer and determine an optimal method for BAG-1 immunohistochemical studies incorporating tumour microarrays using archival tissue from the Tamoxifen Exemestane Adjuvant Multinational (TEAM) trial (van de Velde et al., 2011). The TEAM Trial was a phase III clinical trial involving post-menopausal women from nine countries with hormone receptor-positive breast cancer. The trial demonstrated the aromatase inhibitor Exemestane to be a valid alternative to Tamoxifen or as adjunct after 2-3 years of Tamoxifen. The chapter also addresses some of the limitations of previous biomarker studies and adheres to the criteria outlined by the REMARK study.

5.1.2 Results

5.1.2.1 Whole and tissue microarray sections following antigen retrieval with microwave and standard bath compared with pressure cooker treatment.

To determine the optimal antigen retrieval technique repeat experiments were performed using a variety of methods (Figure 5.1). Initial experiments were conducted using a citrate buffer and microwave (Sharp R272 20L) using the medium power setting (600W) for heat-induced antigen retrieval. There was obvious peripheral damage to some of the paraffin sections, and occasionally a whole section would dislodge after removal from the buffer. This was especially apparent and significantly destructive when using tissue microarrays, as the <1mm cores could be easily damaged and there was no excess tissue to allow for edge effect.

Working under the supervision of Professor John Bartlett in Edinburgh allowed trial of the pressure cooker method of antigen retrieval. The cores were preserved in the vast majority of experiments and there was little evidence of edge effect (Figure 5.1). The pressure cooker

allowed a more even temperature throughout the buffer and less agitation of sections. It was noted that frequently on opening the baths used in the microwave alone method there was bubbling which agitated the tissue. The controlled release of pressure from the pressure cooker seemed to avoid this. The antigen retrieval method of choice for TMA sections was therefore the pressure cooker technique and was adopted for all subsequent IHC using TMA.

5.1.2.2 Choice of blocking reagent for immunohistochemical staining for BAG-1 (3.10G3E2) of TMA sections.

To determine the optimal blocking agents several alternative agents were used in addition to standard 10% foetal calf serum (FCS/FBS) (Figure 5.2). The results using both goat serum and 1% casein did not reveal any improvement on the level of background seen with 10% FCS. Use of the protein-free block (DAKO™) did not significantly improve the clarity of immunoreactivity and some edge effect was seen (Fig 5.2 C). Therefore, 10% FCS was used for the majority of both TMA and whole sections.

5.1.2.3 Titration of BAG-1 antibody using whole sections following blocking with foetal calf serum.

To determine the optimal concentration of 3.10G3E2 antibody consecutive whole sections from one tumour were immunostained with concentrations of 1:600, 1:800 and 1:1000 (diluted with TBS-Tween 0.1%) were used (Figure 5.3). Antigen retrieval using citrate buffer and microwave showed optimal positive immunoreactivity at a 1:1000 (TBS-Tween 0.1%) concentration of anti-BAG-1 antibody, 3.10G3E2.

5.1.2.4 Titration of BAG-1 antibody using TMA sections following blocking with foetal calf serum.

IHC can vary between whole sections and TMA sections for the same tumours as previously discussed, thus the titration to find he optimal concentration of 3.10G3E2 using whole sections was repeated using TMA sections (Figure 5.4)**.** Citrate buffer and pressure cooker antigen retrieval demonstrated clear positive immunoreactivity using a concentration of 1:1000 anti-BAG-1antibody (3.10G3E2). Non-specific stromal background was evident at concentrations greater than this.

5.1.2.5 Titration of BAG-1 antibody using TMA sections following blocking with normal horse serum (1%).

To attempt to improve upon the results seen using 10% FCS and test the results of the previous BAG-1 antibody titration a further titration using horse serum was trialled (Figure 5.5). The optimal concentration of anti-BAG-1 antibody was 1:1000 (TBS-Tween 0.1%). Antigen retrieval was performed using citrate buffer and pressure cooker for TMA sections. 1:600 showed an increased amount of background, whilst more dilute concentrations showed weak immunoreactivity. No significant improvement was seen with horse serum compared to 10% FCS.

5.1.2.6 Optimal length of incubation time with primary antibody for TMA sections.

The incubation time with primary antibody (3.10G3E2) was varied to identify the optimal period of exposure of both whole and TMA sections to 3.10G3E2 (Figure 5.6). Over 12 hours incubation time resulted in an increased amount of non-specific background, All paraffin sections were incubated with anti-BAG-1 antibody (3.10G3E2) for 12 hours overnight at 4° C.

5.1.2.7 The effect of the addition of Tween (0.1%) into TBS for washes following blocking with animal sera.

Tween diluted with TBS (0.1%) provided cleaner positive immunoreactivity and controls (Figure 5.7). TBS-Tween (0.1%) was stirred immediately prior to use and 200µL uniformly added to each slide using a pipette (Figure 5.7B). 200µL of the solution allowed complete coverage of the section and was derived from standardised protocols developed by Mr Ron Lee in the Histopathology Research Unit at Southampton General Hospital.

5.1.2.8 Section of tissue microarray (section kindly provided by Professor John Bartlett, CRUK Centre, Edinburgh).

Test sections from the TEAM trial tissue microarray were processed for expression of BAG-1 (Figure 5.8). Sections were cut from blocks a maximum of 12 hours prior to processing and kept at 4° C. A citrate buffer and pressure cooker were used for antigen retrieval. The

antibody 3.10G3E2 was used at 1:1000 concentration with TBS-Tween (0.1%). Sections were developed using the DAB chromogen and substrate (Vector Labs™) as described in Chapter 2 (2.14.1.4). A clear gradient of both nuclear and cytoplasmic BAG-1 was observed, in addition to sections negative for BAG-1. The results were assessed by Professor John Bartlett who considered the gradient of BAG-1 clear, but the level of non-specific stromal background in some of the TMA cores suboptimal. At lower concentrations the BAG-1immunoreactivity was too weak to assess and higher concentrations of anti-BAG-1 antibody resulted in increased background. Digital analysis of IHC sections can be used to only analyse tumour cells and not the surrounding stroma (Rizzardi et al., 2012) . Use of such software could be a method of analysing BAG-1 levels in tissue sections with stromal background.

5.1.2.9 Construction of 'Virtual tissue microarray' constructed from whole sections of archival breast carcinoma.

'Virtual tissue microarrays' can be digitally constructed to analyse tumour heterogeneity and ascertain the number of cores required to accurately represent the whole tumour. Paraffin-embedded whole sections of breast cancer corresponding to the test cohort from the TEAM Trial were stained using identical protocol to tissue microarray section using a BAG-1(3.10G3E2) antibody dilution of 1:1000 TBS-Tween (0.1%). 7 whole sections from each of 18 tissue blocks were captured overnight using Ariol Imaging System™ and a 'virtual TMA' constructed from raw images using Ariol™ software for each tumour (Figure 5.9). Previous studies have recognised to compensate for tumour heterogeneity, 3-10 cores should be used for each tumour incorporated into a TMA (Eckel-Passow et al., 2010). The number of cores for use in the TMA should be derived from a test cohort of whole sections as can vary from one biomarker to the next. Construction of the 'virtual TMA' also allowed for densitometric analysis resulting in a gradient of immunoreactivity and grouping into low, moderate or high BAG-1 immunoreactivity. The analysis revealed a higher level of background immunoreactivity than desirable for use on the TEAM cohort TMA, and poorly reproducible results. Therefore, further work, possibly involving use of digital analysis to extract stromal background, was deemed necessary on test sections.

5.1.2.10 Immunodepletion using parallel whole sections from a positive control tumour block and pan-isoform anti-BAG-1 antibody 3.10G3E2.

An immunodepletion experiment was performed to assess the specificity of the pan-isoform BAG-1 antibody 3.10G3E2 (Figure 5.10). Paraffin-embedded whole sections of breast tumours were treated with the anti-BAG-1 antibody (3.10G3E2) alone, 3.10G3E2, 3.10G3E2 bound to GST passed through GSH beads, and 3.10G3E2, GST and GSH beads, and TBS-Tween (0.1%). There was positive BAG-1 immunoreactvity as expected with the 1:800 dilution of 3.10G3E2 antibody alone and with sections treated with G310E2, unbound GST, and GSH beads. Sections incubated with 3.10G3E2, 3.10G3E2–GST, and processed using GSH beads showed no BAG-1 immunoreactivity, confirming successful Immunodepletion and hence specificity of the anti-BAG-1 antibody.

5.1.3 The expression of BAG-1L in breast cancer whole sections using a novel isoform-specific antibody, 3C12.

5.1.3.1 The development of a BAG-1L isoform-specific antibody.

A protein surface epitope targeted monoclonal antibody library (SEAL) was used to develop a BAG-1L-specific antibody (experiments performed by Dr Emmanouil Papdakis). Potential protein surface epitopes were selected based on proprietary immunogenicity-enhancement system. Mice were immunised using selected immunogens to produce a high level of IgG immune response. A high throughput hybridoma process was employed to isolate hundreds of high affinity monoclonal and stable clones (Figure 5.11). Western blotting and immunoprecipitation (performed by Dr Emmanouil Papadakis) demonstrated the specificity of the antibody (Figure 5.11A and B). Western blotting demonstrated strong expression of BAG-1L and relatively weak expression of BAG-1M for the 3C12 antibody and was therefore selected for immunohistochemical experiments. Immunohistochemical analysis of BAG-1, using the protocol for whole sections in section 2.12, confirmed nuclear immunopositivity (Figure 5.11C).

5.1.3.2 Immunodepletion using parallel sections from positive control tumour block and BAG-1L-specific antibody 3C12.

An immunodepletion experiment was performed to demonstrate specificity of the BAG-1L-specific antibody 3C12 (Figure 5.12). Positive BAG-1 immunoreactivity was confirmed in whole paraffin-embedded sections incubated with the BAG-1L isoform-specific antibody 3C12 (1:800 dilution with TBS-Tween) and those treated with 3C12, unbound GST, and GSH beads. Immunodepletion confirmed the specificity of the 3C12 antibody as GST-bound 3C12, 3C12, processed through GSH beads was negative for BAG-1.

5.1.3.3 Quick Type-M ™ Monoclonal Antibody Isotyping of 3C12 BAG-1L-specific antibody

Quick Type-M™ Monoclonal Antibody Isotyping Test Kit (MicraTech™) was used to determine the isotype of the BAG-1L-specific monoclonal antibody 3C12. The isotype was shown to be G3κ on 3 repeat tests (Figure 5.13).

5.1.4 Discussion

5.1.4.1 The importance of optimisation of antigen retrieval method.

The optimal method for each antibody must be found prior to any valid immunohistochemical study and this may be a timely, or even unsuccessful process. The method must produce adequate immunohistochemical staining, which preferably shows a gradient between samples, or a distinct cut-off between positive and negative, and show minimal or no background on positive and negative sections. The results must be validated by use of many repeat sections from the same test cohort of tumours to confirm reproducibility. Prior to use in TMA, a correlating test cohort of whole sections should be processed and assessed for congruency.

Heat-mediated antigen retrieval can be achieved by a variety of different methods. Commonly, a microwave oven or pressure cooker are used, but techniques have been described using vegetable steamers, water bath, or heat plates. Paraffin-embedded samples generally require antigen retrieval prior to immunohistochemical staining as fixation in formalin causes formation of methylene bridges which crosslink and therefore mask protein.

Heat-induced antigen retrieval can be replaced by enzymatic retrieval, but enzymatic retrieval is associated with an increase in morphological disturbance of embedded sections. Generally, a Tris/EDTA pH 9.0 buffer can be used, although the sodium citrate (pH 6.0) buffer used in this study is also well-utilised.

The results in this chapter support the advantages of using a pressure cooker for heat-mediated antigen retrieval over microwave oven (Miller et al., 1995). Both require optimisation of process time, but there is overall less variability in using a pressure cooker than a microwave oven. A pressure cooker avoids agitation by violent boiling and buffer containers do not need to be refilled and balanced using this technique. The pressure cooker provides a more even distribution of heat and constant temperature than the microwave oven. Microwave ovens are well-known to produce focal points of high temperature and each microwave oven will do this in a variable way to even one of the same model and manufacturer. Use of the same microwave oven does not wholly eradicate this variable as optimisation of antigen retrieval may take months or even years of processing, in which time the microwave becomes less efficient and less uniform. To improve the microwave oven heat consistency 3 baths of buffer on a carousel were used for all runs, even for a small number of sections.

A microwave oven can achieve boiling therefore a maximal temperature of approximately 100°C in most circumstances. The pressure cooker used in this study (Nordic Ware™) at 12-15 PSI will obtain an even temperature of around 120°C, allowing a shorter process time. Miller et al., have consistently reported an improvement in antibody sensitivity, allowing for use of more dilute antibody stock (Millar et al., 1995). This study shows a similar experience with pressure cooker achieving equal results with an antibody dilution of 1:1200 to those of 1:600, although this was not fully reproduced on transition from cohort whole sections to TMA. Another important variable is the cool down period after pressure cooker use. Washing sections too hastily in running cool distilled water or TBS-Tween will cause tissue loss, therefore a consistent cooling period of 20 minutes employed.

Despite several months of antibody optimisation and results showing clarity and gradient of BAG-1 in a test cohort whole sections (Figure 5.9), this did not translate to use in the test TMA sections from the TEAM trial cohort. Interestingly, previously published images of immunohistochemical staining of BAG-1 in breast cancer show a higher level of background than felt acceptable for study validity (Tang et al., 1999). The laboratory responsible for the TEAM study relocated to Canada during this project, and resolved to not completing

optimisation of the protocol using the 3.10G3E2 BAG-1 antibody. Subsequently, similar work was published by Professor Mitch Dowsett (Afentakis et al., 2013).

5.1.3.2 Tissue microarray use versus whole sections for the validation of BAG-1 as a biomarker in breast cancer.

The results in this chapter demonstrate the number of variables and steps involved in the optimisation of an antibody for use in immunohistochemistry. Optimisation for whole sections can be a convoluted process taking several months involving rigorous validation steps and is well-documented by commercial antibody production companies. These results suggest that a protocol validated using standard paraffin-embedded whole sections is not applicable to the same antibody for TMA sections.

The results show increased background staining, more 'edge-effect' and overall more intense staining at the same antibody dilution. The comparison between a TMA section (Figure 5.8) and a 'virtual TMA' using whole sections (Figure 5.9), which had been previously scored for BAG-1 by an independent pathologist, confirmed significant variation. Approximately 20% TMA cores which showed weak or 1+ staining on whole sections revealed more intense 2+ or 3+ staining for both nuclear and cytoplasmic BAG-1 on a TMA. These findings are not recorded in other immunohistochemical studies of BAG-1 using tissue microarrays (Nadler et al., 2008; Millar et al., 2009). The previous studies do, however, show a lack of standardisation between scoring methods. BAG-1 'positive' has been taken to be above a certain cut-off point if there is bipolar distribution of results, or anything more than negative staining (Tang et al., 1999). Studies performed without automated analysis were subject to both intra- and inter- observer bias. Human inaccuracy can be significantly reduced by incorporation of a correlation coefficient and assessment of reproducibility (Landis et al., 1977), but none of the BAG-1 studies included such measures.

Literature on generic immunohistochemical techniques offers no direct comparison regarding staining for BAG-1 between TMA and whole sections. Many studies discuss problems with tumour heterogeneity and automated analysis of TMA. This project has reviewed the previous literature and revealed some concordance in support of BAG-1 as a molecular marker in breast cancer in both research using microarrays and whole sections. However, a large recent study, not included in the literature review in chapter 1, fails to support a role for BAG-1 as a biomarker in node-positive breast cancer. Molecular analysis of the Breast Cancer International Research Group (BCRIG) 001 Trial used a prospective cohort of 1,350 node-positive patients whom had been randomised to a docataxel-based (TAC) or fluoracil-

based adjuvant chemotherapy regimen (FAC) (Dumontet et al., 2010). The study did not use a microarray but used standard whole sections from representative formalin-fixed paraffin blocks taken and analysed prior to chemotherapy exposure. The study measured 14 pathologic and molecular markers using immunohistochemical techniques. BAG-1 expression was measured in both nuclei and cytoplasm. Samples were deemed as positive for nuclear BAG-1 if staining was >20% and positive for cytoplasmic if >60%. No correlation was found between BAG-1 expression and overall survival following univariate analysis (BAG-1 cytoplasmic p=0.38 and BAG-1 nuclear p=0.36). Shorter overall survival was however correlated with low Bcl-2 expression and HER2 positivity (p=0.005 and p=0.09 respectively).

The Dumontet et al. study is contrasting to previous studies and the results of the work leading to the development of Oncotype DX™ in failing to show any association between BAG-1 in breast cancer and survival outcomes. However, there was a significant amount of patient data missing. 343 of the 1,350 samples were missing data on extra nodal disease, a significant factor in patient survival in node positive disease. Data on multifocal tumours was missing in 164 patients. Another explanation for discrepancy in findings between this and previous studies is that no standardised immunohistochemical scoring system was used, but staining positivity or negativity calculated by dichotomising percentage of BAG-1expression. Referring back to the REMARK Criteria discussed in Chapter 1, the Dumontet et al., study would fail to comply with recommendations.

Proteins with multiple isoforms, such as BAG-1, do prove difficult and are time-consuming to quantify using automated techniques (Camp et al., 2008). The results of chapter 5 suggest that an antibody showing little background when used with whole paraffin-embedded sections, may produce ‘noisy’ TMA sections with an identical protocol. Discrepancy between scoring for intensity between TMA and whole sections may be due to tumour heterogeneity between 0.6mm TMA cores, although this has not been reported in previous TMA studies of BAG-1 (Nadler et al., 2008; Millar et al., 2009).

This chapter has suggested expression patterns exist for BAG-1 expression in breast cancer but failed to add to the clinical significance due to non-specific immunoreactivity observed with use of 3.10G3E2 on TMA. Future work should incorporate a TMA study using the optimal antigen retrieval method identified. Immunohistochemical analysis of the TMA should involve BAG-1, using both pan-isoform 3.10G3E2 and BAG-1L-specic 3C12, in addition to HER2.

5.2 BAG-1 in bladder cancer

5.2.1 Introduction

A single study by Takayama *et al.* (1998) reported weak cytoplasmic BAG-1 expression in normal bladder urothelium. No evidence exists prior to this chapter on the role of BAG-1 in bladder cancer. Recent work using bladder cancer cells has mirrored the findings in of previous chapters in breast cancer, implicating HER2 expression levels as an indicator of aberrant proliferative pathways. A recent study using bladder cell lines T24, HT1376, and UM-UC-3, has shown the HER2-inhibitor Lapatinib in conjunction with mTORC1/2 results in synergistic inhibition of proliferation and anchorage-dependent growth (Becker et al., 2014). No correlation between immunohistochemical analysis of HER2 and tumour stage was found. A review of HER2 overexpression in bladder carcinoma has shown association with increased tumour aggression, lymphovascular invasion and disease-specific mortality (Lammers and Witjes, 2011). However, the authors identified an absence in a standardised cut-off for HER2 positivity in bladder cancer, hence any subsequent correlation with clinical parameters would be rendered potentially inaccurate.

The initial aim of this chapter was to demonstrate BAG-1 overexpression in T24 and UMUC3 bladder cancer cell lines. HER2 expression has been previously described in T24 and UMUC3 bladder cancer cell lines. Western blotting showed HER2 was overexpressed in both T24 and UMUC3 bladder cancer cell lines, although expression was weak relative to HER2 expression in BT-474 cells (Nagasawa et al., 2006). A simple immunohistochemical study was conducted using a TMA of cores from 117 patients who had undergone bladder biopsy and bladder resection (cystectomy) for bladder carcinoma to investigate the clinicopathological significance of BAG-1 expression (Table 5.1). The patient cohort was derived from a larger cohort of 226 patients who had undergone cystectomy for bladder cancer between 2000 and 2011 in a single tertiary centre. 109 patients were excluded from this immunohistochemical study due to incomplete data or damaged or incomplete triplicate TMA cores. Immunohistochemical staining of HER2 was performed for the tumour cohort by Mr James Douglas. Approximately 40% of the tumours were deemed to be HER2-positive by a pathologist using the HERCEP score. TMA sections were correlated with a test cohort of 8 bladder whole sections, which showed a similar pattern of immunohistochemistry for BAG-1 (3.10G3E2).

	Number of patients	Overall Survival at 5 years (%)
Age:		
<55	35	
>55	82	
Gender:		
Male	83	
Female	34	
T1	19	
T2	23	
T3	62	
T4	13	
Node-positive	29	
Node-negative	88	
Distant metastases	19	
Grade:		
Poorly differentiated	44	
Moderately differentiated	28	
Well differentiated	45	
HER2 status:		
HER2-positive	47	42
HER2-negative	70	56
5 year survival (whole cohort)	48%	
Nuclear BAG-1:		
Negative/weak	34	38
Moderate	5	44
Strong	78	46
Cytoplasmic BAG-1:		
Negative/ weak	69	32
Moderate	40	43
Strong	8	29

Table 5.1 Patient demographics of cohort used for immunohistochemical study of BAG-1 expression in bladder cancer. T: Tumour stage from TNM Classification.

5.2.2 Results

5.2.2.1 Western blotting confirms BAG-1 expression in T24 bladder cells.

Repeat experiments showed all BAG-1 isoforms were expressed in HER2–expressing T24 and UMUC3 bladder cancer cells. BT-474 HER2-positive breast cancer cells were used as a positive control (Figure 5.15).

5.2.2.2 Immunohistochemical staining of BAG-1 in a TMA incorporating 117 tumours from patients diagnosed with bladder cancer.

Preliminary analysis shows a trend towards a higher level of nuclear BAG-1 expression in benign and well-differentiated carcinoma with BAG-1 expression correlating inversely with TNM stage. The pattern of expression of BAG-1 in normal bladder surface urothelium was moderate-strong nuclear BAG-1 in over 75% of cores and weak cytoplasmic BAG-1 expression (Figure 5.14). A trend was observed between poorly differentiated bladder carcinoma and weak or negative BAG-1 expression. BAG-1 expression in bladder carcinoma was correlated with patient survival outcomes, but failed to reach statistical significance. HER2-positivity was associated with a shortened 5 year survival (42% versus 56%), but no association was found between BAG-1 immunoreactivity and HER2 status.

5.2.3 Discussion

This chapter has confirmed overexpression of BAG-1 in T24 bladder cells, also known to express HER2 (Nagasawa et al., 2006). Interestingly, Nagasawa et al. (2006) reported similar expression patterns to those in chapter 3 (Figure 3.5) for downstream signalling proteins p-Akt, Akt, p-ERK and ERK following HER2 inhibition by TAK-165, a selective tyrosine kinase inhibitor, in bladder cancer cells. This indicates a potential focus for future study design incorporating BAG-1 expression in HER2-positive bladder cancer.

This chapter has shown a trend towards loss of nuclear BAG-1 expression with increasingly aggressive bladder tumours, with normal bladder urothelium showing moderate-strong nuclear BAG-1 expression. Unfortunately this study failed to reach any statistical significance

with patient survival parameters. The patient cohort was not fully standardised in terms of therapy received. Therefore, this study would fail to reach many of the REMARK criteria previously discussed. A repeat study should incorporate a treatment standardised patient cohort all either undergoing bladder biopsy or cystectomy in the absence of neoadjuvant therapy. A future bladder cancer TMA should be assessed for BAG-1 using both the pan-isoform antibody, 3.10G3E2, and the BAG-1L-specific antibody 3C12, in addition to HER2.

5.3 BAG-1 in oesophageal cancer

5.3.1 Introduction

Systematic review of the expression of HER2 in operable oesophageal cancer has shown an association with poor prognosis (Chan et al., 2012). BAG-1 expression has been previously investigated for correlation with patient survival outcomes or histopathological variables in patients diagnosed with oesophageal adenocarcinoma. In oesophageal squamous cell carcinoma, nuclear BAG-1 expression has been associated with increased tumour invasion and unfavourable prognosis (Noguchi et al., 2003; Takeno et al., 2007), while downregulation of BAG-1 expression by RNAi results in resistance to radiotherapy (Yoshino et al., 2011). More recently, BAG-1 expression has been shown to be higher in oesophageal squamous cell carcinoma than adjacent tissues(Huang et al., 2014). siRNA knockdown of expression of BAG-1 in Eca109 cells showed decreased cell viability and invasion using MTT and Transwell assays respectively. Flow cytometry was used to show the rate of apoptosis in Eca109 cells transfected with BAG-1 siRNA was significantly increased. The expression of Bcl-2 was also down-regulated following siRNA knockdown of BAG-1. This study suggests BAG-1 has a role in development and survival of oesophageal squamous cell carcinoma. This chapter begins to address the current gap in our knowledge with respect to BAG-1 expression in oesophageal adenocarcinoma.

5.3.2 Results

5.3.2.1 Immunohistochemical staining of whole sections from test cohort of patients diagnosed with oesophageal adenocarcinoma.

A test cohort of 16 oesophageal adenocarcinomas selected to represent different stages of oesophageal cancer was used for initial optimisation of BAG-1 immunohistochemical staining. The pan-isoform anti-BAG-1 antibody 3.10G3E2 was used. Strong nuclear and weak cytoplasmic BAG-1 immunoreactivity was seen in 2 sections of moderately differentiated oesophageal carcinoma (Figure 5.16 D and H) compared with negative control sections (Figure 5.16C and H). Breast adenocarcinoma whole sections were used as a positive control for BAG-1 and confirmed strong nuclear and moderate immunohistochemical staining.

5.3.2.2 Description of patient cohort used for TMA construction and study design.

For this retrospective study, a prospectively collected database of consecutive patients undergoing oesophagogastric resection treated at University Hospital Southampton NHS Foundation Trust (UHSFT) between January 2005 and December 2010 was reviewed. All patients were discussed at a specialist multidisciplinary team meeting (MDT). Standard staging investigations included endoscopic ultrasonography, high-resolution computed tomography, integrated fluorodeoxyglucose positron emission tomography/computed tomography (PET-CT) and staging laparoscopy, where indicated. Patients considered suitable for potential surgical resection with tumours staged as T2 N0 M0 or above were considered for neoadjuvant chemotherapy. Neoadjuvant chemotherapy consisted of three 21-day cycles of ECF (Epirubicin 50mg/m^2, Cisplatin 60mg/m^2, both intravenously on day 1 and protracted venous infusion 5-FU 200mg/m^2 per day) or ECX (Epirubicin 50mg/m^2, Cisplatin 60mg/m^2, both intravenously on day 1 and Capecitabine 625mg/m^2 orally twice daily for 21 days) or EOX (Epirubicin 50 mg/m^2 i.v. bolus and Oxaliplatin 130 mg/m^2 i.v. infusion over 2 hours on day 1, Capecitabine 625 mg/m^2 orally twice daily for 21 days).

Surgery was performed at University of Southampton NHS Foundation Trust after initial staging or 4-6 weeks following neoadjuvant chemotherapy. A repeat CT scan was performed for those that received chemotherapy to assess their response to chemotherapy and disease operability. Types of oesophagogastrectomies included Ivor Lewis, left

thoracoabdominal with or without cervical anastomosis and transhiatal oesophagogastrectomy or minimally invasive oesophagogastrectomy (MIO) either 2 stage (MIO-2) or 3 stage (MIO-3) in accordance with recommendations arising from the consensus statement from the Association of Upper Gastrointestinal Surgeons and the Association of Laparoscopic Surgeons for introduction of MIO (http://www.augis.org/newsaugis.guidelines/).

Data recorded included demographics, tumour characteristics, resection type, estimated blood loss (calculated from suction bottles and weighed swabs), and histopathological analysis of the surgical specimen. TNM-7 was used to report tumour stage after analysis of pathology reports (UICC: TNM classification of malignant tumours. Wiley Blackwell 2009). Pathological tumour clearance ("R"-status) was determined according the Royal College of Pathologists system. Pathological response to chemotherapy was assessed using the tumour regression grade (TRG) system developed by Mandard et al. (1994) and by the down-staging of lymph nodes (cN1 to ypN0 or pN0) (Korst et al., 2006; Mandard et al., 1994). TRG was scored by specialist gastrointestinal pathologists initially by one pathologist (ACB) prior to its introduction by all pathologists as part of routine pathological reporting (Table 5.2).

Postoperative complications were graded according to the Clavien-Dindo (CD) classification (Clavien et al., 2009). An anastomotic leak was defined as a leak sufficient to cause symptoms and confirmed by radiology (contrast enhanced multi-detector CT scan with on-table oral contrast or water soluble contrast studies), endoscopy or during surgical exploration. All patients were cared for by a specialist oesophagogastric team who applied a similar perioperative regime to all patients.

Patients were routinely followed-up for 5 years post -surgery according to the following protocol: 2-4 weeks post-discharge, 3 monthly for 1 year, 6 monthly for 2 years and yearly thereafter. Patients were also seen on an "as required" basis if symptomatic. Recurrence of disease during follow-up was defined as the first site or sites of recurrence with radiological or pathological confirmation. For assessment of disease free survival (DFS), recurrence was defined as time from operation to development of local, nodal (regional) and distant metastasis (whichever occurred first). Overall survival (OS) was defined as time from operation to date of death.

5.3.2.3 Kaplan-Meier survival plot for cohort of post-operative patients diagnosed with oesophageal adenocarcinoma.

The Kaplan-Meier survival curve demonstrated cumulative survival with a 95% confidence interval between 3.21-3.76 years (Figure 5.17), which is consistent with other oesophageal cancer studies.

5.3.2.4 Immunohistochemical staining of TMA incorporating 216 tumours from post-operative patients diagnosed with oesophageal adenocarcinoma.

Immunohistochemical staining of BAG-1 in the oesophageal adenocarcinoma TMA showed a clear gradient between negative and positive sections. There was a distinct difference in the pattern of staining between normal gastric and oesophageal mucosa and oesophageal adenocarcinoma (Figure 5.18) with more intense nuclear staining seen in invasive cancer and weaker cytoplasmic BAG-1 (Figure 5.18I).

Tumour cores were independently scored for BAG-1 immunoreactivity by the author and a Consultant Pathologist blind to the associated clinicopathological data. The intensity of nuclear or cytoplasmic BAG-1 immunoreactivity were independently scored as between 1-3 for low, moderate, or high respectively. The percentage of tumour positively stained for nuclear or cytoplasmic BAG-1 was scored independently as 1-4 for 0-25%, >25 but <50%, >50 but less than 75%, and >75% respectively. A ‘quickscore’ was produced by multiplication of intensity (1-3) and percentage of tumour stained (1-4) for both nuclear and cytoplasmic BAG-1 for each core. The mean value of triplicate cores was used for data analysis.

The nuclei of signet ring cells demonstrated dense immunohistochemistry for BAG-1(Figure 5.18E). Signet-ring cell carcinoma is a unique histologic subtype of adenocarcinoma characterized by abundant intracellular mucin accumulation and a compressed nucleus displaced toward one extremity of the cell. The World Health Organisation (WHO), a true signet-ring cell carcinoma is defined as an adenocarcinoma in which the predominant component (>50% tumour) consists of isolated or small groups of signet-ring cells in the stroma. Signet ring cells in oesophageal adenocarcinoma are associated with advanced stage at presentation and poor patient prognosis (Yendamuri et al., 2013).

5.3.2.5 Statistical analysis of results.

Descriptive data are represented as median and interquartile range unless indicated, with Kruskal-Wallis, Mann Whitney U and Pearson's chi-squared test used as appropriate for comparison. Kaplan-Meier (Figure 5.17), univariate and multivariate cox logistic regression modelling were used to assess the relationship between BAG-1 expression with OS, DFS and response to neoadjuvant chemotherapy as outcomes (Table 5.4). All factors that showed statistical significance on univariate analysis were entered to derive the final model. OS and DFS curves of the patients were plotted by using the Kaplan-Meier method and analysed using the Log-rank test. Stratified analyses were performed based on receipt of neoadjuvant chemotherapy and pathological response to chemotherapy. A p value <0.05 was considered statistically significant for all tests. Statistical analysis was performed with SPSS® version 19 (SPSS, Chicago, Illinois, USA) and X-Tile (Yale, US) (Figure 5.19) was used to determine optimal division points for low, moderate and high staining scores.

X-tile plots are constructed by dividing biomarker data into discrete populations: low, middle, and high). Associations can be calculated at each division by the log-rank test for survival. The data are represented graphically in a right-triangular grid where each point represents the data from a given set of divisions. The vertical axis represents all possible "high" populations, with the size of the high population increasing from top to bottom. Similarly, the horizontal axis represents all possible "low" populations, with the size of the low population increasing from left to right. Data along the hypotenuse represent results from a single cut-point that divides the data into high or low subsets. Data points away from the hypotenuse up or to the left represent results from two cut-points that define an additional "middle" population in addition to the high and low subsets. The size of the middle subset increases with greater distances from the hypotenuse. Specifically, a chi-squared value is calculated for every possible division of the population shown on the grid using a color code. The program can be utilised to select the optimal division of the data by selecting the highest chi-squared value.. The calculations done by X-tile have been validated with Statview 5.0.1 (SAS Institute, Cary NC).

Statistical analysis revealed no significant correlation between patient survival or histological tumour grade and BAG-1 staining in this immunohistochemical series. However, a trend was observed between poorly differentiated tumours and increased levels of BAG-1 staining.

Sub-group analysis of those patients who had received neoadjuvant chemotherapy prior to surgery was performed. We identified a significant association between high levels of

nuclear BAG-1 staining and oesophageal tumours with advanced tumour regression grade (p=0.042), hence more aggressive and poorly responsive.

5.3.3 Discussion

Numerous studies report novel cancer biomarkers, but only a minority translate into clinical practice. Our study design adhered to criteria defined by the REMARK (Reporting Recommendations for Tumour Marker and Prognostic Studies), an analytical tool to assess validity of biomarker studies(Mallett et al., 2010).

A discrepancy exists between the correlation with clinicopathological variables and BAG-1 expression in non-breast tumours and breast tumours. Upregulation of BAG-1 in ER+ lymph node negative breast adenocarcinomas conveys improved response to Tamoxifen hormonal therapy, via upregulation of the ERα subunit (Cutress et al., 2003). Previous groups have distinguished between hormone-dependent or hormone-responsive tumours and hormone-independent cancer. An association between increased expression of BAG-1 and aggressive histopathological grade has been reported on gastric and oral squamous cell carcinomas. The gender bias shown by oesophageal adenocarcinoma (male to female ratio ranges from 3:1 to 7:1 worldwide) has instigated research into the role of oestrogen in these cancers (Rashid et al., 2010). The evidence remains conflicting, although several studies showed overexpression of the ERβ subunit was associated with oesophageal adenocarcinoma (Liu et al., 2004). One study reported higher levels of ERβ present in cases reported as Barrett's metaplasia, an aetiological factor for oesophageal adenocarcinoma (Akgun et al., 2002). Interestingly, 2 previous IHC studies found no correlation between ER subunit staining and squamous cell carcinoma, suggesting a distinct role in oesophageal adenocarcinoma.

Preliminary analysis of data shows correlation with clinicopathological parameters supporting a role for BAG-1 as a valid potential prognostic biomarker in oesophageal adenocarcinoma. This chapter suggests increased expression of nuclear BAG-1 in oesophageal adenocarcinoma is associated with advanced tumour regression grade. The results in this chapter show BAG-1 expression has the potential to increase therapeutic stratification, thus recognising the most appropriate and effective treatment for each individual, hence optimising patient survival outcomes.

Future work, in addition to formal data analysis, will involve optimisation of a specific anti-BAG-1L antibody for use in further immunohistochemical TMA studies and comparison with the pan-isoform antibody, 3.10G3E2. We will also look at the relationship between BAG-1 expression and hormonal receptor status in oesophageal adenocarcinoma.

Figure 5.1 Tissue microarray sections following antigen retrieval with microwave and standard bath compared with pressure cooker treatment.

Sections were de-waxed and rehydrated according to standard protocol. The sections were then either placed in a citrate buffer (pH6) in a standard bath and heated at medium power for 25 minutes in a microwave, or underwent pressure cooker treatment. The sections placed in the pressure cooker were only submitted to 5 minutes treatment once the citrate buffer (pH6) had reached boiling point. Sections shown were incubated overnight at 4°C with BAG-1 antibody(3.10G3E2) at a dilution of 1:800, following blocking with foetal calf serum (1%) and washed with TBS-Tween (0.1%).

Figure 5.2 Choice of blocking reagent for immunohistochemistry for BAG-1(3.10G3E2) using TMA sections.
Following antigen retrieval using citrate buffer and pressure cooker sections were blocked initially with 3% hydrogen peroxide for 10 minutes. Cores were then blocked with either 1% normal goat serum, 10% casein, or protein-free block (Dako). 1:600 anti-BAG-1 antibody (3.10G3E2) was used for all TMA sections and washes between steps were with TBS-Tween (0.1%). Sections were incubated overnight (approximately 12 hours) at 4°C with primary antibody and for 1 hour at room temperature with Envision secondary. A: TBS control blocked with normal goat serum. B: TBS control blocked with 10% casein. C: TBS control blocked with protein-free block.

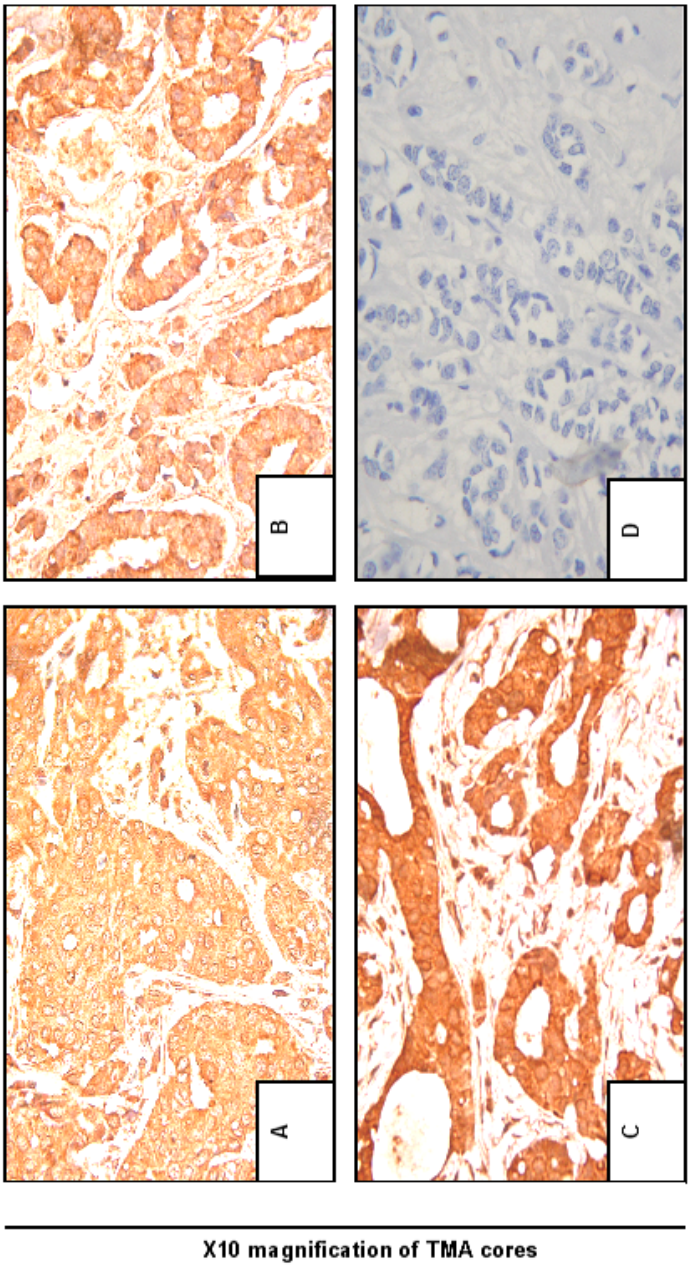

Figure 5.3 Titration of pan-isoform anti-BAG-1 antibody 3.10G3E2 using whole sections following blocking with foetal calf serum.

A: 1:1000; B: 1:800; C: 1:600 anti-BAG-1 antibody 3.10G3E2; D: TBS control. Following antigen retrieval using citrate buffer and microwave with standard bath, sections were blocked initially with 3% hydrogen peroxide for 10 minutes and then for 1 hour with foetal calf serum for one hour. Sections were then incubated in primary antibody for 12 hours overnight at 4°C.

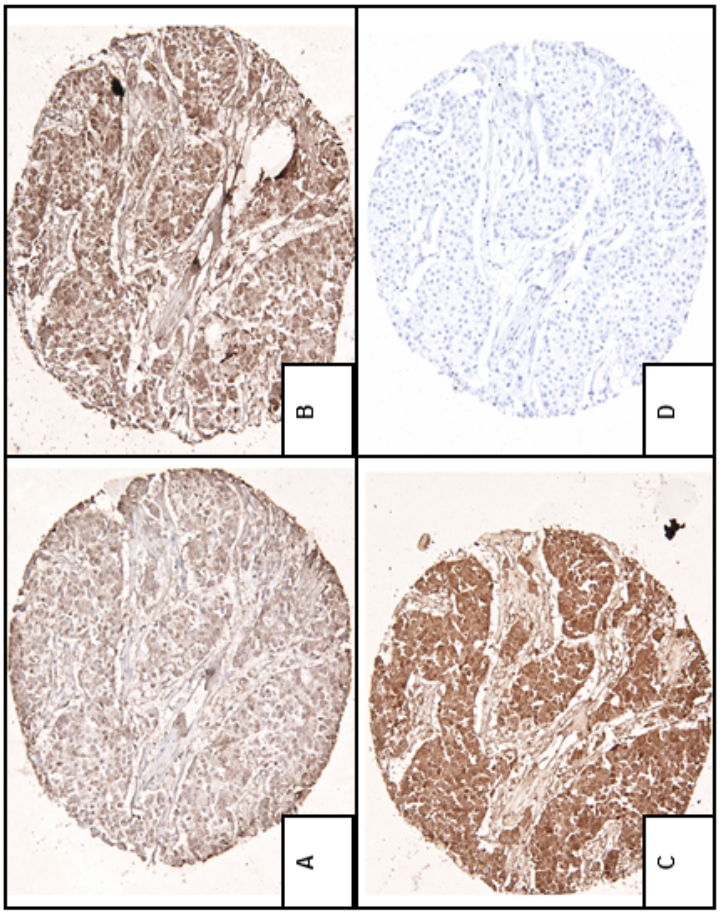

Figure 5.4 Titration of pan-isoform anti-BAG-1 antibody 3.10G3E2 using TMA sections following blocking with foetal calf serum.

A: 1:1000; B: 1: 800; C: 1:600 anti-BAG-1 antibody 3.10G3E2; D: Negative control. Following antigen retrieval using citrate buffer and pressure cooker, all sections were blocked with 3% hydrogen peroxide and 10% foetal calf serum. TMA sections were incubated in primary antibody for 12 hours overnight at 4°C.

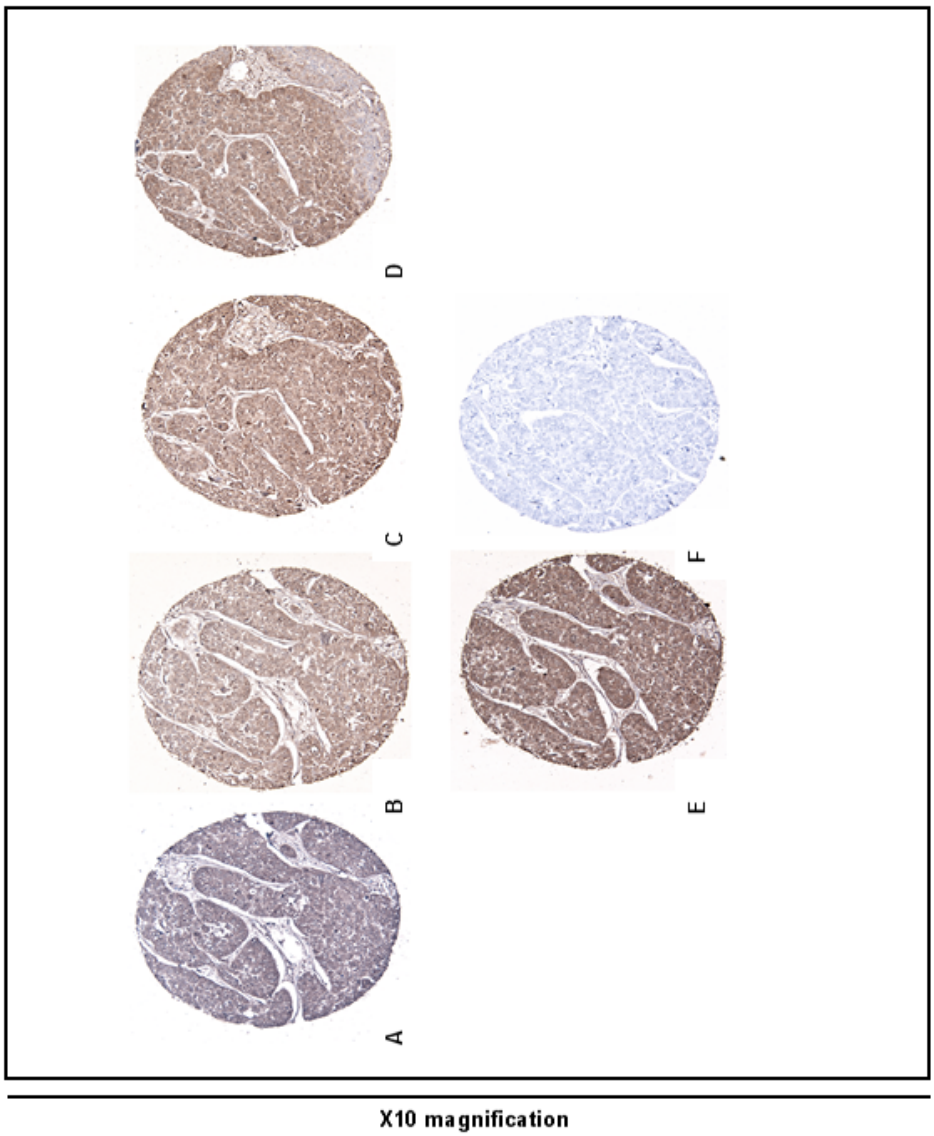

Figure 5.5 Titration of pan-isoform anti-BAG-1 antibody 3.10G3E2 using TMA sections following blocking with normal horse serum (1%).

A: 1:2000; B: 1:1750; C: 1:1500; D: 1:1000; E: 1:600 anti-BAG-1 antibody 3.10G3E2; F: Negative control. Following antigen retrieval with citrate buffer and microwave with pressure cooker, sections were incubated in primary antibody for 12 hours overnight at 4°C.

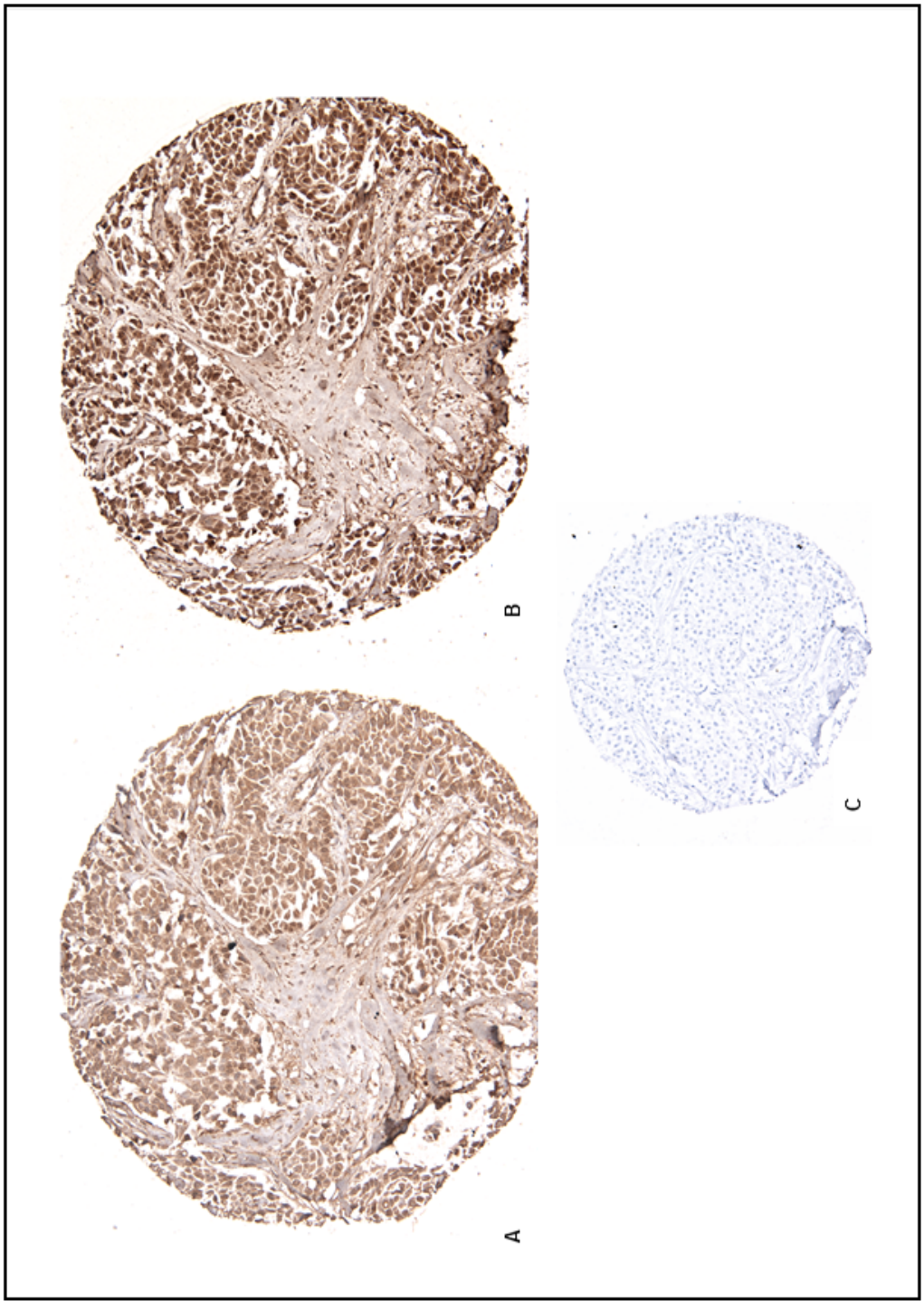

Figure 5.6 Optimal length of incubation time with primary antibody for TMA sections.
TMA cores were incubated for different time periods with 3.10G3E2 anti-BAG-1 antibody after antigen retrieval with microwave pressure cooker and citrate buffer and blocking with 10% FBS.
A: 12 hours; B: 24 hours incubation with 1:1000 anti-BAG-1 antibody 3.10G3E2 at 4°C; C: TBS-Tween (0.1%) control.

Figure 5.7 The effect of the addition of Tween (0.1%) into TBS for washes following blocking with animal sera.

A:TBS alone; B: TBS-Tween (0.1%). Sections shown are negative controls from TMA sections. A similar result was obtained for whole sections (figure not shown). Red box marks area of background observed without the addition of 0.1% Tween uniformly to TMA slide.

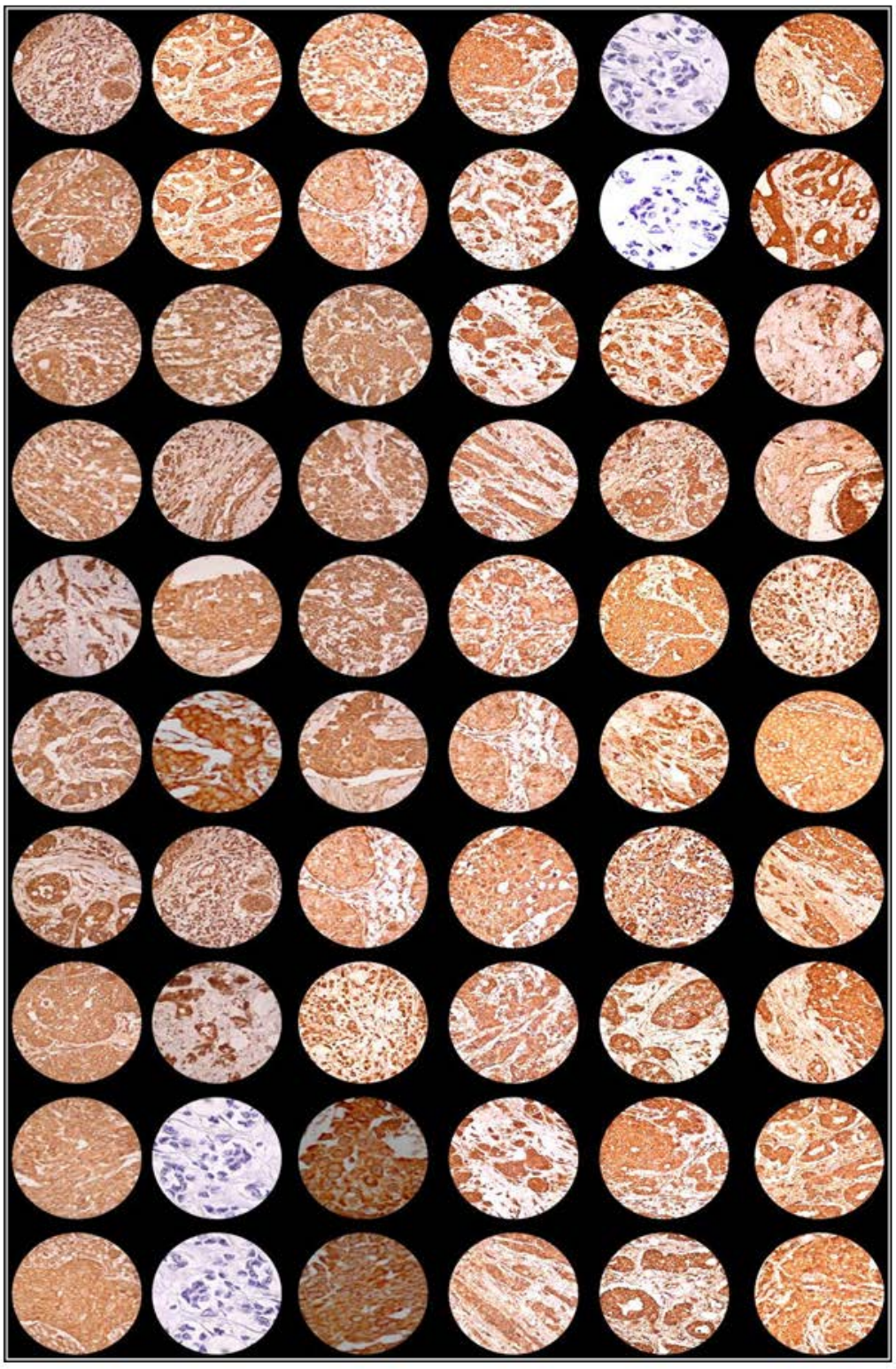

Figure 5.8 Section of tissue microarray (section kindly provided by Professor John Bartlett, CRUK Centre, Edinburgh).

Immunohistochemistry of TMA section for anti-BAG-1 antibody (3.10G3E2) using an antibody dilution of 1:800 TBS-Tween (0.1%) and protocol as per chapter 2. Section imaged using Ariol Imaging System™ and software.

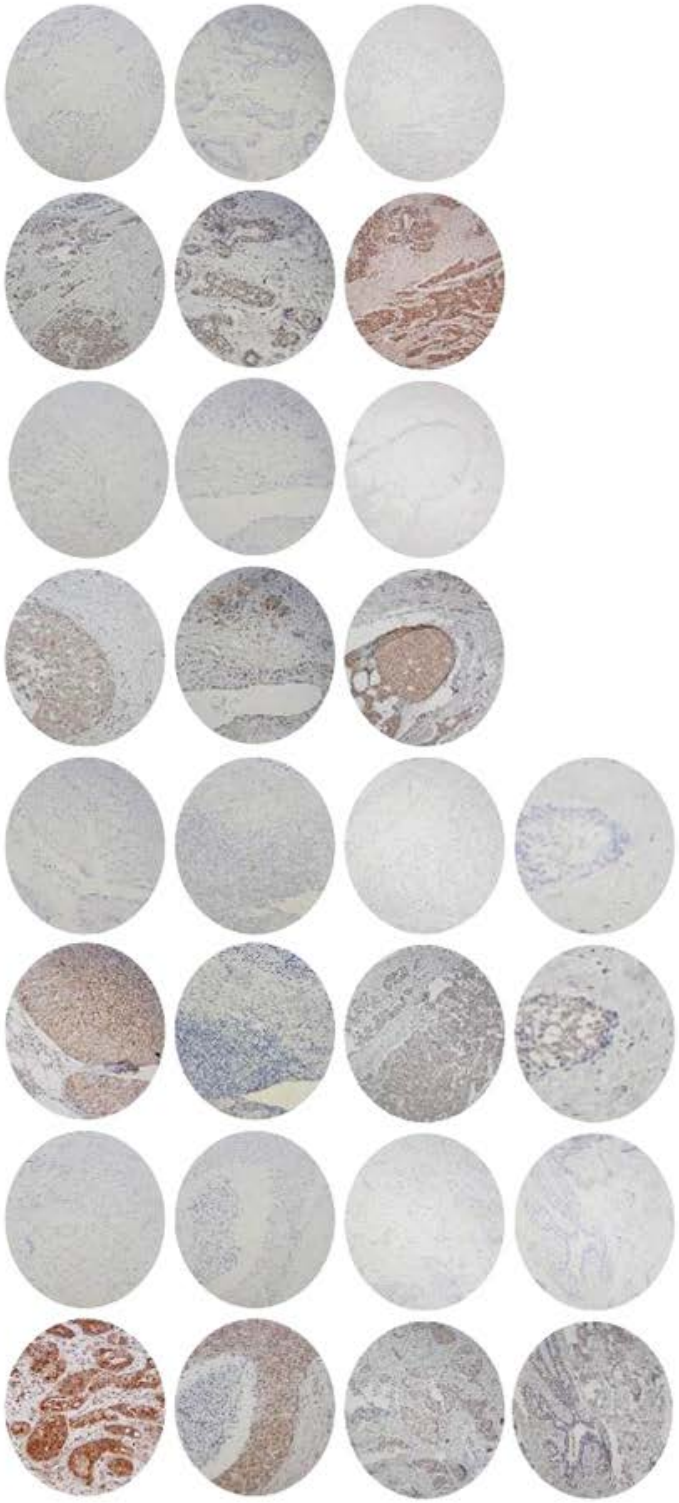

Figure 5.9 'Virtual tissue microarray' constructed from whole sections of archival breast carcinoma.

Immunohistochemistry using whole sections and identical protocol to tissue microarray section (Figure 5.8), using the pan-isoform anti-BAG-1(3.10G3E2) antibody dilution of 1:800 TBS-Tween (0.1%). Individual whole sections imaged using Ariol Imaging System™ and 'virtual TMA' constructed from raw images using Definiens Tissue Studio™ software.

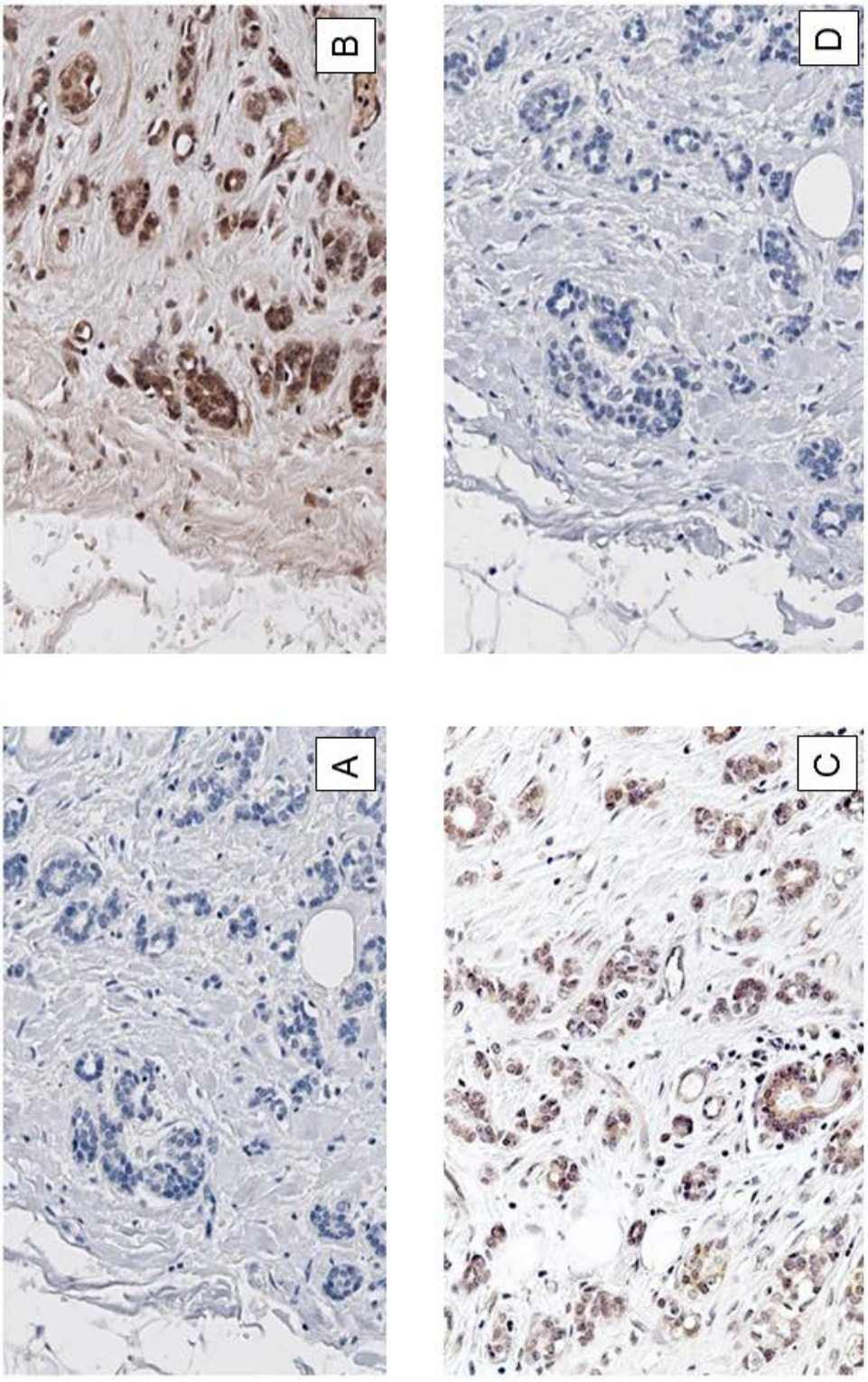

Figure 5.10 Immunodepletion using parallel whole sections and pan-isoform anti-BAG-1 antibody 3.10G3E2.

Antigen retrieval was performed using microwave and standard bath with citrate buffer. All section were blocked with 10% FBS and incubated for 12 hours overnight at 4°. TBS was used as a negative control (A). B: anti-BAG-1 antibody 3.10G3E2, GST and GSH beads; C: anti-BAG-1 antibody 3.10G3E2 (1:800 TBS-Tween 0.1%); D: anti-BAG-1 antibody 3.10G3E2 , GST-BAG-1 (100µg/ml) and GSH beads.

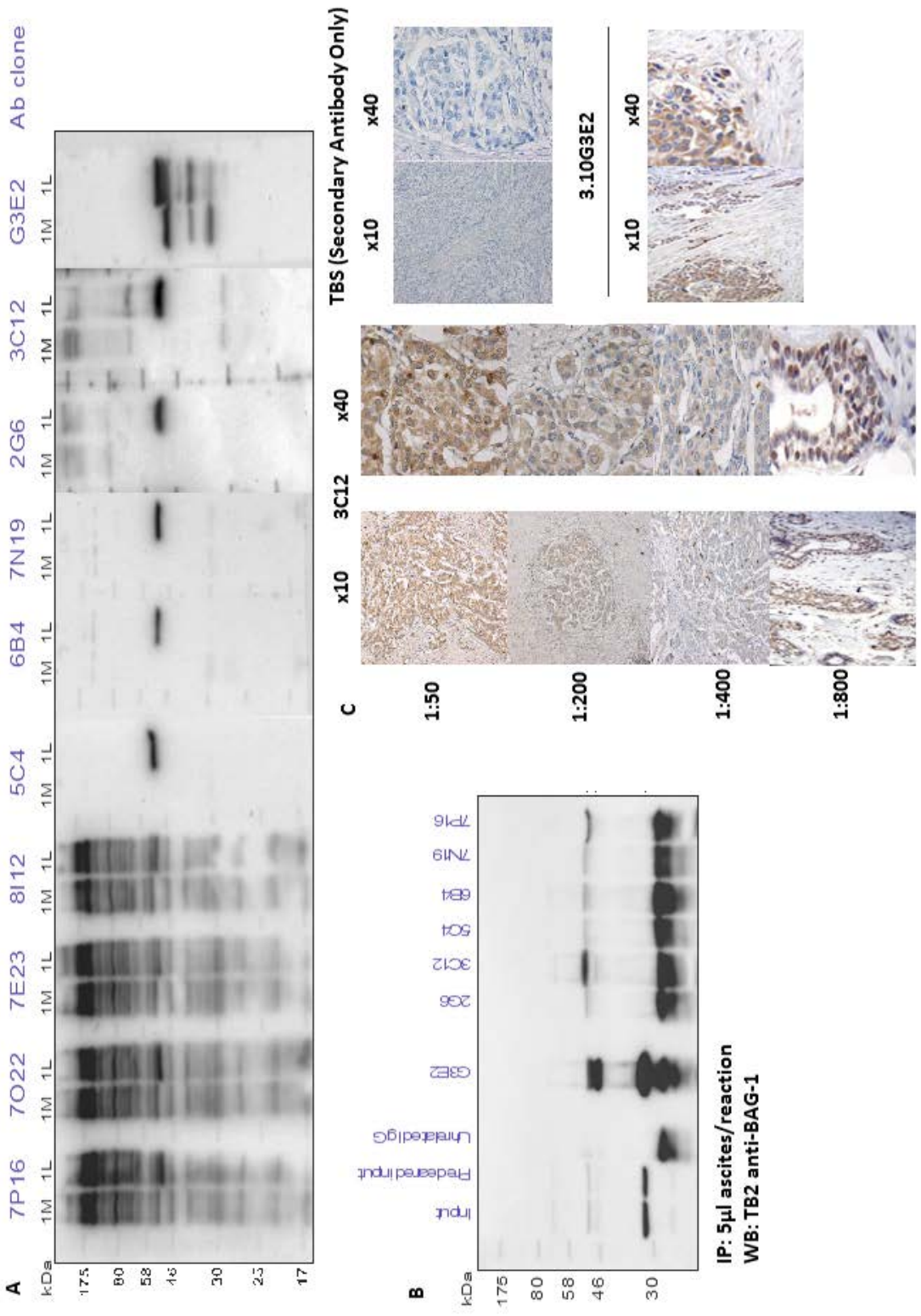

Figure 5.11 Development of an anti-BAG-1L isoform-specific antibody 3C12.
Immunoprecipitation using 8 compounds and the 3.10G3E2 anti-BAG-1 antibody (abbreviated to G3E2) to identify compounds demonstrating BAG1L specificity (A). Western blotting (B) and immunohistochemistry (C) to demonstrate specificity of the anti- BAG-1L antibody 3C12 (Immunoprecipation performed and kindly provided by Dr Emmanouil Papadakis).

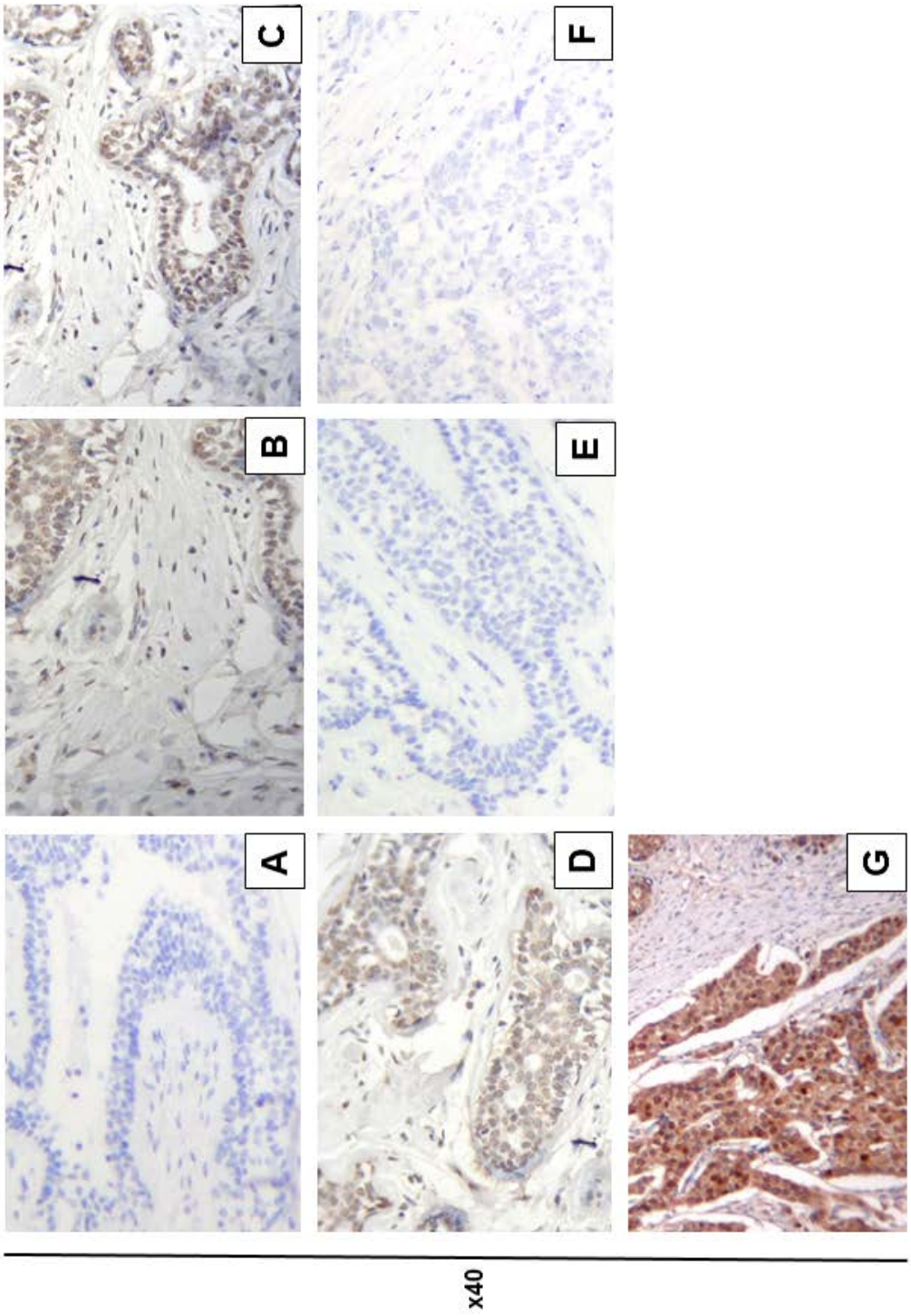

Figure 5.12 Immunodepletion using parallel sections from positive control tumour block and BAG-1L-specific antibody 3C12.
Antigen retrieval was performed using microwave and citrate buffer. All sections were blocked using 10% FCS and incubated for 12 hours overnight at 4°. Immunodepletion was performed using GST-tagged BAG-1 by gentle agitation and centrifugation with GSH beads and anti-BAG-1L antibody 3C12 (2.15.4) A: TBS (2° antibody only, 1:400); B: 3C12 (1:800); C: 3C12 (1:1000); D: 3C12 (1:800) + GST (100µg/ml) + GSH beads; E: 3C12 (1:1000) + GST-BAG-1L (1:800) + GSH beads; F: 3C12 (1:800) + GST-BAG-1M (1:800) +GSH beads; 3.10G3E2 pan-isoform anti-BAG-1 antibody positive control (nuclear + cytoplasmic staining).

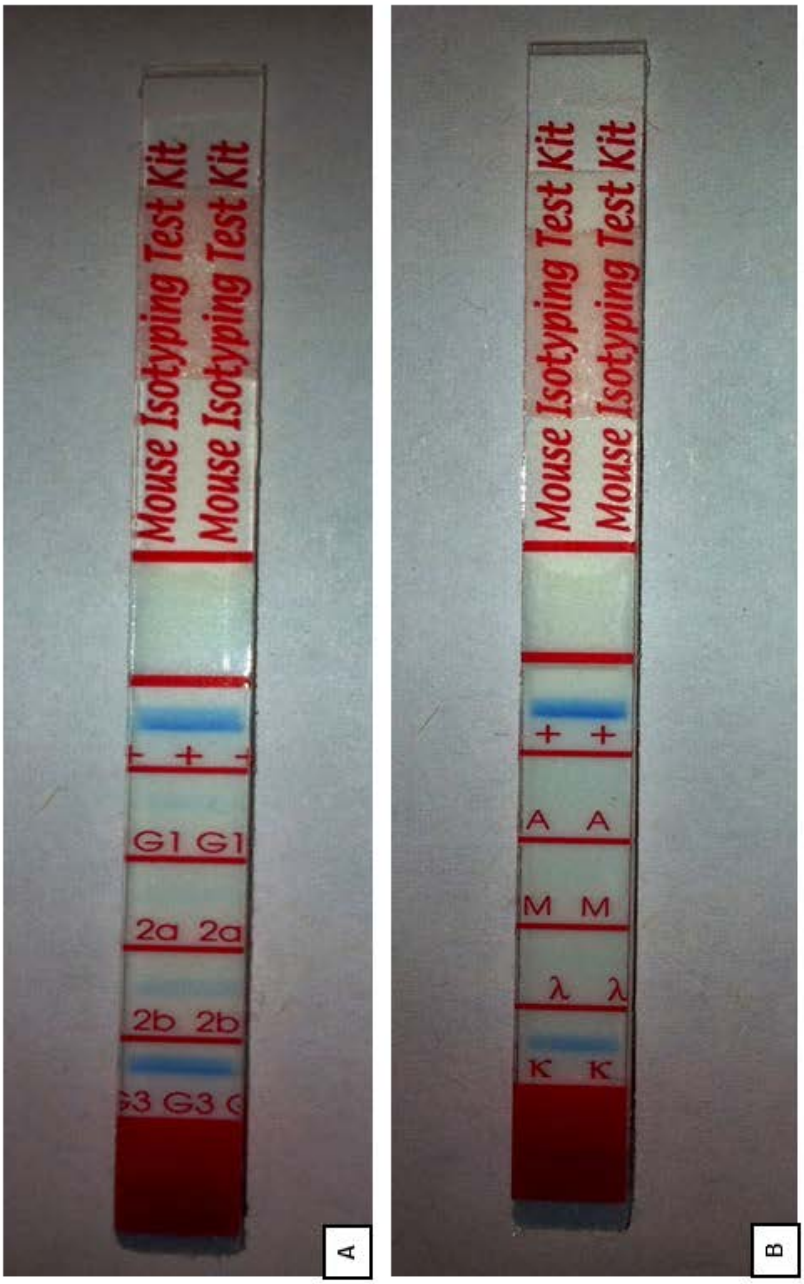

Figure 5.13 Quick Type-M ™ Monoclonal Antibody Isotyping of 3C12 BAG-1L-specific antibody.

Quick Type-M™ Monoclonal Antibody Isotyping Test Kit (MicraTech) was used to elucidate the isotype of the BAG-1L-specific monoclonal antibody 3C12. Samples of 3C12 were diluted to a concentration of 1.0µg/ml in 1% BSA/PBS. 150µL of the diluted sample was then added to the testing kit development tube and incubated at RT for 30 seconds. The tube was briefly vortexed to ensure complete microparticle suspension and one isotyping strip placed in the development tube.

Figure 5.14 Immunohistochemistry for BAG-1 using pan-isoform anti-BAG-1 antibody 3.10G3E2 (1:800) and TMA incorporating 117 patients diagnosed with bladder adenocarcinoma.

A: Haematoxylin and eosin control section of bladder carcinoma; B: Strong cytoplasmic immunoreactivity for BAG-1 in bladder adenocarcinoma x10 magnification to show Brunn's nests; C and G: Negative core for BAG-1 in bladder adenocarcinoma; D: Moderate immunoreactivity of BAG-1 in bladder adenocarcinoma;E: Moderate immunoreactivity for BAG-1 in bladder adenocarcinoma; F: x40 magnification of BAG-1 immunoreactivity in Brunn's nests of bladder urothelium; H: Strong immunoreactivity of BAG-1 in bladder adenocarcinoma.

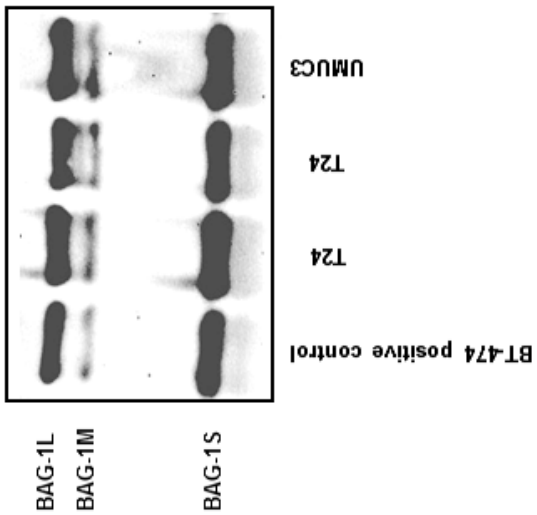

Figure 5.15 Western blot showing BAG-1 expression in HER2+ bladder cells lines.

T24/ UMUC3 bladder cells were seeded 48 hours prior to harvesting and lysate preparation using the standard method described in Chapter 2. Gels were probed for BAG-1 expression using the pan-isoform anti-BAG-1 antibody 3.10G3E2. The image shown is representative of two repeat experiments. HER2+ BT-474 breast cancer cells were used as a positive control.

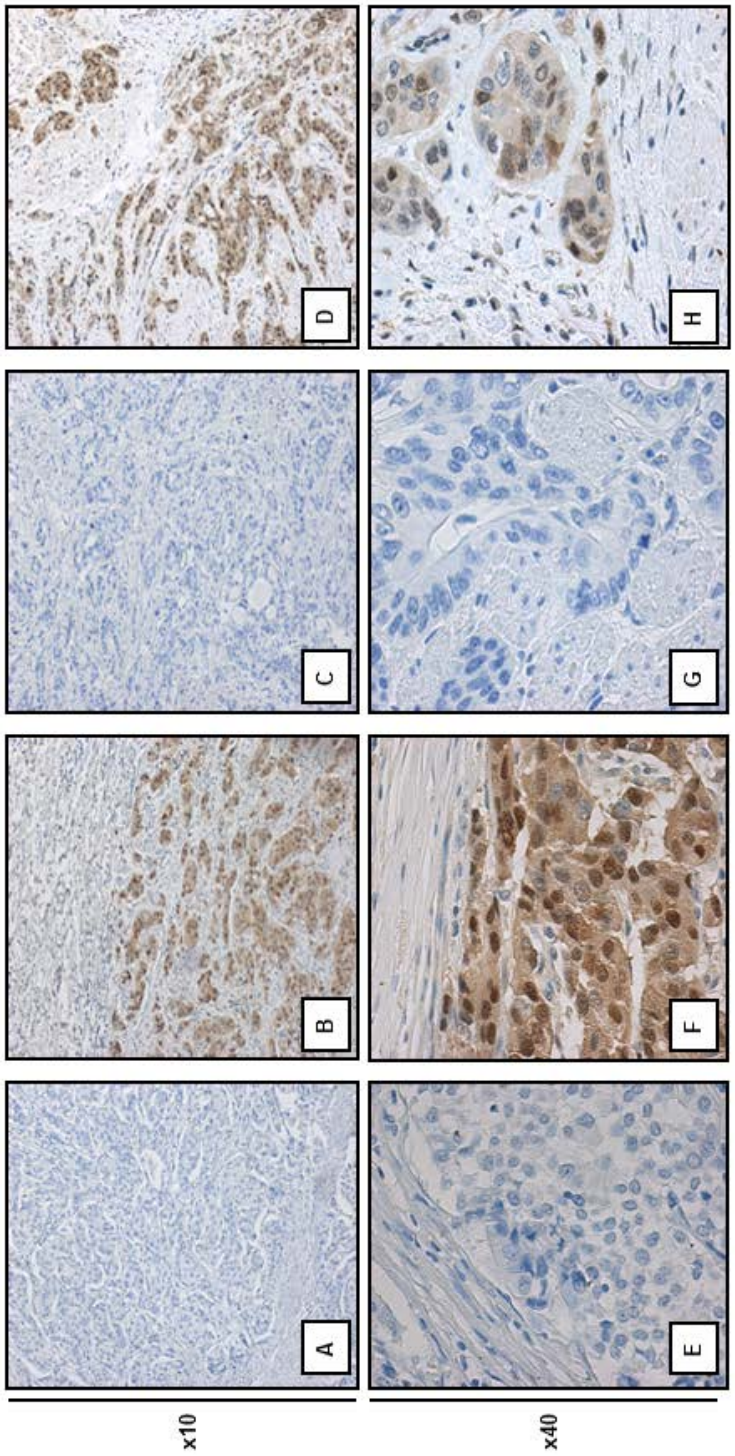

Figure 5.16 Immunohistochemistry using oesophageal adenocarcinoma whole sections and pan-isoform anti-BAG-1 antibody 3.10G3E2 (1:800).

A and E; TBS control in breast adenocarcinoma; B and F Strong nuclear and moderate cytoplasmic immunoreactivity for BAG-1 in breast adenocarcinoma; C and G; TBS control in oesophageal adenocarcinoma; D and H; Strong nuclear and weak cytoplasmic immunoreactivity in oesophageal adenocarcinoma. Sections are shown with whole ER+ breast adenocarcinoma sections as positive controls.

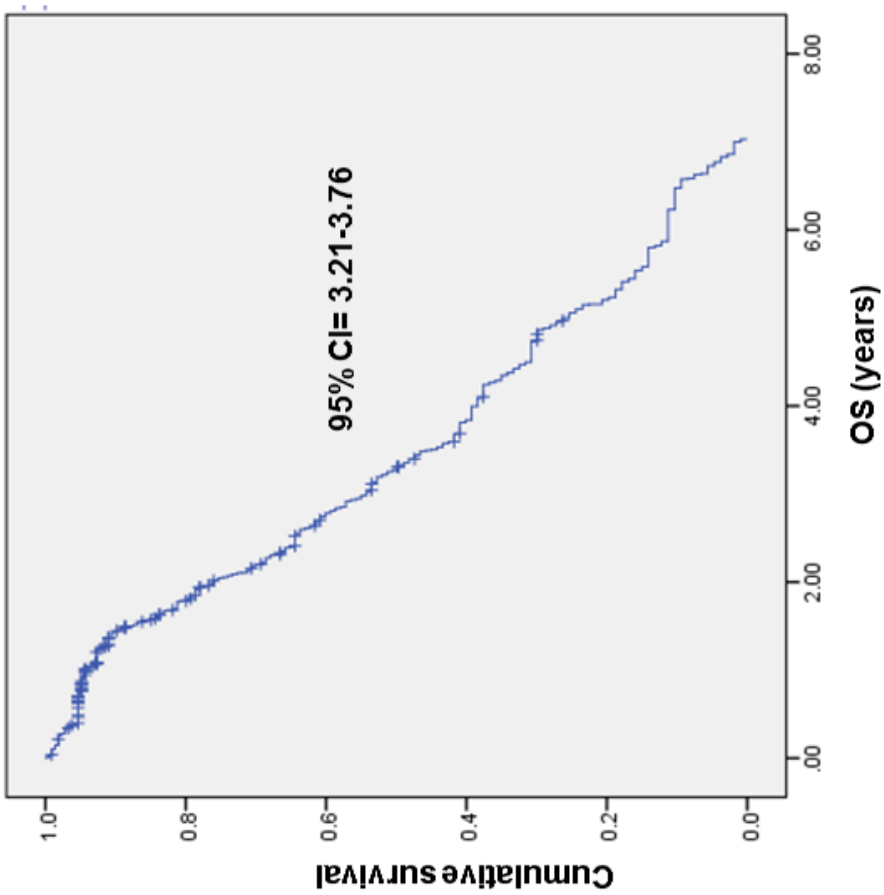

Figure 5.17: Kaplan-Meier curve showing overall survival (OS) and cumulative survival for post-operative oesophageal carcinoma cohort.

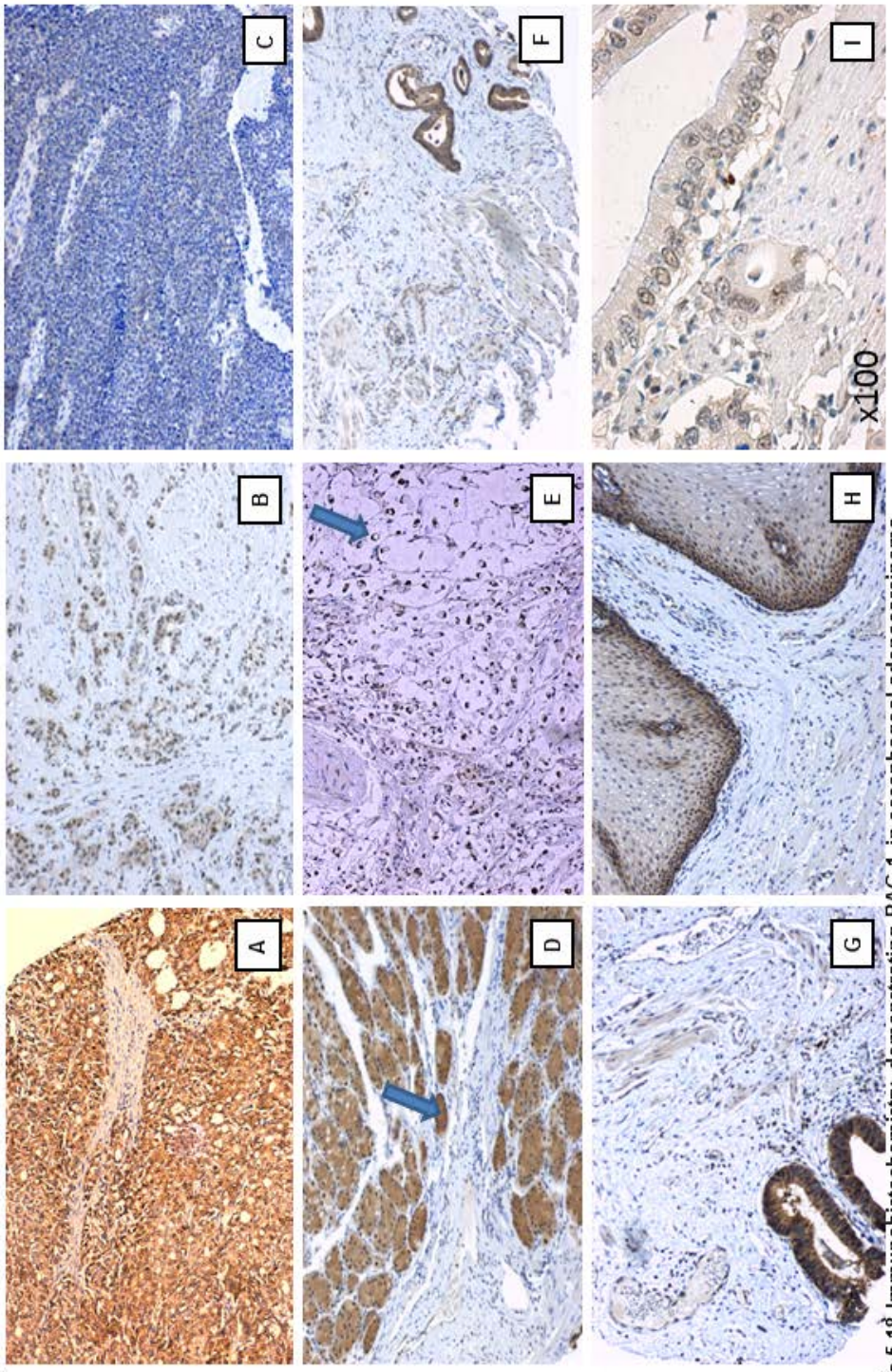

Figure 5.18 Immunohistochemistry demonstrating BAG-1 in oesophageal adenocarcinoma.
Pan-isoform anti-BAG-1 antibody 3.10G3E2 was used to detect BAG-1 expression in tissue microarray sections incorporating tissue from 216 post-operative patients diagnosed with oesophageal adenocarcinoma. Antigen retrieval was performed with microwave pressure cooker and citrate buffer. All sections were blocked using 10% FBS for 30 minutes at room temperature prior to incubation with primary antibody for 12 hours at 4°.A: strong nuclear and cytoplasmic immunoreactivity for BAG-1; B: Strong nuclear immunoreactivity for BAG-1 with weak cytoplasmic immunoreactivity; C: Negative core or BAG-1; D: Moderate cytoplasmic and nuclear immunoreactivity for BAG-1 in deep crypts (arrow) of normal gastric mucosa (crypts are shown in cross section); E Strong nuclear immunoreactivity for BAG-1 in signet ring cells (arrow) in oesophageal adenocarcinoma; F: Strong cytoplasmic and moderate nuclear immunoreactivity for BAG-1 in oesophageal adenocarcinoma; G: Strong cytoplasmic immunoreactivity for BAG-1 in glandular epithelium in oesophageal adenocarcinoma; H: Moderate and weak cytoplasmic immunoreactivity of benign oesophageal basal epithelium; I : x100 magnification of TMA core demonstrating moderate nuclear and weak cytoplasmic immunoreactivity for BAG-1 in oesophageal adenocarcinoma.

Figure 5.19 Xtile™ survival plot.
Xtile™ survival plot using cut-off points derived from statistical analysis of BAG-1 expression in oesophageal TMA to demonstrate survival in patients with high, moderate, and low BAG-1 immunoreactivity (Table A). The Xtile ™ survival plot has been extrapolated beyond 3.5 years as beyond this period <75% of survival data was available for the cohort.

	Count or median	% or range		Count or median	% or range
Age at operation	67.29	(36.99-85.41)			
	181	83.8	Neoadjuvant regime		
Sex Male	35	16.2	EOX	22	17.9
Female			ECX	97	78.9
O-Possum	21	9.8	ECF	3	2.4
ASA 1	180	74.4	CRT	1	0.8
2	35	15.8	TRG1	14	8.5
3	20	9.3	TRG2	17	7.9
Pre-op T0	13	6	TRG3	17	7.9
T1	41	19	TRG4	41	19
T2	134	62	TRG5	33	15.3
T3	8	3.7	Surgery only	94	43.5
T4	56	30.6			
Pre-op N0	150	69.4	Resection Margin		
N1	213	98.6	Positive	41	19
Pre-op M0	3	1.4	Negative	175	81
M1a					
Treatment	93	43.1	Vascular Invasion		
Surgery only			Yes	71	32.9
Neoadjuvant and surgery	123	56.9	No	145	87.1
			Lymphatic Invasion		
Operation	49	22.7	Yes	34	15.7
IVL	50	23.1	No	182	84.3
MIO-2	35	18.2			
MIO-3	41	19			
LTA	1	0.5	Perineural Invasion		
LTA + left cervical	40	18.6	Yes	28	13
Transhiatal			No	188	87
			Tumour Diameter (mm)	25	(0-110)

Table 5.2 Patient data for post-operative oesophageal adenocarcinoma cohort. N = 216

		Nuclear			Cytoplasmic			Overall		
		Quick score	No. Cores	Missing	Quick score	No. Cores	Missing	Quick score	No. Cores	Missing
pT or ypT TNM7	T0	0	2	1	0	2	1	0	2	1
	T1	12	25	7	8	25	7	72	25	7
	T2	8	17	2	12	17	2	64	17	2
	T3	12	31	2	12	31	2	144	31	2
	T4	12	5	0	12	5	0	144	5	0
	TIS		0	0	.	0	0		0	0
pN or ypN TNM7	N0	12	37	7	12	37	7	144	37	7
	N1	12	22	2	12	22	2	144	22	2
	N2	8	13	3	12	13	3	96	13	3
	N3	12	8	0	12	8	0	144	8	0
pM or ypM TNM7	M0	12	80	12	12	80	12	144	80	12
	M1		0	0	.	0	0		0	0
R0 (>1mm)	+ve	12	16	0	12	16	0	144	16	0
R1 (< or =1mm, these are all CRM)	-ve	12	64	12	12	64	12	144	64	12
Vascular Invasion	Yes	12	27	4	12	27	4	144	27	4
	No	12	53	8	12	53	8	144	53	8
Lymphatic Invasion	Yes	12	10	1	8	10	1	64	10	1
	No	12	70	11	12	70	11	144	70	11
Perineural Invasion	Yes	8	9	1	12	9	1	96	9	1
	No	12	71	11	12	71	11	144	71	11
Grade	G1	9	6	1	6	6	1	54	6	1
	G2	12	25	4	12	25	4	108	25	4
	G3	12	49	7	12	49	7	144	49	7
	G4		0	0		0	0		0	0
	GX		0	0		0	0		0	0

Table 5.3 Results of immunohistochemical study of BAG-1 (using 3.10G3E2) pan-isoform anti-BAG-1antibody) and clinicopathological variables in patients diagnosed with oesophageal adenocarcinoma treated with surgery alone. 'Quick score' refers to the maximum quick score calculated by multiplication of intensity of BAG-1 immunoreactivity (1:weak-3:strong) by percentage of immunoreactive tumour (0-25%:1, >25-50%:2, >50-75%:3, >75-100%:4. 'Missing' corresponds to incomplete or damaged cores excluded from data analysis. (CRM: clear radial margin)

Test Statistics [a]

	Nuclear	Cytoplasmic	Overall
Mann-Whitney U	329	485	377
Wilcoxon W	407	563	455
Z	-2.029	-.219	-1.504
Asymp. Sig. (2-tailed)	**.042**	.827	.132

a. Grouping Variable: Response Tumour Regression Grade

Table 5.4 Statistical analysis comparing tumour regression grade and the maximum Quick Score for nuclear, cytoplasmic and overall BAG-1 immunoreactivity of cores taken from post-operative patients diagnosed with oesophageal carcinoma.

Chapter 6: Discussion

Chapter 6: Discussion

6.1 BAG-1 overexpression versus targeting of BAG-1 using siRNA in breast cancer

Following Yang et al.'s (1998) report that BAG-1 overexpression was found in breast cancer an abundance of evidence has arisen in literature to suggest BAG-1 overexpression has a functional role in breast cancer (Yang et al., 1998). The majority of studies in breast cancer cells used BAG-1 overexpression as a tool to propose specific functions for BAG-1 isoforms. Also approximately a dozen studies have performed retrospective analysis of potentially associated clinicopathological parameters and BAG-1 overexpression in breast cancer tissue.

To attempt to characterise BAG-1 isoform function in HER2-positive breast cancer cells, isoforms were transiently overexpressed following transfection of BAG-1 isoform constructs. The results showed increased metabolic activity using MTS assays of cultures overexpressing the longer isoforms BAG-1L and BAG-1M compared to BAG-1S and the pcDNA3 control. Cells from the same population were counted using a CASY® Counter at defined time points over 6 days to show differences between isoforms in cell yield. The cells overexpressing BAG-1L and BAG-1M were associated with a higher cell yield than the cells overexpressing BAG-1S or the pcDNA3 control. In summary, overexpression of BAG-1 isoforms, BAG-1L and BAG-1M, increases both cell viability and cell yield of HER2-positive SKBR3 breast cancer cells (Figure 6.1).

A recent study has shown an increase in BAG-1S is related to decreased cellular proliferation in keratinocycte HaCaT organotypics, whilst BAG-1L overexpression produces an increase in cell layers (Hinitt et al., 2010). The same research group previously published a report showing BAG-1 overexpression in epidermal squamous cell carcinoma is associated with poorly differentiated tumours and resistance to chemotherapy-induced apoptosis (Wood et al., 2011). These results contrast to the improved prognosis associated with BAG-1 expression in breast cancer in both Oncotype Dx™ and previous immunohistochemical studies (Cutress et al., 2003; Sirvent et al., 2004; Tang et al., 2004; Townsend et al., 2003; Turner et al., Nadler et al., 2008; Millar et al., 2009).

BAG-1S may have a distinct role in HER2-positive breast cancer cells due to its predominantly cytosolic localisation. BAG-1S has been reported as the most abundant isoform in MCF-7 cells, and Yang et al. (1999) showed similar results for SKBR3 and BT-474 cells (Yang et al., 1999). This study showed similar levels of endogenous BAG-1S expression in SKBR3, BT-474 and MCF-7 cells. Western blotting was used to demonstrate BAG-1S expression is elevated in SKBR3 cells in response to 50μM trastuzumab. An increase in BAG-1L rather than BAG-1S expression was reported using Western blots at 8 and 24 hours following lapatinib, but no change in expression compared to untreated control at 48 hours. However, exposure of SKBR3 cells overexpressing BAG-1S to 48 hours of lapatinib did result in an increase in cell yield compared to other BAG-1 isoforms or pcDNA3 control. Similar results were not obtained using MTS assays to assess relative cell viability after lapatinib. BAG-1S overexpression in BT-474 cells was shown in this study to be associated with reduced clonogenicity compared to cells overexpressing other BAG-1 isoforms. The ability of a cell to form colonies is essential for survival and an essential part of tissue growth and repair. BAG-1S overexpression in human epidermal HaCaT cells has been shown to decrease cell yield and inhibit wound repair (Hinitt et al., 2010).

BAG-1S is known to competitively bind Hsp/Hsc70 rather than Raf-1 and in response to stress rather than under normal conditions (Townsend et al., 2003). HER2 signalling involves the Raf-1 ERK kinase pathway, rather than interaction with Hsc70/Hsp70. Hs578T (ER-negative, HER2-negative) and MCF-7 breast cancer cells overexpressing BAG-1L and BAG-1M have been shown to resist apoptosis in response to doxorubicin, docetaxel or 5-FU compared to those overexpressing BAG-1S (Liu et al., 2009a). Therefore, if survival mechanisms in HER2 positive cancers rely upon interaction with Raf-1 and mediation of ERK kinase signalling, perhaps BAG-1S has a more dominant role. Wang et al. (1996) showed BAG-1S and Raf-1 interact using binding assays and BAG-1S enhanced Raf-1 kinase activity (Wang et al., 1996). BAG-1 has been shown to form tripartite complexes with Raf-1 and Akt which are important in neuronal and haematopoietic cell survival (Gotz et al., 2005). Therefore, one hypothesis is that BAG-1S moderates the response of HER2 –positive cells to HER2-family inhibition via mediation of Raf-1 and Akt activity.

The initial study design involved producing stable clones overexpressing BAG-1 isoforms, however few previous studies had achieved this and, after several failed attempts, methods were moderated to use transiently overexpressing cells via transfection of BAG-1 isoform constructs. The advantage of using overexpression as a method of functional analysis in

breast cancer cells is that outcomes can be shown to be isoform-specific, in contrast to siRNA studies. Townsend et al. (2003) showed transient overexpression of BAG-1S in MCF-7 cells after transfection of BAG-1S construct resulted in increased tolerance to heat shock (Townsend et al., 2003). Cutress et al. (2003) transiently transfected MCF-7 cells with BAG-1 isoform constructs or pcDNA3 to elucidate the effect of BAG-1 isoform overexpression on ER-dependent transcription (Cutress et al., 2003). Reporter assays showed co-expression of BAG-1L and a luciferase reporter gene markedly increased ER-dependent transcription in the presence of 17-β-oestradiol. MCF-7 cells overexpressing BAG-1L demonstrated increased sensitivity to oestrogen compared to pcDNA3 control or other BAG-1 isoforms (Townsend et al., 2003).

The majority of previous studies have focused on ER-positive breast cancer, none have looked specifically at the role of BAG-1 isoform overexpression in HER2-positive breast cancer cells. Overexpression studies using transient transfection of isoform constructs do have limitations. The overexpression is transient and the length of any effect due to overexpression may be difficult to determine. To address this, Western blots were performed to check BAG-1 overexpression was present at defined time points.

To attempt to strengthen the hypothesis that overexpression of BAG-1 increases cell yield in HER2-positive breast cancer cells, BAG-1 expression was reduced using siRNA. Knockdown experiments using BAG-1 siRNA have previously been performed in breast cancer studies (Berry et al., 2008), but without focus on HER2 positive breast cancer cells. Treatment of SKBR3 cells with either siRNA knockdown of BAG-1 or trastuzumab alone produced decreased relative cell viability on MTS assay and simple cell yield assay. The combination of siRNA targeting of BAG-1 and exposure to trastuzumab produced a greater reduction of cell viability, but no such additive effect was shown by cell yield assay. The inconsistency of results between methods could be explained by cells which are not counted in the simple cell yield assay but produce an adequate reaction with MTS to increase relative cell viability. The limitation of this method was that BAG-1 is derived from a single mRNA, therefore it is impossible to derive further information about isoform-specific function of BAG-1. These results are contrasting to the results of the overexpression studies in Chapter 3, but are limited as are using a single cell line and are inconsistent in different biological assays.

Additional work using siRNA knockdown of HER2 and assessing the levels of BAG-1 expression by Western blotting may provide stronger evidence for the role of BAG-1 in

HER2-positive breast cancer. Ideally, experiments should be performed in a comprehensive assortment of HER2-positive cell lines, with the aim of investigating interesting results in HER2-overxepressing transgenic mice.

6.2 Endogenous BAG-1 expression is increased in response to trastuzumab in HER2-positive SKBR3 and BT-474 cells.

An initial MTS assay performed to ascertain the optimal dose of trastuzumab for use in biological assays produced an unexpected result. Exposure to 25-50μM trastuzumab for over 24 hours produced elevated expression of total BAG-1.This observation implies crosstalk and common signalling pathways may exist between BAG-1 and HER2. Immunocytochemistry investigating BAG-1 following 8-48 hours treatment with trastuzumab confirmed an increase in immunofluorescence relative to BAG-1 in SKBR3 cells. Unsurprisingly, HER2-negative MCF-7 cells showed no change in BAG-1 expression following trastuzumab treatment. This elevation of BAG-1 expression was confirmed by immunoblots, which revealed a specific increase in the p50 isoform with trastuzumab. Interestingly, there was also an increase in BAG-1L in the BT-474 cells treated with trastuzumab and a noticeable pattern of nuclear exclusion in immunofluorescence experiments following lapatinib not shown in SKBR3 cells. Previous studies have shown in HER2 negative, ER positive MCF-7 cells that BAG-1S overexpression results in a survival advantage over controls following cellular stress (Townsend et al., 2003). Townsend et al. (2003) did not investigate BAG-1S expression in HER2-positive cells or ER-negative cells, therefore it is possible that BAG-1S would play an additional role in BT-474 cells which are ER-positive. This study has not focused on the differences seen between ER-positive (BT-474 cells) and ER-negative (SKBR3 cells) HER2-positive cells, but this would be an area to focus on for future experiments as there is known crosstalk between ER and HER2 signalling (Arpino et al., 2008).

The exclusion of BAG-1 from nuclei seen in BT-474 cells following lapatinib suggests a change in protein subcellular localisation. It is possible that the response of BT-474 cells to lapatinib is subsequent to BAG-1-mediated changes in subcellular localisation of Raf-1 and Akt. A lack of BAG-1 expression in mice results in dissociation of BAG-1/Raf-1/Akt tripartite complexes, an absence in phosphorylation of BAD at serine136, and decreased neuronal and haematopoietic cell survival (Gotz et al., 2005). Chapter 4 reports a decrease in

downstream phospho-Akt and phospho-BAD expression following thioflavin S and trastuzumab in BT-474 cells, but these experiments were not repeated using lapatinib. Subcellular fractionation could be performed prior to Western blotting to confirm the localisation of BAG-1 isoforms, Raf-1 and Akt. Propidium iodide and FACS could be used to assess relative cell survival in addition to MTS assays.

The value of these experiments may be limited as endogenous levels of BAG-1 are increased in these cancer cell lines, therefore are also representing BAG-1 overexpression. An alternative method of studying the effects of BAG-1 expression in the response of HER2-positive breast cancer to trastuzumab would be to use trastuzumab-resistant cells (Boyer et al., 2013). Levels of BAG-1 could be assessed by Western blot in trastuzumab-resistant cells and cell yield and viability assays repeated. Immunocytochemistry using immunofluorescence may be used to show no increase in levels of BAG-1 with trastuzumab in trastuzumab-resistant cells compared to wild type.

6.3 Overexpression of all BAG-1 isoforms increases cell yield and viability in HER2-positive SKBR3 breast cancer cells exposed to trastuzumab.

SKBR3 cells transiently overexpressing BAG-1 isoforms result in an increase in cell yield and viability following exposure to trastuzumab compared to cells transfected with pcDNA3 control (Figure 6.2). Previous evidence exists for a role for BAG-1 in proliferation (Song et al., 2001, Sharp et al., 2009) and in survival (Kudoh et al., 2002, Townsend et la., 2003, Liu et al., 2009), as discussed in chapter 1, although no previous study focused on HER2-positive cancers. This study reports a novel effect of BAG-1 isoform overexpression in increasing cell yield and viability on exposure to trastuzumab in SKBR3 cells, thus suggesting BAG-1 may be involved in resistance to anti-HER2 therapy. BAG-1 may be a potential therapeutic target in resistant HER2-positive breast cancer.

6.4 The functional effect of overexpression of BAG-1 is specific to HER2 inhibitors, but increased expression of endogenous BAG-1 occurs with inhibition of other EGFR family receptors.

The increase in BAG-1 expression seen in HER2 positive cells was not specific to trastuzumab, but evident following treatment with lapatinib (Tyverb™) on Western blotting. Lapatinib is an EGFR and HER2 inhibitor, and causes downregulation of signalling pathways

common to both. Chapter 3 shows an interesting pattern of nuclear exclusion of BAG-1 in BT-474 cells following lapatinib not present in SKBR3 cells following trastuzumab. This may be due to the presence of ER in BT-474 cells and perhaps blockage of EGFR (HER1) leading to mediation of BAG-1 interaction with NHR. Further Western blots are required to investigate BAG-1 isoform expression in BT-474 cells following lapatinib. BAG-1L expression was increased compared to untreated vector control at 24 hours in SKBR3 cells following lapatinib. The HER2 receptor has no ligand of its own and relies on forming heterodimers with other EGFR family receptors. To begin to investigate the specific function of EGFR in the response of HER2-positive cells to HER2-targeting therapy, immunoblots following treatment with the EGFR inhibitor, erlotinib were performed. Increased expression of endogenous BAG-1 occurred with exposure to erlotinib, but BAG-1 overexpression did not alter cell yield or cell viability in cells treated with erlotinib (Table 6.1).

BAG-1 expression increased with trastuzumab in SKBR3 cells, but not in MCF-7 cells. These results suggest that the elevation of BAG-1 in HER2-positive cells occurs with inhibition of other EGFR family members and is not exclusive to targeting HER2, but the functional effect of BAG-1 overexpression, increasing cell yield in response to trastuzumab, is unique to exposure to HER2-inhibitors. These results are summarised in Table 6.1.The significance in understanding the individual parts played by EGFR family receptors has become apparent as resistance to monotherapy targeting EGFR or HER2 alone has been shown in breast and other cancers to occur in over 50% of patients (Bardelli et al., 1996; Hynes et al., 2009).

A potential explanation for the results obtained using ER-positive BT-474 cells is the existence of crosstalk between ER and HER2. Shou et al. (2004) used reporter gene assays and immunoblotting of phosphorylated signalling proteins to demonstrate increased crosstalk in Tamoxifen-resistant MCF7/HER2 cells compared to MCF-7 cells (Shou et al., 2004). Tamoxifen resistance and an increase in ER activity may occur in cells overexpressing the co-activator AIB1 or HER2. An increase in activated forms of HER2/EGFR, Akt and MAPK signalling proteins was observed in Tamoxifen-resistant MCF-7/HER2 cells compared to MCF-7 cells. Initial treatment with the EGFR inhibitor Gefitinib prior to Tamoxifen resulted in a decrease in crosstalk, a decrease in ER transcriptional activity, and restoration of tumour growth inhibition. To investigate the role of BAG-1 in ER/HER2 crosstalk, immunoblots of BAG-1, Raf-1, phospho-Akt and phospho-ERK1/2 could be performed using HER2-positive/ ER-positive cell lines and MCF-7s with and without Tamoxifen. Immunoblots of BAG-1 and

upstream and downstream signalling proteins could then be repeated in BT-474 cells treated with EGFR inhibitors, alone, or in combination with, Tamoxifen. A potential hypothesis could be that BAG-1 could potentiate EGFR inhibition via mediation of Akt activation in ER/HER2-positive cells also treated with Tamoxifen. Further cell viability and cell yield assays could be performed to compare the effect of EGFR inhibition on BT-474 cells overexpressing BAG-1 to SKBR3 and MCF-7 cells. BT-474 cells naturally express high levels of AIB 1 and HER2, known to be associated with resistance to Tamoxifen (Shou et al., 2004). BAG-1 overexpression has been shown to increase ER transcriptional activity and response to Tamoxifen (Cutress et al., 2003). Therefore, the decreased response of BT-474 cells to Tamoxifen could also be altered by BAG-1 overexpression and cross-phosphorylation of ER/ HER2 signalling proteins.

6.5 The combination of trastuzumab and thioflavin S shows a decrease in cell viability in HER2-positive breast cancer cells.

Exposure of SKBR3 cells to trastuzumab and the small molecule BAG-1 inhibitor thioflavin S in combination resulted in additive reduction in cell viability shown by crystal violet assay (Figure 6.3). This suggests a common functional signalling pathway exists between BAG-1 and HER2 and an area for potential therapy development.

The PI3K pathway has been implicated in the development and progression of breast cancer by numerous studies and is the target of several drugs in breast cancer (Baselg et al., 2011). Chapter 4 shows additive downregulation of phospho-Akt expression following treatment with a combination of trastuzumab and thioflavin S. Phospho-Akt levels are not decreased by either drug alone. This suggests a common signalling pathway exists and permits crosstalk between BAG-1 and HER2. Previous studies have shown that trastuzumab-resistant BT-474 cells, produced by continuous culture in trastuzumab-containing medium, demonstrate increased expression of phospho-Akt compared to the parental cell line (Chan et al., 2005). The reduction of phospho-Akt seen in this study in the response of BT-474 cells to trastuzumab and thioflavin S suggests activation of Akt is may be moderated indirectly by BAG-1.

6.6 The role of BAG-1 as a biomarker in HER2-positive cancer

Chapter 5 supports previous evidence that fundamental validation steps preceding the use of TMA in biomarker studies in breast cancer should be taken. Chapter 5 demonstrates there may be discrepancy in immunohistochemical studies of BAG-1 between those using TMA and whole tissue sections. Several studies exist validating the use of BAG-1 as a biomarker in breast cancer (Chapter 1), using both whole sections and TMA. None of these studies include detail of optimisation steps, number of cores used per tumour, heterogeneity between cores, or whether results were correlated with a pre-validated cohort of whole sections. The protocols for immunohistochemical study of BAG-1 were optimised in this study for use in whole sections and TMA. Initial validation steps were taken using a treatment-standardised test cohort of whole sections of ER-positive breast cancer from the TEAM Trial. Unfortunately, the optimised protocol still resulted in an excess of non-specific stromal immunoreactivity, prompting assessment of BAG-1 expression in alternative HER2-positive cancers.

Analysis should be automated, ideally pre-validated by several independent human scorers subject to correlation and reproducibility statistics (Kirkegaard et al., 2006). Automated analysis is still somewhat in its infancy, as most systems rely upon tumours being ‘marked’ prior to image analysis by a human observer, and cannot differentiate between a cancer and benign cell. Proteins with multiple isoforms, especially those with both nuclear and cytoplasmic isoforms are also difficult to analyse (Camp et al., 2008), although would be overcome with the development of isoform-specific antibodies.

TMA studies were originally validated for use in breast cancer by Robert Camp in 2000. Camp recommended the use of just 2 cores per breast tumour, reporting in 95% of cases these cores would be representative of whole sections (Camp et al., 2000). Zhang et al. (2003) reported a single 0.6mm core was concordant with, and representative of, protein expression in whole sections of breast tumours (Zhang et al., 2003). Later studies have recommended 3-4 cores per tumour should be used in biomarker studies to account for tumour heterogeneity (Pacifico et al., 2004). However, Tennstedt et al. (2012) have demonstrated that increasing the number of cores does not always lead to strengthened association between biomarkers and cancer phenotype in prostate cancer. Tennstedt et al. (2012) suggest that increasing the number of cores used necessitates an increase in work

and tissue required, and may lead to statistical error if the same amount of tissue is not analysed per core (Tennstedt et al., 2012). There is certainty that biomarker studies using TMA need to incorporate standardised immunohistochemical techniques, be pre-validated on whole sections, ideally with statistics to demonstrate the optimal number of cores required to accurately represent whole sections from the study cohort. Research aiming to validate novel biomarkers should adhere to the criteria outlined in the REMARK study (McShane et al., 2005). To date, there is not an immunohistochemical study of BAG-1 which meets all of these criteria.

Chapter 4 demonstrated a lower cell yield using HER2-positive SKBR3 cells overexpressing BAG-1S than the larger BAG-1 isoforms, findings supported by recent reports on BAG-1 expression in skin keratinocytes (Hinitt et al., 2010). Overexpression of longer BAG-1 isoforms over BAG-1S, and a more significant functional role in proliferation in HER2 positive cancers, may help identify aggressive tumours. This hypothesis needs to be investigated via immunohistochemical study using a BAG-1L-specific antibody of a large cohort of HER2 positive breast cancer patients. To date, one study using microarray analysis of 782 node-negative breast cancer patients has shown positive correlation with HER2 overexpression and Bcl-2 family proteins, including BAG-1 (Petry et al., 2010). Survival outcomes or treatment data were not specifically correlated with BAG-1 expression in this study.

To attempt to assess if BAG-1 expression was associated with clinicopathological outcomes and HER2-status, BAG-1 expression was studied in bladder and oesophageal cancer. BAG-1 expression has been previously associated with an increase in tumour aggression and poor prognosis in oesophageal squamous cell carcinomas (Takeno et al., 2007, Noguchi et al., 2003). Expression of BAG-1 has not been previously studied in oesophageal adenocarcinoma. Downregulation of BAG-1 using siRNA in oesophageal squamous cell carcinoma resulted in resistance to radiotherapy (Yoshino et al., 2010).

This study used tissue from 216 post-operative patients diagnosed with oesophageal adenocarcinoma and followed up for 5 years. Tumours were incorporated into a tissue microarray using triplicate cores and immunohistochemical analysis of BAG-1 was performed using the pan-isoform anti-BAG-1 antibody 3.10G3E2. Sub-group analysis revealed a correlation between increased levels of nuclear BAG-1 expression and advanced

tumour regression grade (p=0.042). An interesting observation was the dense amount of nuclear Bag-1 observed in signet ring cells. Signet ring cells in oesophageal adenocarcinoma are associated with aggressive tumours and poor prognostic outcome (Yendamuri et al., 2013). No correlation could be found between BAG-1 levels and survival outcomes using SPSS.

Analysis of survival data and correlation with IHC score of BAG-1 expression was repeated using Xtile™, Xtile™ allows definition of cut-off points for low, medium and high levels of a biomarker. The IHC data is then analysed according to these cut-off points and correlated with survival data to produce an Xtile™ plot. Log-rank tests are then used to compare each level of biomarker with survival data. Medium versus high levels of BAG-1 showed a significant difference in survival. The limitation of this method is that to reach significance the follow-up period was reduced to 3.5 years and only 87 tumours had both complete follow-up data and triplicate cores from the initial cohort of 216 patients.

In summary, BAG-1 has not been established as a valid biomarker in HER2-positive breast cancer and further work is required. However, a novel BAG-1L isoform-specific antibody has been optimised for use on both breast cancer tissue whole sections and microarray. The pan-isoform anti-BAG-1 antibody requires further optimisation prior to use on a large pre-validated cohort. This work has described immunohistochemical patterns of immunopositivity for BAG-1 in bladder adenocarcinoma, oesophageal adenocarcinoma, and normal gastric and oesophageal glandular epithelium. This study has shown an association between nuclear BAG-1 and advanced tumour regression grade in patients diagnosed with oesophageal adenocarcinoma, suggesting BAG-1 may be a valid biomarker in other cancers known to overexpress HER2.

6.7 Future Work

BAG-1 expression and function has been investigated previously in breast cancer, but not with a focus on HER2 positive cancer. BAG-1 has been shown to have an array of functions within the breast cancer cell and potential mechanisms have been discussed. The role of BAG-1 as a biomarker in breast cancer needs still to be clarified, despite being involved in a

commercial diagnostic tool, Oncotype Dx™. Future studies should employ standardised immunohistochemical techniques, pre-validated whole sections incorporated into tissue microarrays, and focus on specific controlled patient cohorts.

Future immunohistochemical work from this study will aim to first further establish BAG-1 as a biomarker in a controlled cohort of ER positive breast cancer patients, and eventually HER2 positive patients. Prior to this, the 3.10G3E2 antibody must be optimised further. The 3.10G3E2 antibody is from mouse ascites and one proposed mechanism for purification is by using highly purified protein A coupled to agarose beads. The antibody is captured on the resin and unwanted substances are removed by a simple wash procedure. The purified product is then eluted and neutralized. To remove any low molecular weight substances which may interfere with the purified antibody, commercial antibody concentration kits are available which use simple spin columns (Abcam).

To address the problems we have found with the preliminary immunohistochemical experiments incorporating tumour microarrays, we will attempt to purify the current G310E2 BAG-1 antibody, whilst consecutively trialling any alternatives. In addition, the BAG-1L-specifc antibody, 3C12, should be used in a large treatment- standardised cohort of HER2-positive breast cancer patients and correlated with complete survival data. Subcellular fractionation and immunoblotting should be performed to demonstrate the 3C12 antibody is truly nuclear isoform-specific prior to use of a large tissue cohort.

The aim of future work will be to expand upon knowledge of potential functional mechanisms behind the up-regulation of BAG-1L following HER2-targeting therapy. Small molecule inhibitors, developed in our laboratory (the work of E.S. Papadakis), will be used in both cells expressing endogenous BAG-1 alone and those incorporating CMV plasmid-dependent overexpression of BAG-1. Inducible BAG-1 overexpressing HER2+ cells using a Tet-On T-REx System™ (Invitrogen) could be used to assess the impact of BAG-1 upregulation on growth and survival. The effect of synergistic BAG-1 and HER2 targeted therapies on EGFR, MAPK, PI3K, and downstream signalling proteins needs to be further investigated by serial Western blotting. An increase in apoptosis following trastuzumab has been reported in core biopsies from 35 patients with advanced localised HER2-overexpressing breast cancer (Mohsin et al., 2005). Immunohistochemical staining of cleaved caspase 3 was used to

determine apoptotic index in tissue samples. A 35% increase in apoptosis was shown in biopsies after 1 week of trastuzumab therapy. The authors were unable to reproduce these results in experiments using cell lines. Interestingly, this study also found no significant difference in tumour biopsies in phospho-Akt, or Ki67 following trastuzumab. These results contrast with the in vitro work presented in Chapter 3 and may represent the effect of the tumour environment in vivo. There was, however, a small decrease in phospho-MAPK following trastuzumab in breast cancer biopsies after 3 weeks of trastuzumab. This finding is concordant with reports that siRNA knockdown of MAPK1 (ERK2) reverses resistance to trastuzumab (Boyer et al., 2013). These reports offer some support of our earlier hypothesis that a reduction in BAG-1 may potentiate the action of trastuzumab by inhibition of ERK1/2 signalling.

Further MTS assays, immunoblots for caspase and PARP, Annexin V staining and FACS, and immunohistochemistry of proliferation markers, such as BrdU and Ki-67, are required. These experiments preformed on multiple HER2-overxepressing cell lines could clarify the effects of BAG-1 on proliferation and apoptosis following trastuzumab. This study has used several techniques to look at potential functions of BAG-1 following different chemical agents. However, the work has only been hypothesis-generating, and future work should focus initially on one function of BAG-1 following a single agent.

This study has suggested there is up-regulation of BAG-1 following trastuzumab and a subsequent increase in cell yield in SKBR3 HER2-positive cells (Figure 6.2). The inhibition of BAG-1 following thioflavin S was shown to further decrease cell viability in BT-474 cells exposed to trastuzumab. ER-positive BT-474 cells also showed an increase in BAG-1 on exposure to lapatinib and a change in subcellular localisation. Future studies could use siRNA knockdown of HER2 and BAG-1 to investigate cross-talk with the ER signalling pathways in HER2/ ER-positive cells. Use of BAG-1 mutants with mutations in the Hsc/Hsp70 binding domain and immunoblotting for phosphorylation of downstream signalling proteins could help identify a mechanism. Compensatory signalling via the MAPK pathway has been proposed as a mechanism involved in trastuzumab resistance. An extended hypothesis could state that BAG-1 mediates interaction between Akt/ Raf-1 and nuclear receptors in response to HER2 family inhibition, subsequently altering compensatory MAPK signalling. In addition, identifying compensatory signalling mechanisms between EGFR family members and ER may become important in understanding how BAG-1 inhibition may enhance the action of HER2-inhibitors.

Preliminary data and a previous microarray study by Petry et al. (2010) have implicated Bcl-2 family members in cellular response to HER2 signalling (Petry et al., 2010). To elucidate whether the elevation of BAG-1L in HER2 positive cancer cells is due to induction, a change in regulation, or a decrease in protein degradation, future study work should incorporate pulse chase assays or cyclohexamide blocking of translation.

The evidence for correlation between BAG-1 mRNA, BAG-1 protein expression, and survival outcomes is contrasting. The majority of evidence suggests no concordance between BAG-1 mRNA, BAG-1 protein expression and survival data. qPCR was used to demonstrate the increase in BAG-1 protein expression seen following anti-HER2 therapy was accompanied by an increase in BAG-1 mRNA. An increase in BAG-1 mRNA following trastuzumab could be due to an increase in post-transcriptional mRNA stability rather than induction of transcriptional activity (Kaarniranta et al., 1998). Reporter gene assays, microarray analysis, and reverse transcriptase PCR (RT-PCR) could be used to distinguish between an increase in transcription and post-transcriptional increase in mRNA stability (Arava et al., 2003; Tong et al., 1999). Transient transfection of reporter genes into cells and radiolabelled transcripts could be used in pulse-chase experiments to determine mRNA decay (Blaxall and Port, 2000). A further limitation of this study is that mRNA and protein levels are most often concordant in cancer cell cultures (Lundberg et al., 2010). In vivo, cells are subjected to continuous chemical changes in their environment essential for tissue homeostasis and development (Bissell et al., 2002).

Future work would aim to test hypotheses by using tetracycline-inducible expression of BAG-1 in mice xenografts incorporating HER2-positive breast cancer cells. Tumour weight, Ki67, cleaved caspase 3, and BrdU could be assessed in the presence and absence of trastuzumab and, or thioflavin S. The role of BAG-1 following trastuzumab could then be assessed further using HER2-overexpressing mouse models, such as BALB-neuT or FVB-huHER2 (Boggio et al., 1998; De Giovanni et al., 2014; Finkle et al., 2004). Limitations of experiments using such models are expense, time required, and lack of concordance between the BALB-neuT model, which overexpresses the rat HER2 gene, and human HER2 expression. The FVB-huHER2 mouse does overexpress human HER2, but requires 40 weeks to develop mammary carcinomas. BALB-neuT mice develop palpable mammary tumours by 20 weeks.

In summary, this work has suggested trastuzumab increases BAG-1L expression in SKBR3 cells. Cell yield and viability were increased by BAG-1 isoform overexpression in SKBR3 cells compared to vector controls. Immunocytochemistry using ER-positive HER2-positive BT-474 cells demonstrated nuclear exclusion of BAG-1 following lapatinib, suggesting a hypothesis that BAG-1 mediates localisation of HER2/ER signalling proteins. A decrease in relative viability and expression of phospho-Akt(473) following trastuzumab in BT-474 cells was enhanced following addition of the BAG-1 inhibitor thioflavin S, suggesting crosstalk between HER2 and BAG-1 signalling pathways as proposed. Further work is required prior to use of the pan-isoform anti-BAG-1 antibody 3.10G3E2 on tissue from a large cohort of patients diagnosed with HER2-positive breast cancer. BAG-1 may be a potential biomarker in other cancers known to overexpress HER2. Immunohistochemical experiments demonstrated an association between advanced tumour regression grade and levels of nuclear BAG-1 in tissue samples from patients diagnosed with oesophageal adenocarcinoma.

This work supports a role for BAG-1 in HER2-positive breast cancer and the hypothesis that BAG-1 signalling pathways may be targeted to enhance anti-HER2 therapy. Further work is required to more completely understand the mechanisms involved and assist therapeutic design.

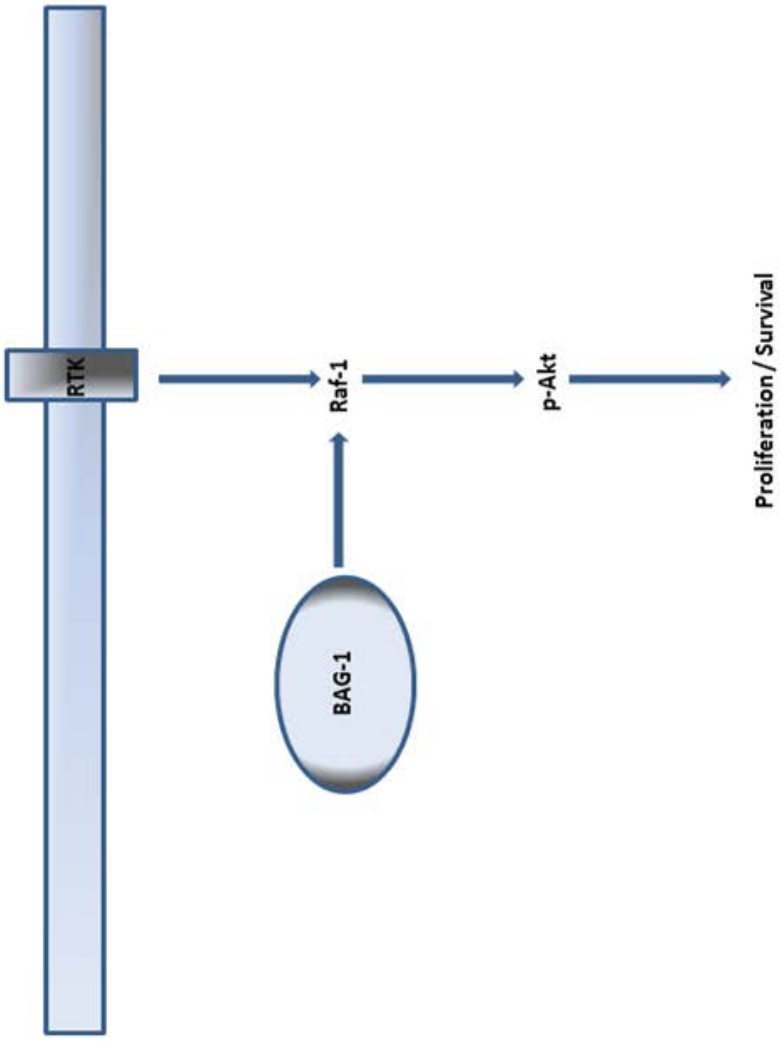

Figure 6.1 Overexpression of endogenous BAG-1 in HER2-positive breast cancer cells increases cell proliferation and viability (Figure 3.1).

Figure 6.2 Overexpression of all BAG-1 isoforms protects cell yield and viability in HER2-positive breast cancer cells exposed to trastuzumab (Figures 3.5 and 3.6)

Figure 6.3 Treatment of BT-474 HER2-positive breast cancer cells with a combination of trastuzumab and thioflavin S down-regulates phospho-Akt and shows synergistic reduction in cell viability (Figures 4.6 and 4.7).

Inhibitor	Effect of BAG-1 overexpression on proliferation	Effect of EGFR family inhibitor on endogenous BAG-1 expression
Erlotinib HER1 (EGFR/ ErbB1)	↔	↑
Trastuzumab HER2 (ErbB2)	↑	↑
Lapatinib HER1 (EGFR/ ErbB1) + HER2 (ErbB2)	↑	↑

Table 6.1 The effects of inhibition of EGFR family receptors on proliferation of SKBR3 cells overexpressing BAG-1 and levels of endogenous BAG-1 expression (Figures 3.5, 3.6, 3.14-3.19).

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