Associations of HSP90 Client Proteins in Human Breast Cancer

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Abstract. *Background: HSP90 has been studied intensively as a therapeutic target, however little is known regarding specific interactions of the large number of HSP90 client proteins. Therefore, this study investigated HSP90 client proteins sensitive to the HSP90 inhibitor geldanamycin in tumour and healthy breast tissue. Materials and Methods: Co-immunoprecipitation and SDS-PAGE were used to investigate protein interactions. Western blotting and LC-MS were used to infer protein identities. Results: HSP90 client proteins were observed in 7 out of 11 breast cancer patients. Further experiments inferred HSP40, -56/FKBP52, -60, -70, -105 and lumican to associate with HSP90 and to belong to this group of geldanamycin-sensitive proteins. In one patient, a cancer-specific group of proteins was identified. In all experiments geldanamycin resistance was observed. Conclusion: HSP90 differentially associated with client proteins and this was patient dependent. Geldanamycin resistance and lack of HSP90 client protein expression may limit clinical applications of HSP90 inhibitors.*

The heat-shock protein 90 (HSP90) molecular chaperone is responsible for the stabilisation of a multitude of cellular pathways and processes (1, 2). HSP90 is essential for the survival of eukaryotic cells and it functions by mediating and stabilizing the activity of other cellular proteins (1, 3). HSP90 is an attractive therapeutic target in the treatment of cancer as many of its client proteins are involved in signal transduction and HSP90 appears to be unique in that its inhibition results in the destabilization of multiple signalling pathways. Specifically, HSP90 is essential for the function of multiple growth and survival pathways that are required for the maintenance of the cancer phenotype (2, 4, 5). Reflecting perhaps the fundamental role that

HSP90 plays in the maintenance and progression of cancer, HSP90 is abnormally expressed in a variety of human cancer types. In breast cancer, HSP90 has been shown to be up-regulated and this correlates with poor patient prognosis (6). Multiple HSP90 inhibitors of the ansamycin type, such as geldanamycin derivatives with more suitable pharmacological profiles, have been evaluated for the treatment of cancer in human clinical trials, including a phase III clinical trial (7) .

Despite the clinical use of HSP90 inhibitors, knowledge of the effect(s) of HSP90 inhibition is limited. It is known that HSP90 interacts with a large number of client proteins, however a thorough knowledge of specific interactions in different biological contexts does not currently exist (1, 3). This study presents a preliminary investigation of HSP90 client proteins sensitive to geldanamycin in human breast tissue. This study included the use of tumour and healthy (non-cancerous) breast tissue from breast cancer patients and healthy breast tissue from a cancer-free individual. In addition to client proteins that bind to HSP90, HSP90 client proteins that associate with HSP40, HSP56, HSP60, HSP70 and HSP105 were examined. Client proteins of these HSPs sensitive to geldanamycin were investigated in an effort to increase the current body of knowledge regarding HSP and HSP client protein associations in cancer and to elucidate the role of HSPs in the assembly of chaperone complexes in health and disease.

Materials and Methods

Extraction and isolation of cellular proteins from breast tissue. Ethics approval was obtained from the University of New England Human Research Ethics Committee (approval no. HE07/145). Both tumour and healthy breast tissue taken from patients during surgical procedures were cooled immediately on ice and subsequently stored at –70°C. Samples were partially thawed and slices excised. Cellular proteins were extracted from breast tissue in a buffer containing 7 M urea (Sigma-Aldrich, Castle Hill, Sydney, NSW, Australia), 2 M thiourea (Riedel-de Haën Sigma-Aldrich, Seelze, Lower Saxony, Germany), 4% CHAPS detergent (ICN Biochemicals MP Biomedicals, Seven Hills, Sydney, NSW, Australia) and PMSF protease inhibitor (Boehringer Mannheim Roche, Dee Why, Sydney, NSW, Australia). The tissues were homogenised with a bladed electric tissue homogeniser (Heidolph Diax900, Schwabach, Bavaria,

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Germany) until a solution of smooth consistency was obtained. This solution was frozen at –70°C overnight. Solutions were thawed and subjected to brief sonication (Branson Ultrasonics, Danbury, CT, USA) and then refrozen at –70°C. Solutions were subsequently thawed, centrifuged and the protein layer removed. Protein fractions were centrifuged twice more to ensure the final extract was free of insoluble contaminants. All steps were performed at 4°C.

Investigation of client proteins. Co-immunoprecipitation was performed under non-denaturing conditions to reveal protein associations. A total of 100 μl of protein extract was incubated with antibody to various HSPs (minimum 2 μg antibody per 100 μl sample) for 1 h at room temperature on a mechanical rocking device. This solution was subsequently incubated with 15-25 μl protein A-coated beads (Protein A ceramic hyperD F product #20078-036; Pall, Cheltenham, Melbourne, Victoria, Australia) overnight at room temperature with gentle rocking in a 1.5 ml membrane filter centrifuge tube (Pall Nanosep MF GHP 0.45 μm, product #ODGHPC34). Protein A beads were washed with washing solution containing 7 M urea (Sigma-Aldrich), 2 M thiourea (Riedel-de Haën Sigma-Aldrich) and 4% CHAPS (ICN Biochemicals, MP Biomedicals) prior to sample addition. Protein extract containing unbound protein was separated from the protein A beads by centrifugation of the membrane filter centrifuge tubes. Following centrifugation, the protein A beads were washed with a minimum of 30 μl washing solution and centrifuged. The protein A beads were washed three times to ensure the removal of any unbound or weakly bound protein. The washing solution was highly stringent to ensure only tightly binding client proteins remained bound, thereby reducing the possibility of detecting non-specifically bound proteins. Protein A beads were washed with 30 μl of washing solution following each geldanamycin treatment (Biomol Enzo Life Sciences, Farmingdale, NY, USA) to remove any remaining protein and prevent sample contamination of subsequent treatments. This procedure was repeated for the two subsequent geldanamycin treatments of 25 and 50 μg/ml. Following the geldanamycin elution and washing steps, beads were treated with a denaturing solution to thoroughly disrupt any remaining protein interactions [10% glycerol (Promega, Alexandria, Sydney, NSW, Australia), 5% mercaptoethanol (BDH VWR, Radnor, PA, USA) and 2.3% sodium dodecyl sulphate (SDS) (Sigma-Aldrich) all w/v in ddH$_2$O]. This solution was collected as described above and stored at 4°C until SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separation. SDS-PAGE separation of eluted proteins was performed the same day as immunoprecipitation. Selected experiments were repeated to confirm the initial results. With each immunoprecipitation experiment, a set volume of total protein extract from each sample was loaded onto an SDS-PAGE gel to ensure consistent protein loading with different extracts in each experiment.

SDS-PAGE separation of proteins. Precast 4-15% gradient Tris-HCl polyacrylamide mini-gels were used to separate protein samples (product #161-1122, used with the Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, Gladesville, Sydney, NSW, Australia). To 15-20 μl of each sample, 3 μl of 3.75× concentrated denaturing solution (described above) containing bromophenol blue tracking dye (BDH VWR) was added. Sample tubes were boiled in water for 3 min followed by rapid cooling on ice to mitigate residual protease activity. Samples were loaded and electrophoresis performed at 4°C with running buffer [14.4% glycine (ICN

Biochemicals MP Biomedicals) 3.0% tris (Sigma-Aldrich) and 1.0% SDS (Sigma-Aldrich) all w/v at 30 mA per gel until the tracking dye had migrated to the bottom of the gel (typically after 1-1.5 h). Kaleidoscope pre-stained protein standards were run on each gel (Bio-Rad product #161-0324).

ECL Western immunoblotting. Following electrophoresis, proteins from SDS-PAGE gels were transferred to nitrocellulose membranes (Hybond C; GE Life Sciences, Rydalmere, Sydney, NSW, Australia) using a semi-dry transfer method by applying 120 mA for 90 min (Multiphor II Novablot; GE Life Sciences). Membranes were blocked in 5% skim milk powder (Diploma Fonterra, North Ryde, Sydney, NSW, Australia) phosphate-buffered saline Tween 20 (Sigma-Aldrich) (SMP PBS-T) at 4°C overnight and washed according to the manufacturer's instructions in PBS-T before Western blotting. Primary antibody was diluted in SMP PBS-T and incubated with the membrane for 1 h. Membranes were washed according to the manufacturer's instructions in PBS-T and appropriate horseradish peroxidase (HRP)-conjugated secondary antibody added and incubated as per primary antibody. Following incubation with secondary antibody, proteins were visualised in a dark room. Membranes were incubated with HRP substrate according to the manufacturer's instructions. Hyperfilm enhanced chemiluminescence (ECL) film (GE Life Sciences) was used to detect labelled proteins. A GE Life Sciences ECL kit was used which contained HRP substrate and HRP-conjugated secondary antibodies.

Staining of acrylamide gels. Gels were stained for at least 12 h with a highly sensitive Coomassie G250 protein stain (8) (0.12% dye (ICN Biochemicals MP Biomedicals), 10% ammonium sulphate (BDH VWR), 10% phosphoric acid (Sigma-Aldrich) and 20% methanol (Merck, Kilsyth, Melbourne, Victoria, Australia) all w/v).

Sample acquisition and study population. Samples were obtained with informed consent from patients undergoing surgery in the treatment of breast cancer or breast reduction surgery and consisted of those from ten breast cancer patients and one cancer-free individual undergoing breast reduction surgery. For three patients, both tumour and healthy breast tissue were available.

Liquid chromatography-mass spectrometry (LC-MS). LC-MS was performed on excised stained protein bands by the Bioanalytical Mass Spectrometry Facility at the University of New South Wales, Sydney. Samples were analysed with a Q-TOF Ultima mass spectrometer (Micromass/Waters) and peptides corresponding in sequence to known database proteins were identified using Mascot MS software.

Antibodies used. HSP105: Rabbit polyclonal clone N-187 (product #SC-6241; Santa Cruz Biotechnology, Santa Cruz, CA, USA). HSP90α: Mouse monoclonal clone Hyb-K41009 (product # SMC-108D; Stressmarq, Victoria, BC, Canada). Goat polyclonal clone N-17 (product #SC-1055; Santa Cruz Biotechnology). HSP70: Mouse monoclonal clone C92F3A-5 (product # SPA-810; Stressgen Enzo Life Sciences, Farmingdale, NY, USA). Mouse monoclonal clone C92F3A-5 (product #SMC-100B; Stressmarq). HSP56: Rabbit polyclonal anti-FKBP59/HSP56 (Biomol Enzo Life Sciences). HSP40: Goat polyclonal clone N-19 (product #SC1801; Santa Cruz Biotechnology).
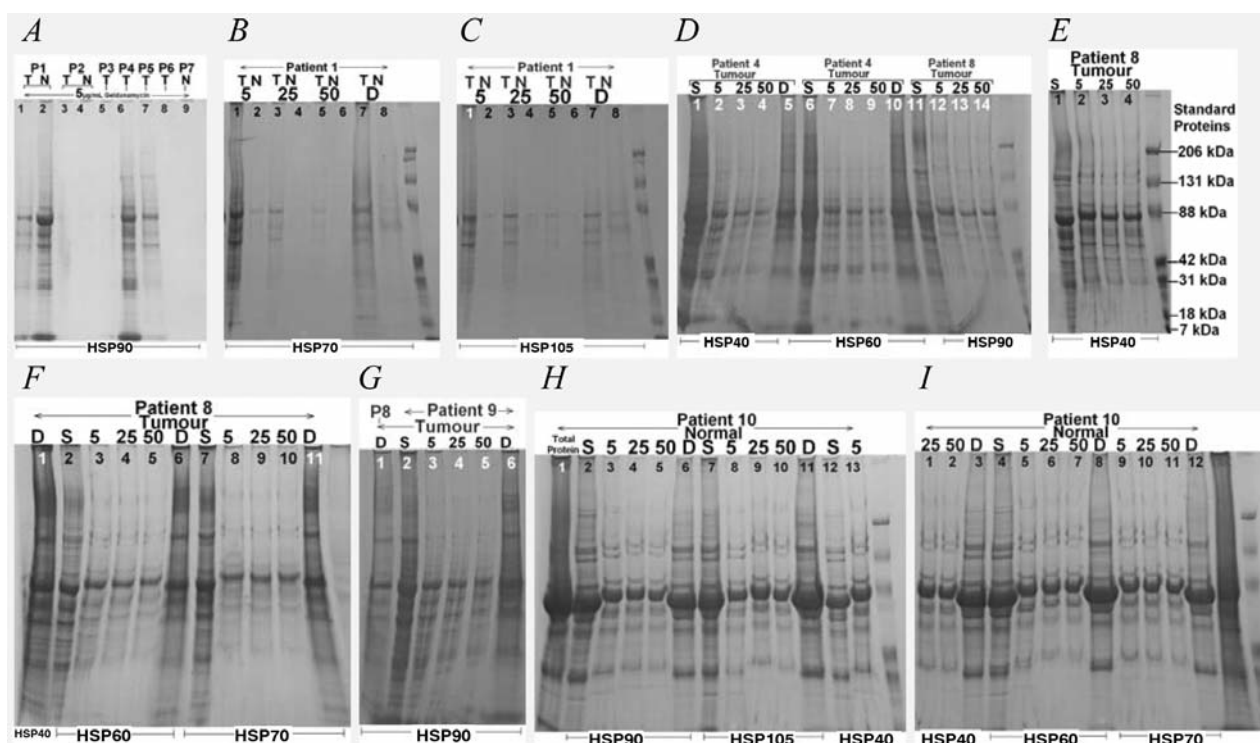
Figure 1. *Geldanamycin-sensitive proteins in breast cancer patients. Tumour and healthy breast tissue extracts were immunoprecipitated with HSP antibodies and HSP90 client proteins eluted with geldanamycin. P, Patient number; T, tumour breast tissue sample; N, healthy breast tissue sample; S, supernatant (unbound protein after immunoprecipitation); D, denaturation applied to protein A beads; 5/25/50, geldanamycin concentration (μg/ml); HSP, immunoprecipitation performed with the noted HSP antibody.*

Results

HSP90 client proteins in breast cancer patients. Protein extracts of tumour and healthy breast tissue from ten breast cancer patients (three matched for tumour and healthy tissue) and one cancer-free individual were screened for HSP90 client proteins using co-immunoprecipitation followed by geldanamycin treatment. Since geldanamycin is a specific HSP90 inhibitor, proteins eluted from the immuno-precipitated protein complexes, following treatment with geldanamycin, were taken as representing a select subset of HSP90 client proteins. HSP90α client proteins eluted by geldanamycin were identified in 7 out of 11 patients. Among the seven patients in which HSP90 client proteins were identified (Figure 1A lanes 1-2 and 6-7, D lanes 11-14, G, H lanes 2-6; one patient data not shown), two were matched for tumour and healthy tissue and no differences in the client proteins between tissue types were observed for either patient. HSP90 client proteins sensitive to geldanamycin were not identified in 4 out of the 11 patients (Figure 1A lanes 3-5 and 8-9). Treatment of these four patient samples with denaturant did not result in any observable protein,

suggesting that these samples either had an undetectable level or an absence of HSP90α client proteins (denaturation data not shown).

Among the seven patients in which HSP90α client proteins were identified, the majority (5/7) displayed the same protein group consisting of approximately 20 proteins, most of which migrated to a position corresponding to 90 kDa or less. One of these seven patients (patient 10, Figure 1H lanes 2-6) was unique in displaying a different group of proteins compared to the other patients. It is noteworthy that this was the only sample of healthy tissue obtained from a cancer-free individual. Despite this, at least three protein bands in common to all patients were identified, as shown in Figure 3. All client proteins were sensitive to geldanamycin at a minimum concentration of 5 μg/ml.

HSP90 associates with HSPs 40, 56, 60, 70 and 105 in human breast cancer. To investigate the role of other HSPs associating with HSP90 in breast cancer, the original immunoprecipitation experiments were repeated using antibodies to HSP 40, -60, -70 and -105 in place of anti-HSP90 and followed by elution with geldanamycin, as
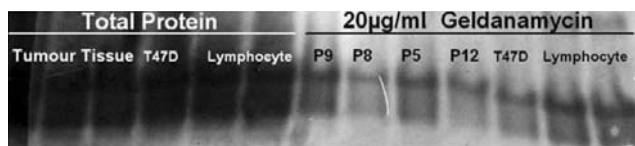
Figure 2. *Western blotting inferred HSP56 as an HSP90 client protein. Breast tumour tissue extracts from four breast cancer patients and the T47D breast tumour cell line were immunoprecipitated with HSP90 antibodies and eluted with geldanamycin. Western blotting with anti-HSP56 was subsequently performed on the eluted proteins. P, Patient number.*

Figure 3. *Geldanamycin-sensitive HSP90 client proteins common to all patients and both tissue types were sequenced by LC-MS. Arrows indicate bands analysed. Top arrow indicates lumican. S, Supernatant (unbound protein after immunoprecipitation); 5/25/50, geldanamycin concentration (μg/ml).*

before. Client proteins sensitive to geldanamycin (inferred as HSP90 client proteins since they were eluted from the immunoprecipitates by geldanamycin) were observed in association with all antibodies to HSPs tested in samples from all five patients (Figure 1B, C lanes 1-8, D lanes 1-10, E lanes 1-4, F lanes 1-11, H lanes 7-13 and I lanes 1-12). In four out of the five patients, these proteins were the same as those identified in experiments with HSP90 antibodies. Therefore, these experiments suggested that these HSPs associate with HSP90 in human breast cancer and that they assemble in a complex with the same proteins sensitive to geldanamycin identified using HSP90 antibodies as the target for immunoprecipitation. All eluted proteins were sensitive to geldanamycin at a minimum concentration of 5 μg/ml.

One of the five patients (patient 1, Figure 1A lanes 1-2, B and C lanes 1-8) showed differences in the association of HSP90 client proteins between the tumour and normal tissue samples. While the tumour sample displayed the same group of client proteins sensitive to geldanamycin for each HSP, the normal sample only displayed this group of proteins in experiments with HSP90 antibodies. By contrast, in experiments using HSP40 (data not shown), HSP60 (data not shown), HSP70 and HSP105 antibodies, a single protein band was detected in the healthy tissue. These experiments implied that in this patient there was a selective association of HSP90 client proteins with HSPs-40, -60, -70 and -105 in the breast tumour tissue, while this association was absent from the healthy breast tissue.

Patient 10 (healthy tissue sample from a cancer-free individual, Figure 1H and I) had a different group of client proteins by comparison with the other patients. This result was consistent with the experiments performed with HSP90 antibodies.

Immunoprecipitation with HSP56 antibody was performed with protein extracts from patients 1 and 9 (data not shown). Client proteins sensitive to geldanamycin were observed in both patients. To confirm this result, HSP90-bound geldanamycin-sensitive proteins from four breast cancer patient samples and the T47D tumour cell line were transferred to membranes and probed with HSP56 and HSP90
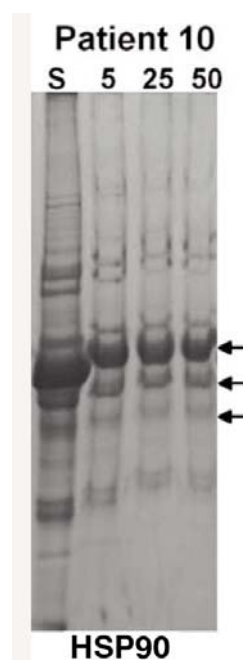
antibodies using Western immunoblotting (Figure 2 and 4). The presence of HSP56 and HSP90 was observed in all samples. Taken together, these data provided two independent sources of evidence for an association between HSP56 and HSP90 in breast cancer. These data also implied that HSP56 is a member of the geldanamycin-sensitive protein group.

Lumican is an HSP90 client protein in human breast tissue. A select number of HSP90 client proteins eluted with geldanamycin after immunoprecipitation with anti-HSP90 were also observed to be present in immunoprecipitation using antibodies against HSPs-40, -60, -70 and -105. Three of these common proteins were excised (Figure 3, patient 10) and sequenced. The same three protein bands were evident in all samples (both tumour and healthy) where HSP90 client proteins were observed. Lumican, a protein that plays an important role in breast stromal tissue, was identified as one of these common HSP90 client proteins and, thus, as a member of the geldanamycin-sensitive group of proteins.

A subset of HSP90 displays resistance to geldanamycin in breast cancer tissue. In immunoprecipitation experiments, bound protein complexes were treated with geldanamycin solutions of increasing concentration (5, 25 and 50 μg/ml). This
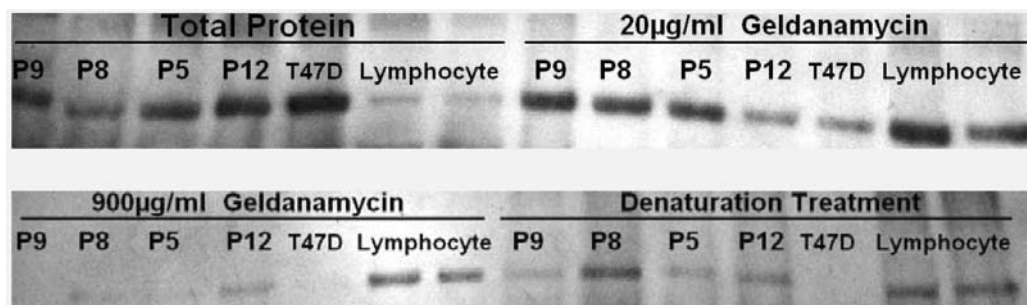
Figure 4. *HSP90 displays resistance to geldanamycin in human breast tissue. Four breast tumour tissue samples and the T47D breast tumour cell line were immunoprecipitated with HSP90 antibodies and eluted with geldanamycin. Western blotting with HSP90 antibodies was subsequently performed on the eluted proteins. P, Patient number.*

was followed by application of a denaturing solution to ensure all protein associations had been disrupted, thereby resulting in the removal of all client proteins. In the example shown in Figure 1B the healthy tissue sample eluted protein only by the 5 μg/ml geldanamycin treatment condition (Figure 1B, lane 2). The higher concentration solutions failed to elute protein from the immunoprecipitated protein complexes (Figure 1B, lanes 4 and 6). This suggests all HSP90 and HSP90 client proteins had been eluted from the immunoprecipitated protein complexes. However, treatment of this sample with a denaturant resulted in the elution of the same protein band (Figure 1B, lane 8). This was a feature of all immunoprecipitation experiments and was further investigated using Western immunoblotting. In the Western blots shown in Figure 4, an HSP90 immuno-precipitation was performed with four breast cancer patient (tumour) samples and the T47D tumour cell line. The immunoprecipitated protein complexes were treated with solutions containing 20 and 900 μg/ml geldanamycin before being treated with a denaturant.

The samples were transferred to nitrocellulose membranes and probed with an HSP90 antibody using Western immunoblotting. The geldanamycin solution (20 μg/ml) eluted a single band in all breast cancer samples. Increasing the concentration to 900 μg/ml did not result in further elution of this band in patients 5 and 9. However, when these samples were treated with a denaturant, a reappearance of this same protein band was seen. These data suggest that HSP90 is at least partially resistant to geldanamycin. It is not known whether this was due to reduced sensitivity or resistance to geldanamycin or whether two distinct pools of HSP90 exist, one sensitive to geldanamycin and the other insensitive.

Discussion

HSP90 client proteins in breast cancer patients. HSP90 and its client proteins are involved in the growth of cancer cells and, hence, are used as therapeutic targets (2, 4). As such,

the degree to which HSP90 and HSP90 client proteins are expressed may predict patient response to HSP90 inhibitors. Furthermore, it is not known whether expression of HSP90 client proteins correlates with expression of HSP90. Client proteins were identified in 7 out of the 11 patients screened in this study. Subsets of breast cancer patients with reduced expression of HSP90 or its client proteins thus may not respond to HSP90-targeted drugs as favourably, if at all. To date, studies have focused on the expression of HSP90 or a small number of well-characterized client proteins. No studies have investigated the spectrum of HSP90 client proteins in cancer tissue as performed here and no clinical studies have investigated the expression of HSP90 or of its client proteins in relation to response to HSP90 inhibitors. In addition, it remains to be determined whether the observed absence of client proteins was due to down-regulation or an absence of these proteins. These aspects of the HSP90 chaperone machinery should be investigated in future studies.

HSP90 associates with HSPs-40, -56, -60, -70 and -105 in human breast cancer. This study is the first to report the association of HSP90 with these HSPs in human breast tumour tissue. The specific effect(s) of HSP90 inhibition remains a pressing question. It is known that HSP90 functions as a member of a multi-protein chaperone complex with other HSPs (3, 9). These chaperone complexes are delicately balanced and transiently dynamic in their function (3, 10). A change in the activity of one protein within the complex is likely to result in a cascade of repercussions for the functionality of the complex as a whole. As an example of the intimate and co-dependent role these HSPs possess, HSP90 inhibition by geldanamycin has been shown to induce HSP70 expression (11). As a consequence, the chaperoning of client proteins is likely to be altered and this may be a major mechanism of action of HSP90 inhibitors *in vivo*. HSP56, otherwise known as FKBP52, is an important co-

chaperone of HSP90 that possesses peptidyl-prolyl isomerase activity (12). This is the first report of an association between FKBP52 and HSP90 in breast cancer, however, FKBP52 has previously been reported to be up-regulated in breast tumour tissue (13). Given that HSP90 is also up-regulated in breast cancer (6, 14), increased expression and association of these two proteins may be a supporting mechanism for the growth of breast tumour cells. This study has provided evidence for the inherent complexity of the HSP-chaperone system in breast cancer and for the co-chaperones and client proteins whose activity may be altered by HSP90 inhibitors in breast cancer.

One patient displayed a select group of HSP90 client proteins that was only present in immunoprecipitation experiments using antibodies to HSPs-40, -60, -70 and -105 in the tumour tissue, while the healthy tissue from the same patient did not contain these proteins. These data provided evidence of a cancer-specific group of proteins that may be used as a therapeutic target to specifically deliver a drug to cancerous breast cells. However, to qualify as a clinically useful drug, this cancer-specific group needs to be present in a substantial proportion of the breast cancer patient population which is known to be highly diverse in both causative mechanisms and prognostic outcomes.

Lumican is an HSP90 client protein in human breast tissue. Lumican mRNA has previously been demonstrated to be up-regulated in breast cancer patients (15). However, this study is the first to report the association between lumican and HSP90. As this protein band was identified in HSP90 immunoprecipitates and eluted by geldanamycin in breast tumour and healthy tissue extracts, it can be inferred that lumican associates with HSP90 in breast tissue. Leygue *et al.* (15) reported that increased expression of lumican is associated with higher tumour grade and increased HSP90 expression in breast cancer patients is predictive of a poor prognosis. These findings taken together with the present study reporting that lumican associates with HSP90 in breast tissue suggest that these proteins may function in tandem to facilitate the progression of breast cancer. Given the results of the immunoprecipitation experiments using antibodies to other HSPs, the association of lumican with HSPs-40, -60, -70 and -105 can be inferred and these proteins are likely to support the progression of breast cancer through their chaperoning action. Indeed, increased HSP70 expression has been reported to be associated with decreased survival in breast cancer patients (16). By further mapping HSP90 client proteins whose expression is associated with the progression of breast cancer, a range of biomarkers may become available and this may allow a more accurate prediction of a patient's prognosis and, as such, may have consequences for the therapeutic management of breast cancer patients as decided by oncologists on a patient-by-patient basis. Therefore, this work contributes at a preliminary preclinical level to the growing trend of personalised cancer care. In addition, this avenue of research will give greater insight to the progression of breast cancer at the biochemical level, which may, in turn, lead to the development of more effective therapies by revealing new families of HSP-associated biochemical targets.

A subset of HSP90 displays resistance to geldanamycin in breast cancer tissue. A subset of HSP90 was observed to be resistant to the effects of geldanamycin, even at a high physiological concentration of 0.9 mg/ml. This observation may be important to the clinical application of HSP90 inhibitors. The ability of some HSP90 to resist inhibition may be a limitation of these drugs. In cancer patients treated with HSP90 inhibitors, the proportion of HSP90 that is resistant to geldanamycin may increase in an attempt by the tumor to continue to grow. Consequently, the proportion of cells resistant to HSP90 inhibitors may increase in response to treatment, a potential mechanism for the development of resistance.

Acknowledgements

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