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Additive viability-loss following *hsp70*/*hsc70* double interference and Hsp90 inhibition in two breast cancer cell lines

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Abstract. Hsp70 is an anti-apoptotic protein over-expressed in breast, lung, rectum, endometrial and bladder cancers. In this study, we investigated the impact of Hsp70 protein expression, and its close family member, Hsc70, on breast cancer cell viability and on the activity of the Hsp90/Hsp70 chaperone complex, a complex whose activity is important for the survival of cancer cells and tumours. The simultaneous blockade of Hsp70, Hsc70 and Hsp90 was most efficient in reducing breast cancer cell viability, as compared to their respective separate blockades. However, while the Hsp90 inhibition alone correlates with lost cell viability and reduced Hsp90/Hsp70 chaperone complex activity, the single or mixed reduction in Hsp70 and Hsc70 expression displayed no ability to reduce the activity of the Hsp90/Hsp70 chaperone complex. The results suggest that targeting both the Hsp70 family, as well as the Hsp90 protein will have an additive negative effect on cancer cell survival, even though their pathways of action are separate.

Introduction

Several proteins that can promote the proliferation of tumours by protecting cells from entering apoptosis have been identified in the past decade. These include members from the heat shock protein (HSP) family, Hsp70 and Hsp90, the Bcl-2 protein family, and the inhibitor-of-apoptosis protein family (1). Among the HSP family, the increased level of the Hsp70 protein is linked to bad prognosis in breast cancer (2,3), lung, rectum, endometrial, bladder and oral cancers (4-8). The over-expression of the Hsp70 protein in tumour models enhances proliferation and protects against cell death (3,9-12). The reduced Hsp70 expression induced by antisense-*hsp70* cDNA, provokes cell death in cultured cells and in orthotopic xenografts in mice (13,14).

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The anti-apoptotic property of Hsp70 has been investigated in tumour cell lines, heat shock-induced cell death, and in TNFα, LPS/IFNγ and nitric oxide/CHOP-mediated apoptosis (15-20). Hsp70 expression blocks apoptosis at several levels, including protecting the mitochondria membrane-integrity and the release of Cytochrome C into the cytosol, apoptosis-inducing-factor (AIF) translocation to the nucleus, caspase-3 processing and by inhibiting late caspase-dependent events. The intrinsic anti-apoptotic properties of Hsp70 depend on both the ATPase domain as well as the C-terminal EEVD domain (18-20). The depletion of different Hsp70 related proteins arrests cell growth at distinct phases of the cell cycle, thus indicating functional specialization in the regulation of apoptosis, cell growth and survival (21).

The inducible Hsp70 protein is a member of the Hsp70 chaperone family comprising of at least nine members. The closest family member, Hsc70, is constitutively expressed and shares 85% protein similarity and is structurally related to Hsp70. Both Hsp70 and Hsc70 are expressed in the cytosol and the nucleus. The Hsp70 family members are molecular chaperones that bind unfolded proteins and enable the folding and structural rearrangements of polypeptides during processes such as protein synthesis, ligand-protein binding, membrane translocation, oligomerization and protein degradation. Following disease or environmental stress stimuli, the induced expression of Hsp70 can assist Hsc70 in responding to the increased demand for such chaperone activities.

In general, chaperones fall into six major families: The Hsp110-, Hsp90-, Hsp70-, Hsp60-, Hsp40-/DnaJ-, and the small HSP family. Individual chaperones collaborate in a dynamic cohort of other chaperones. The Hsp90 and Hsp70 chaperone systems cooperate in the maturation, activation and inactivation of several client proteins by forming a super-chaperone complex that functions between an 'open' and a 'closed' state (reviewed in ref. 22). In the open state, the client protein is loaded into Hsp90 with the help of co-chaperones such as Hsp70, Hsp40, Hop and Hip. In the closed and mature state, p23 and immunophilins replace the original co-chaperones and mediate the folding of the client protein into an active state. With respect to anti-cancer treatments, the Hsp90 protein has become an important target due to Hsp90's and the super-chaperone's role in regulating the activity of hormonal receptors and various kinases that are involved in functions including apoptosis, survival and growth (23,24).

The ligand-activated tyrosine kinase Her2 is a client protein for the cytosolic Hsp90 chaperone complex and

plays a critical role in cell proliferation (25). The selective suppression of Her2 has been utilized in various anti-cancer treatments (e.g. by the recombinant monoclonal Her2 antibody, trastuzumab). However, as with many other single target treatments, the results illustrate that a tumour cell can circumvent the inhibition of one target (such as Her2) by activating alternative pathways to support cell proliferation (24). Her2 can be co-precipitated with both Hsp70 and Hsp90 in the breast cancer cell line, SKBr3 (26,27). Following the administration of the natural and specific Hsp90 inhibitor, geldanamycin (GA), Her2 is ultimately degraded. It has been suggested that Her2 degradation is mediated by the increased expression of and the association to Hsp70, and CHIP E3 ubiquitin ligase activity (26-28).

In order to study the effects of Hsp70 and Hsc70 protein suppression on cancer cell viability, we designed siRNA molecules for the selective degradation of *hsp70* and *hsc70* mRNA transcripts. Reduced cell viability was observed upon Hsp70 and mixed Hsp70/Hsc70 suppressions in MCF-7. In both the MCF-7 and SKBr3 cell lines, *hsp70/hsc70* interference-mediated viability loss added to, or facilitated, the cell viability loss triggered by the Hsp90 inhibition. As Hsp70 and Hsp90 cooperate in a super-chaperone complex that is critical for cancer cell viability, we wanted to determine whether both the Hsp90 inhibition and Hsp70/Hsc70 suppressions could mediate cell viability loss by controlling the activity of the Hsp90/Hsp70-chaperone complex. The activity of the chaperone complex was measured by its ability to process and expose the cell surface form of its client protein, Her2. The two breast cancer cell lines, MCF-7 and SKBr3, expressing low and high levels of mature Her2, respectively, were used in the study.

Materials and methods

Cell cultures. The breast cancer cell lines (MCF-7 and SKBr3) were obtained from the American Type Culture Collection (ATCC). The MCF-7 cells were grown in RPMI (Gibco, France) and the SKBr3 cells in MCCOY's 5A medium (Gibco). Both media were supplemented with 10% (v/v) fetal bovine serum and 4 mM L-glutamine. Cultures were performed at 37˚C in a 5 vol-% CO_2/air incubator.

siRNA materials. Reverse phase HPLC purified *hsp70*-siRNA, *hsc70*-siRNAs, *her2*-siRNA and *non-silencing* (ns)-siRNA were purchased from both Proligo (Europe) and Ambion (France): *hsp70*: sense, 5'- GAAUCUGUUGGGGCGCUU CdTdT -3'; antisense, 5'- GAAGCGCCCCAACAGAUU GdTdT -3'. *hsc70*: sense, 5'- GGACCUAAAUUCGUA GCAdTdT -3'; antisense, 5'- UGCUACGAAUUUAGGUCC UdTdT -3'. *Non-silencing*: sense, 5'- AUACGACGGUAC GUACGAUdTdT -3'; antisense, 5'- dTdTUAUGCUGCGAU GCAUGCUA -3'. *CyclinD1*: sense, 5'- AGUAGCAGCG AGCAGCAGAdTdT -3'; antisense, 5'- UCUGCUGCUC GCUGCUACUdTdT -3'. The *gapdh*-siRNA and the *non-silencing* siRNA labelled with FITC were obtained from Ambion and Qiagen (France), respectively.

Oligofectamine transfection protocol. Double-stranded RNAs were transfected into cells in 6-, 12- or 96-micro well plates with Oligofectamine™ Reagent (Invitrogen Life Technologies, France) according to the manufacturer's recommendations. Transfection-efficiency was analysed after 24 h on a FACSCalibur™ Flow Cytometer (BD systems): The fluorescent signal of the FITC-*non-silencing* siRNA was compared to that of the oligofectamine-treated cells alone. For the verification of non-toxic effects, all types of manipulations were analysed by the Cell Proliferation Reagent WST-1 (Roche, France) or by cell number counting (as described for the *PI*/FDA protocol).

Protein extraction and immunoblotting. Proteins were extracted by lysing the cells in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.4 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1% NP-40, 1 mM pefablock) containing complete protease inhibitor (one tablet per 10 ml RIPA; Roche). The lysed cells were incubated for 15 min on ice and centrifuged at 13,000 x g for 10 min. Protein concentrations in the supernatant were measured by the Bio-Rad Protein Assay Dye Reagent (Bio-Rad, France). Samples of 15 μg protein were loaded onto and separated on NuPAGE 4-12% Bis-Tris Gels (Invitrogen). Proteins were blotted onto PolyScreen PVDF Transfer Membrane membranes (PerkinElmer, France). Hsp70 and Hsc70 detection and semi-quantification were performed by using 1/500 diluted anti-hsp70 (BioVison, France) and 1/10000 diluted anti-Hsc70 (Stressgen, France) antibodies, respectively. The anti-ßactin antibody (1/10000; Abcam, France) was used as a protein-loading control marker. Green and red fluorescent secondary antibodies were used for the detection and semi-quantification of immunoblots: goat anti-rabbit and goat anti-mouse (Alexa 680; Molecular Probes), goat anti-rabbit (IRDye™ 800; Rockland)

PI/FDA assay and viable cell counting. The cells were trypsinised and collected by centrifugation at 1500-2000 rpm for 5 min. The pellet was resuspended in 500 μl PBS containing propidium iodide (PI, 20 μg/ml; Sigma-Aldrich, France) and fluorescein diacetate (FDA, 0.25 μg/ml; Sigma-Aldrich) and 0.15-0.25 μl 10 μ microspheres (Polybead-Polystyren beads; Biovalley, France), and incubated/maintained at 4˚C for 0.5 to 3 h. The solution was passed through a FACSCalibur™ Flow Cytometer (BD systems) using the FL1 and FL3 detectors for detecting fluorescein (viable cells) and PI-stained cells (late apoptotic cells), respectively. The relative cells/beads-ratios obtained after a given manipulation was used to measure the relative loss of cells and viability as compared to the control manipulation or sample.

For the statistical presentation of the cellular fate, cell viability was used and related to FDA signalling. Late apoptotic cells, detected by PI, were difficult to maintain in solution, thus leading to a high variance in the data (points).

mRNA extraction, reverse transcription and quantitative real-time PCR. RNA extraction was performed from 96-well plates on a Qiagen 8000 workstation with the RNeasy 96 BioRobot 8000 kit (Qiagen) following manufacturer's instructions. Half of the total RNA extracted was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems). mRNA expression was analyzed via a semi-quantitative PCR-based assay (Taqman fluorescence

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ogy). The real-time PCR assay was performed in rism 7900 Sequence Detector System (Applied Biosystems). This method used a dual-labelled non-extendable oligonucleotide hydrolysis (Taqman) probe in addition to the two amplification primers. The probe was contained in 5' 6-carboxy-fluorescein (FAM) as a fluorescent reporter dye, and in either 3' 6-carboxytetramethyl-rhodamine (TAMRA) as a quencher for its light emission spectrum, or as a nonfluorescent quencher conjugated with MGB molecule that stabilizes the oligoprobe-target duplex by folding into the minor groove of the double-stranded DNA. To normalize the gene expression, the housekeeping gene, ß-2-microglobulin, was also amplified in duplex. cDNA (5 μl) was added to 15 μl reaction mix, resulting in a final concentration of 900 nM (each) target gene forward and reverse primer, 100 nM (each) ß-2-microglobulin forward reverse primer, 200 nM FAM-labelled Taqman MGB or TAMRA of each probe, and 1X Taqman universal master mix (Applied Biosystems). The amplification conditions were as follows: 50˚C for 2 min to activate AmpliTaq Gold DNA polymerase, 95˚C for 10 min to denature DNA, then 40 cycles of 95˚C for 15 sec (denaturation step), and 60˚C for 1 min (a combined annealing-extension step). The sequences of primers and probes used in this study are : *gapdh*: sense, 5' AGGAGTGGGTGTCGCT GTTG -3'; antisense, 5'- AAGGGCATCCTGGGCTACA -3'; probe (MGB), 5'- AGTCAGAGGAGACCACC -3'. *hsc70*: sense, 5'- GCAAATCTTGAACATTCTCAGACTTG -3'; antisense, 5'- GGAAAAGAACTGAATAAGAGCATCAAC -3'; probe (MGB), 5'- CTGCCTGGACAGCTGCACCATA AGC -3'; *hsp70*: sense: 5'- GCCGGAGGCGTGATGA -3'; antisense, 5'- GAAGATCTGCGTCTGCTTGGT -3'; probe (MGB), 5'- TGATCAAGCGCAACTC -3'. *Hsp90alpha*: sense, 5'- ATTCATCAGATGCATTGGACAAA -3'; antisense, 5'- TGCAGCTCTTTCCCAGAGTCTAAT -3'; probe (MGB), 5'- AGCTTGACAGATCCCAGTAA -3'.

Her2 immunolabelling and Hsp90/Hsp70 chaperone activity assay. The MCF-7 and SKBr3 cells were trypsinised, collected by centrifugation and incubated with the anti-HER2 c-ErbB2/c-neu (Ab-5) monoclonal antibody, against the extra cellular domain (Oncogene Res. Products, ref. OP39) at 2 μg/ml, 250 μl volume, for 30 min at room temperature. After washing twice with PBS, the cells were incubated with the secondary antibody goat F(ab')2 fragment anti-mouse linked to FITC (Immunotech, ref. IM0819) at a dilution of 1/200 in 250 μl PBS for 30 min at room temperature (light protection) and washed again with PBS twice. Finally, the cells were resuspended in 0.5ml PBS and passed through the FACSCalibur™ Flow Cytometer to measure the green fluorescence FITC signal. The FITC signal correlated with the level of the Her2 expression and was used to measure the activity of the Hsp90/Hsp70 chaperone activity as further described in 'Results'.

Hsp90 inhibition. The appropriate medium with serum was prepared having three different GA (10 μM in DMSO) concentrations: 225, 75 and 25 nM and 45, 15, and 5 nM for MCF-7 and SKBr3, respectively. The lowest GA concentration was used to inhibit Hsp90 in combination with *hsp70*- and *hsc70*-siRNA transfections. Twenty-four hours after the siRNA transfections, the transfected and control cells were rinsed with serum-free medium or Optimem once before being incubated with the GA containing medium. The control samples were incubated with the vehicle buffer (diluted DMSO) and all the samples contained DMSO corresponding to the DMSO concentrations in the 225 and 45 nM GA samples for MCF-7 and SKBr3, respectively. The level of Hsp90 inhibition was set according to its ability to mediate Her2 cell surface expression and was thus given as relative changes in the Her2 immunolabelling signals between the compared samples.

Statistical analysis. All experiments were repeated at least 5 or 6 times, and the statistical analyses shown in 'Results' were performed with n=3 or as stated. The student's two-tailed t-test was used for the statistical analysis and standard error means (S.E.M.) were used for statistical presentation. Statistical significant values for $p<0.05$ were marked by an asterisk (*).

Results

hsp70- but not hsc70-siRNA decreases tumour cell viability. In order to analyse the effects of Hsp70 and Hsc70 suppression on cancer cell viability, two *hsp70* and three *hsc70* specific siRNAs were designed and assayed for the knockdown of their respective targets in the MCF-7 and SKBr3 cell lines. The degradation of the *hsp70* and *hsc70* mRNAs was determined by TaqMan quantitative reverse transcription (RT)-PCR 48 h post transfection. Corresponding protein knockdowns were verified by semi-quantitative immunoblotting using Hsp70 and Hsc70 specific antibodies (48 and 72 h post siRNA transfection).

The most efficient *hsp70*-siRNA reduced *hsp70* mRNA by 70% and 90% in MCF-7 and SKBr3, respectively (Table I) (Fig. 1). *hsc70* mRNA expression was reduced by 70% in both cell lines following transfections with the most efficient *hsc70*-siRNA. *hsc70* suppression induced both *hsp70* mRNA and Hsp70 protein expression by 2-3 fold as compared to the *non-silencing (ns)* controls. In order to concomitantly suppress *hsp70* and *hsc70* mRNA expressions, a transfection protocol with a mix of the two corresponding siRNAs (named *mix70*) was defined (Fig. 1). Molar excess of *hsp70*-siRNA to *hsc70*-siRNA was shown to be required for optimal *mix70* interference. A molar *hsp70:hsc70*-siRNA ratio of 5:1 and 7:1 was used in MCF-7 and SKBr3, respectively. *mix70* silenced 60-70% *hsc70* mRNA, whilst the maximal *hsp70* mRNA suppression was determined to be 45-55% of the normal levels in both cell lines.

A *gapdh*-siRNA was used to control optimal cellular and transfection conditions (Table I). Cell viability following control and *ns*-siRNA transfection was verified by the WST-1 assay 48 h post siRNA transfections.

Changes in the relative fractions of apoptotic and viable cells in response to Hsp70 and Hsc70 protein suppression on MCF-7 and SKBr3 were analysed by flow cytometry on non-fixed cells stained by PI and fluorescein diacetate (FDA). *mix70* interference consistently generated an apoptotic response in MCF-7, starting 72 h post siRNA transfection (Fig. 2A). The suppression of Hsp70 alone had in general a slightly weaker apoptotic effect than the *mix70* interference

Table I. Quantitative real-time PCR analysis of *hsp70*, *hsc70*, *hsp90* and *gapdh* expression in MCF-7 and SKBr3 cells 48 h after siRNA transfections.

Cells	Control/siRNA	FC *hsp70*	FC *hsc70*	FC *hsp90*	FC *gapdh*
MCF-7	Transfectant	1.24±0.07	1.03±0.05	0.90±0.05	1.09±0.07
	ns	1.15±0.05	0.99±0.05	0.94±0.09	0.94±0.09
	gapdh	1.29±0.07	1.13±0.07	0.87±0.07	0.15±0.01 ↓
	hsp70	0.36±0.03 ↓	1.20±0.10	1.07±0.07	1.01±0.98
	hsc70	4.59±0.20 ↑	0.28±0.05 ↓	1.68±0.06 ↑	0.98±0.13
	mix70	0.63±0.04 ↓	0.42±0.03 ↓	2.23±0.21 ↑	0.87±0.07
SKBr	Transfectant	0.93±0.17	0.86±0.09	0.74±0.02	1.16±0.16
	ns	0.89±0.11	0.64±0.02	0.61±0.02	0.83±0.10
	gapdh	0.75±0.03	0.64±0.05	0.54±0.05	0.07±0.01 ↓
	hsp70	0.09±0.01 ↓	0.54±0.06	0.44±0.05	0.92±0.27
	hsc70	2.59±0.34 ↑	0.20±0.03 ↓	0.70±0.02	0.81±0.13
	mix70	0.39±0.04 ↓	0.19±0.02 ↓	0.85±0.13	0.68±0.05

FC, fold change; *ns*, *non-silencing*. The FC expression value for a gene is calculated relative to its respective expression value in normal untreated cells. The arrows denote the statistical significance of the up- (↑) and down- (↓) regulation of a gene, as compared to the *ns*-siRNA transfection control (t-test, $p<0.001$).

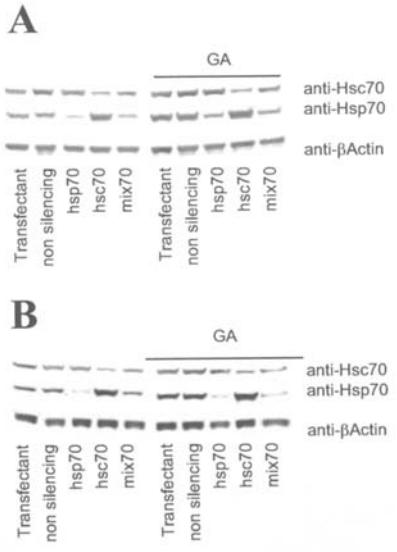

Figure 1. Immunoblot analysis of Hsp70 and Hsc70 expression in MCF-7 (A) and SKBr3 (B) homogenates collected from cultures transfected with *hsp70*-, *hsc70*-, *non-silencing (ns)*- and *gapdh*-siRNAs. Proteins were extracted 72 h post siRNA transfections. Anti-ß-Actin was used as a loading marker. The siRNA-mediated knockdown of the Hsp70 and Hsc70 proteins was also verified in co-treatments with geldanamycin (GA) (marked with the solid line labelled GA).

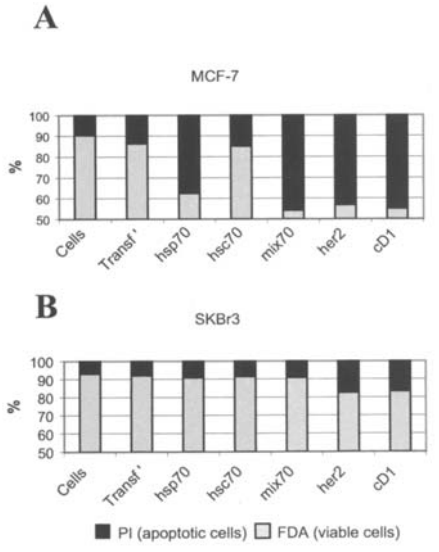

Figure 2. Relative quantification of viable and apoptotic MCF-7 and SKBr3 cells transfected with *hsp70*-, *hsc70*-, *her2*- and *cyclinD1*-siRNAs, and the controls. (A) The MCF-7 cells were analysed 72 h post siRNA transfection. An increased fraction of apoptotic MCF-7 cells was observed in response to the *hsp70*-, *mix70*-, *her2*- and *cyclinD1*- (cD1) siRNAs. (B) At 96-h post siRNA transfection, the SKBr3 cells responded by an increased fraction of apoptotic cells in response to the *her2*- and *cD1*-siRNAs. The black and grey bars illustrate the respective fractions of apoptotic and viable cells.

(Fig. 2A), while Hsc70 suppression alone did not result in apoptosis.

CyclinD1 or Her2, two proteins whose loss of function or suppression leads to reduced cell viability (29-31), were used as the controls. As expected, an increase in apoptosis was observed for the MCF-7 cells treated by *cyclinD1* or *her2*-siRNA (Fig. 2A).

In SKBr3, we observed no reproducible viability loss or apoptosis upon Hsp70, Hsc70 or Mix70 suppression (six independent experiments were performed). In the control experiments, the suppression of CyclinD1 and Her2 resulted in increased apoptosis 96 h post siRNA transfection (Fig. 2B).

Hsp70 or Hsc70 suppressions do not reduce the activity of the Hsp90 chaperone protein complex. Both Hsp70 and Hsc70 are known to interact with Hsp90 to form a complex able to chaperone many client proteins (32-34). In tumour cell lines, the inhibition of Hsp90 activity by GA leads to a

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Changes in the Her2 cell surface levels and cell viability of MCF-7 and SKBr3 in response to GA concentration gradients.

	MCF-7				SKBr3		
	24 h	48 h	72 h		24 h	48 h	72 h
A		Reduced Her2 expression (%)					
25 nM GA	12±3	19±4	9±4 *ns*	5 nM GA	28±8	19±4	24±4
75 nM GA	34±1	37±4	34±9	15 nM GA	62±10	43±9	55±5
225 nM GA	66±2	66±3	60±15	45 nM GA	90±3	81±6	81±3
B		Cell viability (%)					
Control cells	90±2	89±5	87±4	Control cells	94±2	88±2	84±6
25 nM GA	66±6	53±7	64±4	5 nM GA	92±2 *ns*	69±7	56±6
75 nM GA	36±3	14±2	14±4	15 nM GA	83±5 *ns*	47±2	30±3
225 nM GA	23±10	8±1	8±2	45 nM GA	82±8 *ns*	40±5	21±4

GA, geldanamycin. (A) The cells were immunolabelled with anti-Her2 antibodies 24, 48 and 72 h after replacing the media with GA-containing growth media. Changes in the Her2 levels are given as the percentage loss of expression relative to the Her2 expression level in the vehicle control cells [MCF-7 and SKBr3 cells incubated with the vehicle (DMSO) buffer-containing media]. (B) Percentage viable (FDA-positive) MCF-7 and SKBr3 cells relative to the vehicle control cells. All changes in the Her2 expression and viability were found to be statistically significant relative to the control cells (n=4, t-test, $p<0.05$), except those denoted as ns (non-significant).

decrease in both proliferation and cell viability. Her2 is a well-known Hsp90 client protein whose cell surface expression is decreased upon GA treatment in a time- and dose-dependent manner (35). We used the cell surface expression level of Her2 as a measurement of the chaperone activity and investigated the importance of the Hsp70 and Hsc70 proteins in this chaperone complex. Hsc70 and Hsp70 protein levels were suppressed by the use of *hsc70*- and *hsp70*-siRNAs, with or without GA co-treatment.

First we established an immunolabelling assay in order to enable us to follow Her2 cell surface expression. Cells were exposed to GA concentration gradients (MCF-7: 25, 75 and 225 nM GA; SKBr3: 5, 15 and 45 nM GA). Subsequent Her2 cell surface levels were detected by immunolabelling 24, 48 and 72 h after GA exposure (Table II). The lowest GA dosage used led to Her2 depletions of about 10-25% in both cell lines. Maximum Her2 depletion with the highest GA dosage was 66% and 90% in MCF-7 and SKBr3, respectively. GA-mediated Her2 depletion persisted for at least three days in both cell cultures, except with the lowest GA dosage (25 nM) in MCF-7 which was not found statistically significant on the last day. We also showed in the control experiment that the Her2 cell surface expression on MCF-7 and SKBr3 could be reduced by 30% using the *her2*-siRNA (Fig. 3, *her2* bars).

The correlation between GA concentrations and cell viability is shown in Table IIB. For further applications of GA in this study, we used dosages inhibiting MCF-7 and SKBr3 cell viability by 47±7% (2 days incubation) and 44±6% (three days incubation), respectively (MCF-7, 25 nM GA; SKBr3, 5 nM GA). These GA dosages suppressed Her2 surface expression consistently by 10-25%. When GA was used in combination with the siRNAs, GA was added to the growth media 24 h post siRNA transfection and the cells were left for an additional 24, 48 and 72 h before analysis.

The role of the Hsp70 or Hsc70 proteins in the Her2 chaperoning was investigated by transfecting MCF-7 and SKBr3 with the *hsp70*- or *hsc70*-siRNA. In MCF-7, the addition of *hsp70*- or *hsc70*-siRNAs resulted in a significant increase in Her2 expression at the cell surface (20-40% in independent experiments), 72 and 96 h post siRNA transfection (Fig. 3A and B). 'Mix70' had a comparable impact as opposed to the single siRNA treatments 72 h post siRNA transfection (Fig. 3A and B, *mix70*). In contrast, the suppression of Hsp70, Hsc70 or 'Mix70' in SKBr3 exhibited no significant effect on Her2 surface expression (Fig. 3C and D). The elevated intrinsic Her2 level in SKBr3 could explain this finding. In conclusion, suppressing the Hsp70 family partners of the Hsp90 super-chaperone complex does not lead to a reduced Hsp90 chaperone activity.

We then wanted to determine whether the Her2 cell surface expression could be altered by Hsp70/Hsc70 interferences and the partial co-inhibition of Hsp90 (Fig. 3, GA exposed samples shown in the dark-grey bars). Co-treatment with GA was attempted as Hsp90 mRNA and protein expression levels were observed to increase 2-3-fold in the *hsc70* and *mix70* suppressions in MCF-7, as shown by real-time PCR and immunoblotting (Fig. 4) (Table I). This indicates an increased Hsp90 chaperone activity which in turn could lead to elevated Her2 cell surface levels. A similar increase in Hsp90 protein expression was also observed in the control cells, *hsp70*-, *hsc70*- and *mix70*-siRNA transfected MCF-7 that were co-treated with 25 nM GA (Fig. 4B). However, despite the GA-mediated increase in Hsp90 protein levels, we have shown that 25 nM GA significantly reduced the overall Hsp90 chaperone activity (Table IIB).

In MCF-7 co-treated with GA, the Her2 expression increased 72 h post *hsp70*-, *hsc70*- and *mix70*-siRNA transfections, as compared to the GA/transfectant control cells

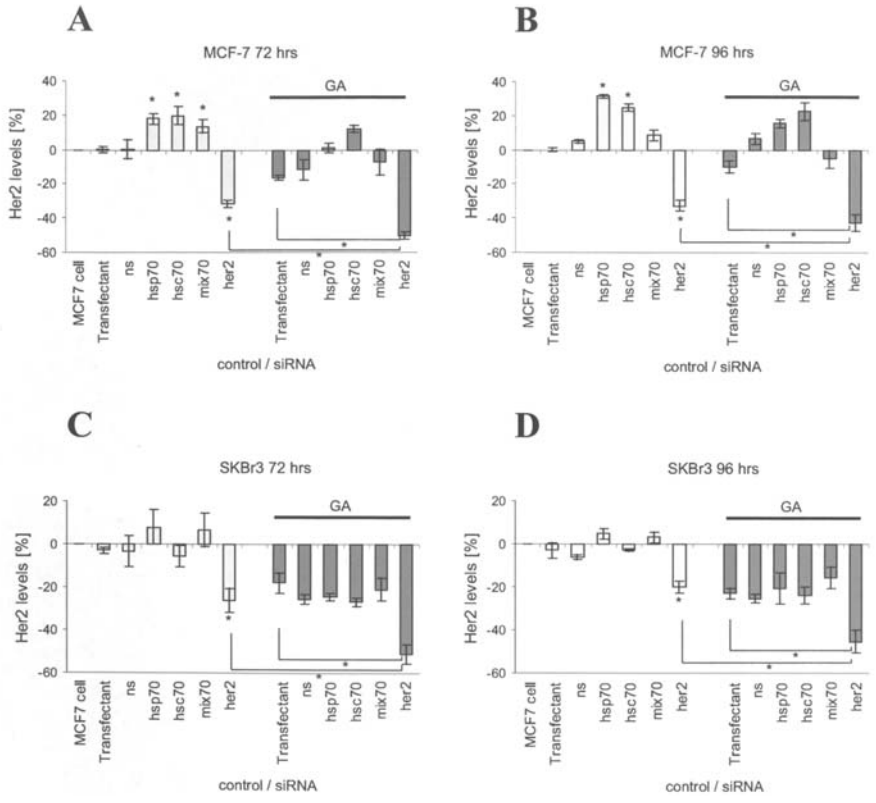

Figure 3. Her2 immunolabelling of MCF-7 and SKBr3 cells after transfection with *hsp70*-, *hsc70*-, *mix70*-, *her2*- and *non-silencing (ns)*-siRNAs and co-treatment with geldanamycin (GA). MCF-7 and SKBr3 cells were exposed to 25 and 5 nM GA, respectively. Her2 expression levels are shown relative to the Her2 expression level in non-treated cells. For MCF-7, changes in Her2 levels are shown 72 (A) and 96 h (B) post siRNA transfections. In MCF-7 *hsp70*-, *hsc70*- and *mix70*-siRNA transfections increased Her2 cell surface expression levels. The *her2*-siRNA significantly reduced the level of Her2 cell surface expression. In the co-treatment with GA we observed an accumulative reduction on Her2 cell surface expression only for the *her2*-siRNA. Similar results were shown for the *her2*-siRNA and GA/*her2*-siRNA treatments in SKBr3 72, as investigated 72 (C) and 96 h post siRNA transfection. *hsp70*-, *hsc70*- and *mix70*-siRNA transfections in SKBr3 had no impact on Her2 cell surface levels. Standard error means are shown by error bars (n=3) and statistically significant values are denoted by asterisks (*). Similar results were shown in at least 4 independent experiments.

(Fig. 3A and B). A similar effect was observed for the Hsc70 suppressions 96 h post siRNA transfections. Thus, partial inhibition of the Hsp90 chaperone activity by GA cannot prevent the Hsp70 and Hsc70 suppression-mediated increase in the Her2 cell surface expression level. This indicates that the Hsp70/Hsc70 suppression is a major block on the Her2 protein degradation pathway, leaving the remaining Hsp70/Hsc70 proteins to present immature Her2 proteins to Hsp90 for chaperoning and subsequent cell surface expression.

In the *hsp70*- and/or *hsc70*-siRNA transfected SKBr3 cells, the addition of GA had no impact on Her2 cell surface expression, as compared to the GA/transfectant or GA/*ns*-siRNA control cells. Also, in contrast to MCF-7, *hsp70*- and *hsc70*-siRNA transfections in SKBr3 did not change Hsp90 mRNA or protein levels (Fig 3C and Table I, *hsp90* RT-PCR assay; protein data not shown).

In a control experiment with siRNA-mediated Her2 suppression and partial Hsp90 inhibition we demonstrated the additive loss of Her2 cell surface expression in both MCF-7 and SKBr3, 72 and 96 h post siRNA transfection (Fig. 3). This shows that *her2*-mRNA levels and Hsp90 chaperone activity both can regulate the expression of Her2 on the cell surface in two independent steps, most likely by controlling the amount of Her2 proteins exposed to the Hsp90 chaperone system and by regulating the chaperone activity itself, respectively.

Thus, in MCF-7 as well as in SKBr3, the Hsp70 and/or Hsc70 suppression, without or combined with partial Hsp90 inhibition, does not impair the chaperoning activity on Her2 beyond the effect mediated by GA exposure alone. This suggests that Hsp70 and Hsc70 do not have a rate-limiting control on the Hsp90 chaperoning activity.

MCF-7 and SKBr3 viability loss upon Hsp70 and Hsc70 suppression, and co-treatment with partial Hsp90 inhibition. We have shown that both the GA-mediated Hsp90 inhibition and Hsp70/Hsc70 suppression, respectively, had the ability to reduce cell viability (Table I) (Fig. 2). We then wanted to determine the impact of the concomitant Hsp90 inhibition and Hsp70/Hsc70 suppression on cell viability. This was performed by transfecting MCF-7 and SKBr3 with *hsp70/hsc70*-siRNA one day prior to the addition of GA, as described above. Treated MCF-7 and SKBr3 cultures were analysed by PI/FDA at 72 and 96 h post siRNA transfection (48- and 72-h exposure to GA). Six independent experiments were performed and the experiment shown here is based on triplicate sample sets.

Mix70 suppression combined with the Hsp90 inhibition protocol resulted in the accumulative reduction in cell viability in MCF-7 and SKBr3 (Fig. 5A and B). *mix70*-siRNA transfection reduced MCF-7 cell viability by 44%, as compared to the *ns*-siRNA transfection controls (Fig. 5A). GA exposure

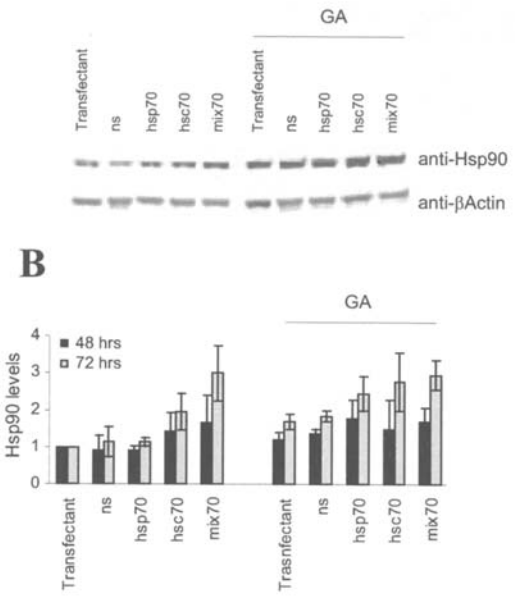

Figure 4. Immunoblot analyses of Hsp90 protein expression in MCF-7 48 and 72 h post *hsp70*-, *hsc70*-, *mix70* and *non-silencing (ns)*-siRNA transfections, and in co-treatments with geldanamycin (GA). (A) Immunoblot analysis of Hsp90 and ß-Actin. (B) Diagram showing relative changes in Hsp90 protein expression relative to the cells exposed to the transfectant alone. Relative Hsp90 expression levels 48 and 72 h post siRNA transfections are shown by the black and grey bars, respectively. While Hsp90 was increased following Hsc70 or Mix70 suppression alone, all MCF-7 cells treated with GA displayed elevated Hsp90 levels. A trend in which GA exposure combined with *hsp70*-, *hsc70*- or *mix70*-siRNAs gave the highest elevated Hsp90 levels, was observed.

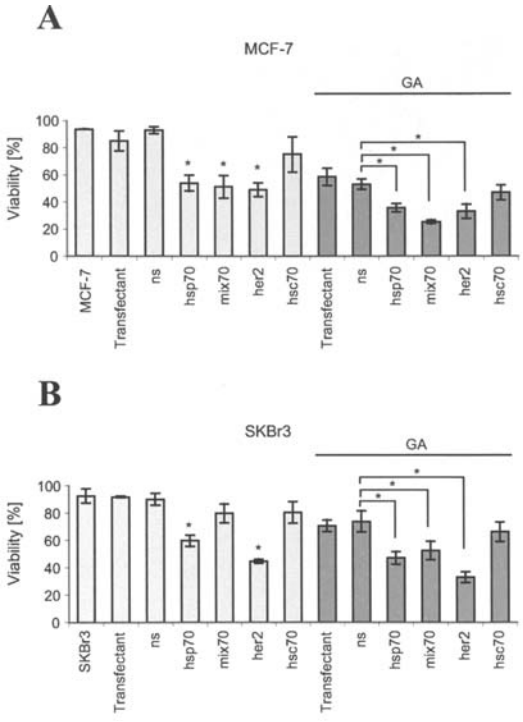

Figure 5. Quantification of viable MCF-7 and SKBr3 cells by the PI/FDA/beads assay following transfections with *hsp70*-, *hsc70*-, *her2*- and *non-silencing (ns)*-siRNAs, and in co-treatments with geldanamycin (GA). The bars illustrate the percentage number of viable (FDA-positive) cells, as compared to the non-treated cells. (A) MCF-7 was analysed 72 h post siRNA transfection and showed significantly reduced viability in response to *hsp70*-, *mix70*- and *her2*-siRNA transfections. In the co-treatment with GA, an accumulative loss of cell viability was observed for the same siRNA transfections. The *mix70*-siRNA yielded the strongest viability loss when combined with GA. *hsc70*-siRNA transfection alone, or in combination with GA, had no effect on cell viability. (B) SKBr3 was analysed 96 h post siRNA transfection and showed a reduced viability in response to *hsp70*-, and *her2*-siRNA transfections. No response was seen following *mix70*- and *hsc70*-siRNA transfections. In the co-treatment with GA, an accumulative loss of cell viability was observed for all siRNA transfections, except *hsc70*. The *mix70*-siRNA yielded the strongest viability loss when combined with GA. *hsc70*-siRNA transfections when combined with GA exposure had no additional effect on SKBr3 viability. Standard error means are shown in the error bars (n=3) and statistically significant values ($p<0.05$) are denoted by asterisks (*). Similar results were observed in 5-6 independent experiments, except for the *hsc70*-siRNA transfections in SKBr3 (see text for details).

to the *ns*-siRNA transfected control cells resulted in 48% loss of cell viability. *mix70*-siRNA transfection combined with GA exposure resulted in 47% loss of cell viability relative to the GA/*ns*-control. Thus, *mix70*-siRNA blocked MCF-7 proliferation equally well in the absence or presence of GA, and the combined anti-proliferative effect of the Mix70 suppression and Hsp90 inhibition is therefore most likely, additive. Single *hsp70*- and *her2*-siRNA transfections reduced cell viability by about 40%, respectively, as compared to the *ns*-siRNA control. In combination with GA they were slightly less efficient resulting in about 30% reduced cell viability, as compared to the GA/*ns*-siRNA control. Hsc70 suppression did not result in any significant loss of cell viability either following single *hsc70*-siRNA transfections, or in combination with GA. This was possibly due to a functional rescue by the induced Hsp70 expression that could be mediated by *hsc70* interference and/or Hsp90 inhibition (Table I, *hsc70*-siRNA rows).

In SKBr3, we did not obtain a consistent anti-proliferative effect in response to *hsp70*- or *mix70*-siRNA transfections, as described above (Fig. 2B). In two separate experiments (out of six), *hsp70*- and *mix70*-siRNA transfections showed about a 30% loss of SKBr3 viability, respectively. In contrast, in the co-treatments with GA, SKBr3 cells sensitized to Mix70 and Hsp70 suppressions in terms of reduced cell viabilty (Fig. 5B). In the GA/*mix70*-siRNA co-treatments we observed 30% reduced viability, as compared to the GA/*ns*-siRNA control. Thus, we cannot rule out the possibility of a synergetic anti-proliferative effect by the two treatments. In Fig. 5, the effect of *hsp70*- and *her2*-siRNA transfections was shown to reduce cell viability by 33 and 50% as compared to the *ns*-siRNA transfection control, respectively. A similar efficiency in reducing cell viability was observed in the co-treatment with GA, as compared to the GA/*ns*-siRNA control. For Her2 suppression, this additive effect on cell-viability-loss in combination with Hsp90 inhibition is consistent with the additive effect the two treatments have on Hsp90/Hsp70 chaperone-mediated Her2 cell surface expression (Fig. 3C and D). Also in SKBr3, Hsc70 suppression did not result in reduced cell viability, alone or in combination with GA.

Thus, while Her2 suppression and Hsp90 inhibition reduce cell viability through the reduced action of the Hsp90/Hsp70-chaperone complex, Mix70 suppression is likely to trigger cell-viability-loss through other molecular mechanisms.

Discussion

In this report, we investigated the cell viability of two breast cancer cell lines following the depletion of the chaperones, Hsp70 and Hsc70. Dissecting the role of Hsp70-family members has become an important strategy in order to identify the proteins responsible for the anti-apoptotic and cell survival properties associated with this protein family (21). The Hsp70 chaperone system is known to cooperate with the Hsp90 chaperone system in a Hsp90 super-chaperone complex which is responsible for the maturation, activation and inactivation of numerous client proteins. Many of the Hsp90 clients encode factors involved in apoptosis, growth, survival and the maintenance of transformed cellular states (23,36). In order to address the functional relationship between the Hsp70 family and Hsp90, we blocked the expression of Hsp70 and Hsc70 proteins and monitored the cell viabilities as well as the chaperoning activity of Hsp90. Our results showed beneficial effects in terms of reduced cell viabilities following the simultaneous blockade of the two chaperone systems. However, Hsp70 and Hsc70 chaperone levels did not have a rate-limiting effect on the activity of the Hsp90/Hsp70 chaperone complex.

The *hsp70*-siRNA was designed to specifically recognize the two intronless Hsp70 transcripts, *hsp70*A1A and *hsp70*A1B. The siRNA does not recognize the *hsp70-Hom* and *hspA2*-mRNAs that encode the testis abundant Hsp70t and Hsp70-2 proteins, respectively. Hsp70t and Hsp70-2 share 91 and 84% protein similarity with Hsp70, respectively. While the Hsp70-2 and Hsp70t expressions are low or non-detectable in normal tissues, the *hspA2*-mRNA expression is detected in human cancer cell lines, including MCF-7 (21,37). However, no induction of the Hsp70-2 protein was reported in the cells depleted for the Hsp70 and Hsc70 proteins. Limited information is available for the Hsp70t expression patterns, although it has been shown that heat shock-induced stress in HeLa cells can trigger the translocation of Hsp70t from the cytoplasm to the nucleus without increasing the total Hsp70t expression level (37). Thus, we cannot rule out that HspA2 or Hsp70t have a functionally redundant property in the cells depleted for Hsp70 and Hsc70 proteins. However, their expressions are low and are unlikely to be induced in response to Hsp70 or Hsc70 depletion in normal or tumour cells. Therefore, the loss of viability in MCF-7 and SKBr3 cells that we observed in various treatments with Hsp70 and Hsc70 depletion, most likely reflects the specific loss of functions of the two proteins (Fig. 2).

When addressing the anti-cancer potential of down-regulated HSP proteins it is important to evaluate the effects on related members of this protein family: Potential inhibitors are likely to recognize structurally similar protein domains, and could induce other HSP proteins in a stress-dependent manner. For instance, it has recently been shown that Hsp90 inhibition increased Hsp70 levels, and that *hsp70* interference could sensitise K-562 cells to Hsp90 inhibition-mediated apoptosis (38). We observed a similar induction of *hsp70* in our two breast cancer cell lines and showed an additive reduction in cell viability in response to the Hsp90 and Hsp70 blockade (Figs. 1 and 5). In our report, we further demonstrate that Hsc70 suppression induces the expression of Hsp70 and Hsp90 (Table I) (Figs. 1 and 4). Both Hsp70 and Hsp90 could compensate for the loss of Hsc70. Such a rescue could depend on the increased activity or capacity of the Hsp90 super-chaperone complex, or on the increased amounts of the Hsp70 protein that simply substitute for and replace lost Hsc70 proteins. This functional compensation is likely to explain the unchanged cell viability and lack of apoptosis seen in MCF-7 and SKBr3 cells transfected with the *hsc70*-siRNA (Figs. 2 and 5). Alternatively, it has recently been proposed that Hsp70 and Hsc70 have respective roles in the regulation of cell growth and survival (21). It has been suggested that Hsc70 is important for cell survival processes while Hsp70 (and HspA2) is critical for cell growth. Thus, we cannot rule out that the differences in cell viability effects observed in response to Hsp70 and Hsc70 depletions are encoded in the cells, implicating that they could depend more on the Hsp70 cell growth mechanisms than on the Hsc70 survival mechanisms.

Hsp70 suppression had no effect on Hsp90 and Hsc70 mRNA or protein expression levels (Figs. 1, 2 and 5). This is in agreement with the observation made with the pluripotent embryonal carcinoma cell line, P19, transfected with an *hsp70* antisense cDNA (39). When we combined Hsp70 and Hsc70 suppression by transfecting a mixed *hsp70/hsc70*-siRNA pool, an increased Hsp90 and Hsp70 expression was observed. This could rely on the known property of Hsc70 and Hsp90 to associate with and regulate the transcriptional activity of the stress regulated heat shock factor HSF1 (40-42). In response to various stimuli or stress, hyperphosphorylated homotrimeric HSF1 activates the expression of inducible heat shock genes, including Hsp70, by binding to specific heat shock elements (HSEs) in the promoter regions. In non-stressed cells, Hsc70 is shown to associate with inactive homodimeric HSF1, while GA-mediated inhibition of Hsp90 has been demonstrated to enhance HSF1 DNA binding and transcriptional activity. Thus, blocking the expression or activity of both Hsc70 and Hsp90 is likely to enhance *hsp70* gene expression via an HSF1-dependent mechanism.

When Hsp90 was inhibited by GA in MCF-7 and SKBr3 cells, and depleted for Hsp70 and/or Hsc70 proteins, both cell lines responded with the accumulative blockade of cell proliferation in response to the two respective treatments. In particular, SKBr3 was clearly sensitized to respond more strongly and consistently towards Hsp70 and Mix70 depletions if the Hsp90 activity was blocked as well (Fig. 5). This indicates the critical role of Hsp70 and Hsc70 expression levels upon additional stress stimuli, at least in SKBr3. However, the limitation for SKBr3 to respond to *hsp70*- and *hsc70*-siRNA transfections alone (without GA exposure) cannot be taken as evidence for SKBr3's independence from the two targeted proteins. SKBr3 cell cultures grow slower than the MCF-7 ones and the siRNA-induced protein depletion occurs at a lower rate than that in MCF-7 (data not shown). Thus, it is possible that *hsp70* and *hsc70* interference protocols lasting for longer time periods could induce SKBr3 cell death.

Hsp90 is already a promising anti-cancer drug target. The mechanisms behind its anti-apoptotic effects, cell growth and survival are well characterized (23,24). For instance, the Hsp90 client protein, Survivin, is over-expressed in almost

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's and can elevate the cells' threshold for entering as well as promoting cell proliferation (43). Hsp90 can also interfere with the intrinsic caspase apoptotic pathway by associating with Apaf-1, thereby inhibiting the formation of a functional apoptosome (44). Hsp90 is able to chaperone proteins whose disregulation favours transformation, e.g. Her2, Raf-1, Akt, Cdk4, mutant P53 and mutant B-Raf. However, it is unclear as to what extent Hsp70 or Hsc70 expression levels influence the maturation and activation of various Hsp90 chaperone client proteins. We characterized the expression of the active state of the Hsp90 client protein, Her2, following the suppression of Hsp70 or Hsc70 (Fig. 3). The Her2 expression level on the cell surface corresponds to its active state and is a suitable marker for the Hsp90 protein activity. Her2 is shown to be dependent on the cytosolic Hsp90 chaperone system (25), and to correlate with the degree of GA-inhibited Hsp90 activity (35,45).

In our study, the Her2 protein expression level was not blocked following the suppression of Hsp70, Hsc70 or mixed Hsp70/Hsc70 expression. In contrast, MCF-7, but not SKBr3, showed a weak increase in Her2 levels. This could be due to functional redundancy by other HSP family members leading to the increased activation of the Hsp90/Hsp70 chaperone complex. However, as described above, we find it unlikely that Hsp70 family members other than the described enhancement of the *hsp70* gene expression, compensate for lost Hsp70/Hsc70 levels in our system (Table I) (Fig. 1). The Hsp90-mRNA and protein were shown to be elevated in Hsc70 and Mix70 depletions, but not in the Hsp70 depletions (Fig. 4). Thus, we are not aware of a common chaperone protein that can rescue the loss of both Hsp70 and Hsc70. Enhanced Her2 levels in response to Hsp70 and Mix70 depletions are therefore more likely due to the association of Hsp70 and Hsc70 with the protein degradation pathway. The Hsp90 and Hsp70 chaperone systems interact with the proteasome pathway via the E3 ubiquitin ligase CHIP. CHIP is a chaperone-interacting protein that contains an N-terminal tetratricopeptide (TPR) and a C-terminal U-box domain (46,47). TPR and U-box bind to Hsp70/Hsc70 and Hsp90 and are shown to mediate the degradation of Her2 (and others) in response to the application of GA (27,28). It is possible that the suppression of Hsp70 and/or Hsc70 partially blocks the Her2 degradation, thus leading to elevated Her2/ErbB2 expression levels.

In order to investigate whether the Hsp90 super-chaperone system could be linked to the accumulative block of cell proliferation following the combined Hsp70/Hsc70 depletion and Hsp90 inhibition, we analysed the expression level of the active client protein in the co-treated cells (Fig. 4). MCF-7 and SKBr3 did not show any accumulative reduction in Her2 expression when the GA-treated cells were combined with Hsp70 and/or Hsc70 depletions. GA exposure was the only treatment that blocked the expression of the Hsp90/Hsp70 chaperone client protein. This indicates that the Hsp70 and Hsc70-mediated blockade of cell viability is linked to other cellular pathways. It also demonstrates that the Hsp70 and Hsc70 protein levels are not rate-limiting for the chaperone activity.

In conclusion, we have shown that reduced Hsp70/Hsc70 protein levels, in combination with a partial Hsp90 inhibition is a promising tool for reducing cancer cell viability. We have further shown that Hsp70 and Hsc70 protein expression levels are less likely to determine the expression levels of Hsp90 client proteins, such as Her2. Future studies should concentrate on identifying the specific pathways and mechanisms behind the loss of cancer cell viability in response to partially blocking the Hsp70 family members.

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