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Induction of heat shock protein HSPA6 (HSP70B′) upon HSP90 inhibition in cancer cell lines

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A B S T R A C T

Genome-wide transcript profiling to elucidate responses to HSP90 inhibition revealed strong induction of HSPA6 in MCF-7 cells treated with 17-AAG. Time- and dose dependent induction of HSPA6 (confirmed by qPCR and Western Blots) occurred also upon treatment with Radicicol, another HSP90 inhibitor. HSPA6 was not detectable in untreated cells or cells treated with toxins that do not inhibit HSP90, or upon applying oxidative stress. Thus, HSPA6 induction is not a general response to cytotoxic insults. Modulation of HSPA6 levels by siRNA-mediated inhibition or recombinant expression did not influence 17-AAG mediated cell death. HSPA6 induction as a consequence of HSP90 inhibition occurs in various (but not all) cell lines and may be a more specific marker for HSP90 inhibition than induction of other HSP70 proteins.

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1. Introduction

Geldanamycin is a small molecule that inhibits the function of HSP90 family proteins [1,2]. As HSP90 is overexpressed in many tumors, Geldanamycin is a candidate for anti-cancer therapies [3], and efficacy of Geldanamycin derivatives is evaluated in numerous clinical trials [4–6]. Preliminary results suggest that growth of HER2-positive breast cancers is particularly sensitive towards HSP90 inhibition by the Geldanamycin derivative 17-AAG (Tanespimycin) [7]. This observation might be explained by the dependency on HER2 expression of this cancer subset as HER2 is a "client" protein of HSP90 [8]. HSP90 is a master regulator of HER2 protein stability, and inhibition of HSP90 function results in degradation of HER2 [8]. It appears, however, that some members of the Heat shock 70 protein family become induced in Geldanamycin treated cells which interferes with the therapeutic

potency of Geldanamycin [9]. In complete agreement with that, a recent report demonstrates enhanced anti-cancer efficacy upon dual targeting of HSP90 and HSP70 in some cancer subtypes [10]. Therefore, a more detailed understanding of the interplay between members of the cellular HSP network in the context of HSP90-inhibition may improve anti-cancer therapies.

The HSP70 family comprises at least 13 members [11], of which HSPA6 (HSP70B′) appears evolutionary unique as it is not conserved in rodents [12,13]. HSPA6 expression is not detectable in most cells under normal conditions. It becomes induced upon severe stress conditions and might mediate cytoprotective functions in a cell-type and context-dependent manner [11,14,15]. The potential influence of drug-mediated HSP90 inhibition on expression of HSPA6 has not been analyzed so far. This work describes the specific induction of HSPA6 upon treatment of cancer cells with HSP90 inhibitors.

2. Results

2.1. Geldanamycin induces the expression of HSPA6 in MCF-7 breast cancer cells

A genome-wide analysis of transcriptional responses in MCF-7 breast cancer cells was performed upon exposure to respective

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IC50 concentrations of the HSP90 inhibitor 17-AAG, in comparison to other cytotoxic compounds with cellular targets other than HSP90: these included alpha-amanitin (ama, targets RNA-Pol), saporin or cycloheximide (sap, CHX, both target the ribosome). We were able to recapture previously described Geldanamycin-mediated effects, such as the induction of various HSP70 and HSP40 family members [16] (Table 1), thus validating our experimental work-flow. Surprisingly, we also observed that HSPA6, a poorly characterized member of the HSP70 family became induced to higher levels (more than 3000-fold) than any other gene (up to 50-fold), even though induction of HSPA6 expression upon Geldanamycin treatment has not been described before. HSPA7 is very closely related to HSPA6 (the putative HSPA7 protein is 94% identical to HSPA6), but has been characterized as a pseudogene [13,17]. Therefore, the mRNA hybridization signals could have been derived from HSPA6 and/or HSPA7. To differentiate between these two transcripts, we performed qPCR with three different primer sets at different time points following 17-AAG treatment. Each of these primer sets amplified regions of HSPA6/HSPA7 with at least one nucleotide mismatch between HSPA6 and HSPA7 sequences. We were subsequently able to distinguish between HSPA6/HSPA7 transcripts by sequencing transcript fragments derived from qPCRs at 4 h time point (=maximal induction). This revealed that all amplified fragments exclusively contained HSPA6 sequences, indicating exclusive (or at least predominant) induction of HSPA6 and not HSPA7 (Fig. 1). These qPCR analyses also revealed that induction of HSPA6 transcripts is time-dependent. It peaks around 4 h and declines to low levels between 4 and 8 h after 17-AAG treatment (Fig. 1). Further, HSPA6 mRNA induction is dose-dependent and saturation is observed with concentration of 17-AAG between 125 and 250 nM (Fig. 1C). Next, we performed Western Blot analyses to test

if GA-induced HSPA6 transcription translates to HSPA6 protein production. We separated 17-AAG treated and untreated MCF7 cell extracts on SDS/PAGE followed by immunoblotting with a HSPA6-specific antibody. HSPA6 protein is not detectable in untreated, but in 17-AAG-treated cells (Fig. 2A). Similar to our observations for HSPA6 mRNA levels, HSPA6 protein levels reach a plateau at around 4 h following 17-AAG treatment (Fig. 2A) and induction is concentration-dependent up to 500 nM 17-AAG (Fig. 2B). In contrast to other HSP70 proteins (the HSP70-antibody used in this study detects HSP72, HSPA1L as well as HSPA8), which remain significantly enriched even 24 h after 17-AAG treatment, HSPA6 protein levels decline rapidly between the 8 h and 24 h time points (Fig. 2A).

Taken together, we conclude that exposure of MCF-7 cells to 17-AAG leads to a transient induction of HSPA6 protein expression.

### 2.2. HSPA6 expression is specifically induced by HSP90 inhibitors

A comparison of the genome wide mRNA profiles of MCF7 cells treated with various toxins reveals that HSPA6 signals become induced only by 17-AAG exposure, but not by toxins with targets other than HSP90 (Table 1). This indicates that HSPA6 induction is not a general response towards cytotoxic insults, but instead either a specific consequence of treating cells with the substance 17-AAG, or triggered by inhibition of its molecular target HSP90.

In order to determine if induction of HSPA6 is a direct consequence of HSP90-inhibition, we examined the effects of Radicicol, another HSP90-inhibitor, on HSPA6 expression in MCF-7 cells. Similar to 17-AAG, Radicicol induced HSPA6 mRNA (Fig. 3) in a concentration-dependent manner. Maximum levels of induced HSPA6 transcripts appear somewhat lower in Radicicol- compared to 17-AAG-treated cells. This may be caused by the previously

Table 1

Induction of HSPA6 in 17-AAG treated MCF-7 cells. Cells were exposed for the indicated time periods to IC50 concentrations of toxins that either inhibit protein synthesis (Sap = Saporin, CHX = Cycloheximide), protein (re-)folding (17-AAG = 17-N-allylamino-17-demethoxygeldanamycin), or transcription (Ama = alpha-amanitin). Listed are log change values, i.e. fold changes are calculated according to the formula (Euler Constant)^{value}. Changes were observed with at least 2 probes for HSPA6/HSPA7, DNAJ and HSP90. HSP70A, HSP70B, and HSP70L were detected with one specific probe for each mRNA and one probe that detects HSP70A as well as HSP70B. Increased expression is indicated green (positive values), decreased expression in red (negative values), minor or no changes in yellow.

	17-AAG 2h	17-AAG 4h	17-AAG 7h	Ama 2h	Ama 4h	Ama 7h	Sap 2h	Sap 4h	Sap 7h	CHX 2h	CHX 4h	CHX 7h
HSPA6/HSPA7 (HSP70B')	7.03 6.53	8.30 7.90	7.01 6.50	-0.03 -0.10	-0.03 -0.01	0.06 0.00	-0.02 -0.04	0.17 -0.03	0.31 0.09	-0.15 -0.12	-0.13 -0.18	-0.09 -0.10
DNAJB1 (HSP40)	2.23 2.16	3.00 2.62	2.51 2.33	0.07 -0.21	0.19 -0.13	-0.12 -0.83	0.12 -0.11	0.52 0.31	0.57 0.27	-0.84 -0.92	-1.36 -1.36	-1.48 -1.51
DNAJA4 (HSP40)	1.50 1.21	3.01 2.43	3.92 2.98	-0.52 -0.25	-0.41 -0.31	-0.48 -0.24	-0.17 -0.15	-0.14 -0.01	0.62 0.24	-0.21 -0.31	-0.77 -0.57	-0.78 -0.84
DNAJB4 (HSP40)	2.17 1.84	3.24 2.88	3.06 2.69	-0.59 -0.57	0.08 0.23	0.31 -0.10	-0.40 -0.27	0.04 0.12	0.94 0.54	-0.44 -0.40	0.12 -0.06	0.41 0.17
HSPA1L (HSP70L)	2,07	2,81	2,14	-0,32	-0,03	0,06	-0,03	0,25	0,22	-0,77	-1,12	-1,39
HSPA1A/A1B (HSP70A+B)	1,65	1,89	1,95	-0,11	-0,14	0,25	-0,03	0,17	0,30	-1,85	-2,77	-2,77
HSPA1B (HSP70-B)	1,29	1,54	1,77	-0,19	0,10	0,19	-0,09	0,073	0,33	-1,33	-2,10	-2,29
HSPA1A (HSP70A/HSP72)	0,86	1,04	1,21	-0,10	0,02	0,06	-0,03	0,06	0,28	-1,31	-2,23	-2,41
Hsp90AA1 (HSP90A)	0,12 0,11	0,24 0,19	0,40 0,32	-0,07 -0,04	0,05 0,01	-0,06 -0,02	-0,04 -0,04	-0,02 -0,01	0,10 0,08	-0,06 -0,01	-0,07 -0,08	-0,11 -0,05
HSP90B1 (Grp94)	0,05 0,25	0,18 0,32	0,37 0,69	-0,09 -0,09	-0,04 -0,18	0,09 0,27	-0,01 -0,03	-0,04 -0,05	-0,06 -0,04	-0,06 -0,22	-0,16 -0,43	-0,18 -0,33

> +1.0	0.5 - 1.0	-0.5 – 0.5	-0.5 – -1.0	< -1.0
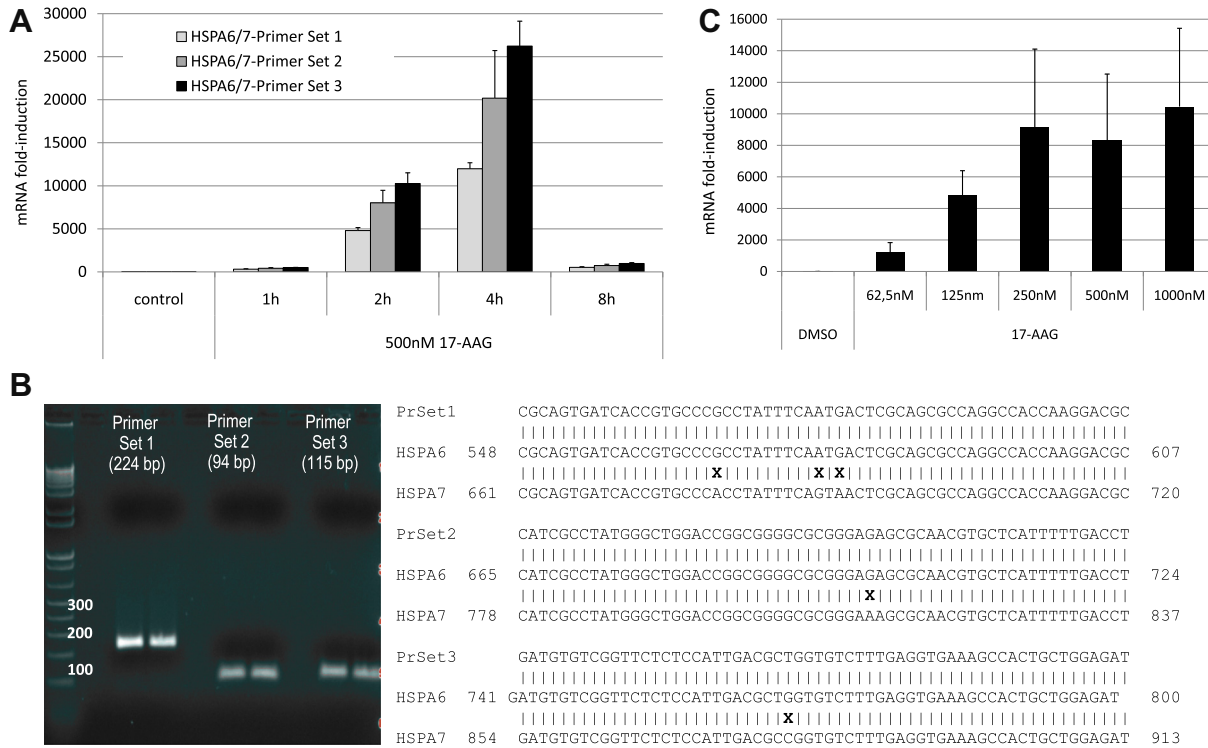
Fig. 1. Time- and dose-dependent induction of HSPA6, but not HSPA7, mRNA by 17-AAG. (A) MCF-7 cells were treated with 500 nM 17-AAG and mRNA extracted at different time points, as indicated, after treatment. Following reverse transcription, qPCR assays were performed using 3 different primer sets for HSPA6/HSPA7. Amplification of the housekeeping gene GAPDH served as an internal control and reference. Values for HSPA6/HSPA7 represent fold-changes compared to expression in untreated cells. Statistical analyses (one-way ANOVA, unpaired 2-tailed t-test) reveal significant time-dependent differences of HSPA6 induction vs control with for all time points vs each other and primer sets ($P < 0.01$ for all steps and primers except primer set 2 for 2–4 h $P < 0.05$). Induction levels decrease significantly after 4 h ($P < 0.01$ 4 h vs 8 h for all primer sets). (B) PCR products derived from qPCR assays performed on samples from cells treated for 4 h with 17-AAG treatment (see (A)) were separated on a 2% Agarosegel, extracted, column-purified and sequenced. Each primer set generated a single PCR-product of the expected size (224 bp for primer set1, 94 bp for primer set2, and 115 bp for primer set3). Sequencing confirmed the specific amplification of HSPA6 as all sequences aligned to 100% with the reference sequence of HSPA6 and positions with mismatches (marked 'x') for HSPA6 and HSPA7 did not amplify any signals, which could be derived from HSPA7 transcripts. The cDNA sources for HSPA6 were NCBI refseq NM_002155 and Genbank_AF093759 for HSPA7. The numbering of the HSPA6 and HSPA7 sequences relate to the nucleotide numbers in these sequences. (C) MCF-7 cells were treated for 4 h with different concentrations of 17-AAG, as indicated. QPCR assays were performed with HSPA6 primer set3 and GAPDH to determine fold-changes of HSPA6 transcripts compared to untreated cells. Statistical analyses (one-way ANOVA, unpaired 2-tailed t-test) reveal significant dose-dependent increases of HSPA6 induction vs control (DMSO) with $P < 0.03$ for 62.5 nM, $P < 0.02$ for 62–125 nM. Induction reaches saturation after 125 nM (therefore induction levels do not increase further in a significant manner).
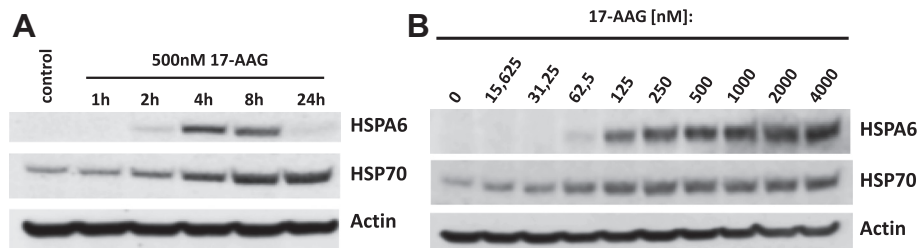
Fig. 2. Time- and dose-dependent induction of HSPA6 protein by 17-AAG. (A) MCF-7 cells were treated with 500 nM 17-AAG and cells lysed at different time points, as indicated, after treatment. Samples were separated on SDS–PAGE, proteins transferred to a PVDF membrane and HSPA6, HSP70 and Actin proteins detected with appropriate antibodies. (B) MCF-7 cells were treated for 8 h with different concentrations of 17-AAG, as indicated, cells lysed and processed as described in (A).

described mRNA destabilization effects mediated by Radicicol derivatives [18].

These results indicate that the specific trigger of HSPA6 induction is the inhibition of HSP90.

2.3. HSPA6 is not induced by Thapsigargin- or BrefeldinA-mediated induction of the unfolded protein response

Cytotoxins, other than 17-AAG, used in our genome-wide transcriptional analysis did not significantly induce Heat shock 70 family proteins (Table 1). Therefore, our data do not indicate if HSPA6 represents a specialized, differentially regulated arm of the HSP70 family or if HSPA6 is commonly co-induced along with other HSP70 proteins. 17-AAG has been reported to cause ER stress and to induce genes of Unfolded Protein Response (UPR) pathway, including the HSP70 family member GRP78/BiP (also known as HSPA5 or HSP70-5) [19,20]. Further, GRP78/BiP is induced by other ER stress/UPR inducing compounds with modes of action other than HSP90 inhibition, e.g. Thapsigargin [21,22] and BrefeldinA [23,24]. Thus, we were wondering if treatment of cells with Thapsigargin or BrefeldinA may also result in induction of HSPA6. Interestingly, only 17-AAG and Radicicol, but not Thapsigargin or BrefeldinA
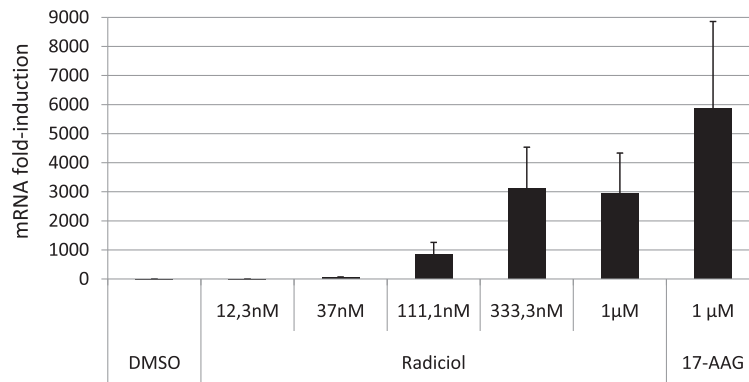
Fig. 3. Dose dependent induction of HSPA6 mRNA by Radicicol. MCF-7 cells were treated for 4 h with different concentrations of Radicicol, as indicated, or 1 μM of 17-AAG. Subsequently, qPCR assays were performed as described in Fig. 1. Shown are fold-changes for HSPA6 compared to untreated cells. Statistical analyses (one-way ANOVA, unpaired 2-tailed t-test) reveal significant dose-dependent increases of HSPA6 induction vs lowest AAG concentration (12.3 nM) with $P < 0.02$ for 37 nM, $P < 0.03$ for 37–111 nM, $P = 0.054$ for 333 nM and higher. Induction reaches saturation after 111 nM (therefore induction levels do not increase further in a significant manner).
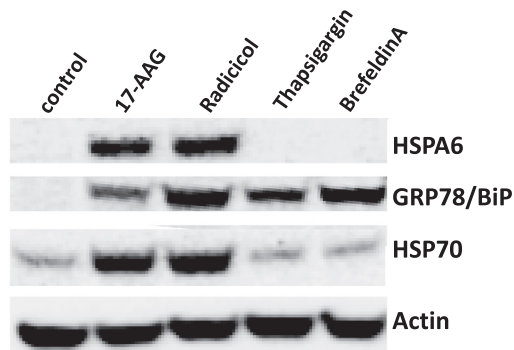

Fig. 4. HSPA6 induction is specifically triggered by HSP90 inhibition. MCF-7 cells were treated for 6 h with approximately 4xIC50 concentrations of 17-AAG (1 μM), Radicicol (1 μM), Thapsigargin (30 nM) or BrefeldinA (0.1 μg/ml). Following cell lysis, samples were separated on SDS–PAGE, proteins transferred to a PVDF membrane and HSPA6, GRP78/Bip, HSP70 and Actin detected with appropriate antibodies.

can induce HSPA6 protein expression (Fig. 4). In contrast, the UPR marker protein GRP78/BiP is strongly upregulated following treatment with any of the 4 compounds tested (Fig. 4). This indicates

that the trigger of HSPA6 induction is specifically and directly linked to HSP90 inhibition, and not a consequence of UPR induction. Also, expression of the HSP70 family proteins HSPA6 and GRP78/BiP does not seem to be co-regulated. However, increased expression of "HSP70" ("HSP70" = simultaneous detection of HSP72, HSPA1L and HSPA8) is observed following treatment with 17-AAG and Radicicol, but not Thapsigargin or BrefeldinA. Thus, induction of HSPA6 expression correlates with upregulation of one or more other HSP70 family proteins (Fig. 4 and Table 1). Like other HSP70 family members, HSPA6 becomes induced upon applying heat-shock (Fig. 9 and Suppl. Fig. 5), most likely as consequence of HSF-dependent transcription.

### 2.4. Induction of HSPA6 expression following HSP90 inhibition in different cancer cell lines

Is HSPA6 induction upon HSP90 inhibition a specific feature of MCF-7 (breast cancer) cells, or does it represent a more general response that can be observed in other cells? To address this question, we treated 12 different cancer cell lines with their respective IC50 concentrations of 17-AAG (Fig. 5, Table 2 and Suppl. Data S1) and determined HSPA6 levels by Western Blotting. Of the 7 breast cancer cell lines that we analyzed, 4
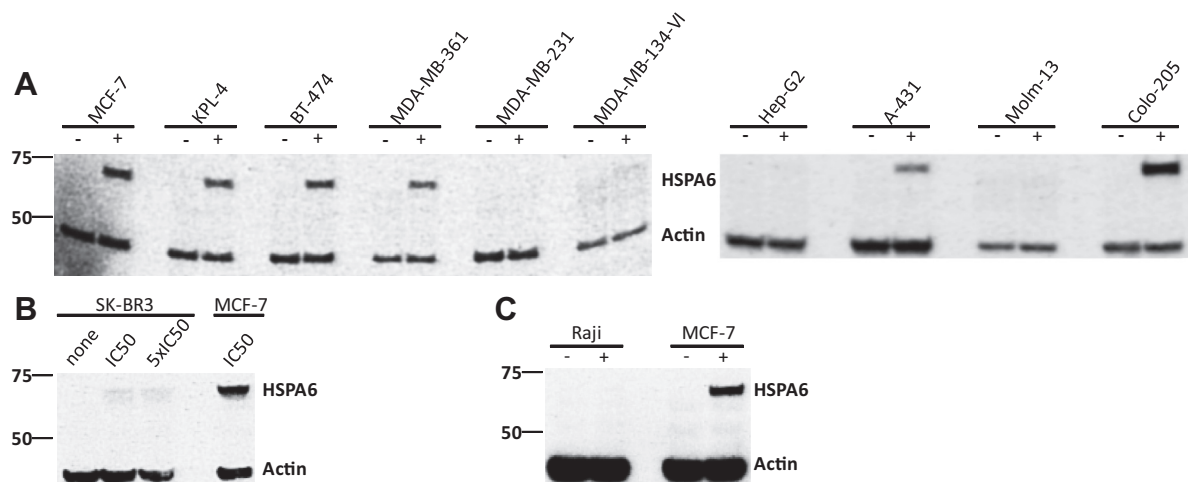
Fig. 5. Induction of HSPA6 by 17-AAG in various cancer cell lines. (A–C) Cancer cell lines were treated for 6 h with their respective IC50 concentration of 17-AAG (see Fig. S1 and Table 2). HSPA6 and Actin protein levels were determined by Western Blot analysis. (B) In addition, 5xIC50 concentrations of 17-AAG have been applied to SK-BR3 cells to determine if lack of HSPA6 induction is cell line or concentration dependent. (C) Raji and MCF-7 cells have been treated with 20 μM 17-AAG (=estimated IC50 for Raji) for 6 h to exclude detrimental effects on HSPA6 induction with very high concentrations of 17-AAG.
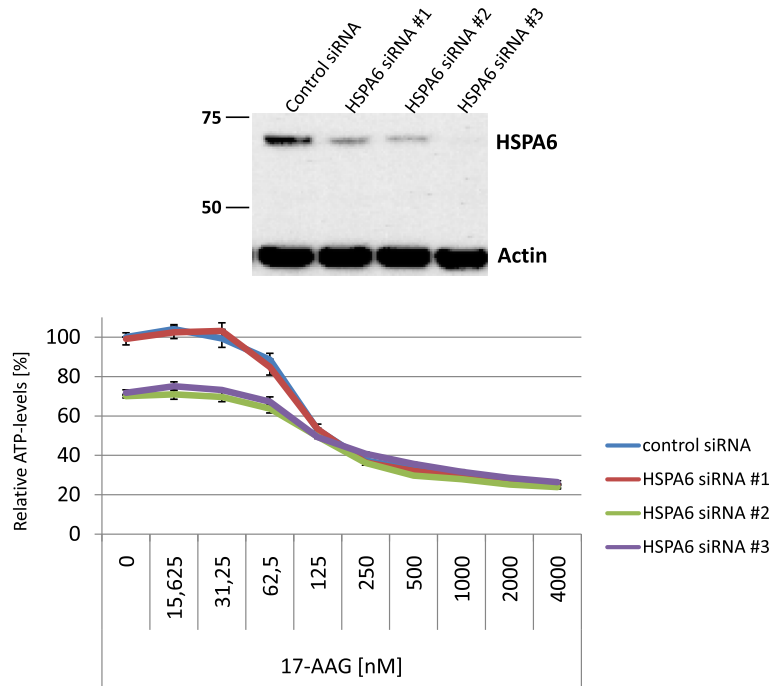
Fig. 6. ATP levels in MCF-7 cells following RNA Interference to blank HSPA6 induction in response to 17-AAG. MCF-7 cells were transfected with non-targeting (control) or HSPA6-specific (#1–3) siRNA duplexes. (Upper panel), 4 h after transfection, cells were treated with 1 μM 17-AAG. 4 h after 17-AAG treatment cells were lysed, samples separated on SDS–PAGE, and Actin and HSPA6 proteins detected by Western Blotting. (Lower panel), 4 h after transfection, cells were treated with increasing concentrations of 17-AAG, as indicated. 72 h after 17-AAG treatment, Cell Titer Glo assays were performed.
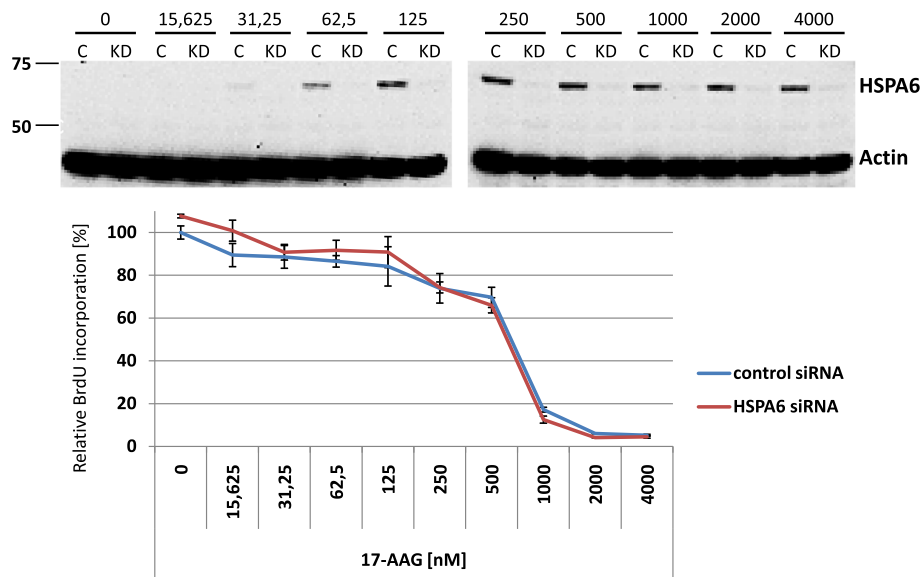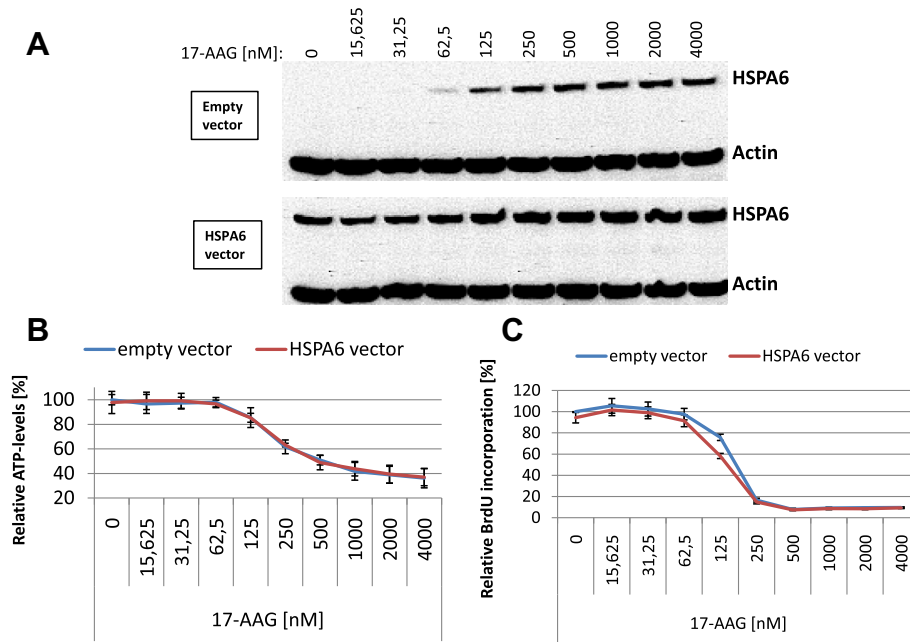

Fig. 7. BrdU incorporation in MCF-7 cells following RNA Interference to blank HSPA6 induction in response to 17-AAG. MCF-7 cells were transfected with control siRNA ("C") or HSPA6-siRNA#3 ("KD"). (Upper panel), 4 h after transfection, cells were treated with increasing concentrations of 17-AAG, as indicated. 4 h after 17-AAG treatment cells were lysed, samples separated on SDS–PAGE, and Actin and HSPA6 proteins detected by Western Blotting. (Lower panel), 4 h after transfection, cells were treated with increasing concentrations of 17-AAG, as indicated. 24 h after 17-AAG treatment, BrdU incorporation assays were performed.

(MCF-7, KPL4, BT-474, MDA-MB-361) demonstrated a strong induction of HSPA6 protein. The other three breast cancer cell lines (MDA-MB-134-VI, MDA-MB-231, SK-BR3) showed no induction of HSPA6 protein upon HSP90 inhibition (Fig. 5). Five additional cell lines with tissue origins other than breast were also examined. Among these, 17-AAG exposure does result in strong HSPA6 induction in A-431 (epidermoid carcinoma) and Colo-205 (colon cancer) cells, but not in Hep-G2 (liver cancer), Molm-13 (Acute Myeloid Leukemia) and Raji (Burkitt's Lymphoma) cells (Fig. 5). Interestingly, the 3 cell lines

MDA-MB-231, SK-BR3 and Raji cells which are rather unresponsive to 17-AAG and require high concentrations of 17-AAG to induce cell death do not induce HSPA6. Three of six cell lines with medium sensitivity also lack HSPA6 induction. In contrast, the three cell lines that are most sensitive to 17-AAG (MDA-MB-361, Colo-205 and BT-474) are HSPA6 inducers (Table 2 and Suppl. Fig. 2). However, lack of HSPA6 induction may not reflect a general defect in mediating effects of HSP90 inhibition as induction of HSP70, but not HSPA6, is observed in Raji cells (Suppl. Fig. 4).

Fig. 8. ATP levels and BrdU incorporation in MCF-7 cells following HSPA6 overexpression and 17-AAG exposure. MCF-7 cells were transfected with pCMV6-entry ("empty vector") or pCMV6-HSPA6-untagged ("HSPA6 vector") plasmids. 4 h after transfection medium was replaced. Another 20 h later, 17-AAG was added to increasing final concentrations, as indicated. (A), 4 h after 17-AAG exposure, cells were lysed, samples separated on SDS–PAGE, and Actin and HSPA6 proteins detected by Western Blotting. (B), 72 h after 17-AAG treatment, Cell Titer Glo assays were performed. (C), 24 h after 17-AAG treatment, BrdU incorporation assays were performed.
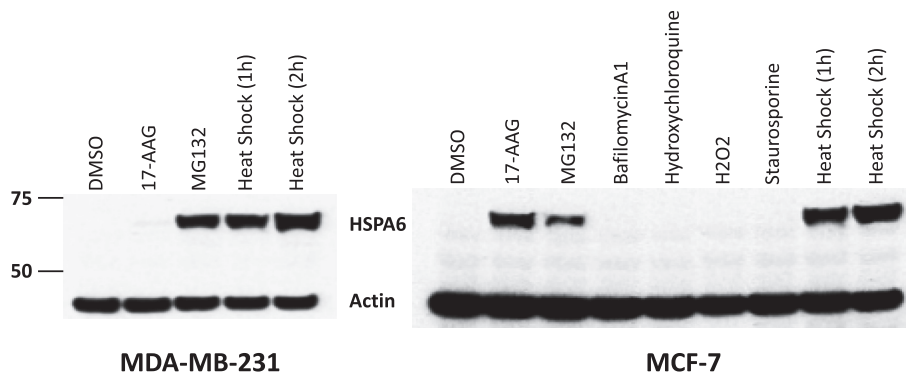
Fig. 9. MDA-MB-231 or MCF-7 cells were treated for 6 h respective 4xIC50 concentration of various compounds, as indicated. HSPA6 and Actin protein levels were determined by Western Blot analysis.

Table 2
IC50 concentrations of 17-AAG and HSPA6-responsiveness for all cell lines used in this study. (*) Fold-induction of HSPA6 protein is estimated based on serial dilution of HSPA6 signals in 17-AAG-treated Colo-205 cells (Suppl. Fig. 3).

Cell line	Cancer	IC50 (nM)	HSPA6 induction (*)
MDA-MB-361	Breast	55	+(>8)
BT-474	Breast	100	+(>16)
Colo-205	Colon	125	+(>100)
KPL-4	Breast	250	+(>16)
MCF-7	Breast	250	+(>32)
MDA-MB-134-VI	Breast	250	–
Molm-13	Acute Myeloid Leukemia	300	–
A-431	Epidermoid carcinoma	375	+(>4)
Hep-G2	Liver	375	–
MDA-MB-231	Breast	2 000	–
SK-BR3	Breast	15 000	–
Raji	Burkitt's lymphoma	20 000	–

2.5. Expression levels of HSPA6 do not affect cellular sensitivity to 17-AAG

The observation that the three cell lines that were least sensitive to 17-AAG lacked HSPA6 induction and three cell lines that were most sensitive to 17-AAG showed strong HSPA6 induction, raises the question if HSPA6 levels directly affect the cytotoxicity of 17-AAG. To this end, we transfected a scrambled siRNA or 3 different HSPA6-specific siRNAs into MCF-7 cells. All HSPA6-specific siRNA sequences efficiently blocked induction of HSPA6 upon treatment with 1 µM 17-AAG (Fig. 6, upper panel). Ablation with HSPA6-siRNA#3 demonstrated an efficient suppression of HSPA6 induction even at concentrations of 17-AAG up to 4 µM (Fig. 7). Upon transfection of two siRNAs (HSPA6-siRNA#2 and 3) a modest 'non-specific' reduction of cell metabolism (ATP content, Fig. 6) was observed in the absence of 17-AAG. However, HSPA6-siRNAs did not affect cell proliferation rates (BrdU incorporation, Fig. 7).

Furthermore, exposure of HSPA6-siRNA treated cells to increasing concentrations of 17-AAG revealed no difference in cellular sensitivity between cells that induced HSPA6 and cells that did not induce HSPA6 (Figs. 6 and 7). Next, we were wondering if HSPA6 function might be relevant under conditions of very severe stress. Therefore, we repeated our knock down experiments and challenged cells by heat shock at 42 °C with and without additional 17-AAG treatment. Heat shock and 17-AAG treatment alone or in combination resulted in induction of HSPA6 protein, which could be virtually blanked by our HSPA6-siRNA (Suppl. Fig. 5, upper panel). Surprisingly, although HSPA6 induction seems to be very strong upon heat stress in combination with 17-AAG challenge (Suppl. Fig. 5, upper panel), ablation of HSPA6 does not affect cellular sensitivity to both insults at any concentration of 17-AAG (Suppl. Fig. 5, lower panel). Taken together, ablation of HSPA6 induction did not protect against 17-AAG or 17-AAG plus heat shock mediated decline of cell proliferation or viability rates in MCF-7 cells.

In addition, we also analyzed the effect of ectopic expression of HSPA6 on 17-AAG sensitivity in MCF-7 cells. Transfections conditions were optimized to generate steady-state levels of overexpressed HSPA6 comparable to 17-AAG-induced levels of endogenous HSPA6 (Fig. 8, see Section 4 for transfection details). Under the conditions used, ectopic expression of HSPA6 did not alter cell proliferation and viability in the absence or presence of 17-AAG (Fig. 8). Further, MDA-MB-231 cells, which do not induce endogenous HSPA6 in response to 17-AAG, do not show an altered sensitivity towards 17-AAG when modified to stably overexpress HSPA6 (Suppl. Fig. 6). Taken together, HSPA6 induction correlates to some extent with 17-AAG responsiveness, but does not seem to represent a survival factor or component that ameliorates 17-AAG toxicity.

2.6. MG132 and heat stress induce HSPA6 in cells that do not induce HSPA6 in response to 17-AAG

HSPA6 induction following heat stress or treatment of cells with the proteasome inhibitor MG132 have been described before. As these triggers do not act via HSP90 inhibition, we were wondering if these stimuli are effective for HSPA6 induction in cells that do not induce HSPA6 in response to 17-AAG. MDA-MB-231 cells fail to induce HSPA6 in response to 17-AAG, but show a prominent induction of HSPA6 following treatment with 4xIC50 concentration of MG132 (Suppl. Fig. 7) or exposure of cells to 42 °C for 1 h or 2 h (Fig. 9). In contrast, MCF-7 cells induce HSPA6 protein in response to MG132, heat shock and 17-AAG (Fig. 9). This may suggest, that transcriptional upregulation of HSPA6 in response to 17-AAG is not solely dependent on the Heat Shock Element (HSE) within the HSPA6 promoter region. In addition, we tested additional cytotoxic compounds, that act in a HSP90-independent manner. No induction of HSPA6 could be observed following exposure of MCF-7 cells to respective 4xIC50 concentrations (Suppl. Fig. 7) of the autophagosome-lysosome-fusion inhibitor BafilomycinA1, the lysosome inhibitor Hydroxychloroquine, the oxidative stress inducer Hydrogenperoxide or the protein kinase inhibitor Staurosporine (Fig. 9). This further supports the notion that HSPA6 induction is not a general response to cytotoxic insults, but rather controlled in a very defined, yet complex, manner.

3. Discussion

Geldanamycin derivatives, such as 17-AAG, are drug candidates for anticancer therapy [4–6]. The target of Geldanamycin, HSP90, is overexpressed in many cancer tissues studied to date [3]. However, cancer cells seem to be able to escape cytotoxic effects of HSP90

inhibition by a (possibly compensatory) upregulation of other heat shock proteins, particularly members of the HSP70 family [9,10]. Thus, a detailed knowledge of the interplay between members of the cellular HSP network in the context of HSP90-inhibition might help in the design of strategies that avoid the activation of escape mechanisms by cancer cells. We report that in various cancer cell lines, expression of HSPA6 protein is induced following administration of 17-AAG. This effect appears to be a specific consequence of HSP90 inhibition, as only HSP90 inhibitors (17-AAG and Radicicol), but not other cytotoxic compounds with different mode of actions, result in a similar induction of HSPA6. In MCF-7 cells and several other cell lines, HSPA6 induction on mRNA and protein level is a time- and concentration-dependent consequence of treatment with 17-AAG or Radicicol. Some other cell lines, however, do not elicit a 'HSPA6 response', suggesting that cell line dependent factors may be implicated in the transcriptional control of HSPA6 downstream of HSP90. The molecular pathways of HSPA6 transcriptional control are only beginning to be elucidated and control of HSPA6 transcription is still poorly understood. A recent report suggests the existence of positive as well as negative regulatory elements in the HSPA6 promoter [25]. It is possible that one or more HSP90 client protein(s) might be involved in the transcriptional regulation of HSPA6. Our data point towards a transcriptional control of HSPA6 following HSP90 inhibition that is different from the induction of HSPA6 in response to the proteasome inhibitor MG132 or heat stress. While heat shock induction of HSPA6 is mediated by the heat shock element (HSE), induction of HSPA6 in response to MG132 is not defined on a molecular level. HSE mediated transcription cannot fully explain HSPA6 regulation, because some cell lines are fully competent to induce HSPA6 during heat stress, but do not induce HSPA6 upon HSP90 inactivation. Recently a functional AP-1 site within the HSPA6 promoter region was described and MG132 is known to induce transcriptional activity from AP-1 promoter sequences [25]. Thus, HSP90 inhibition, proteasome inhibition and heat shock might mediate HSPA6 induction by distinct mechanisms, of which only signaling downstream of HSP90 appears to be highly cell type specific. It would be of interest to determine if the postulated promoter elements C/EBP or ZFX may be involved in HSPA6 induction downstream of HSP90 [25].

It has been suggested that HSPA6 can form a complex with HSP90, but no function has been assigned to this putative HSPA6/HSP90 complex. Interestingly, formation of HSPA6/HSP90 complexes is Geldanamycin-dependent [26]. Given the absence of HSPA6 expression in untreated cells, it is tempting to speculate that Geldanamycin-dependency of this complex formation is simply reflected by Geldanamycin-dependent expression of HSPA6.

MG132, a proteasome inhibitor representing another class of anti-cancer drugs also induces expression of HSPA6 in human cells [27,28]. In this example, HSPA6 induction was suggested to be a cellular 'pro-survival' response, because increased sensitivity towards MG132-induced cell death has been reported for human cell lines lacking a 'HSPA6 response' [27]. In contrast, our data indicate that cell lines with high tolerance for 17-AAG (SK-BR3, Raji and MDA-MB-231) do not induce HSPA6 expression (Fig. 5). Furthermore, ablation of HSPA6 induction in 17-AAG sensitive MCF-7 cells did not alter sensitivity towards 17-AAG-mediated cell death (Figs. 6 and 7). A similar finding has been reported for heat shock induced cell death, where neither knock down nor overexpression of HSPA6 did affect cell viability [11]. Thus, cell type specific and context dependent factors might contribute to the function and localization of HSPA6. Interestingly, localization to the extracellular milieu [29], cell surface [28], centrioles [30] and the nucleus [31], has been described for HSPA6.

Molecular markers to track HSP90 inhibition as well as more detailed information about the consequences of HSP90 inhibition

are desired for basic research as well as in the clinic. HSPA6 mRNA and protein are virtually undetectable in untreated cells, but highly induced in 17-AAG treated cells (up to 7000-fold induction of HSPA6 mRNA in MCF-7 cells, Fig. 1C). In contrast to general induction of HSP70 proteins (with background levels and general UPR-induction (Fig. 2), determination of HSPA6 levels (mRNA or protein) can represent a very clean and highly sensitive readout for successful HSP90 inhibition. Especially at early time points (2–8 h) following 17-AAG administration, fold-change is much higher for HSPA6 as compared to other 17-AAG-responsive genes/proteins, e.g. other HSP70 proteins (Table 1 and Fig. 2A).

A restriction in applying HSPA6 induction as general marker for HSP90 inhibition is our observation that not all cell lines or cell types induce HSPA6 following 17-AAG exposure. Thus, an initial evaluation step of HSPA6-responsiveness in a given cell type/cell line is required before applying HSPA6 as sensitive marker for HSP90 inhibition.

4. Materials and methods

4.1. Cell culture

MCF-7 [ATCC, HTB-22], KPL-4 [Kawasaki Medical School], Hep-G2 [ATCC, HB-8065], Molm-13 [DSMZ, ACC-554], BT-474 [ATCC, HTB-20], A-431 [ATCC, CRL-1555], Colo-205 [ATCC, CCL-222], MDA-MB-231 [ATCC, HTB-26], MDA-MB-361 [ATCC, HTB-27], MDA-MB-134-VI [ATCC, HTB-23], Raji [ATCC, CCL-86] and SK-BR3 [ATCC, HTB-30] cells were cultured at 37 °C in 5% CO_2 atmosphere with 80% humidity. Following base media were used: RPMI (MCF-7, Colo-205, BT-474, Molm-13, Raji), DMEM (Hep-G2, A-431, KPL-4), L-15 (MDA-MB-134-VI, MDA-MB-231, MDA-MB-361) and McCoy's 5a (SK-BR3). Media contained $NaHCO_3$ at concentrations of 0.7 g/l (L-15 media), 2.0 g/l (RPMI media) or 2.2 g/l (DMEM media). Further, all cell culture media were supplemented with 10% fetal bovine serum and 2 mM Glutamine. For culture of Molm-13 cells additional fetal bovine serum was added (final concentration = 20%) and culture media for BT-474 cells was supplemented with human Insulin (final concentration = 10 μg/ml).

4.2. Affymetrix

Transcriptional profiling was performed by chip-based (Affymetrix chip) hybridization analyses of mRNA isolated from MCF-7 cells as previously described [32]. In brief, cells were seeded and grown to subconfluency in 6-well plates and exposed to 17-AAG or other toxins, each at IC50 concentration. At different time points thereafter, RNAs were prepared from these cells and subjected to Affymetrix chip hybridization experiments according to the manufacturer's protocol. RNA from untreated MCF-7 cells served as reference. All analyses were performed in triplicates, and passed quality controls for mRNA/cDNA preparation.

4.3. RT-PCR

For quantitative real time analysis of RNA transcripts, MCF-7 cells were seeded at 180000 cells per well on a 12-well plate. The day after, test compounds were added to cells. At different time points, as indicated, cells were lysed and RNA extracted applying the RNeasy Mini kit (Qiagen). Following removal of genomic DNA (TURBO DNA-free kit, Ambion), RNA was reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche). CDNA samples were mixed with SYBR Green Master Mix (Light Cycler RNA Master SYBR Green I kit), and specific amplification primers. QPCR was performed on a Lightcyler (Roche) applying 3 different primer pairs for HSPA6 [(HSPA6-Primer Set1, forward: CAAGGTGCGCGTATGCTAC; reverse: GCTCATTGATGATCCGCA ACAC), (HSPA6-Primer Set2, forward: CATCGCCTATGGGCTG GAC; reverse: GGAGAGAACCGACACATCGAA), (HSPA6-Primer Set3, forward: GATGTGTCGGTTCTCTCCATTG, reverse: CTTCCATGAAGT GGTTCACGA)] and the primers GAPDHfor (GAAGGTGAAGGT CGGAGTCA) and GAPDHrev (GAAGATGGTGATGGGATTTCCA) to amplify the GAPDH housekeeping gene as normalization standard. In subsequent experiments, HSPA6-Primer Set3 was used to quantify HSPA6 transcripts.

4.4. Western Blotting, antibodies and recombinant proteins

For protein detection by Western Blotting, 600000 cells were plated on 6-well plates. The day after, test compounds were added to cells. At different time points, as indicated, cells were lysed in RIPA buffer (Sigma) supplemented with complete protease inhibitor cocktail (Roche). Cell lysates were normalized for total protein amounts (BCA protein assay kit, Pierce) and resolved by SDS/PAGE (4.12% NuPAGE SDS–PAGE, Life Technologies).

Following antibodies were used for immunodetection: Anti-HSPA6 (OriGene Technologies, mouse monoclonal, clone 1D12), Anti-Beta-Actin (Sigma, mouse monoclonal, clone AC-74), HSP70 (Cell Signaling), Anti-BiP (Cell Signaling, rabbit mAb, clone C50B12), and HRP-coupled anti-mouse or anti-rabbit secondary antibody (Dako, goat polyclonal). Recombinant HSPA1A and HSPA8 used to determine specificity of HSPA6 and HSP70 antibodies were purchased from origene (Catalog number TP10001 and TP302209, respectively). For Western Blot analysis 500 ng of recombinant protein was loaded per lane. The specificity of the HSPA6 antibody was proven by several lines of evidence: knockdown experiments applying HSPA6 mRNA specific reagents (gene specific sequences) prevents generation of antibody-signals in otherwise responsive cells, '17AAG unresponsive' cell lines can induce other HSP70 proteins upon 17AAG treatment but are still negative for HSPA6 (crossreactivity of the HSPA6 antibody with other HSPs would generate signals under such conditions), and Western Blots with HSP70 proteins HSPA1A (Hsp70-1) and HSPA8 (Hsc70) side by side with HSPA6 induced cell extracts revealed no reactivity with both proteins.

4.5. RNAi and recombinant expression

For RNA Interference the following stealth siRNAs from LifeTechnolgies were used: scrambled control siRNA (siRNA Negative Control Med GC Duplex #2, catalog number 12935-112), HSPA6-siRNA#1 (HSS105080), HSPA6-siRNA#2 (HSS179391) and HSPA6-siRNA#3 (HSS179392). MCF-7 cells were transfected with 30 pmol (60000 cells, 24 well format) or 5 pmol (10000 cells, 96 well format) of respective siRNA duplexes with Lipofectamine 2000 according to manufacturer's manual. 4 h after transfection cells were exposed to different concentrations of 17-AAG, as indicated. For overexpression experiments plasmids encoding for no protein, herein referred to as "empty vector" (pCMV6-entry, origene, SKU RC207795) or HSPA6 without any tags (HSPA6 (untagged), origene, SKU SC118784) were used. 400 ng (60000 cells, 24well format) or 66,7 ng (10000 cells, 96 well format) total DNA were transfected with Lipofectamine 2000 according to manufactureŕs manual. DNA mixes for HSPA6 overexpression contained 1/8 of HSPA6 vector and 7/8 of empty vector. 4 h after transfection medium was replaced and another 20 h later 17-AAG was added.

4.6. Quantification of cell numbers and cell proliferation

Cell numbers were detected based on total ATP levels (Cell Titer Glo assay, Promega) and BrdU incorporation was used to quantify cell proliferation rates (BrdU, ELISA etc. kit, Roche). Cell Titer Glo and BrdU incorporation assays were performed 72 h and 24 h, respectively, after exposure of cells to 17-AAG according to manufacter's instructions.

Conflict of interest

The authors are employed by Roche Pharma Research and Early Development. Roche has an interest in the development of novel cancer therapies.

Acknowledgment

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.04. 053.

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