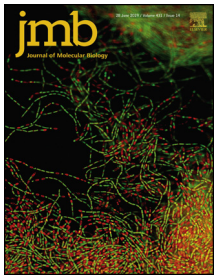

# Beta-sheet-specific interactions with heat shock proteins define a mechanism of delayed tumor cell death in response to HAMLET

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## Abstract

As chaperones, heat shock proteins (HSPs) protect host cells against misfolded proteins that constitute a by-product of protein synthesis. Certain HSPs are also expressed on the surface of tumor cells, possibly to scavenge extracellular unfolded protein ligands and prevent them from becoming cytotoxic. HAMLET—a complex of partially unfolded alpha-lactalbumin and oleic acid—is relying on its N-terminal alpha-helical domain to perturb tumor cell membranes, and the cells die as a consequence of this interaction. Here we show that in parallel, cell surface HSPs bind the beta-sheet domain of alpha-lactalbumin and activate a temporarily protective loop, involving vesicular uptake and lysosomal accumulation. Later, HAMLET destroys lysosomal membrane integrity, and HAMLET release kills the remaining tumor cells. HSPs were identified as HAMLET targets in a proteomic screen and Hsp70-specific antibodies or shRNAs inhibited HAMLET uptake by tumor cells, which showed increased Hsp70 surface expression compared to differentiated cells. The results suggest that HAMLET engages tumor cells by two parallel recognition mechanisms, defined by alpha-helical- or beta-sheet domains of alpha-lactalbumin and resulting in an immediate death response, or a delay due to transient accumulation of the complex in the lysosomes. This dual response pattern was conserved among tumor cells but not seen in normal, differentiated cells. By two different mechanisms, HAMLET thus achieves a remarkably efficient elimination of tumor cells.



## Introduction

Complex cellular quality control systems are in place to survey protein synthesis and ensure that erratic or unfolded products are detected and degraded. Molecular chaperones are essential for the surveillance of endogenous, unfolded protein species and assist their refolding to a native and functional state. As a result, molecular chaperones influence many different aspects of cellular homeostasis and longevity. Such chaperones include the heat shock proteins (HSPs), which support protein folding and protein translocation across membranes [1,2]. The HSP70 family is important for the surveillance of endogenous, unfolded protein species and their refolding to a native and functional state. Besides Hsp70, members of the HSP70 family include Hsc70, the mitochondrial protein Grp75, and the endoplasmic reticulum localized Grp78 [3]. The Hsp70 proteins also co-operate with Hsp40s, Hsp60s, and Hsp90s, to maintain cellular integrity and assist in the recognition of unfolded protein ligands in different cellular compartments [2,4].

The versatility of Hsp70s as chaperones reflects the binding and release of hydrophobic segments of unfolded polypeptide chains in an ATP-dependent reaction cycle [4]. The peptide-binding domain of Hsp70 recognizes unfolded protein motifs and the ATPase domain hydrolyses ATP, providing energy for the conformational switch required for refolding and for the lid domain to act as cap, hindering ligand dissociation. Switching between a low-affinity, peptide-binding conformation and a high-affinity conformation is essential to maintain ligand interaction

 

and for transport of the refolded protein into the cytosol [1].

Global profiling of cancer cell proteomes has shown that Hsp70, Grp75, and Hrp90 are highly expressed in cancer cells compared to non-malignant cells, consistent with the high rate of protein synthesis in those cells [5]. Moreover, increased surface expression of Hsp70, Grp75, Grp78, and Hsp90 has been detected in cancer cells compared to non-transformed cells [6,7] and associated with resistance to therapeutic agents and poor prognosis [8,9]. Besides protecting tumor cells from proteotoxic stress, Hsp70 mediates aggregation and clearance of extracellular amyloid beta and intracellular tau proteins, as well as the internalization of granzyme B, leading to a perforin-independent cell death pathway [10].

In this study, we show that plasma membrane Hsp70 recognizes the beta-sheet domain of HAMLET (human alpha-lactalbumin made lethal to tumor cells), a complex of partially unfolded alpha-lactalbumin and oleic acid that kills tumor cells [11,12] and shows therapeutic efficacy in several cancer models [13–15]. Hsp70 recognition is shown to drive vesicular HAMLET uptake and transport to the lysosomes. Interestingly, this vesicular uptake pattern, which occurred in about 50% of the tumor cells, was defined by the beta-sheet domain and was distinct from a second uptake pattern defined by the alpha helical domains of the same protein, which showed a different uptake pattern and triggered rapid tumor cell death. The Hsp70-dependent translocation of the complex to the lysosomes protected a subset of tumor cells from the immediate cytotoxic effects of the HAMLET complex, but later, lysosomes released the HAMLET complex and the tumor cells died. The findings illustrate how different protein domains may engage functionally distinct cellular targets and trigger several concomitant response mechanisms in a single-cell population.

## Results

### HAMLET is internalized into two distinct populations of tumor cells

We used real-time confocal imaging to characterize the dynamics of HAMLET uptake by tumor cells. After exposure to Alexa-Fluor 568-labeled HAMLET (Alexa-HAMLET), the majority of adherent lung carcinoma cells (A549) rapidly internalized the complex but two distinct cellular patterns were detected after 1 h. In Population I (47%) staining was exclusively cytoplasmic, with a predominately vesicular pattern. In population II (30%), cytoplasmic HAMLET staining was diffuse rather than vesicular and HAMLET was detected in the nuclei of these cells, suggesting rapid nuclear translocation. In addition, a minority of cells showed filament-like distribution (2%) as expected from cytoskeletal interactions [16]. The uptake of HAMLET was time and dose dependent, as shown by Western blot analysis of whole-cell extracts (Fig. 1B, C).

The cytoplasmic vesicles in population I were identified as lysosomes by staining with Lysotracker, which showed strong co-localization with Alexa-HAMLET (Fig. 1D). The ring-like structure in the nuclear periphery in population II was identified as nuclear speckles, by staining with antibodies directed against the nuclear speckle marker SC-35 (Fig. 1E). Nuclear speckles reside in the inter-chromatin space of eukaryotic nuclei and serve as important nodes in the splicing of pre-mRNA and transport of spliced RNA [17,18].

The results distinguish two cellular responses to HAMLET in tumor cells, defined by divergent translocation of the complex either to lysozomes in the cytoplasm or spliceasomes in the nuclei. Importantly, these staining patterns were only detected in unfixed cells or after mild fixation (2% PFA), explaining why they have not previously been detected and studied. These two populations were also detected in HAMLET-treated human kidney carcinoma cells (A498) and murine bladder carcinoma cells (MB49). In contrast, significant HAMLET uptake was not observed in differentiated keratinocytes (Fig. S1A–B).

### Hsp70 family proteins interacts with HAMLET

To identify cellular targets involved in HAMLET uptake, we performed a protein microarray comprising 8000 human proteins based on the *in vitro* ProtoArray technology [19]. Target proteins were detected by fluorescence following incubation of the target protein-coated array with Alexa-HAMLET. A subset of nucleotide-binding proteins was identified as top hits [19]. Here, HSPs were further investigated as potential HAMLET binding partners. Eleven HSPs were identified HAMLET targets (Fig. 2A), with a binding intensity significantly correlated to the Alexa-HAMLET concentration used in the screen (5 and 50 ng/ml, $R^2$ = 0.74 (Fig. 2B). The HSP proteins recognized by HAMLET were structurally related, forming two evolutionarily conserved clusters by ClustalW, including Grp75, Hsp70, Hsp701L, Hsp74 and Hsc70 in cluster I, Hsp90α, Hsp90β, HspB7, HspB9, and Hsp40 in cluster II. Hsp60 was phylogenetically different from the proteins present in both clusters I and II (Fig. S2A).

Binding of HAMLET to HSPs identified in the proteomic screen was confirmed, using a dot blot assay. Recombinant Hsp70, Hsc70, Hsp60, Grp75, or Hsp90β were spotted on polyvinylidene fluoride membranes and overlaid with HAMLET. Binding of HAMLET was detected by staining with bovine anti-α-lactalbumin primary antibody and goat anti-bovine HRP-conjugated secondary antibody, compared to a negative background control (Fig. 2C).

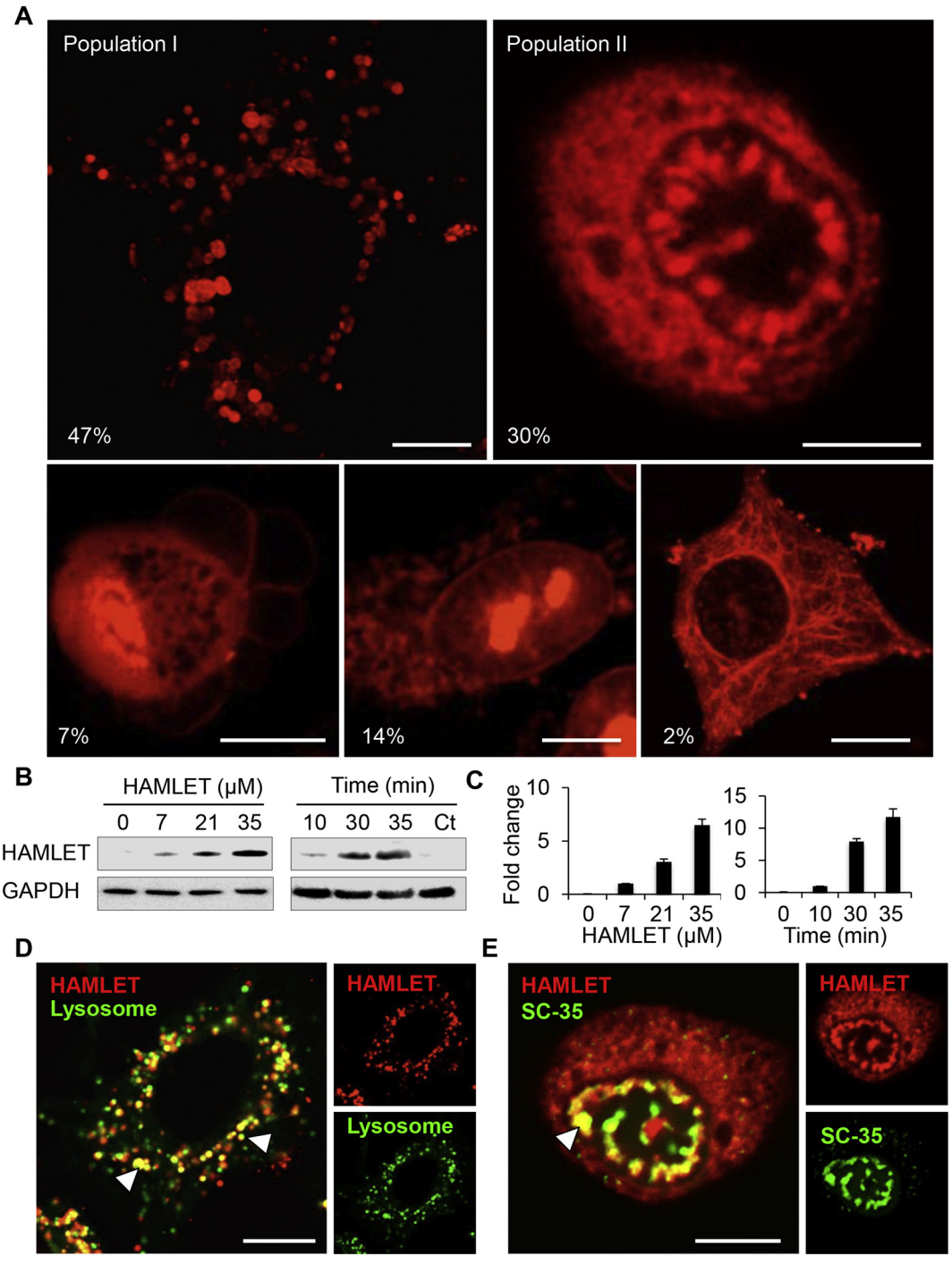


**Figure 1.** Two distinct patterns of subcellular localization: HAMLET accumulates in lysosomes or nuclear speckles. (A) Two cell populations were detected in lung carcinoma cells treated with Alexa-HAMLET for 60 min. Population I showed vesicular cytoplasmic staining (left panel). Population II showed diffuse cytoplasmic staining and nuclear uptake into a ring-like structure. (B) Western blot analysis, demonstrating dose- and time-dependent HAMLET uptake. GAPDH was the loading control. (C) Quantification of western blots. (D) Cytoplasmic vesicles were identified as lysosomes (green) by staining with lysotracker, which co-localized with HAMLET (red). (E) Nuclear HAMLET was localized to nuclear speckles by staining with SC-35, which co-localized with HAMLET (red). The scale bar represents 10 μm.

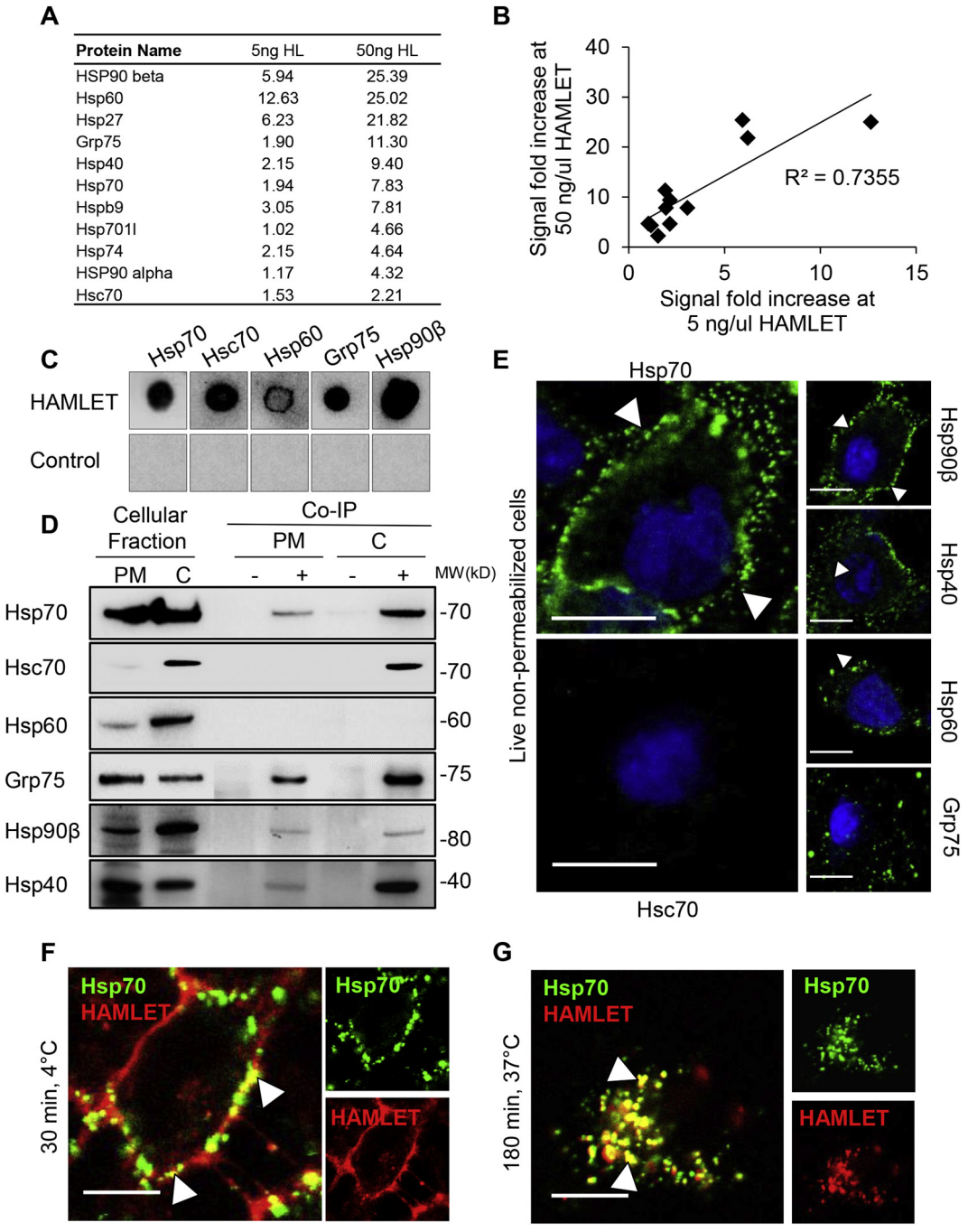


**Figure 2.** Identification of HSPs as HAMLET interaction partners. (A) HAMLET interacting HSPs identified by protoarray. Binding is determined by dose dependent fold increase in fluorescence intensity of Alexa-Fluor-568 labeled HAMLET. (B) Signal fold increase at 5 ng/µl is strongly correlated to 50 ng/µl HAMLET. (C) Dot blots of HAMLET binding to Hsp90β, Hsp60, Grp75, Hsp70, and Hsc70. (D) Co-immunoprecipitation of plasma membrane- and cytoplasmic fractions from HAMLET-treated tumor cells (A549). HSPs interacting with HAMLET were pulled down using α-lactalbumin specific antibodies and identified by Western blot analysis, using HSP-specific antibodies. MW markers indicate the sizes of individual HSPs. (E) Detection of HSPs at tumor cell membrane. The scale bar represents 10 µm. (F) Lung carcinoma cells treated with HAMLET (red) co-localizes with membrane bound Hsp70 (green, anti-Hsp70 full length) at 4 °C. (G) Co-localization of HAMLET (red) and Hsp70 (green) in cytoplasmic vesicles at 37 °C. Arrowhead indicates co-localized clusters. The scale bar represents 10 µm.

### Cell surface HSPs interact with HAMLET

Binding of HAMLET to HSPs in tumor cells was demonstrated by co-immunoprecipitation. Tumor cells were exposed to HAMLET (35 μM, 1 h), and total cell extracts were subjected to sub-cellular fractionation. Plasma membrane fractions and cytoplasmic fractions were precipitated, using goat anti-bovine alpha-lactalbumin antibody. HSPs bound to HAMLET were identified by Western blot analysis using HSP-specific antibodies (Fig. 2D).

In the plasma membrane fraction, Hsp70, Grp75, Hsp90β, and Hsp40 were identified and Hsp70 and Grp75 were precipitated, indicating a positive interaction with HAMLET. In the cytoplasmic fractions, Hsp70, Hsc70, Hsp60, Grp75, Hsp90β, and Hsp40 were identified and Hsp70, Hsc70, Grp75, and Hsp40 were precipitated, indicating that HAMLET may interact with a larger number of HSPs, once internalized. Hsc70, which is structurally homologous to Hsp70, was present only in the cytoplasm, suggesting that it may not act as a receptor, due to its localization. Hsp90β and Hsp40 showed very weak interactions.

Cell surface expression of Hsp70, Hsp60, Grp75, Hsp90β, and Hsp40 was confirmed by live cell imaging of non-permeabilized lung carcinoma cells after staining with HSP-specific antibodies (Figs. 2E and S2B). Hsp70 and the other HSP were shown to co-localize with Alexa-HAMLET in distinct membrane domains, which were resolved at 4 °C (Figs. 2F and S3). At 4 °C, ATP-dependent endocytosis process is halted, preventing the immediate internalization of Hsp70 upon HAMLET binding. When the temperature was raised to 37 °C, the same cells rapidly internalized HAMLET and Hsp70 into cytoplasmic vesicles (Fig. 2G).

The results suggest that HAMLET interacts with Hsp70 at the plasma membrane of tumor cells, indicating that Hsp70 might be involved in the vesicular uptake of HAMLET by cells in population I.

### Heterogenous HSP expression by tumor cells

Hsp70 expression was subsequently quantified in A549 lung carcinoma cells, after staining with increasing concentrations of anti-Hsp70 antibody (Fig. 3). By confocal microscopy, a dose-dependent signal was recorded and a population of cells with elevated HSP staining was detected, comprising about 60% of all cells (Fig. 3B). This result was confirmed by flow cytometry, which detected a dose-dependent right-shift of the Hsp70 positive population (Figs. 3C and S4). Hsp70 expression was further quantified in differentiated keratinocytes after staining with anti-Hsp70 antibodies. By confocal microscopy, lower Hsp70 staining was detected in the keratinocytes than in the tumor cells (Fig. S5).

The results suggest that high HSP expression may be a marker of the HAMLET-responsive subset of tumor cells and that the cell fate might be influenced by the abundance of cell surface Hsp70. Sorting the cells by Hsp70 expression prior to HAMLET exposure was not technically achievable, however.

### Hsp70 is essential for vesicular HAMLET uptake

To confirm the involvement of Hsp70 in HAMLET internalization, A549 lung carcinoma cells were pre-incubated with antibodies to the full-length Hsp70 protein and challenged with Alexa-HAMLET (60 min, 35 μM). The internalization of HAMLET was quantified by confocal imaging and Western blots (Fig. 4). Cells pre-treated with the anti-Hsp70 antibody showed markedly reduced HAMLET internalization and lysosomal uptake, compared to IgG2a-isotype controls ($p < 0.01$), (Figs. 4A–C and S6B–S6C). This effect was confirmed by transient transfection of the A549 lung carcinoma cells with HSP70-specific shRNA (48 h). Transfection reduced Hsp70 expression compared to scrambled control shRNA and HAMLET internalization was inhibited ($p < 0.01$, Fig. 4D-F). Furthermore, HAMLET internalization was inhibited by quercetin and phenylethynesulfonamide (PES), (Figs. 6A and S6D), which have been used in previous studies to regulate HSP expression [20].

The results suggest that Hsp70 recognition activates HAMLET internalization and vesicular transport to the lysosomal compartment in population I. In contrast, the Hsp70 antibodies and inhibitors did not affect the cells in population II (Figs. 6E and S6E).

### Hsp70 domains potentially involved in HAMLET recognition

The peptide-binding domain of Hsp70 recognizes unfolded protein motifs and the ATPase domain hydrolyses ATP, to support conformational adjustments to ligand binding [1]. To further examine if specific Hsp70 domains are recognized by HAMLET, tumor cells were exposed to Alexa-HAMLET and stained with antibodies to the full-length Hsp70, the ATPase domain or the peptide-binding domain, respectively (Fig. S7). Cytoplasmic co-localization with HAMLET was observed for the full-length antibody and the antibody to the ATPase domain, supporting HAMLET binding to Hsp70. Unexpectedly, HAMLET treatment abrogated the peptide binding domain (PBD) staining, suggesting that HAMLET may bind to the PBD and competitively inhibit the antibody from recognizing these domains. The data are preliminary, and more studies are needed to further define this mechanism.

### Peptide-specific HAMLET recognition by Hsp70

To examine if specific peptide domains act as HSP ligands, we synthesized peptides corresponding to

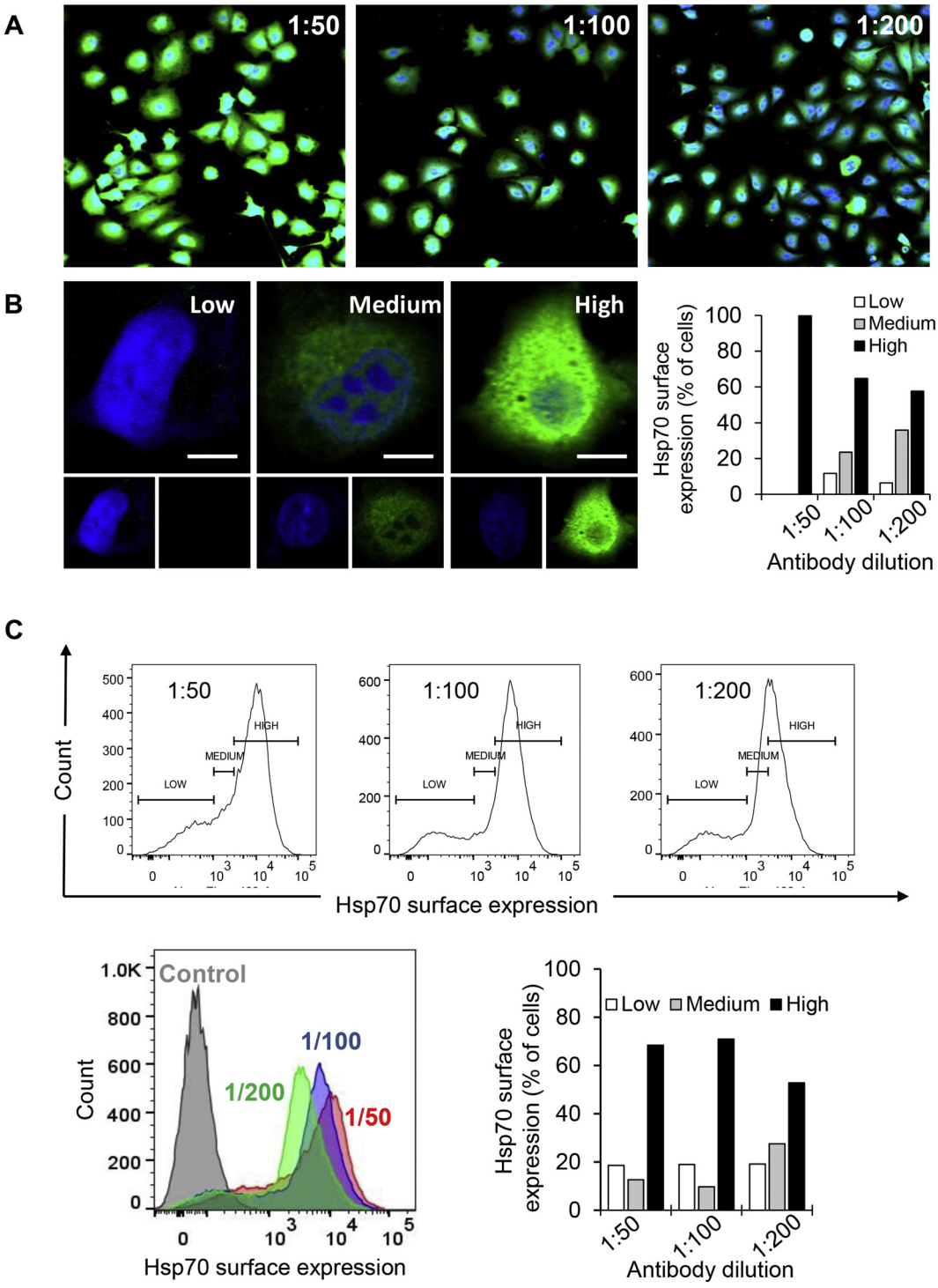


**Figure 3.** Variable Hsp70 surface expression in lung carcinoma cells. The surface expression of Hsp70 was quantified in A549 lung carcinoma cells, by staining of with anti-Hsp70 antibodies after fixation with 2% PFA without permeabilization. (A) A concentration-dependent increase in staining was detected by confocal microscopy. (B) Higher magnification (×63; the scale bar represents 10 μm) of representative cells in panel A. (C) HSP staining quantified by flow cytometry (20,000 cells/sample). Anti-human Hsp70 antibody was used at dilutions 1/50, 1/100, and 1/200 and IgG2a was the isotype control (Supplementary Fig. S4). An increase in the right-shift of peak expression levels and was detected with increasing antibody concentrations. The frequency of cells with low, medium, and high Hsp70 levels is shown in the histogram.

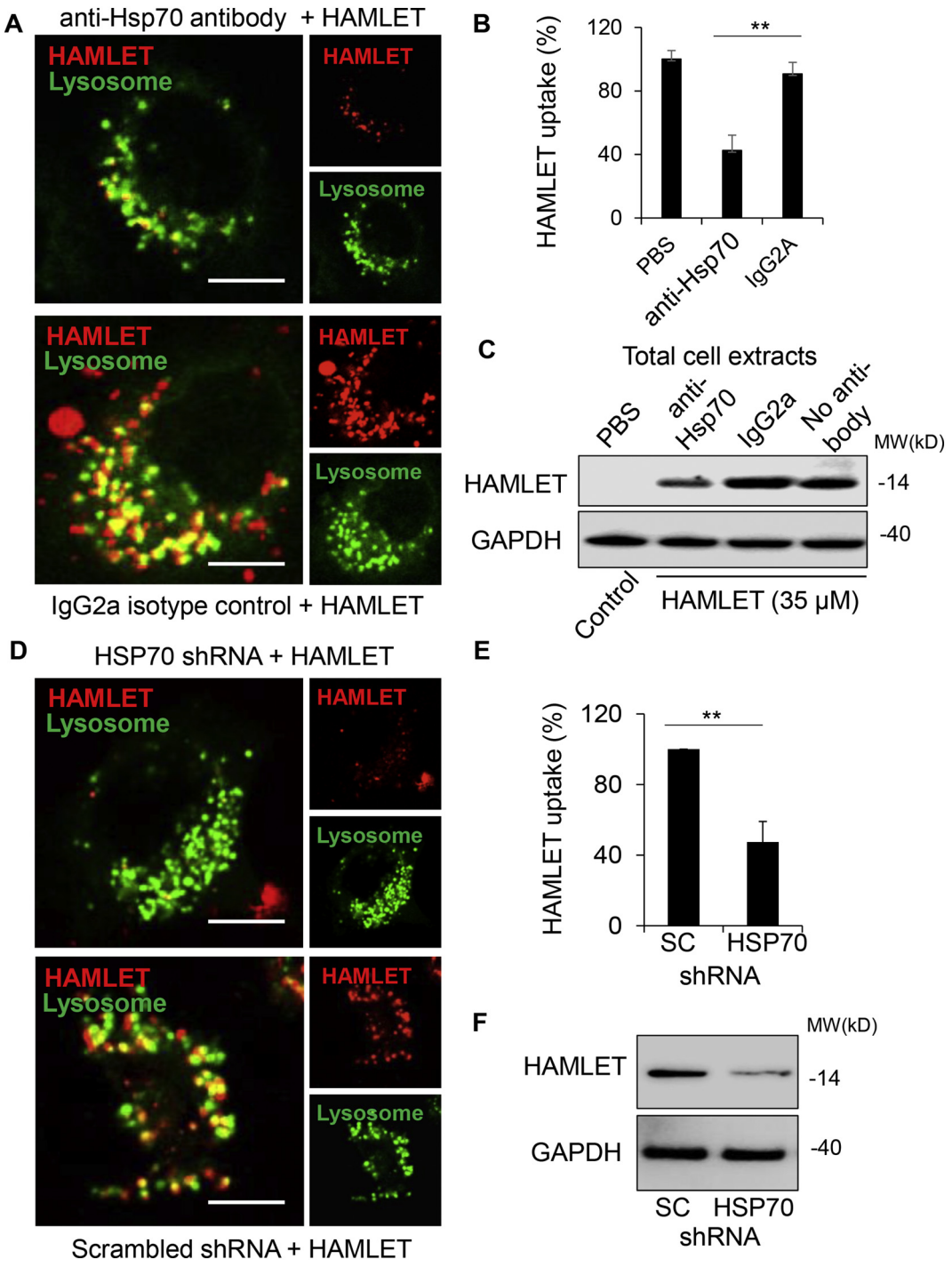


**Figure 4.** Hsp70 is involved in vesicular HAMLET uptake. (A) Significant reduction in HAMLET uptake and lysosomal accumulation in lung carcinoma cells after pretreatment with polyclonal anti-Hsp70 antibodies. The scale bar represents 10 μm. (B) Quantification of images in panel A ($p < 0.01$). (C) Reduction in HAMLET uptake and lysosomal accumulation confirmed by Western blot analysis. MW markers indicate the sizes of individual protein. (D) Significant reduction in HAMLET uptake and lysosomal accumulation in lung carcinoma cells after transfection with HSP70-specific shRNA, compared to scrambled shRNA control. The scale bar represents 10 μm. (E) Quantification of HAMLET uptake in panel D ($p < 0.01$). (F) Western blot analysis confirming the reduction in HAMLET uptake after transfection with HSP70-specific shRNA. MW markers indicate the sizes of individual protein.

the N-terminal alpha1 domain (residues 1–39), the beta sheet (40–80), or the C-terminal alpha2 domain (residues 81–123) of alpha-lactalbumin, the globular, 14.2k-Da protein constituent of HAMLET [21] (Fig. 5A, B). By real-time imaging, the beta peptide was shown to accumulate in cytoplasmic vesicles, where it co-localized with Lysotracker. Interestingly, this process was unaided by oleate, suggesting that the interaction of the beta peptide with Hsp70 is sufficient for internalization to occur (Fig. S8). The alpha-peptides, in contrast, reproduced the population II pattern with rapid accumulation in nuclear speckles (Fig. 5C). The initial membrane integration phase was peptide-specific, but the subsequent internalization and nuclear accumulation of the alpha1- and alpha2 peptides required sodium oleate (Fig. S8). The difference in subcellular distribution of the peptides was confirmed by Western blot analysis of cytoplasmic and nuclear fractions from cells treated with the peptide oleate complexes (Fig. 5D).

### The alpha-lactalbumin beta peptide serves as an HSP ligand

The beta peptide was subsequently shown to bind to the HSPs in the dot blot assay, with a pattern similar to HAMLET (Figs. 5E and S9). Furthermore, pretreatment of the cells with anti-FL Hsp70 antibodies significantly reduced internalization and lysosomal uptake of beta-oleate complexes compared to the IgG2a isotype control ($p < 0.05$, Fig. 5F, G). Quercetin and PES, which inhibited the internalization of HAMLET, also reduced the internalization of the beta-oleate complexes ($p < 0.05$, Fig. 6A, B).

The results suggest that the beta-sheet domain of alpha-lactalbumin serves as an Hsp70 ligand and that the peptide is delivered to the lysosomal compartment by an HSP-dependent internalization mechanism. The Hsp70 inhibitors did not affect the uptake of HAMLET in population II, confirming specificity (Fig. 6E).

### Endocytosis inhibitors reduce vesicular HAMLET and beta-oleate uptake

HSPs are essential constituents of clathrin-coated pits that identify internalize extracellular cargo by endocytosis [22]. We therefore pretreated A549 lung carcinoma cells with pharmacological inhibitors, known to affect different aspects of endocytosis, including monensin, which blocks the secretory function and disrupts traffic between endosomes and lysosomes, chloroquine, which prevents the fusion of endosomes and lysosomes, dynasore, which affects the GTPase activity of dynamin and dansylcadaverine, which stabilizes clathrin-coated vesicles. As controls, we used the clathrin-independent endocytosis inhibitor filipin, which disrupts caveolar structure and function [23–27].

Monensin, chloroquine, and dynasore pretreatment (30 min) markedly reduced vesicular HAMLET and beta-oleate uptake ($p < 0.01$; Fig. 6C, D), identifying endosome–lysosome fusion as an essential step in this process. In contrast, dansylcadaverine and filipin showed no significant effect, suggesting that the uptake mechanism does not directly involve clathrin or caveolin. These findings are consistent with previous observations of macropinocytosis in HAMLET-treated cells [28].

Interestingly, inhibitors that reduced the vesicular uptake of the beta-peptide in population I did not affect the uptake of the alpha-oleate complexes by cells in population II, supporting the existence two parallel cellular recognition mechanisms (Figs. 6E and S10).

### HSP internalization delays tumor cell death

We subsequently used the Hsp70 inhibition strategies described above to investigate if Hsp70 recognition influences the tumoricidal effect of HAMLET. Anti-Hsp70 antibody pretreatment or HSP-specific shRNA transfection did not inhibit cell death in response to HAMLET, quantified as total cellular ATP levels or PrestoBlue staining. The pharmacological Hsp70 inhibitors PES and quercetin showed a similar pattern, and the endocytosis inhibitors monensin, chloroquine, dansyl-cadaverine, or filipin had no significant effect on tumor cell death (35 μM, 3 h; Fig. 7A–D), suggesting that the rapid cell death response to HAMLET does not involve Hsp70 recognition. This conclusion was supported by control experiments, showing that the HSP or endocytosis inhibitors do not reduce the tumoricidal activity of the alpha-peptide oleate complex.

To further address the functionality of the HSP interaction, cells were subjected to heat shock, at 43 °C for 3–24 h. An increase in Hsp70 expression was detected by Western blot analysis of whole-cell extracts (Fig. S11). A moderate reduction in the susceptibility of the cells to HAMLET was detected, consistent with a protective effect.

In previous studies, the bovine alpha-lactalbumin complex (BAMLET) was shown to first accumulate in the lysosomes and then re-enter the cytoplasmic compartment, following lysosomal membrane degradation. These observations suggested that the complex might be inactivated by lysosomal sequestration but then released from the lysosomes, to reinitiate cell death [29]. We therefore examined the stability of the lysosomal compartment by time-lapse confocal live cell imaging technology, using Lysotracker staining as the end point (Fig. S12A, B). Interestingly, lysotracker staining showed less reduction for at least 60 min in cells treated with the beta-oleate complex. HAMLET-treated cells, in contrast, showed more reduction in Lysotracker staining intensity, suggesting that the

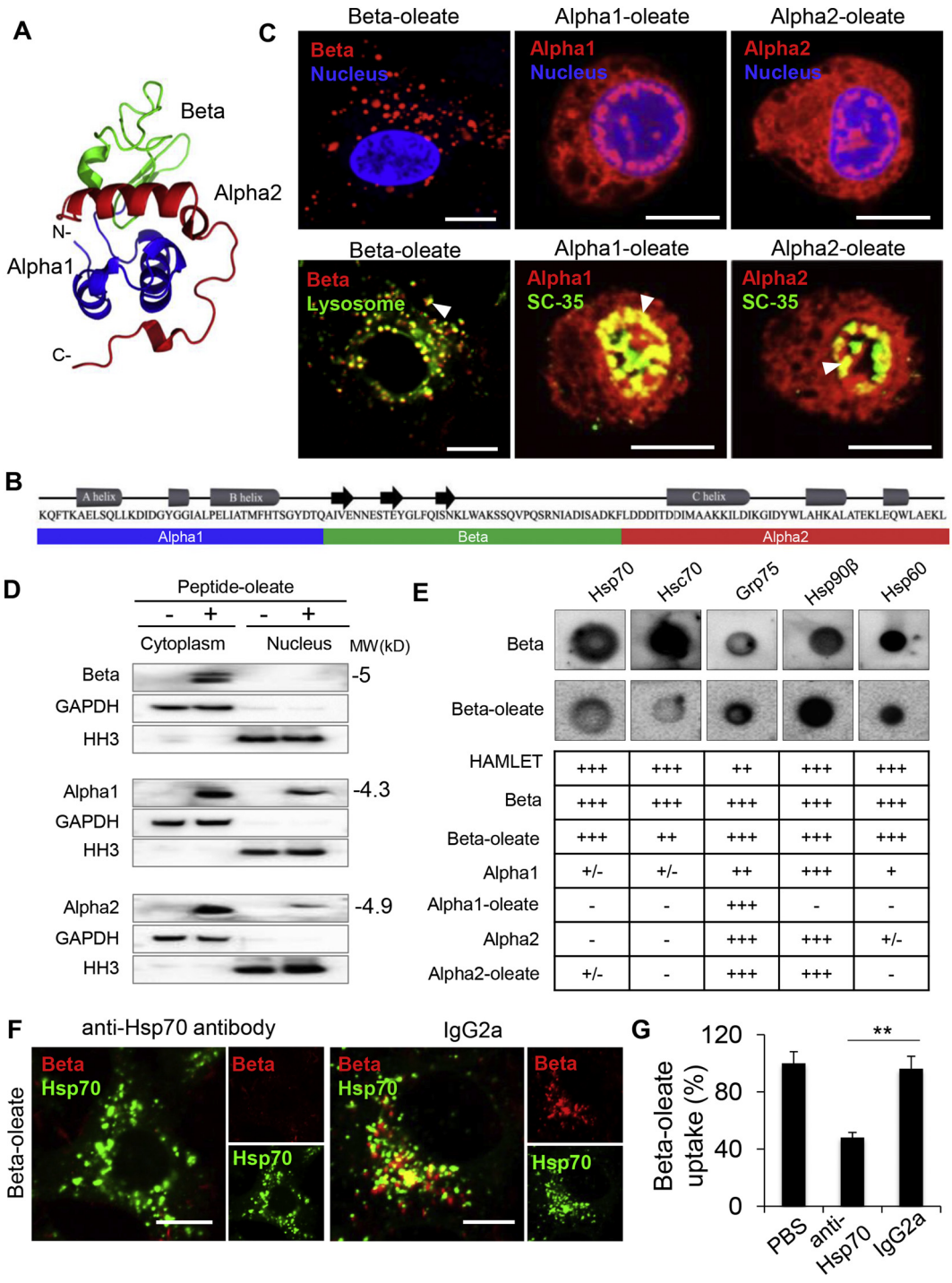


**Figure 5.** Hsp70 interacts with the beta domain of alpha-lactalbumin. (A) Crystal structure of human α-lactalbumin, indicating the alpha1 (blue), beta sheet (green) and alpha2 (red) domains (PDB ID: 1B9O). (B) Amino acid sequence and secondary structure of the peptide domains alpha1, alpha2, and beta domain of α-lactalbumin. (C) Lung carcinoma cells treated with beta peptide-oleate complexes showed accumulation in cytoplasmic vesicles. In contrast, the alpha1- or alpha2-oleate complexes were accumulated in a nuclear ring-like structure in the nuclear periphery. Colocalization of beta-oleate (red) with the lysosomal marker Lysotracker (green). Colocalization of alpha1- and alpha2-oleate (red) with SC-35 (green). The scale bar represents 10 μm. (D) Subcellular fractionation, localizing beta, alpha1 and alpha2 in the cytoplasm and/or nucleus. MW marker indicates size of the peptide or individual protein. (E) Dot blots showing binding of beta- and beta-oleate complexes to Hsp70 and Hsc70, Grp75, Hsp90β, and Hsp60. Quantifications are shown in the lower panel (– to +++). Dot blots for alpha1-oleate and alpha2-oleate are shown in Fig. S9. (F) Lung carcinoma cells pretreated with anti-Hsp70 antibodies showed reduced lysosomal uptake of beta-oleate complexes. The scale bar represents 10 μm. (G) Quantification of images in panel F indicating significant reduction ($p < 0.01$).

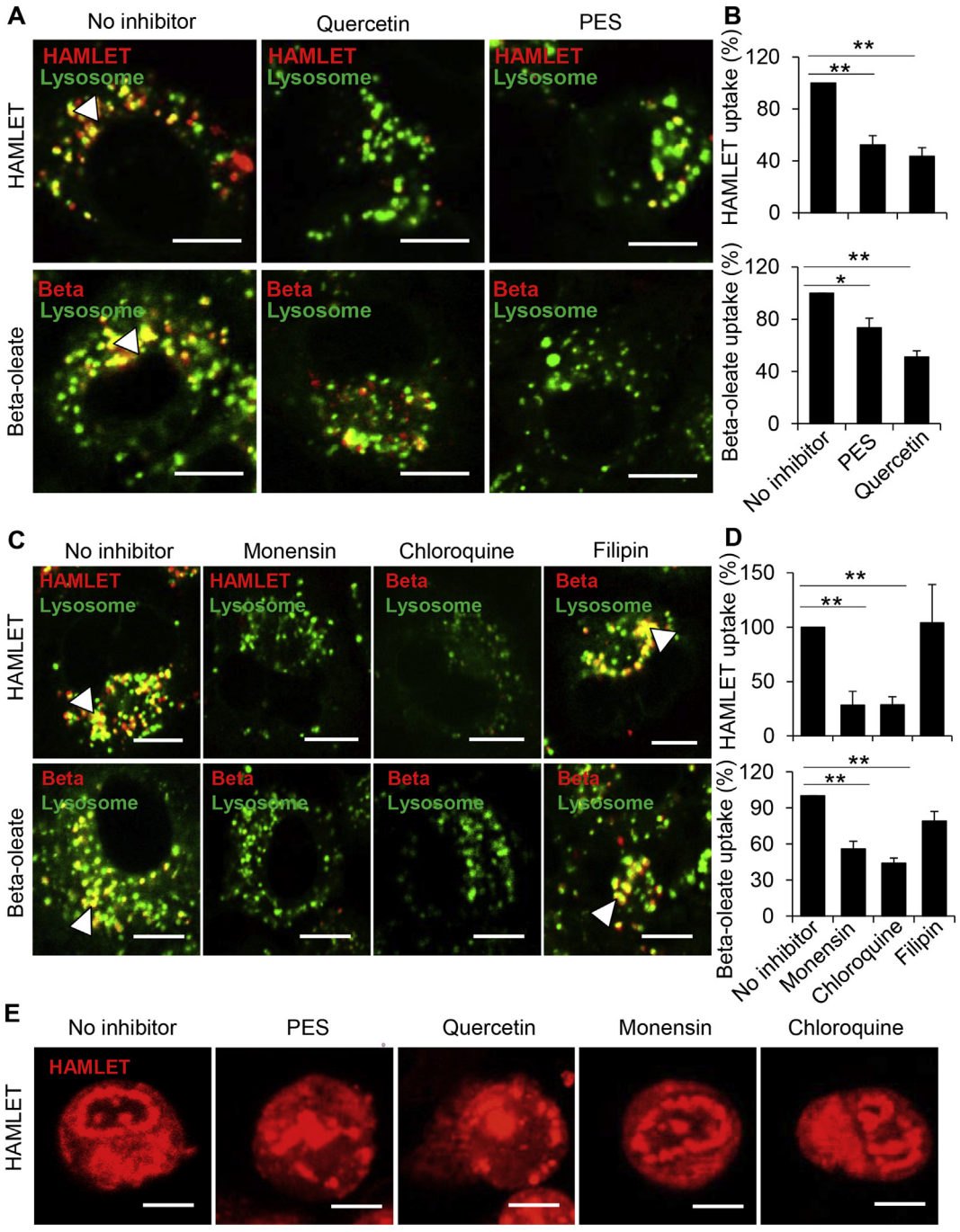


**Figure 6.** Effects of endocytosis inhibitors on the uptake and lysosomal accumulation of HAMLET and beta-oleate. (A, B) The pharmacological Hsp70 inhibitors PES and querecetin reduced HAMLET uptake by lung carcinoma cells and the translocation to the lysosomes (*$p < 0.05$). A similar effect was obtained for the beta-oleate complexes. Data represent the mean ± SEM, $n = 20$ for each data set with significant differences indicated (*$p < 0.05$). The scale bar represents 10 µm. (C, D) Monensin and chloroquin inhibited HAMLET uptake, but Filipin had no significant effect. Histogram indicates quantification of HAMLET uptake. (mean ± SEM, 20 cells per sample). The scale bar represents 10 µm. (E) PES, Quercetin, monensin, and chloroquine had no effect on HAMLET uptake by cells with the Population II phenotype. The scale bar represents 10 µm.

alpha1 domain might be involved in this effect. This hypothesis was supported using alpha1-oleate, which caused an immediate reduction in Lysotracker staining (Fig. S12A, B). The results suggest that the membrane-perturbing effect of the alpha1-oleate complex might extend to the lysosomes, and account for an "inside out" membrane damage in lysosomes that have sequestered HAMLET.

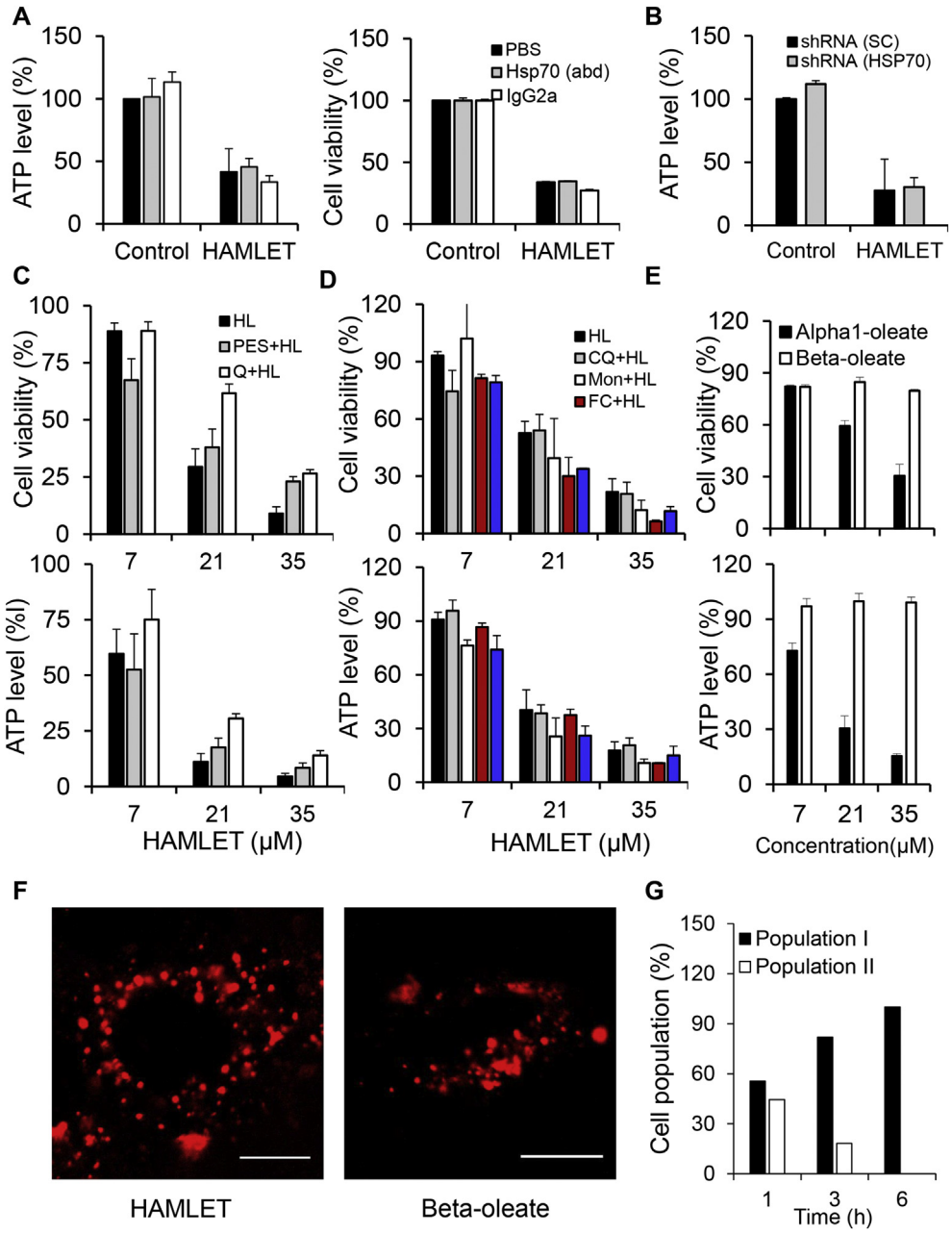


**Figure 7.** Hsp70 inhibition does not reduce HAMLET-induced tumor cell death. Inhibition of Hsp70 by (A) anti-Hsp70 antibody or (B) shRNA has no significant effect on HAMLET induced cell death measured by reduction in cellular ATP levels and/or PrestoBlue staining. (C, D) Endocytosis inhibitors has no significant effect on HAMLET induced tumor cell death. (E) Rapid, dose dependent tumor cell death in response to alpha1-oleate but not beta-oleate complexes (3 h). Data represent mean ± SEM, n = 3. (F) Phenotype of cells surviving HAMLET treatment (3 h), showing a predominance of the vesicular phenotype (population I). Cells treated with beta-oleate were used as positive controls. The scale bar represents 10 μm. (G) Kinetics illustrating the change in frequency of cells with the HAMLET uptake pattern characteristic of population I or population II. Enrichment of the population I phenotype.

We also observed that cells surviving HAMLET treatment for 3 h were enriched for the vesicular phenotype, suggesting improved survival of cells that recognize HAMLET by the beta sheet compared to cells that recognize the alpha1 domain (Fig. 7E–G). After 6 h, however, the number of cells with the vesicular phenotype was also reduced and a further reduction after 24 h indicated that the cells died (Fig. S12C, D).

These observations distinguish at least two distinct patterns of HAMLET recognition by tumor cells, defined by the alpha-helical peptides or beta-sheet domains of alpha-lactalbumin, respectively.

## Discussion

The HAMLET complex, formed by partially unfolded alpha-lactalbumin and oleic acid, has potent tumoricidal activity and apparently lacks toxicity for normal differentiated cells and healthy tissues. The molecular basis of tumor recognition by the complex is not fully understood, however. The present study was initiated when we detected two distinct cellular response patterns in lung carcinoma cells after HAMLET exposure. In population I, staining was exclusively cytoplasmic and contained in numerous vesicles that were defined as lysosomes. Cells in population II, in contrast, underwent a rapid death response, initiated by membrane perturbations, followed by diffuse cytoplasmic uptake and translocation to the nuclei. Using synthetic peptides, we were able to attribute the rapid cell death response to the N-terminal alpha-helical peptide domain and the vesicular uptake to the beta sheet domain of alpha-lactalbumn; the protein constituent of HAMLET. In cells treated with HAMLET, both cellular response patterns were detected but interestingly, cells with vesicular uptake and lysosomal accumulation showed prolonged survival. We speculate that HAMLET triggers a "double-hit" death response, where alpha-helical peptide-oleate complexes immediately kill about half of the cell population, while remaining cells internalize HAMLET by an HSP70 dependent mechanism and die at a later time.

A network of protein folding and clearance mechanisms (the proteostasis network) is proposed to maintain a healthy proteome for normal cellular function [30,31]. Central to the proteostasis network are molecular chaperones and co-chaperones, a diverse group of proteins that modulate the synthesis, folding, transport, and degradation of proteins [31]. The conformations of aggregation-prone proteins are subject to multiple layers of regulation by the proteostasis network; however, as evidenced by the widespread pathologies of protein conformational diseases, the aggregation propensity of proteins associated with these diseases ultimately overwhelms the proteostasis machineries, thus initiating a cascade of cellular dysfunctions [32–37].

The HSP family of chaperones is essential to prevent the consequences of protein misfolding and amyloid formation during protein synthesis. An increasing body of evidence suggests that certain HSPs may have evolved to also scavenge unfolded proteins from the extracellular environment and convert them to a source of nutrition, through lysosomal degradation [36]. These include Hsp70 and the Aβ peptide co-immunoprecipitates several chaperone proteins including Hsp70. The interaction of Aβ1–42 with Hsp70 appears to be highly specific [36]. Hsp70 overexpression has also been shown to reverse polyglutamine-repeat-dependent toxicity in a *Drosophila* model [33] and in mice. Furthermore, a human HspA1-class Hsp70 was recently shown to suppress α-synuclein-dependent toxicity in a Parkinson's disease model [32], supporting a protective potential of HSP recognition.

In contrast to the amyloid protein species, HAMLET lacks tissue toxicity and despite extensive studies, amyloid-like aggregates have not been observed *in vitro* or in different tissues. While alpha-lactalbumin is partially unfolded and exposes the beta-sheet domain, the molecule also retains alpha-helical structure, in a structurally fluid state, where binding to oleate can occur [38]. This study identifies Hsp70 as a potential scavenger of HAMLET, suggesting that mechanisms may be in place to take up and degrade the complex and prevent it from forming tissue aggregates. This mechanism also delayed tumor cell death, as shown by the enrichment of cells with the vesicular phenotype after 3 and 6 h. After 6 h, the cells gradually died, however, when maintained under *in vitro* conditions. As a result, this potentially protective, scavenging mechanism may develop into a Trojan horse for the tumor, where destruction of the lysosomal membranes and leakage of the complex to the cytoplasm [29] may provide a second-hit mechanism for cells that have survived the immediate death response.

Native protein structure is often regarded as a prerequisite for biological function, as specific epitopes exposed in the native state define molecular fitness for a finite number of cellular targets or by domain-specific, conformational changes. This study illustrates how an increase in structural flexibility can allow a single protein to gain new functions, not found in the native protein. We use alpha-lactalbumin as model, where, by partial unfolding and exposure of alternative domains the protein gains tumoricidal activity by forming a fatty acid complex. Alpha-lactalbumin is the most abundant protein in human milk and a homologue of lysozyme, proposed to have evolved by gene duplication [39]. The native protein is essential for the survival of mammals, due to its role in lactose synthesis and for feeding the offspring. The added tumoricidal activity is a fascinating example of how proteins can gain functional diversity, in this case the ability to form a protein-lipid complex that kills cancer cells, by relinquishing the native structure and gaining flexibility.

## Materials and Methods

### Chemicals

Dimethyl sulfoxide, formaldehyde, Triton X-100, Tween-20, sodium dodecyl sulfate (SDS), sodium deoxycholate and Fluoromount were from Sigma (St. Louis, MO). EDTA (ethylenediaminetetraacetic acid), and Tris (hydroxymethyl) aminomethane were from VWR (Volumetric solutions, BDH Prolabo) and

DRAQ-5 was obtained from eBioscience (San Diego, CA; horseradish peroxidase).

### Cell culture

Lung carcinoma cells (A549) and kidney carcinoma cells (A498) were procured from American Type Culture Collection (ATCC), and murine bladder carcinoma cells (MB49) were maintained in RPMI-1640 medium supplemented with 1 mM sodium pyruvate (Fisher Scientific), non-essential amino acids (1:100) (Fisher Scientific), 50 μg/ml gentamicin (Gibco, Paisley, UK), and 5% fetal calf serum (FCS). Cells were cultured at 37 °C temperature, 90% humidity, and 5% $CO_2$. Cells were grown in 96 well plates overnight (for PrestoBlue™ and ATP assays), in 6-well plate (for Western blots), and in 75-mm flasks (for co-immunoprecipitation). Neonatal human epidermal keratinocytes (nHEK, Life technologies, No. C0015C) were maintained in serum free keratinocyte medium (M154, Life Technologies, No. M154500) containing growth supplement (HKGS, Life Technologies, No. S0015). For keratinocytes differentiation 2 days after initial passage, cell culture media was replaced with fresh medium containing 10% serum without HKGS. Cells were differentiated for 5 days before performing HAMLET treatment.

### Antibodies

Anti-SC35 antibody [SC-35]—nuclear speckle marker (ab11826, Abcam), anti-Grp75 (ab129201, Abcam), anti-Hsp70 (ab79852, Abcam), anti-Hsp70 [5A5] (Ab2787, Abcam), anti-Hsp70 (C92F3A/5, Santa Cruz), anti-Hsp90β (ab80159, Abcam), anti-Hsp40 (ab69402, Abcam) or Hsp60 (ab13532, Abcam) and anti-Hsc70 (sc-7298, Santa Cruz Biotechnology).

### Confocal microscopy

For immunocytochemistry, cells were grown on 8-well glass chamber slide (Lab-Tek, Chamber Slide, Thermo Fisher Scientific) at a concentration $2.5 \times 10^4$ cells per well overnight at 37 °C. The co-localization of HAMLET and SC35 (nuclear speckle marker) was detected by treatment of lung carcinoma cells with Alexa-HAMLET, followed by fixation (2% PFA, 30 min), permeabilization (0.025% TritonX, 15 min). After PBS washing (three times), cells were incubated with anti-SC35 antibodies (1:100 dilution in 10% FCS PBS, overnight at 4 °C, ab11826), washed with PBS (three times) followed by incubation with Alexa-488-conjugated secondary antibodies (1:200 dilution in 10% FCS PBS).

For cell surface localization of HSPs, cells were incubated with respective HSP antibodies (5 μg/ml in 10% FCS PBS, 1 h, 4 °C), washed with ice cold PBS (twice), followed by incubation with respective secondary antibodies (1:200 dilution in 10% FCS PBS, 1 h, 4 °C). After antibodies incubation cells were washed with ice cold PBS (twice), fixed with PFA (2%, 30 min at RT). DRAQ-5 (eBioscience, San Diego, CA) was used as nuclear stain (1:500 dilution in 10% FSC PBS, 10 min at RT). Cells were mounted with mounting media (Fluoromount, Sigma), and fluorescence was detected in a LSM 510 META confocal microscope (Carl Zeiss).

For cell surface co-localization experiments, cells were washed twice with ice-cold PBS and treated with Alexa-HAMLET (4 °C, 30 min in RPMI-1640 serum-free media). Unbounded HAMLET was washed with PBS (twice) followed by incubation with respective HSPs antibodies (5 μg/ml in 10% FCS PBS, 1 h, 4 °C). After primary antibodies, incubation cells were incubated with respective Alexa-labeled secondary antibodies (1:200 dilution in 10% FCS PBS, 1 h, 4 °C) and washed with ice-cold PBS twice. The cells were visualized by live confocal microscopy. The same cells were kept in incubator (37 °C and 5% $CO_2$) for 3 h and imaged live by confocal microscopy.

For intracellular staining of HSPs lung carcinoma cells seeded on 8-well chamber slide were fixed with PFA (2%, 30 min at RT) and permeabilized (0.025% TritonX, 15 min). After PBS washing (three times), cells were incubated with HSP-specific antibodies (1:100 dilution in 10% FCS PBS, overnight at 4 °C, ab11826), washed with PBS (three times) followed by incubation with Alexa-488 conjugated secondary antibodies (1:200 dilution in 10% FCS PBS).

For intracellular co-localization of HAMLET and HSPs, lung carcinoma cells were treated with Alexa-HAMLET in serum free RPMI-1640 media for 1 h, washed with PBS and fixed with PFA (2%, 30 min at RT), washed with PBS (three times). After fixation, cells were incubated with respective HSP antibodies (1:100 dilution in 10% FCS PBS, 1 h at RT). For co-localization of HAMLET with individual domain of Hsp70, after fixation cells were incubated with anti-Hsp70 antibodies corresponding to the full-length protein or its individual domain, that is, ab79852 (full length), 5A5 (ATP binding domain), or C92F3A/5 (PBD)), followed by Alexa-Fluor 488 or 568-labeled secondary antibodies (1:200, 1 h, Molecular Probes).

DRAQ-5 (eBioscience, San Diego, CA) was used as nuclear stain (1:500, 10 min). Cells were mounted with mounting media (Fluoromount, Sigma) and fluorescence was detected in a LSM 510 META confocal microscope (Carl Zeiss).

### HAMLET uptake

For uptake experiments, lung carcinoma cells were treated with Alexa-488 or 568 labeled HAMLET, washed and visualized under confocal microscope live. HAMLET in lysosomes was detected by pre-labeling the cells with lysotracker or in nucleus with DRAQ5.

For HAMLET uptake inhibition experiments, lung carcinoma cells were pre-treated with anti-Hsp70 5A5 (ATP binding domain, Abcam), ab79852 (full length, Abcam), C92F3A/5 (PBD, Santa Cruz), or IgG2A (R&D systems) antibodies. After pre-treatment cells were washed with PBS and treated with Alexa-568-labeled HAMLET, washed and visualized under confocal microscope. The experiments with Hsp70 inhibitors (PES; 25 μM and quercetin; 25 μM), CME inhibitors (monensin; 50 μM, chloroquine; 200 μM, dynasore; 30 μM, dansylcadaverine; 50 μM) and CIE inhibitor (filipin; 5 μg/ml) were performed by pre-treatment of lung carcinoma cells with individual inhibitor for 30 min followed by Alexa-HAMLET treatment for 1 h. Cells were washed and visualized live under confocal microscope LSM 510. Images were quantified with LSM 510 software, ImageJ, or Photoshop CS5.

### Co-immunoprecipitation and Western blot

A549 cells treated with HAMLET (35 μM, 60 min) were lysed with mammalian NP-40 lysis buffer. For extraction of different cell compartments Qproteome cell compartment kit (Invitrogen) or plasma membrane extraction kit (abcam) was used according to the manufacture's instructions. Protein content for each sample was measured with Pierce 660-nm nano-drop method according to the manufacture's instructions. Bovine serum albumin (BSA) was used as standard. Cell lysates were incubated with goat anti-bovine α-lactalbumin antibody, and complexes were collected with Dynabeads® (magnetic) Protein G (Life technologies AS, Oslo). Proteins were separated on SDS-PAGE 4%–12% gradient gel (invitrogen) and transferred to PVDF membranes, blocked with 5% non-fat dry milk or 5% BSA, incubated with primary antibodies including anti-Grp75, Hsp70, Grp78, Hsp90β, Hsp40, Hsp60, and Hsc70 (1:1000–1:10,000, 5 % BSA) followed by HRP-conjugated secondary anti-rabbit or anti-mouse antibodies (1:4000, 5 % NFDM) at room temperature (1 h) and visualized using ECL detection reagent (Amersham Biosciences, Piscataway, NJ).

### Surface HSP analysis by fluorescence microscopy

A549 cells were seeded in an 8-well chambers plates ($2.5 \times 10^4$ cells per well) and were incubated overnight at 37 °C with 5% $CO_2$. The cells were fixed for 20 min in 2% paraformaldehyde at room temperature. After washing, the cells were incubated overnight with primary anti-Hsp70 antibody diluted in PBS (Abcam, 1:50, 1:100 or 1:200). The chamber slides were washed twice with PBS and incubated with goat-anti-rabbit Alexa-488 secondary antibody for 1 h. Nuclei were counter-stained with Draq5 (1:1000, Abcam) for 15 min. The cells were washed thrice with PBS, coverslipped using Fluoromount (Sigma), and visualized by confocal microscopy (ZEISS-META-510).

### Flow cytometry

A549 cells were seeded in 400 μl of serum-free medium at a concentration $5 \times 10^5$ cells/ml. Each FACS tube was centrifuged at 400*g* for 10 min and transferred to V-shape 96-well plates, fixed with 100 μl of 2% paraformaldehyde, and incubated for 20 min at room temperature followed by centrifugation at 1000*g* for 6 min and washing with 100 μl of 10% FCS/PBS. Subsequently, cells were incubated with anti-Hsp70 antibody (1 mg/ml) (Abcam, UK) in 100 μl 10% FCS/FBS overnight at 4 °C. After centrifugation and washing, Alexa-Fluor 488-labeled secondary antibodies were added (1:200, Molecular Probes) in 100 μl 10% FCS/FBS and incubated for 1 h at room temperature. Cells were washed, suspended in 10% FCS/FBS, and collected for flow cytometry measurement by FACS Canto II (BD Biosciences).

### Statistical analysis

Results are presented as a mean ± SEM. Statistical analysis was performed using Student's *t*-test at different statistical levels of significance, $*p < 0.05$ and $**p < 0.01$.

## CRediT authorship contribution statement

**Aftab Nadeem:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **James Ho:** Conceptualization, Investigation, Visualization, Writing - review & editing. **Tuan Hiep Tran:** Data curation, Validation, Methodology, Writing - review & editing. **Sanchari Paul:** Data curation, Validation, Methodology, Writing - review & editing. **Victoria Granqvist:** Data curation, Validation, Methodology, Writing - review & editing. **Nadege Despretz:** Data curation, Validation, Methodology, Writing - review & editing. **Catharina Svanborg:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

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## Appendix A. Supplementary data

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†C.S. holds shares in HAMLET Pharma, as a representative of scientists in the HAMLET group.

***Abbreviations used:***
HSP, heat shock protein; HAMLET, Human Alpha-lactalbumin Made LEthal to Tumour cells; PES, phenylethynesulfonamide; PBD, peptide binding domain; BSA, bovine serum albumin.

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