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FAST TRACK

HAMLET (human α-lactalbumin made lethal to tumor cells) triggers autophagic tumor cell death

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HAMLET, a complex of partially unfolded α-lactalbumin and oleic acid, kills a wide range of tumor cells. Here we propose that HAMLET causes macroautophagy in tumor cells and that this contributes to their death. Cell death was accompanied by mitochondrial damage and a reduction in the level of active mTOR and HAMLET triggered extensive cytoplasmic vacuolization and the formation of double-membrane-enclosed vesicles typical of macroautophagy. In addition, HAMLET caused a change from uniform (LC3-I) to granular (LC3-II) staining in LC3-GFP-transfected cells reflecting LC3 translocation during macroautophagy, and this was blocked by the macroautophagy inhibitor 3-methyladenine. HAMLET also caused accumulation of LC3-II detected by Western blot when lysosomal degradation was inhibited suggesting that HAMLET caused an increase in autophagic flux. To determine if macroautophagy contributed to cell death, we used RNA interference against Beclin-1 and Atg5. Suppression of Beclin-1 and Atg5 improved the survival of HAMLET-treated tumor cells and inhibited the increase in granular LC3-GFP staining. The results show that HAMLET triggers macroautophagy in tumor cells and suggest that macroautophagy contributes to HAMLET-induced tumor cell death.

Key words: HAMLET; cell death; macroautophagy; cancer therapy; protein folding; α-lactalbumin

Macroautophagy serves to degrade long-lived proteins and organelles. During macroautophagy, cytoplasm and organelles are enwrapped in membrane sacs forming double-membrane-enclosed autophagosomes. These fuse with lysosomes where the contents are degraded for reutilization.[1] Macroautophagy occurs at basal levels in most normal cells[2] and plays a role in development, differentiation[3] and immune defense.[4] Furthermore, macroautophagy has been proposed to suppress early tumorigenesis[5] and macroautophagy-inducing drugs are in clinical trials for cancer therapy.[6] Macroautophagy is increased by cellular stress such as starvation and organelle damage,[2] and excessive macroautophagy may cause a form of programmed cell death called autophagic/Type II cell death. However, the role of macroautophagy in cell death is still a matter of debate.[7–9]

Macroautophagy is controlled by phosphatidylinositol 3-kinases (PI3Ks).[10] Class III PI3K forms a complex with Beclin-1 which initiates autophagosome formation, while the Class I PI3K/Akt/mammalian target of rapamycin (mTOR) pathway inhibits macroautophagy. In addition, autophagosome formation requires 2 conjugation cascades involving several Autophagy-related proteins (Atgs).[1,7,11] Inhibition of Beclin-1 and Atgs by RNA interference is therefore used to inhibit macroautophagy.[12–15]

HAMLET is a complex of partially unfolded α-lactalbumin and oleic acid that kills tumor cells and embryonal cells but spares healthy differentiated cells. Studies in a rat xenograft model of human glioblastoma and in patients with skin papillomas or bladder cancer have confirmed that HAMLET eliminates tumor cells also *in vivo*.[16–18] Furthermore, no adverse effects from the HAMLET treatment were observed in these studies. However, the mechanism(s) of cell death in response to HAMLET remain unclear.

We have proposed that HAMLET identifies common, conserved death pathways in tumor cells, based on the broad activity spectrum and on the similarity of the response to HAMLET between cell lines[19,20] and have used the "hydra" metaphor to depict HAMLET as a substance with many targets in sensitive cells.[21] HAMLET translocates from the cell surface to the nuclei, where it binds histones and disrupts the function of chromatin.[22] In early studies, HAMLET was shown to affect the mitochondria and to cause an apoptotic response with Cytochrome c release, low caspase activation, phosphatidylserine exposure and DNA fragmentation.[19,23–25] Apoptotic changes were also observed *in vivo* in HAMLET-treated tumors.[17,18] Nevertheless, tumor cell death does not depend on classical apoptosis. Cells proceeded to die in the presence of caspase inhibitors and neither over-expression of Bcl-2 nor loss of p53 function prevented cell death.[25] Recently, HAMLET has been shown to trigger a rapid ER stress response (Storm, unpublished data) and to inhibit proteasome activity.[26]

During a morphologic analysis of HAMLET-treated cells by electron microscopy we observed double-membrane-enclosed

Conflict of interest: Catharina Svanborg is a shareholder in NatImmune, a biotech company which is developing HAMLET as a drug.

Abbreviations: 3MA, 3-methyladenine; AMPKα, 5′-AMP-activated protein kinase catalytic subunit alpha; Atg, Autophagy-related protein; Bcl-2, apoptosis regulator Bcl-2; EBSS, Earle's balanced salt solution; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HAMLET, human α-lactalbumin made lethal to tumor cells; LC3, microtubule-associated proteins 1A/1B light chain 3; MAL, multimeric α-lactalbumin; MAPK, mitogen-activated protein kinase; MPT, mitochondrial permeability transition; mTOR, mammalian target of rapamycin (FKBP12-rapamycin complex-associated protein); p53, cellular tumor antigen p53; p70 S6K, ribosomal protein S6 kinase beta-1; PI3K, phosphatidylinositol 3-kinase; zVAD-fmk, Z-Val-Ala-Asp(OMe)-fluoromethylketone

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vesicles suggestive of macroautophagy. In this study, we have linked macroautophagy to HAMLET-induced tumor cell death. HAMLET caused LC3 translocation and inhibition of macroautophagy by Beclin-1 and Atg5 siRNAs significantly reduced HAMLET-induced cell death. The results indicate that HAMLET causes macroautophagy in tumor cells and suggest that this process contributes to cell death.

Material and methods

HAMLET production

α-Lactalbumin was purified from human milk and converted to HAMLET by removal of calcium and binding to oleic acid as previously described.[27] Concentrations are based on the weight of the protein component only (14,078.1 Da).

Cell lines and culturing

The A549 lung carcinoma cell line (American Type Culture Collection (ATCC), no. CCL-185) and the Jurkat T-cell leukemia cell line (ATCC, no. TIB-152) were cultured in RPMI 1640 medium with 5% FCS, nonessential amino acids (1:100), 1 mM sodium pyruvate and 50 μg/ml Gentamicin (Gibco, Paisley, UK). The MDA-MB-231 LC3-GFP breast adenocarcinoma cell line was cultured in DMEM (high glucose) with 5% FCS, 1 mM sodium pyruvate, 3.7 mM L-Glutamine and 200 μg/ml G418 (Sigma-Aldrich, St. Louis, MO).

Assays for cell death and protein and RNA extraction

Cells were detached with Versene (140 mM NaCl, 2.4 mM KCl, 8 mM Na_2HPO_4, 1.6 mM KH_2PO_4, 0.5 mM EDTA, pH 7.2), washed, diluted to 10^6 cells/ml in serum-free RPMI 1640 medium (with nonessential amino acids, sodium pyruvate and Gentamicin) and plated in 24-well or 96-well culture plates (TPP, Trasadingen, Switzerland) or 96-well clear-bottom plates (Corning Incorporated, Corning, NY). If required, cells were pretreated with Z-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk, BioMol International, Butler Pike, PA). HAMLET was added and cells were incubated at 37°C. FCS (5%) was added after 1 hr.

Viability was quantified by trypan blue exclusion (Chroma Gesellschaft Schmid & Co, Stuttgart, Germany) counting at least 100 cells per sample or by measuring ATP levels (ViaLight Plus Kit, Cambrex, East Rutherford, NJ or ATPlite Kit, PerkinElmer, Boston, MA) in a LUMIstar Luminometer (BMG LABTECH, Offenburg, Germany).

Electron microscopy

A549 cells were treated with HAMLET, fixed in 4% paraformaldehyde and 0.1% glutaraldehyde and pelleted at 4°C. Pellets were dehydrated in ethanol for 1 hr and embedded in Lowicryl. Ultra-thin sections were mounted onto nickel grids and examined with a JEM 1230 transmission electron microscope (Jeol, Tokyo, Japan) operated at 60 kV accelerating voltage and a Gatan Multiscan 791 CCD camera (Gatan, Munich, Germany).

Western blot

Cells were washed with PBS containing 0.2 mM PMSF, 1 μg/ml Pepstatin A, 5 μg/ml Leupeptin (Sigma-Aldrich) and Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and lysed in modified RIPA buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 50 μM ZnCl, 1% NP-40, 0.1% deoxycholate, 0.1% SDS) containing the same protease inhibitors. Protein concentrations were measured with the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). For Western blots for siRNA experiments cells were detached with Versene and the same number of cells from each sample was lysed in NP-40 buffer (20 mM Tris HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing Complete protease inhibitor cocktail. Equal amounts of protein or, for siRNA Western blots, equal volumes of lysates derived from the same number of cells were separated by SDS-PAGE and blotted onto PVDF membranes. Membranes were saturated with nonfat dry milk (phospho-mTOR, mTOR, phospho-p70 S6K, p70 S6K, phospho-AMPKα, AMPKα) or BSA (Beclin-1, GAPDH) and incubated with rabbit anti-phospho-mTOR (S2448), anti-mTOR, anti-phospho p70 S6K (T389), anti-p70 S6K, anti-phospho-AMPKα (T172), anti-AMPKα, anti-Beclin-1 (all 1:500–1,000, Cell Signaling Technology, Danvers, MA) or mouse anti-GAPDH antibody (1:3,000–5,000, Novus Biologicals, Littleton, CO). Bound antibodies were detected with HRP-conjugated swine anti-rabbit (1:2,000, DakoCytomation, Glostrup, Denmark) or rabbit anti-mouse antibody (1:50,000-200,000, Novus Biologicals) using ECL Plus Western Blotting Reagent (GE Healthcare, Little Chalfont, UK) and GelDoc equipment (Bio-Rad Laboratories). To quantify protein levels, band intensity was measured with ImageJ software[28] and normalized against GAPDH. If required, membranes were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), blocked and reprobed with new antibodies.

For LC3 Western blot, cells were first washed and incubated in DMEM medium without FCS in the presence of Insulin Transferrin Selenium A (ITS, 1X) (Invitrogen, Carlsbad, CA) or, for the starvation control, in Hank's balanced salt solution with calcium and magnesium (Invitrogen). Then, cells were pretreated with Bafilomycin A1 (100 nM) or E64d and Pepstatin A (both 10 μg/ml, Sigma-Aldrich) for 30 min, treated with HAMLET for 3 hr and lysed in Laemmli buffer (12.5 mM Na_2HPO_4, 15% glycerol, 3% SDS). Protein concentrations were measured as described above. A total of 100 μg proteins for each sample were separated by SDS-PAGE and blotted onto PVDF membranes. Membranes were saturated with TNB buffer (10 mM Tris HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 0.1% Tween-20, 3% BSA, 0.5% gelatin) and incubated with mouse anti-LC3 (1:1,000, NanoTools Antikörpertechnik, Teningen, Germany) or rabbit anti-p44/42 MAPK antibody (1:2,000, Cell Signaling Technology, as loading control). Membranes were washed with TNT buffer (10 mM Tris HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween-20) and bound antibody was detected with HRP-conjugated anti-mouse (1:3,000, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-rabbit antibody (1:10,000, Santa Cruz Biotechnology) using the Enhanced Chemiluminescence detection system (PerkinElmer) and Hyperfilm (GE Healthcare).

LC3-GFP translocation

For transient transfection with the LC3-GFP plasmid[29] (kind gift by Dr. Marja Jäättelä and Dr. Maria Høyer-Hansen, Apoptosis Department and Centre for Genotoxic Stress Research, Institute of Cancer Biology, Danish Cancer Society, Copenhagen, Denmark) A549 cells were grown on 8-well chamber slides (Nalge Nunc, Rochester, NY) to 80–90% confluence and transfected using Lipofectamine 2000 (Invitrogen). After 24 hr cells were pretreated with 3-methyladenine (3MA, Sigma-Aldrich) and treated with HAMLET.

Stably transfected MDA-MB-231 LC3-GFP cells were grown on 8-chamber slides, washed, incubated with HAMLET or Earle's balanced salt solution (EBSS) (1.8 mM $CaCl_2 \cdot H_2O$, 0.8 mM $MgSO_4$, 4.0 mM KCl, 26.2 mM $NaHCO_3$, 117.2 mM NaCl, 1.0 mM NaH_2PO_4, 5.6 mM D-Glucose) with or without 3MA and fixed in 4% paraformaldehyde.

LC3-GFP translocation was examined by manual counting (LSM 510 META microscope, Carl Zeiss, Oberkochen, Germany).

RT-PCR

RNA was prepared with the RNeasy kit (QIAGEN, Hilden, Germany), treated with DNAse I (DNAse I (Invitrogen or QIAGEN) and cDNA synthesized using the Superscript III first-strand RT-PCR system (Invitrogen). Real-time PCR was performed on a Rotorgene 2000 instrument (Corbett Life Science, Sydney, Australia) using QuantiTect Primer Assays for Beclin-1, Atg5 and Atg7 (QIAGEN) and a GAPDH assay (Applied Biosystems, Foster City, CA).

RNA interference

siRNAs against Beclin-1 (sense sequence CUCAGGAGAG GAGCCAUUU) and Atg5 (sense sequence GCAACUCUG GAUGGGAUUG)[12] were used (QIAGEN). AllStar Negative Control siRNA-Alexa Fluor 488 was used to control transfection efficiency and AllStar Negative Control siRNA as negative control (QIAGEN). Cells were grown in 24-well plates (TPP) and transfected with siRNAs using Lipofectamine 2000 (Invitrogen). After 42 hr knockdown was examined by RT-PCR and Western blot (for Beclin-1) and cells were used for experiments.

Statistical analysis

InStat software (Version 3.06, GraphPad, San Diego, CA) was used to perform One-way ANOVA on the results from the A549 LC3-GFP experiments, Repeated measures ANOVA on the results from RNA interference cell death experiments and MDA-MB-231 LC3-GFP experiments and paired *t*-tests on the results from RT-PCR experiments examining regulation of macroautophagy components.

Results

HAMLET-induced cell death is accompanied by morphological changes compatible with macroautophagy

The death response to HAMLET was quantified by trypan blue exclusion and ATP levels and HAMLET was shown to kill A549 cells in a time- and dose-dependent manner (Fig. 1*a*). Morphological changes accompanying cell death were examined by electron microscopy after 30 min and 6 hr of HAMLET treatment (Fig. 1*b*, CT and 1*c*, Untreated). A549 cells (Fig. 1*b,* CT and 1*c,* Untreated) showed normal morphology but after exposure to HAMLET most cells showed extensive cytoplasmic vacuolization (Fig. 1*b* and 1*c*, Vacuolated) and sometimes nonapoptotic chromatin changes.[30,31] The frequency of cells with vacuoles increased from 22% in the control to 62% after 30 min and 6 hr of HAMLET treatment. At a higher magnification of HAMLET-treated cells, double- and multi-membrane structures compatible with macroautophagy were observed (Fig. 1*d*). These contained cytoplasm, organelles or material resembling chromatin. In addition, a few necrotic cells were seen (Fig. 1*b* and 1*c*, Necrotic) at a frequency of 6 and 13% after 30 min and 6 hr of HAMLET treatment. These cells showed ruptured plasma membranes, loss of cellular material, resolution of nuclear membranes and merging of nuclear contents with the cytoplasm. In some cases, the remaining cytoplasm contained remnants of vacuoles suggesting that necrosis might be secondary to vacuolization.

HAMLET causes mitochondrial damage

HAMLET and a HAMLET-containing milk fraction (multimeric α-lactalbumin, MAL) have previously been shown to cause mitochondrial swelling, depolarization and permeabilization.[23,24] Since macroautophagy may be triggered by mitochondrial damage[32,33] mitochondrial integrity was examined by electron microscopy. Drastic changes in mitochondrial structure were observed 30 min after HAMLET treatment of A549 cells (Fig. 2*a*). Mitochondria were swollen with disrupted membranes and the organization of the cristae was lost. In contrast, control cells showed intact mitochondrial morphology with densely packed and ordered cristae (Fig. 2*a*).

HAMLET decreases mTOR activity

Macroautophagy is inhibited by mTOR[2,34] and phosphorylation at S2448 correlates with mTOR activity.[35–37] To determine if HAMLET affects mTOR, we treated A549 cells with HAMLET for 3 hr and quantified total mTOR protein and S2448 phosphorylation by Western blot. At 36 μM of HAMLET the level of phosphorylated mTOR decreased to 42% of the control (Fig. 2*b* and 2*c*). This suggests that mTOR is inactivated in tumor cells in response to HAMLET.

To confirm the inactivation of mTOR we examined the phosphorylation of the mTOR substrate p70 S6K at T389 which is frequently used as a marker for mTOR activity.[38] HAMLET treatment for 3 hr reduced the level of phosphorylated p70 S6K (Fig 2*b* and 2*c*). At lower HAMLET concentrations this reduction was solely due to a loss of phosphorylation since the total p70 S6K level was unchanged. At the highest HAMLET concentration total p70 S6K levels were decreased as well. The results show that HAMLET reduces p70 S6K phosphorylation and suggest that this may be due to the reduction in mTOR activity.

AMP-activated protein kinase (AMPK) is an important upstream inhibitor of mTOR which is mainly activated by an increase in the AMP/ATP ratio.[38,39] To determine if the reduction in mTOR activity was caused by increased AMPK signaling, we examined the phosphorylation of the catalytic α-subunit at T172 which is required for AMPK activation.[38] HAMLET caused a reduction in AMPKα phosphorylation (Fig. 2*b* and 2*c*), however, indicating that the inactivation of mTOR is not caused by AMPK.

HAMLET causes LC3 translocation to autophagosomes

LC3 translocation is a well-established marker of autophagosome formation.[40] LC3 normally resides in the cytoplasm as LC3-I but associates with autophagosomal membranes during macroautophagy after modification to LC3-II by attachment of phosphatidylethanolamine.[40] LC3 translocation in response to HAMLET was first investigated in A549 cells transiently transfected with LC3-GFP (30% transfection rate) (Fig. 3*a* and 3*b*). HAMLET changed staining from a diffuse (LC3-I) to a granular pattern (LC3-II). After HAMLET treatment 41% of the successfully transfected cells showed granular staining compared to 20% in the control ($p < 0.001$) and this effect was reduced to control levels by 3-methyladenine (3MA), which blocks PI3Ks and inhibits macroautophagy ($p < 0.001$).

LC3 translocation was further investigated in MDA-MB-231 breast adenocarcinoma cells stably transfected with LC3-GFP. HAMLET caused a dose- and time-dependent increase in granular LC3-GFP staining (Fig. 3*c* and 3*d*). After 10 min of HAMLET treatment (36 μM) granular LC3-GFP staining had increased from 38% to 65% and after 4 hr 78% of the HAMLET-treated cells (36 μM) showed granular staining compared to 47% of the control cells ($p < 0.05$). The effect of HAMLET was similar to that of amino acid starvation with Earle's buffered salt solution (EBSS) ($p < 0.01$, compared to control), which was used as the positive control. The granular staining was reduced by 3MA in all cells. The results show that HAMLET triggers rapid LC3 translocation in lung and breast carcinoma cells.

HAMLET causes LC3-II accumulation in the presence of lysosomal inhibitors

LC3-II accumulation during macroautophagy can be detected by Western blot. However, LC3-II levels decline during prolonged macroautophagy since LC3-II is degraded after autophagosomal-lysosomal fusion. Therefore, the flux through the macroautophagic system is best measured by comparing LC3-II levels in the presence or absence of lysosomal inhibitors that partially prevent LC3-II degradation.[41,42] To examine if HAMLET caused an increase in autophagic flux or a block in the macroautophagic system, A549 cells were treated with HAMLET for 3 hr and lysosomal degradation of LC3-II was inhibited by Bafilomycin A1 or Pepstatin A and E64d,[41,42] (Fig. 3*e*). Amino acid-starved cells were used as positive control. In the absence of lysosomal inhibitors no changes in LC3-II levels were detected between untreated, starved and HAMLET-treated cells. However, when lysosomal LC3-II degradation was blocked a large increase in LC3-II was observed in starved and HAMLET-treated cells but not in untreated cells. These results suggest that HAMLET increases the autophagic flux.

Knockdown of Beclin-1 rescues HAMLET-treated cells

To examine if macroautophagy contributes to HAMLET-induced cell death we inhibited macroautophagy by RNA in-

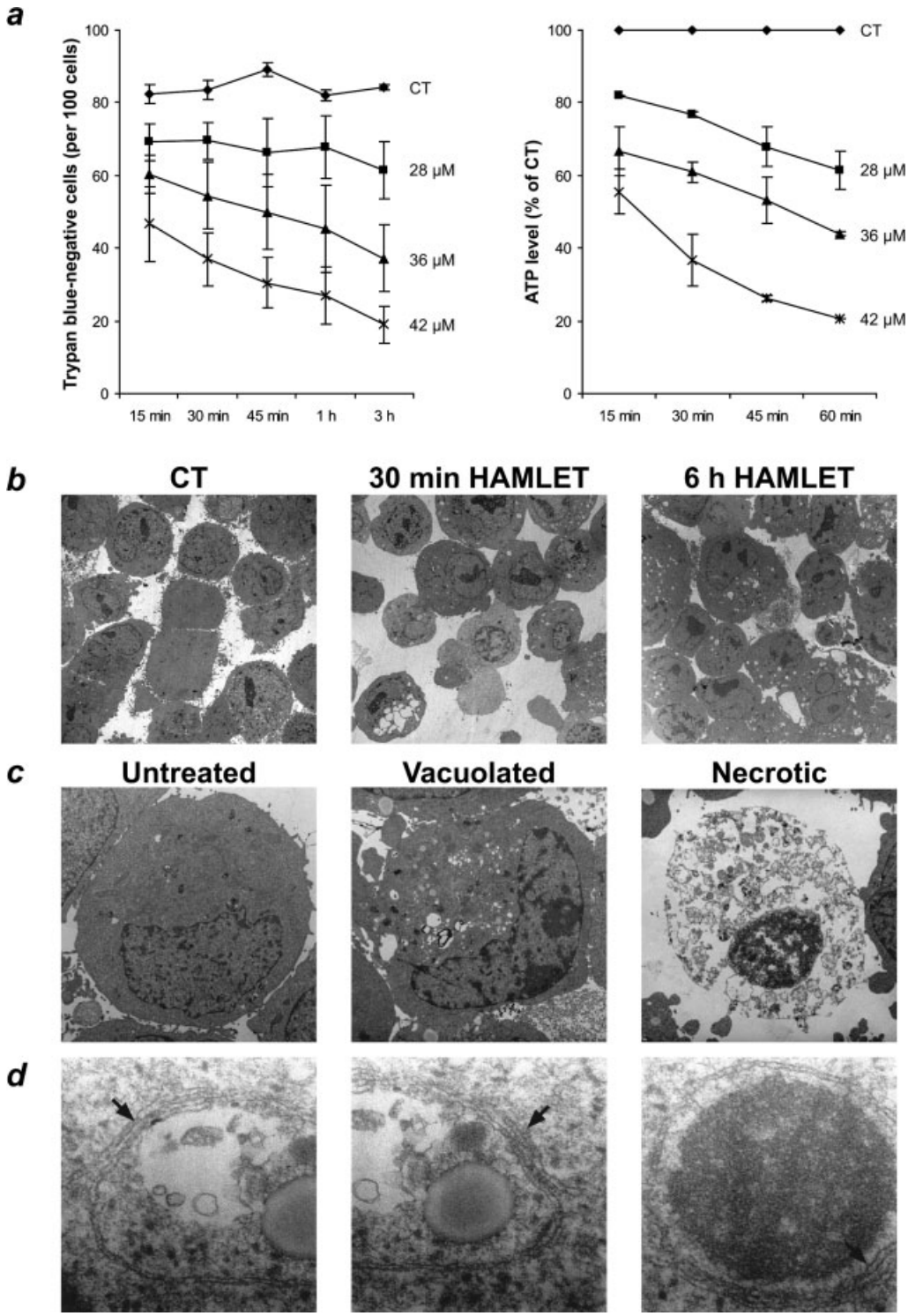

Figure 1 – Cell death and morphological changes in HAMLET-treated A549 cells. (*a*) HAMLET caused dose- and time-dependent cell death. A549 cells were treated with HAMLET (28–42 μM) and viability was quantified by trypan blue exclusion (left) or by ATP levels in % of the control at each time point (right). Means ± SEMs of 6 (trypan blue) or 2 (ATP) independent experiments are shown. (*b–d*) A549 cells were treated with HAMLET (36 μM) and examined by electron microscopy. (*b*) Electron micrographs showing changes in cell morphology after 30 min and 6 hr of HAMLET treatment. (*c*) Typical cell morphologies before and 6 hr after HAMLET treatment. (*d*) Double-membrane and multi-membrane structures (indicated by arrows) characteristic of macroautophagy in HAMLET-treated cells after 6 hr.

terference targeting Beclin-1. A549 cells were transfected with Beclin-1 siRNA or nontargeting control siRNA (CT siRNA) for 42 hr. Knockdown was confirmed by RT-PCR and Western Blot. Beclin-1 siRNA-transfected cells showed a 64% reduction in Beclin-1 mRNA compared to untransfected cells and a 76% reduction compared to CT siRNA-transfected cells (Fig. 4*a*). Beclin-1 protein levels were also reduced (Fig. 4*b*).

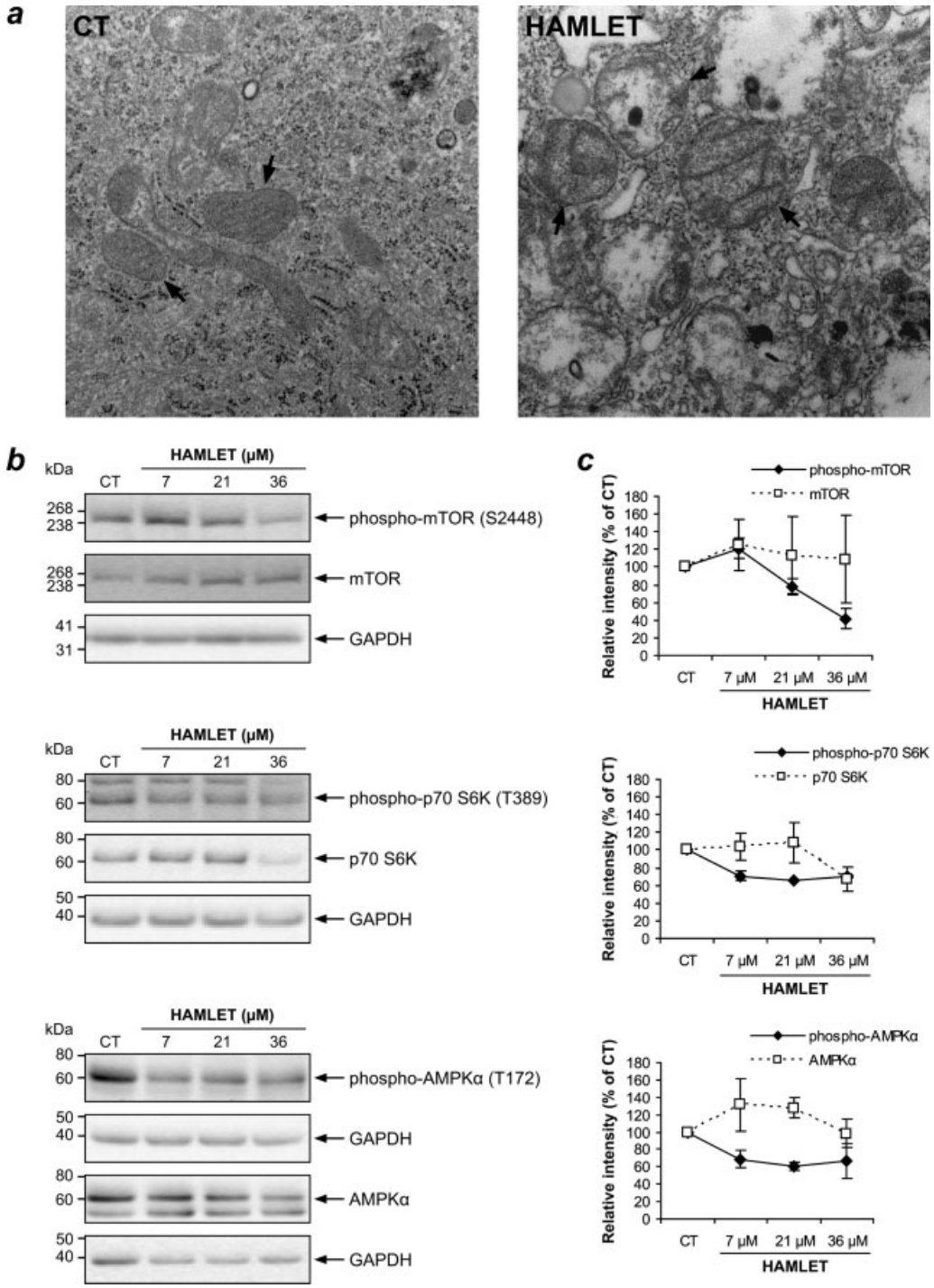

Figure 2 – Possible initiators of macroautophagy. (*a*) Mitochondria (indicated by arrows) were swollen and damaged in HAMLET-treated cells after 30 min but not in control cells. A549 cells were treated with HAMLET (36 μM) and examined by electron microscopy. (*b, c*) Levels of mTOR phosphorylated at S2448 (phospho-mTOR), p70-S6K phosphorylated at T389 (phospho-p70 S6K) and AMPK phosphorylated at T172 (phospho-AMPK) were reduced in HAMLET-treated A549 cells (3 hr). (*b*) Western blots showing phosphorylated and total proteins using GAPDH as loading control. Membranes were stripped and reprobed. (*c*) Quantification of phosphorylated and total proteins showing relative levels (*e.g.*, phospho-mTOR/GAPDH intensity ratio in % of this ratio in PBS-treated control cells) as means ± SEMs of 2 or 3 (phospho-AMPK) independent experiments.

A549 cells transfected with Beclin-1 or CT siRNA were subsequently exposed to different HAMLET concentrations for 3 hr and cell death was quantified by trypan blue exclusion and ATP levels (Fig. 4*d*). Beclin-1 siRNA caused a significant rescue from HAMLET-induced cell death. At 36 μM of HAMLET cell death measured by trypan blue exclusion was reduced to 45% compared to more than 70% in control cells ($p < 0.001$). In addition, Beclin-1 siRNA inhibited the increase in granular LC3-GFP staining in HAMLET- or EBSS-treated MDA-MB-231 cells (Fig. 5).

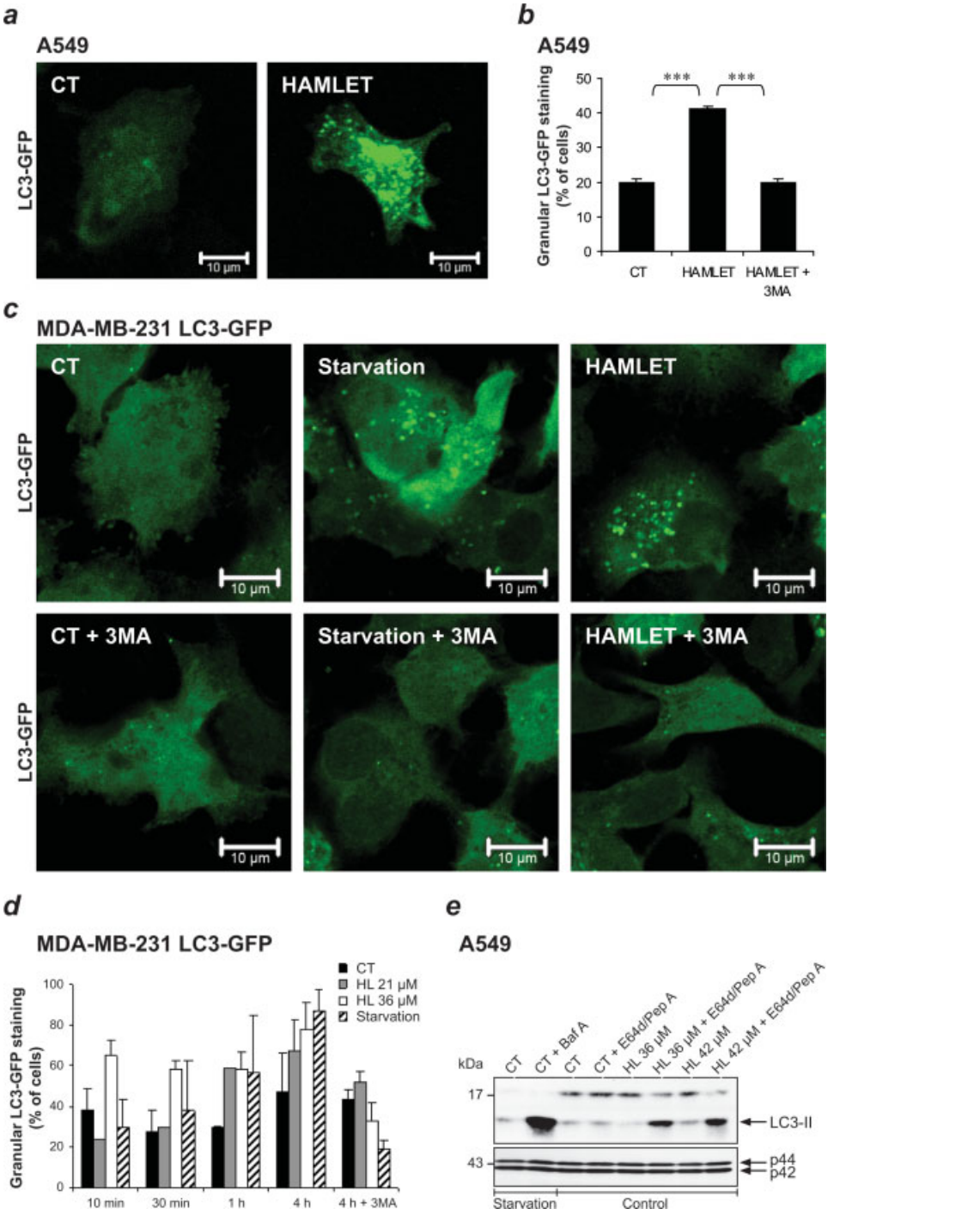

FIGURE 3 – LC3 translocation and accumulation. (*a–d*) HAMLET treatment and starvation increased granular LC3-GFP (green) staining indicating LC3 translocation to autophagosomes. This effect was inhibited by 3MA. (*a, b*) A549 cells were transiently transfected with LC3-GFP and treated with HAMLET (36 μM, 3 hr) with or without 3MA (10 mM, 12 hr pretreatment). (*a*) Granular staining in a HAMLET-treated cell but uniform staining in a control cell. (*b*) Quantification of granular LC3-GFP staining (defined as cells with large LC3-GFP dots, counting at least 50 cells). Means + SEMs of 3 independent experiments are shown. (*c, d*) Stably LC3-GFP-transfected MDA-MB-231 cells were treated with HAMLET (36 μM) or were amino acid-starved with EBSS for different times. 3MA was used as a macroautophagy inhibitor (10 mM, no pretreatment). (*c*) Granular staining after 4 hr in HAMLET-treated (36 μM) cells and in starved control cells but more uniform staining in untreated control cells and 3MA-treated cells. (*d*) Quantification of granular LC3-GFP staining (defined as more than 5 LC3-GFP dots per cell, counting at least 50 cells per sample). Means + SEMs of 1–3 independent experiments are shown. (*e*) Western blot showing LC3-II accumulation in HAMLET-treated A549 cells (3 hr) when lysosomal LC3-II degradation was inhibited. Amino acid-starved cells were used as a positive control. Lysosomal LC3-II degradation was inhibited by Bafilomycin A1 (Baf A) or Pepstatin A (Pep A) and E64d. A p42/p44 Western blot is shown as loading control.

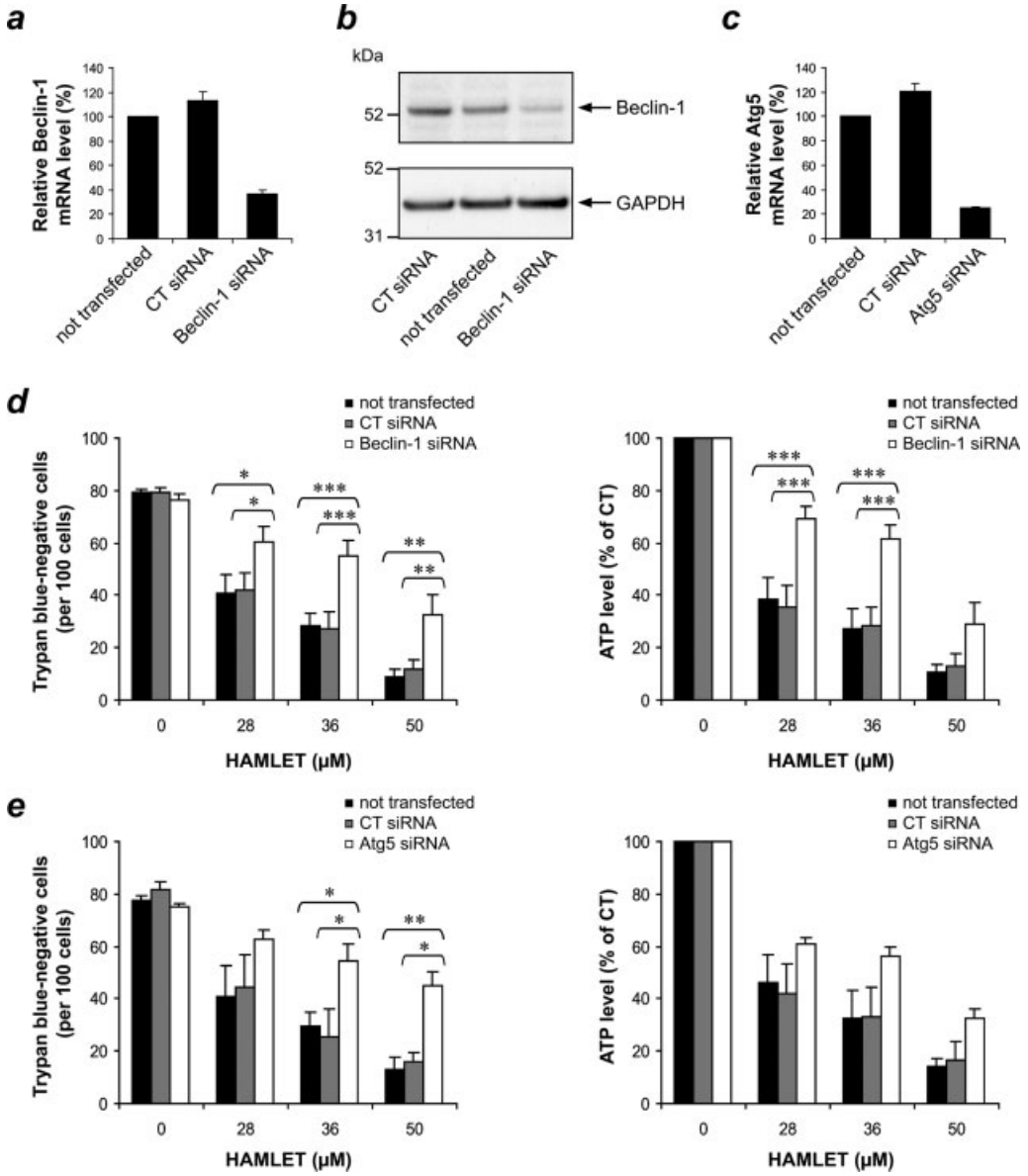

Figure 4 – Inhibition of HAMLET-induced cell death by siRNA interference with Beclin-1 and Atg5 expression. A549 cells were transfected with Beclin-1 siRNA (*a, b, d*), Atg5 siRNA (*c, e*) or nontargeting siRNA (CT siRNA). (*a, c*) RT-PCR results confirming the reduction in Beclin-1 (*a*) and Atg5 (*c*) mRNA levels after 42 hr. Relative mRNA levels (*e.g.,* Beclin-1 mRNA/GAPDH mRNA in % of this ratio in untransfected cells) are shown as means + SEMs of 7 (Beclin-1) or 4 (Atg5) independent experiments. (*b*) Western Blot confirming the reduction in Beclin-1 protein levels. Equal volumes of lysate derived from the same number of cells were loaded in each lane. A GAPDH Western blot of duplicate samples was performed as loading control. (*d, e*) Beclin-1 and Atg5 siRNAs protected cells from HAMLET-induced cell death. Viability was quantified by trypan blue exclusion (left) or by ATP levels in % of the respective control (not transfected, CT siRNA-transfected and Beclin-1 or Atg5 siRNA-transfected cells) (right). Means + SEMs of 7 (Beclin-1, trypan blue), 6 (Beclin-1, ATP) or 4 (Atg5) independent experiments are shown (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Note that ATP levels in untreated control cells were 67–92% of levels in untreated cells transfected with Beclin-1 or Atg5 siRNA.

Knockdown of Atg5 rescues HAMLET-treated cells

To further examine if macroautophagy contributes to HAMLET-induced cell death we used siRNA to inhibit Atg5, which is also required for autophagosome formation.[11] Atg5 siRNA treatment for 42 hr reduced Atg5 mRNA levels by 75 and 95% compared to untransfected or CT siRNA-transfected A549 cells, respectively (Fig. 4*c*). Transfected cells were exposed to HAMLET for 3 hr and cell death was quantified by trypan blue exclusion and ATP levels (Fig. 4*e*). Atg5 siRNA caused a significant rescue from cell death which was similar to that observed after Beclin-1 siRNA treatment. At 36 μM of HAMLET cell death measured by trypan blue exclusion was reduced from 70% in control cells to 46% ($p < 0.05$). In addition, Atg5 siRNA inhibited LC3-GFP translocation in HAMLET- or EBSS-treated MDA-MB-231 LC3-GFP cells (Fig. 5).

The Beclin-1 and Atg5 siRNA experiments suggest that macroautophagy contributes to HAMLET-induced cell death.

HAMLET increases Beclin-1, Atg5 and Atg7 mRNA levels, and Beclin-1 protein levels

To study the effect of HAMLET on the macroautophagy machinery, Beclin-1, Atg5 and Atg7 expression was examined by RT-PCR using GAPDH for normalization. In A549 cells Beclin-1

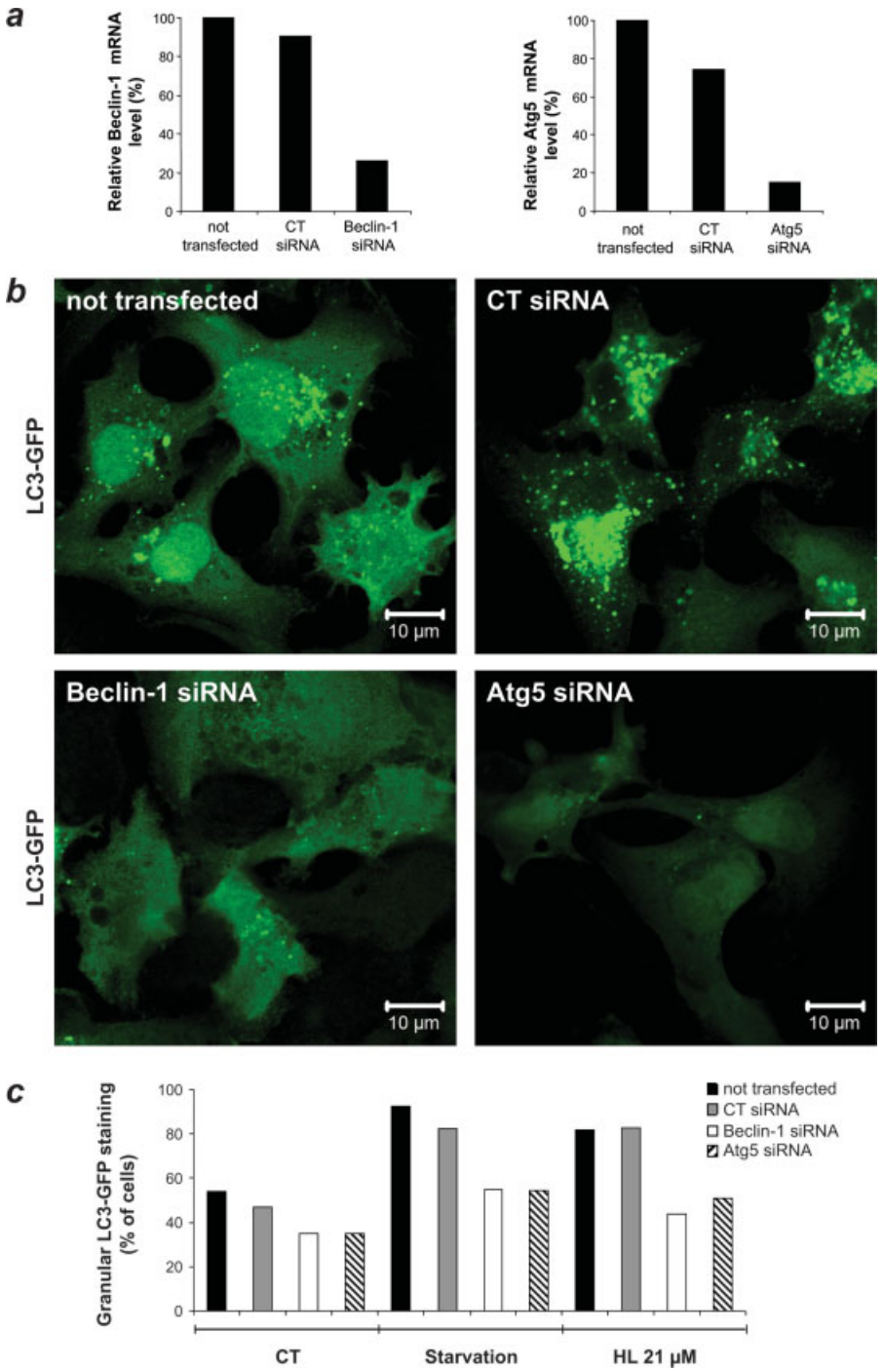

FIGURE 5 – Inhibition of HAMLET-induced LC3-GFP translocation by Beclin-1 and Atg5 siRNA. Stably LC3-GFP-transfected (green) MDA-MB-231 cells were transfected with Beclin-1 siRNA, Atg5 siRNA or nontargeting siRNA (CT siRNA). (*a*) RT-PCR confirming the reduction in Beclin-1 (left) and Atg5 (right) mRNA levels after 42 hr. Relative mRNA levels (*e.g.,* Beclin-1 mRNA/GAPDH mRNA in % of this ratio in untransfected cells) are shown. (*b, c*) Beclin-1 and Atg5 siRNA inhibited the increase in granular LC3-GFP staining caused by HAMLET (21 µM) or by amino acid starvation with EBSS (both 4 hr). (*b*) Granular staining in control cells but more uniform staining in Beclin-1 and Atg5 siRNA-transfected cells after HAMLET treatment. (*c*) Quantification of granular LC3-GFP staining (defined as more than 5 LC3-GFP dots per cell, counting at least 40 cells per sample).

and Atg7 mRNA levels had increased after 1 hr of HAMLET treatment (Fig. 6*a*) and after 3 hr, Atg5 mRNA levels were increased as well (Fig. 6*b*). In addition, levels of all 3 mRNAs had increased in Jurkat cells after 3 hr (Fig. 6*c*).

Changes in Beclin-1 were confirmed at the protein level by Western blot. Low concentrations of HAMLET (7 µM) increased the Beclin-1 level already and at 36 µM an increase to 154% of the control level was observed (Fig. 6*d*). These results suggest that components of the macroautophagy machinery are upregulated in response to HAMLET.

Simultaneous inhibition of apoptosis and macroautophagy in HAMLET-treated cells

To investigate if macroautophagy and caspase-dependent apoptosis might cooperate to promote death, both pathways were inhibited simultaneously with Beclin-1 siRNA and the pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk). zVAD-fmk was used at a concentration of 50 µM which is slightly higher than the concentration previously shown to inhibit caspase 3 activity and the cleavage of caspase substrates in A549 cells treated with MAL.[23] Viability was quantified by trypan blue

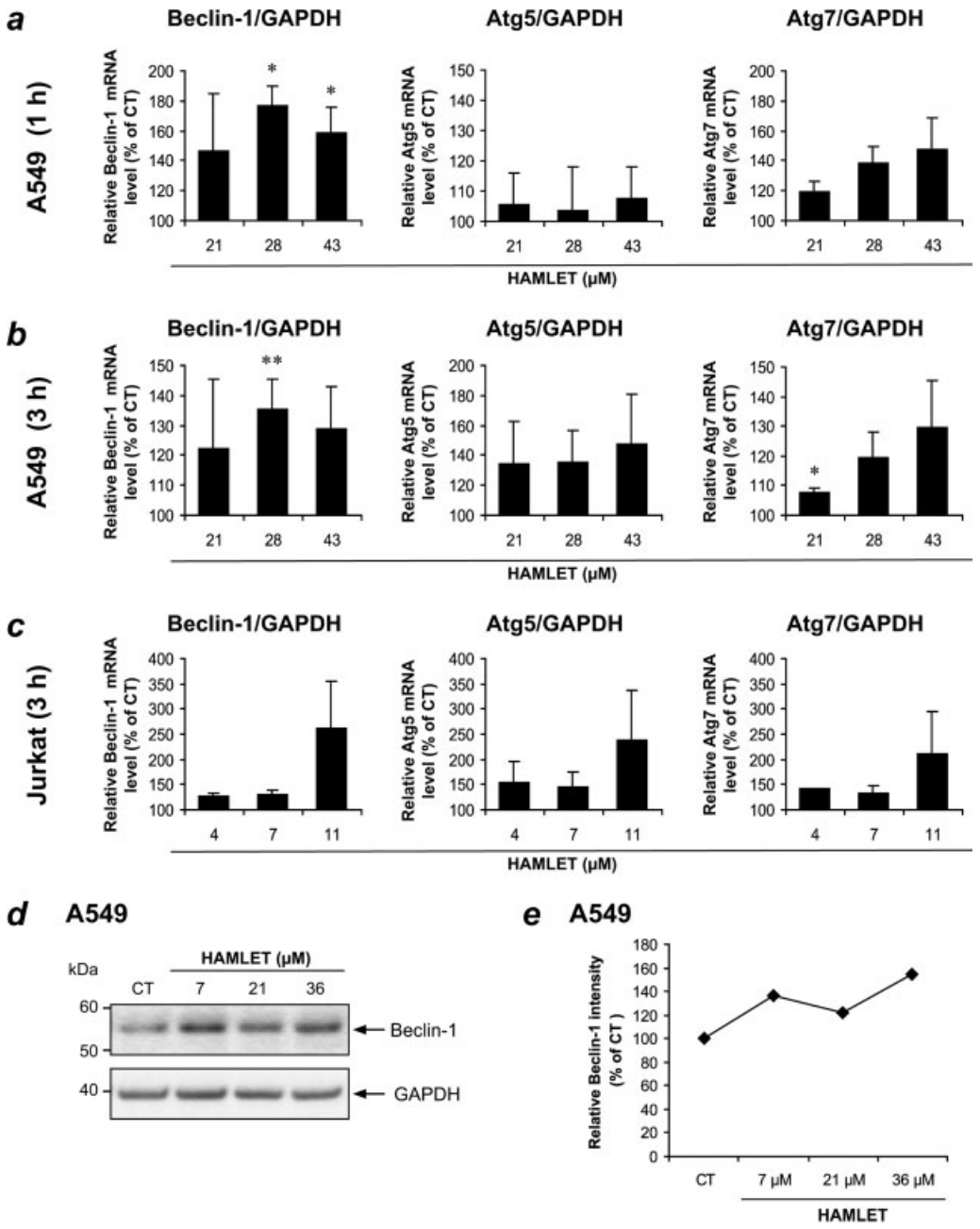

FIGURE 6 – Upregulation of Beclin-1, Atg5 and Atg7 mRNA and Beclin-1 protein by HAMLET. (*a–c*) HAMLET increased Beclin-1, Atg5 and Atg7 mRNA levels in A549 and Jurkat cells. Relative mRNA levels are shown (*e.g.*, Beclin-1 mRNA/GAPDH mRNA in % of this ratio in PBS-treated control cells) as means + SEMs of 2 (Jurkat 4 and 11 μM), 3 (A549 Atg5 and Atg7, Jurkat 0 and 7 μM) or 4 (A549 Beclin-1) independent experiments. Significant differences compared to the control cells are indicated (*$p < 0.05$, **$p < 0.01$). Note that *y*-axes have different scales and do not cross the *x*-axes at 0%. (*d*) Western blot showing increased Beclin-1 protein levels in A549 cells treated with HAMLET (3 hr). The blot was stripped and reprobed with GAPDH antibody as loading control. (*e*) Quantification of Beclin-1 protein levels. Relative levels (Beclin-1/GAPDH intensity in % of this ratio in PBS-treated control cells) from one Western blot experiment are shown.

exclusion and ATP levels (Fig. 7). No additional rescue was observed with trypan blue exclusion after zVAD-fmk treatment of Beclin-1 siRNA-transfected cells but a small additional increase in ATP levels was observed.

Discussion

Macroautophagy serves as a basal cellular recycling mechanism and is upregulated by cellular challenges like starvation or organelle damage. It has also been proposed as a mechanism of cell death. HAMLET is a complex of partially unfolded α-lactalbumin and oleic acid that causes rapid death of tumor cells derived from different tissues and species but not of healthy differentiated cells. HAMLET retains its anti-tumor activity *in vivo* without causing apparent side effects[16–18] but so far the mechanism of HAMLET-induced tumor cell death has remained unclear. This study shows that HAMLET triggers a rapid macroautophagy response and suggests that this response contributes to tumor cell death.

A connection between mitochondrial damage and macroautophagy has previously been reported in yeast[43] and mam-

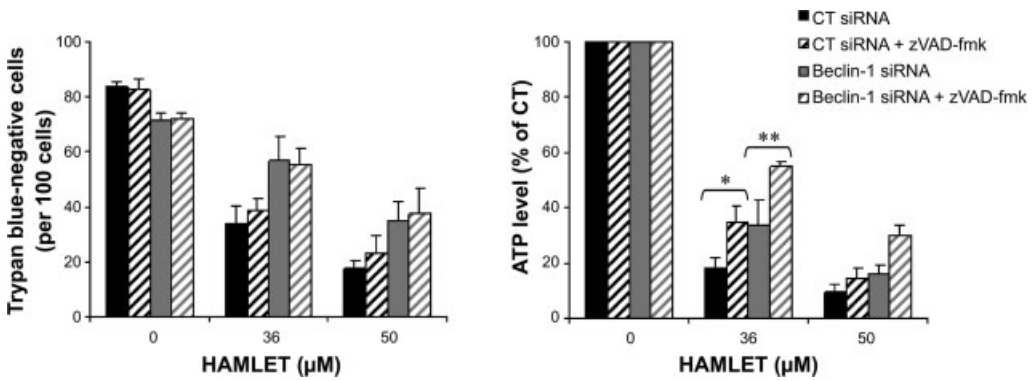

FIGURE 7 – Simultaneous inhibition of macroautophagy and caspases. A549 cells were transfected with Beclin-1 siRNA (42 hr), pretreated with zVAD-fmk (50 μM, 1 hr) and incubated with HAMLET (3 hr). Viability was quantified by trypan blue exclusion (left) or by ATP levels in % of the respective control (CT siRNA-transfected ± zVAD-fmk or Beclin-1 siRNA-transfected cells ± zVAD-fmk) (right). zVAD-fmk did not cause an additional reduction of cell death quantified by trypan blue exclusion but caused a small increase in ATP levels compared to Beclin-1 siRNA alone. Means + SEMs of 5 (trypan blue) or 3 (ATP) independent experiments are shown (*$p < 0.05$, **$p < 0.01$).

mals.[32,33] Elmore *et al*.[32] found that depolarized mitochondria enter the lysosomal compartment in rat hepatocytes undergoing macroautophagy. Inhibition of mitochondrial permeability transition (MPT) prevented mitochondrial depolarization and expansion of the lysosomal compartment suggesting that MPT triggers mitochondrial degradation by macroautophagy. In the present study, electron microscopy showed disrupted, swollen mitochondria in HAMLET-treated cells after 30 min. This is consistent with earlier studies showing that HAMLET causes rapid mitochondrial damage with MPT, loss of mitochondrial membrane potential, mitochondrial swelling and Cytochrome c release in isolated mitochondria.[24] Cytochrome c release was also observed in tumor cells exposed to a crude HAMLET-containing milk fraction.[23] Accordingly, the mitochondrial damage and MPT induction caused by HAMLET may help initiate the macroautophagy response.

mTOR is the central negative regulator of macroautophagy.[34] mTOR is activated through the Class I PI3K/Akt pathway in response to growth factor signaling but inactivated under cellular stress such as nutrient and energy depletion, hypoxia, osmotic stress or DNA damage.[38] mTOR inhibition by cellular stress, rapamycin or mTOR siRNA can induce macroautophagy and may, under some conditions, lead to autophagic cell death.[2,34,44] We show that HAMLET caused a reduction in mTOR activity and suggest that mTOR is an essential regulator of macroautophagy and cell death in response to HAMLET.

In other studies, dying cells have often shown a macroautophagic morphology but few studies have provided conclusive evidence that macroautophagy actually causes cell death (reviewed in Refs. 7–9). Yu *et al*.[15] showed that Beclin-1 and Atg7 siRNA inhibited zVAD-fmk-induced death in mouse fibroblastic cells and human monocytoid cells. Similarly, knockdown of Beclin-1 and Atg5 reduced death induced by staurosporin and etoposide in embryonic fibroblasts from Bax/Bak double knock-out mice[14] and death by a short mitochondrial isoform of p19ARF.[13] Interestingly, in the latter study cell death was also preceded by a reduction in mitochondrial membrane potential. We found that Beclin-1 and Atg5 siRNAs inhibited LC3 translocation and reduced cell death in response to HAMLET thereby suggesting a direct link between macroautophagy and cell death.

Increased levels of macroautophagy components have been shown to activate or facilitate macroautophagy and under some conditions autophagic cell death.[9] For example, Atg5-Atg12 conjugates and Beclin-1 were elevated during etoposide-induced autophagic death in embryonic fibroblasts from Bax/Bak double knock-out mice[14] and ectopic expression of Atg5 was sufficient to trigger cell death in HeLa and MCF7 cells.[45] HAMLET was found to increase the levels of Beclin-1, Atg5 and Atg7 mRNAs and Beclin-1 protein, all of which are involved in the induction of macroautophagy.[11] The upregulation of macroautophagy components might contribute to the activation of autophagic cell death by HAMLET.

Apoptosis and macroautophagy have been proposed to regulate each other (reviewed in Ref. 46). Macroautophagy may prevent the release of proapoptotic molecules by scavenging damaged mitochondria and as a consequence inhibition of macroautophagy might increase apoptosis. On the other hand, an increase in macroautophagy is frequently observed when apoptosis is inhibited suggesting that it constitutes a backup system for apoptosis which is normally preferred.[46] In previous studies, HAMLET was shown to cause an apoptotic response but in contrast to macroautophagy its inhibition did not prevent tumor cell death.[25] In this study, cell death measured by trypan blue exclusion was not further reduced by simultaneous caspase inhibition in Beclin-1 siRNA-treated cells confirming our previous results.[25] However, using ATP levels to quantify cell death a small rescue effect of zVAD-fmk was detected. This is consistent with studies showing that caspases can cleave enzymes involved in ATP production such as pyruvate kinase[47] and a component of the respiratory chain,[48] and that zVAD-fmk may temporarily permit continued ATP production by the mitochondria after Cytochrome c release.[49] HAMLET-induced cell death may thus, be an unusual example where macroautophagy is preferred over apoptosis for the execution of cell death even though apoptosis is activated.

Macroautophagy plays a dual role in cancer.[5,50] It can be a survival mechanism in the poorly vascularized environments of advanced tumors and promote growth and therapy resistance. Under normal conditions macroautophagy is less active in tumor cells than in normal cells, however, and avoidance of macroautophagy seems to be a survival strategy for tumor cells. The *beclin-1* gene is often monoallelically deleted in breast, ovarian and prostate tumors[5] and in breast cancer cell lines.[51] In addition, heterozygous *beclin-1* knock-out mice show increased spontaneous tumor formation suggesting that *beclin-1* is a haplo-insufficient tumor suppressor gene[52,53] and *beclin-1* reconstitution suppresses tumorigenicity of MCF7 cells.[54] Accordingly, macroautophagy has become a target in cancer therapy. The focus has been on rapamycin-derived mTOR inhibitors, so-called rapalogues.[6,55] Clinical trials have shown success in certain tumor types such as renal cell carcinomas[56] but general use against a wide range of tumors seems to be limited due to resistance.[56,57] This study proposes that HAMLET acts more broadly than rapa-

logues that target only mTOR. The multi-facetted death response to HAMLET including macroautophagy might help to explain the broad tumoricidal effect of HAMLET.

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