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 during 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
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. 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, 250 μg/ml G418 (Sigma-Aldrich, St. Louis, MO) or, for the harvesting of conditioned media, in RPMI medium with 25% FCS (5%) was added after 1 hr. The A549 lung carcinoma 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, 250 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 Na2HPO4, 1.6 mM KHPO4, 0.5 mM EDTA, pH 7.2), washed, diluted to 108 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 trypan blue exclusion (Chromatex, New York, NY) or by measuring ATP levels (ViaLight Plus 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 PMSE, 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 ZnCl2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS) containing the complete protease inhibitors. Protein concentrations were measured with the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). For Western blots for 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 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 Laemml buffer (12.5 mM Na2HPO4, 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, NaneTools Antibody Technology, 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 (kind gift by Dr. Marja Jäättelä and Dr. Maria Hoyer-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, Denmark) and lysed in modified RIPA buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 50 μM ZnCl2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS) containing the complete protease inhibitors. Protein concentrations were measured with the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). For Western blots for 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 and normalized against GAPDH. If required, membranes were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), blocked and reprobed with new antibodies.

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**RT-PCR**

RNA was prepared with the RNasey kit (QIAGEN, Hilden, Germany), treated with DNase I (DNase I (Invitrogen or QIA-GEN) 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 CUCAGGGAG GAGCCAUUU) and Atg5 (sense sequence GCAACUCUG GAUGGGAGU) 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. 1a). Morphological changes accompanying cell death were examined by electron microscopy after 30 min and 6 hr of HAMLET treatment (Fig. 1b, CT and 1c, Untreated). A549 cells (Fig. 1b, CT and 1c, Untreated) showed normal morphology but after exposure to HAMLET most cells showed extensive cytoplasmic vacuolization (Fig. 1b and 1c, Vacuolated) and sometimes nonapoptotic chromatin changes. 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. 1d). These contained cytoplasm, organelles or material resembling chromatin. In addition, a few necrotic cells were seen (Fig. 1b and 1c, 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. Since a HAMLET decreases mTOR activity phophorylated mTOR decreased to 42% of the control (Fig. 2a).

AMP-activated protein kinase (AMPK) is an important upstream inhibitor of mTOR which is mainly activated by an increase in the AMP/ATP ratio. 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. HAMLET caused a reduction in AMPKα phosphorylation (Fig. 2b and 2c), 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. LC3 normally resides in the cytoplasm as LC3-I but associates with autophagosomal membranes during macroautophagy after modification to LC3-II by attachment of phosphatidyethanolamine. LC3 translocation in response to HAMLET was first investigated in A549 cells transiently transfected with LC3-GFP (30% transfection rate) (Fig. 3a and 3b). 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. 3c and 3d). 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 autophagosome-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. 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 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. 2b and 2c). 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. HAMLET treatment for 3 hr reduced the level of phosphorylated p70 S6K (Fig. 2b and 2c). 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.

Knockdown of Beclin-1 rescues HAMLET-treated cells

To examine if macroautophagy contributes to HAMLET-induced cell death we inhibited macroautophagy by RNA in-
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. 4a). Beclin-1 protein levels were also reduced (Fig. 4b).

**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.
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. 4d). 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 Batilomycin A1 (Baf A) or Pepstatin A (Pep A) and E64d. A p42/p44 Western blot is shown as loading control.
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. \(^1\) 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. 4c). Transfected cells were exposed to HAMLET for 3 hr and cell death was quantified by trypan blue exclusion and ATP levels (Fig. 4e). 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
and Atg7 mRNA levels had increased after 1 hr of HAMLET treatment (Fig. 6a) and after 3 hr, Atg5 mRNA levels were increased as well (Fig. 6b). In addition, levels of all 3 mRNAs had increased in Jurkat cells after 3 hr (Fig. 6c).

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. 6d). 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...
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 effects17–18 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 yeast13 and mam-

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**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.
Evidence that macroautophagy actually causes cell death  

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.13 showed that Beclin-1 and Beclin-1B double knock-out mice 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.35 mTOR inhibition by cellular stress, rapamycin or mTOR siRNA can induce macroautophagy and may, under some conditions, lead to autophagic cell death.2,3,4,4 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. 

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

Acknowledgements

We thank Dr. Marja Jäättelä and Dr. Maria Hoyer-Hansen (Apoptosis Department and Centre for Genotoxic Stress Research, Institute of Cancer Biology, Danish Cancer Society, Copenhagen, Denmark) for kindly providing the LC3-GFP plasmid, Dr. Masahiro Shibata (Department of Cell Biology and Neurosciences, Osaka University Graduate School of Medicine, Osaka, Japan) for a LC3 antibody, Jenny Pettersson (Institute of Laboratory Medicine, Section of Microbiology, Immunology and Glycobiology, Lund University, Lund, Sweden) for assistance with RNA preparation and Lennart Philipson (Department of Cell and Molecular Biology, Karolinska Institute, Stockholm, Sweden) for helpful comments on the manuscript.

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