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Research Article

Molecular Cancer Therapeutics

BAMLET Activates a Lysosomal Cell Death Program in Cancer Cells

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Abstract

A complex of human α-lactalbumin and oleic acid (HAMLET) was originally isolated from human milk as a potent anticancer agent. It kills a wide range of transformed cells of various origins while leaving nontransformed healthy cells largely unaffected both *in vitro* and *in vivo*. Importantly, purified α-lactalbumins from other mammals form complexes with oleic acid that show biological activities similar to that of HAMLET. The mechanism by which these protein-lipid complexes kill tumor cells is, however, largely unknown. Here, we show that complex of bovine α-lactalbumin and oleic acid (BAMLET), the bovine counterpart of HAMLET, kills tumor cells via a mechanism involving lysosomal membrane permeabilization. BAMLET shows potent cytotoxic activity against eight cancer cell lines tested, whereas nontransformed NIH-3T3 murine embryonic fibroblasts are relatively resistant. BAMLET accumulates rapidly and specifically in the endolysosomal compartment of tumor cells and induces an early leakage of lysosomal cathepsins into the cytosol followed by the activation of the proapoptotic protein Bax. Ectopic expression of three proteins known to stabilize the lysosomal compartment, i.e. heat shock protein 70 (Hsp70), Hsp70-2, and lens epithelium-derived growth factor, confer significant protection against BAMLET-induced cell death, whereas the antiapoptotic protein Bcl-2, caspase inhibition, and autophagy inhibition fail to do so. These data indicate that BAMLET triggers lysosomal cell death pathway in cancer cells, thereby clarifying the ability of α-lactalbumin:oleate complexes to kill highly apoptosis-resistant tumor cells. *Mol Cancer Ther; 9(1); 24–32. ©2010 AACR.*

Introduction

An ideal anticancer agent should be toxic to malignant cells with minimum toxicity towards normal cells. Currently there are limited numbers of such agents available for clinical use. Thus, development of novel tumor selective drugs is an important and challenging task. The caspase-mediated apoptosis is the main mechanism of action of most current anticancer treatments. Defects in apoptosis signaling pathways are, however, among the major hallmarks of cancer, and apoptosis-inducing therapies further select for highly apoptosis refractory tumor cell clones (1, 2). Accordingly, new strategies to kill cancer cells by nonapoptotic mechanisms have flourished during the past decade, and many inducers of alternative caspase-independent cell death pathways have been identified (3–6). Among them is a lipid:protein complex composed of oleic acid and human α-lactalbumin that was originally purified from human milk and termed HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cells) based on its ability to effectively kill transformed cells while leaving nontransformed cells largely unaffected (7, 8). In addition to the broad antitumor activity *in vitro*, the therapeutic effect of HAMLET has been confirmed in a human glioblastoma xenograft model in rats as well as in human patients with skin papillomas and bladder cancer (9–11).

In spite of the significant tumoricidal effects observed both *in vitro* and *in vivo*, the cytotoxic mechanism induced by HAMLET is as yet largely unclear. Although HAMLET can activate apoptotic caspases in some target cells, these enzymes are not required for its cytotoxic effect (12, 13). The nonapoptotic nature of HAMLET-induced cytotoxicity is further emphasized by its ability to kill tumor cells that have acquired resistance to classic apoptosis pathway either by overexpressing the antiapoptotic protein Bcl-2 or by carrying mutations in the p53 tumor suppressor protein (12). Instead, HAMLET has been suggested to act by numerous unrelated mechanisms including (*a*) direct induction of mitochondrial outer membrane permeabilization (14), (*b*) histone binding and subsequent DNA damage (15, 16), (*c*) unfolded protein response (17), and (*d*) autophagic cell death (18).

α-Lactalbumins are structurally conserved among species. Amino-acid sequences of human, bovine, equine,

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caprine, and porcine show 71% homology and 63% identity with the highest sequence conservation surrounding the high affinity Ca^{++}-binding site (19). Accordingly, ion exchange chromatography on oleic acid–conditioned matrixes allows the formation of protein:lipid complexes of nonhuman α-lactalbumins with yields comparable with HAMLET (19). Importantly, all of these complexes have been reported to have tumoricidal activity as assessed by their ability to kill L1210 mouse lymphoma cells, indicating that α-lactalbumins from different species can be converted into active cytotoxic protein-lipid complexes. The possibility to prepare the cytotoxic protein-lipid complexes using nonhuman milk (e.g., cow milk) is of great importance for the putative future use of such compounds in clinics, because it will circumvent the problem of poor availability of human milk for large-scale production.

The present study aimed at determining the cellular mechanism of action of complex of bovine α-lactalbumin and oleic acid (BAMLET), the bovine counterpart of HAMLET consisting of oleic acid and α-lactalbumin purified from cow's milk. Importantly, comparative analysis of BAMLET and HAMLET showed similar efficacy and specificity towards malignant cells. Furthermore, our results show that BAMLET accumulates in the endolysosomal compartment where it induces destabilization of the lysosomal membrane causing leakage of lysosomal content (e.g., cathepsins and other lysosomal hydrolases) into the cytoplasm, which ultimately leads to activation of the caspase-independent lysosomal cell death pathway. These results encourage the development of BAMLET for the treatment of highly apoptosis-resistant cancers.

Materials and Methods

Cell Culture

Cell lines used included L1210 (murine lymphocytic leukemia; ATCC, CCL-219), HeLa (human cervical epithelial adenocarcinoma; kindly provided by Karl Agger, Biotech and Research Innovation Centre, Copenhagen, Denmark), J82 (human urinary bladder transitional cell carcinoma; ATCC, HTB-1), RT4 (human urinary bladder transitional cell papilloma; ATCC, HTB-2), PC-3 (human prostate adenocarcinoma; ATCC, CRL-1435), U118 MG (human glioblastoma; ATCC, HTB-15), MCF-7 (S1 subclone of MCF-7 human breast carcinoma; ref. 20), U-2-OS (human osteosarcoma; kindly provided by Jiri Bartek, Danish Cancer Society), and NIH-3T3 (murine embryonic fibroblast). WEHI-Vector (WEHI-vector-2) and WEHI-Hsp70 (WEHI-hsp-2) are vector- and Hsp70-transfected single cell clones of WEHI-S murine fibrosarcoma cells (21, 22). The MCF7-eGFP-LC3 cell line is a single cell clone of MCF-7 cells expressing a fusion protein consisting of enhanced green fluorescence protein (eGFP) and rat LC3 (23). MCF-7-Bcl-2 cells are a pool of Bcl-2–transfected cells (23). MCF-7-Casp3 cells are a pool of Casp3-transfected MCF-7 cells (24). MCF-7-LEDGF cells are a pool of two previously described single cell clones (clone 5 and 20) of MCF-7 cells transfected with lens epithelium-derived growth factor (LEDGF; ref. 25). To create MCF-7-Hsp70-2 cells, MCF-7 cells were transfected with pcDNA-Hsp70-2 (26) and single cell–cloned. Two clones with high expression of Hsp70-2 were pooled and used in these experiments. All transfected cells were compared with cells transfected with an empty plasmid in parallel. Atg5−/− murine embryonic fibroblasts (MEF) and appropriate wild-type control MEFs were kindly provided by Noboru Mizushima (Japan) and atg7+/− and −/− MEFs were kindly provided by Masaaki Komatsu (Japan; refs. 27, 28).

Production of BAMLET AND HAMLET

Concentrated milk plasma was prepared from bovine milk (ultrafiltration with 100 kDa cutoff, followed by ultrafiltration with 5 kDa cutoff to remove low-mass milk constituents). The milk protein fraction was then depleted for β-lactoglobulin by an anion exchange chromatography step, which retained β-lactoglobulin, and afterward applied to a hydrophobic interaction chromatography, to isolate bovine α-lactalbumin from residual β-lactoglobulin. After addition of EDTA, bovine α-lactalbumin was mixed with oleic acid and the mixture was then applied on an anion exchange column. During this process, the complex was formed. The product was eluted from the column using a salt gradient. Following the conversion step, the preparation was filtered through a prenanofilter (100 nm) and subsequently through a nanofilter (20 nm) to deplete any potential virus burden. The filtered BAMLET preparation was concentrated by ultrafiltration using a Proflux TFF system (Millipore). HAMLET was produced by a similar process from human α-lactalbumin prepared from whole human milk (kindly provided by Cathrina Svanborg, Lund University, Sweden). The lipid:protein ratio in the complexes is 1:1.

Viability and Cell Death Assays

The cells were seeded at 5,000 cells/well on 96-well plates and incubated for 24 to 48 h before the treatment. The viability of cells was analyzed by the MTT reduction assay (M5655 Sigma-Aldrich) according to the manufacturer's prescription. The optical density was analyzed with a Versamax microplate reader (Molecular Devices Ltd.). The cell death was assessed by lactate dehydrogenase release assay (Roche) essentially as described previously (29).

For the ViaLight Plus Cell Proliferation and Cytotoxicity Bioassay (Cambrex), 140,000 cells in 50 μL serum-free RPMI were plated on white 96-well plates (Cambrex) preplated with the indicated concentrations of HAMLET, BAMLET, or bovine [α]-lactalbumin in 20 μL 0.9% NaCl solution. After 1 h at 37°C, fetal bovine serum was added to the final concentration of 5% and the cells were incubated for 1 h at 37°C. The relative ATP concentrations were determined according to the manufacturer's instructions using BMG Lumistar Optimar luminometer. Similar results were obtained by Trypan blue exclusion assay.

Apoptotic cells were determined in an Olympus IX microscope with the UV channel by counting condensed

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Table 1. Cytotoxicity of HAMLET and BAMLET on cancer cell lines

Cell line	LC_{50} (mg/mL)		
	bLA*	HAMLET	BAMLET
L1210; murine lymphocytic leukemia	>6.7	0.05	0.04
HeLa; human cervical epithelial adenocarcinoma	>3.5	0.20	0.12
J82; human urinary bladder transitional cell carcinoma	>3.5	0.27	0.15
RT4; human urinary bladder transitional cell papilloma	>1.0	0.44	0.32
PC-3; human prostate adenocarcinoma	>3.5	0.17	0.25
U118; human glioblastoma	>3.5	0.40	0.34

NOTE: Cell viability was assessed by ViaLight Plus Cell Proliferation and Cytotoxicity Bioassay.
Abbreviation: bLA, bovine [α]-lactalbumin
*Maximum concentration tested.

nuclei in cells stained with cell-permeable Hoechst 33342 (Sigma-Aldrich) for 10 min. A minimum of 100 randomly chosen cells were counted for each sample.

z-Val-Ala-DL-Asp-fmk (zVAD-fmk) was from Bachem, z-Phe-Ala-fluoromethylketone (z-FA-fmk) was from Enzyme Systems, 3-methyladenine was from Sigma-Aldrich, and recombinant human tumor necrosis factor was from R&D Systems.

Cathepsin and Caspase Activity Measurements

The total and cytoplasmic (digitonin-extracted) cathepsin activities and total caspase-3/7–like activities were measured using zFR-AFC and zDEVD-AFC (Enzyme System Products) probes, respectively, as described previously (29). The substrate hydrolysis was analyzed using a VersaMax tunable photospectrometer (Molecular Devices).

LC3 Translocation Assay

Autophagosomes were detected in MCF7-eGFP-LC3 cells fixed in 4% formaldehyde (v/v), by counting the percentage of cells with a minimum of five eGFP-LC3–positve dots (a minimum of 2 × 100 cells/sample) applying Olympus BX60 fluorescence microscope.

BAMLET Localization

Confocal fluorescence microscopy was done with Zeiss LSM 510 META and normal fluorescence microscopy with Zeiss Axiovert 200 M. The cells were seeded 24 h before experiment in the 4-chamber Lab-Tek cover glass system with 75,000 cells/well and treated as indicated with BAMLET coupled to Alexa 488. For the last 25 min, 1 μg/mL Hoechst 33342 (Molecular Probes), 62.5 nmol/L Lysotracker-Red, or 62.5 nmol/L Mitotracker-Red (Molecualr Probes) were added. Representative images are presented.

Lysosomal Leakage and Bax Activation

MCF7-cells were plated at a density of 10×10^5 cells per glass cover slip, and were washed and fixed in 4% formaldehyde in PBS for 20 min at 25°C followed by 2 min in 0.2% Triton X-100 in PBS (permeabilization). Samples were blocked with 20% FCS in PBS for 30 min. Antibodies used were antihuman cathepsin L (Transduction Laboratories) and anti-Bax (active conformation #2772, Cell Signaling) and the appropriate Alexa-488- and Alexa-594-coupled secondary antibodies (Molecular Probes). Glass cover slips were mounted with antifade gold kit (Molecular Probes), and fluorescence images were taken with Zeiss 510 laser-scanning microscope with Axiovert 100 M. For quantification of cells with cathepsin released to the cytosol or Bax activation, 100 representative cells were counted per cover slip.

Immunoblotting

Primary antibodies used included murine monoclonal antibodies against phospho-$p70^{S6K}$ (Thr389; #9206, Cell Signaling Technology, Inc.), LEDGF (Clone 26, BD Biosciences Pharmingen), GFP (sc-9996, Santa Cruz), and β-tubulin (Biogenesis) as well as rabbit antibodies against $p70^{S6K}$ (#9202), AMP-activated protein kinase (AMPK;

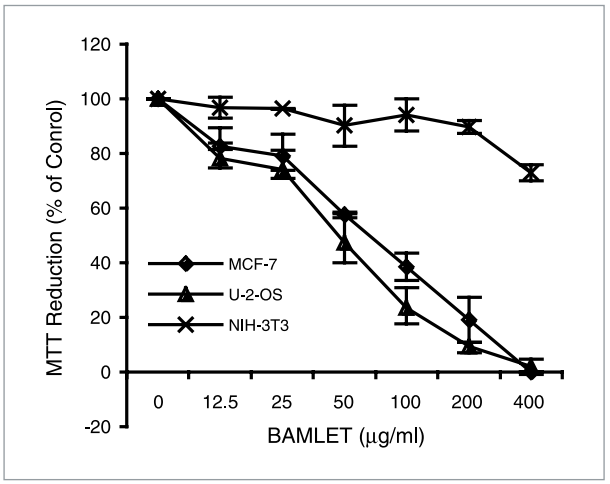

Figure 1. BAMLET kills cancer cells more effectively than nontransformed cells. MCF-7 breast cancer cells, U2-OS osteosarcoma cells and NIH-3T3 murine embryonic fibroblasts were plated at 5,000 cells/well on 96-well plates and treated 24 h later with indicated concentrations of BAMLET for 24 h (first 1 h without serum). The viability of the cells was assessed by the MTT assay and the values represent means of representative triplicate measurements.

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#2630) and p-AMPK (#2535) from Cell Signaling Technology and Hsp70-2 (26). Immunodetection of proteins separated by 10% to 12% SDS-PAGE and transferred to nitrocellulose was done using appropriate peroxidase-conjugated secondary antibodies from Dako, ECL Western blotting reagents (Amersham), and Luminescent Image Reader (LAS-1000Plus, Fujifilm).

Statistical Analysis

The statistical significance of the results was assessed using the unpaired two-tailed *t*-test.

Results and Discussion

BAMLET Induces Caspase-Independent Apoptosis-like Cell Death in Cancer Cells

We produced the BAMLET protein:lipid complex, the bovine counterpart of HAMLET, by large-scale purification of bovine α-lactalbumin from cow's milk and subsequent incubation with oleic acid. Whereas the purified bovine α-lactalbumin alone failed to induce any cytotoxicity, the BAMLET complex exhibited cytotoxic activity similar to that of HAMLET in L1210 mouse lymphoma cells as reported earlier (19), and in seven additional cancer cell lines tested with LC_{50} values ranging from 0.05 to 0.34 mg/mL (Table 1 and Fig. 1). Importantly, the non-transformed NIH-3T3 murine embryonic fibroblasts were practically unaffected by the treatment with BAMLET at concentrations that effectively eradicated all MCF-7 breast cancer cells and U-2-OS osteosarcoma cells (Fig. 1).

HAMLET has been reported to induce apoptotic cell death characterized by cytochrome c release and caspase-dependent chromatin condensation (13). However, further studies have revealed that ectopic expression of the antiapoptotic protein Bcl-2 and inhibition of caspases by zVAD-fmk fail to rescue the cells from HAMLET-induced cytotoxicity (12). As analyzed by the Hoechst staining of the nuclei, treatment of MCF-7 cells with

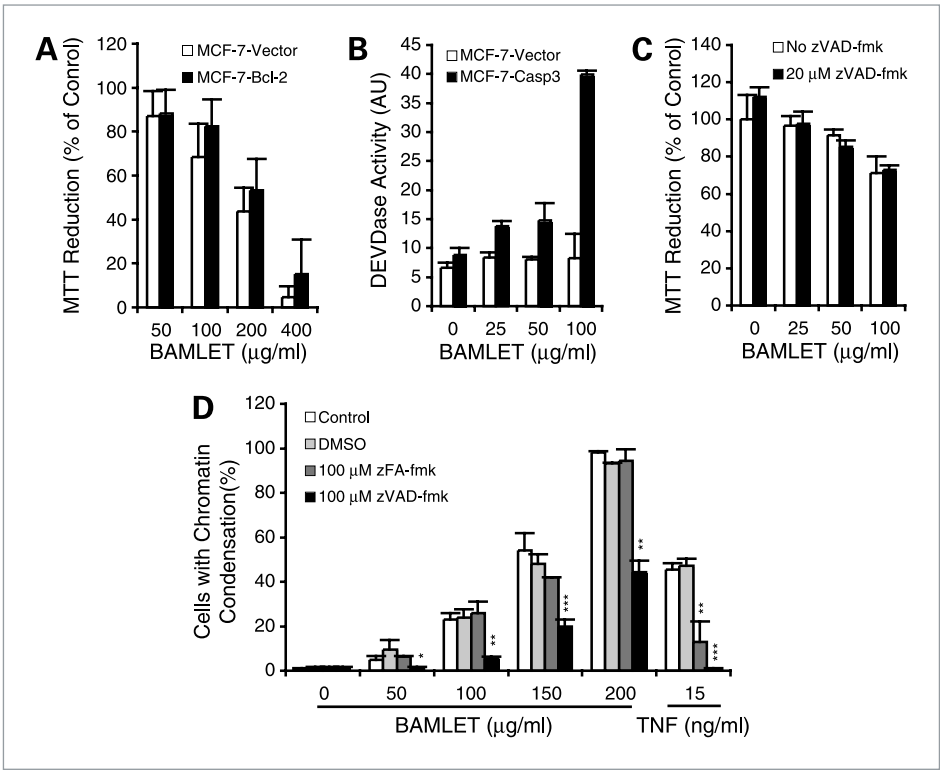

Figure 2. Bcl-2 and caspase inhibition fails to inhibit BAMLET-induced cytotoxicity in MCF-7 cells. **A,** Bcl-2–expressing MCF-7 cells (MCF-7-Bcl-2) and appropriate vector-transfected control cells (MCF-7-Vector) were treated with indicated concentrations of BAMLET for 24 h (first 1 h without serum). The viability of the cells was assessed by the MTT assay and the values represent means of three independent triplicate measurements ± SD. The differences between the two cell lines were not significant ($P > 0.05$, Student's *t*-test). **B,** caspase-3–expressing MCF-7 cells (MCF-7-Casp3) and appropriate vector-transfected control cells (MCF-7-Vector) were treated with indicated concentrations of BAMLET for 24 h (first 1 h without serum) before the analysis of caspase-3/7 activity (DEVDase) in the cell lysates. The values are DEVDase activities corrected to the lactate dehydrogenase activity in the samples and they represent means of a representative triplicate experiment ± SD. **C,** MCF-7-Casp3 cells were treated with indicated concentrations of BAMLET for 24 h (first 1 h without serum) alone or with 20 μmol/L zVAD-fmk. The viability of the cells was assessed by the MTT assay and the values represent means of a representative triplicate experiment ± SD. **D,** MCF-7 cells were treated with indicated concentrations of BAMLET for 24 h (first 1 h without serum) or with 15 ng/mL tumor necrosis factor (TNF) in complete medium. When indicated, the cells were cotreated with vehicle (DMSO), 100 μmol/L zFA-fmk, or 100 μmol/L zVAD-fmk. Apoptosis-like cell death was assessed by counting the Hoechst-stained cells with condensed nuclei. The values represent means of three independent experiments with a minimum of 100 cells counted ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared with control or cells cotreated with DMSO.

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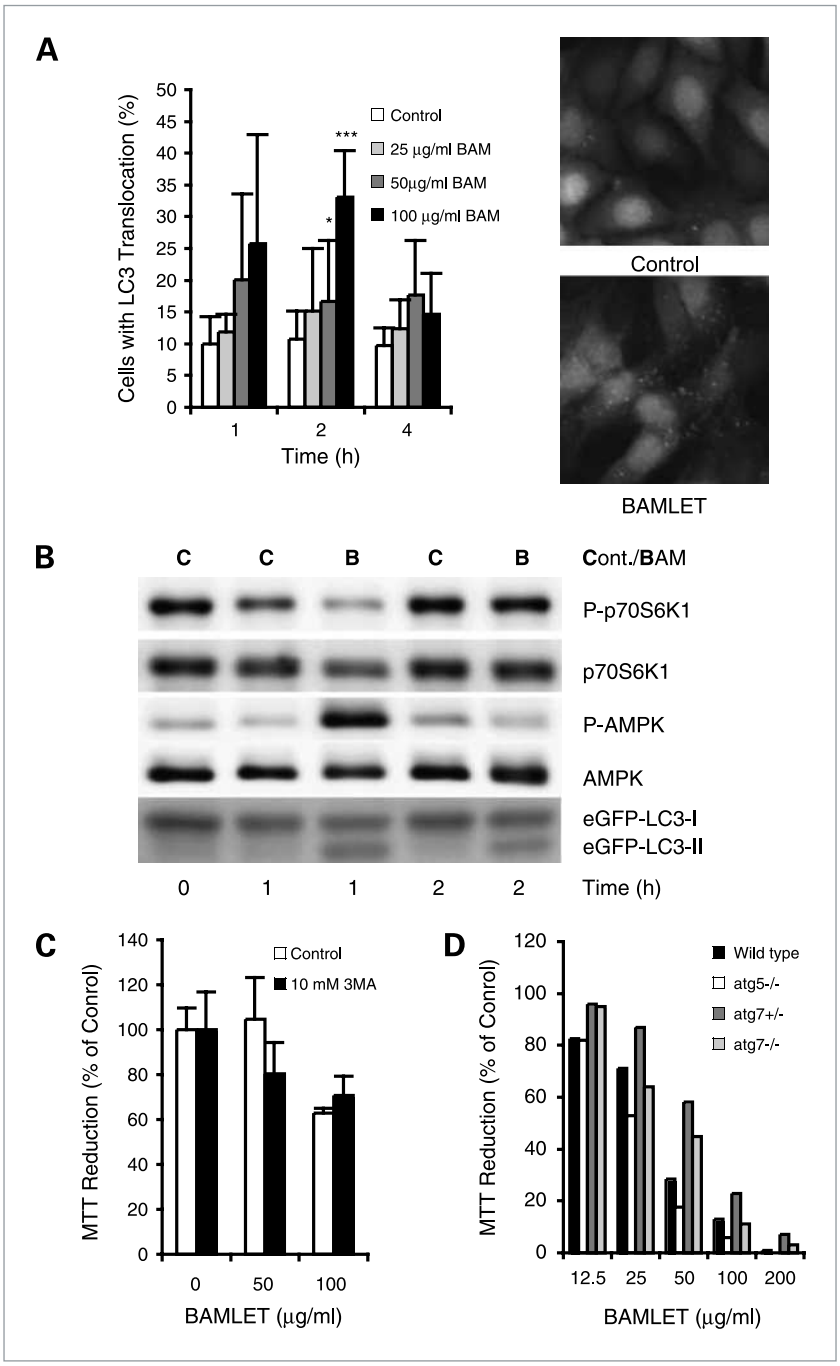

Figure 3. Autophagy does not mediate BAMLET-induced cell death. **A,** MCF-7-eGFP-LC3 cells were left untreated (control) or treated with indicated concentrations of BAMLET for indicated times (first 1 h without serum). Histograms with percentages of green cellular cross-sections with over five LC3-positive dots are shown. The values represent mean ± SD for three triplicate experiments with a minimum of 200 cells counted per sample. *, $P < 0.05$; ***, $P < 0.001$ as compared with untreated control cells. *Right*, representative images of control and BAMLET-treated MCF-7-eGFP-LC3 cells. **B,** a representative immunoblot of phospho-p70^{S6K1} (P-p70S6K1), p70^{S6K1}, phospho-AMPK (P-AMPK), AMPK, and GFP-LC3 in MCF-7-eGFP-LC3 cells left untreated (**C**) or treated with 50 μg/mL BAMLET (**B**) for indicated times (first 1 h without serum). GFP-LC3-I and GFP-LC3-II mark the nonlipidated and lipidated forms of the protein, respectively. **C,** MCF-7 cells were treated with indicated concentrations of BAMLET for 24 h (first 1 h without serum). When indicated cells were cotreated with 10 mmol/L 3-methyladenine (3MA). The viability of the cells was assessed by the MTT assay and the values represent means of a triplicate representative experiment ± SD. **D,** wild-type, atg5–/–, atg7+/–, and atg7–/– MEFs at low confluence were left untreated (Control) or treated with indicated concentrations of BAMLET for 19 h (first 45 min without serum). The viability of the cells was assessed by the MTT assay.

BAMLET induced a dose-dependent condensation of chromatin and shrinkage of the cells, two hallmarks of apoptosis-like cell death (Fig. 2D and data not shown; ref. 3). Thus, we tested the role of Bcl-2 and caspases in BAMLET-induced death of MCF-7 cells. In concordance with the published data on HAMLET, BAMLET-induced cytotoxicity was not significantly affected by ectopic Bcl-2 expression (Fig. 2A), which protects these cells from various apoptotic stimuli (20, 23). Furthermore, BAMLET failed to activate effector caspases in MCF-7 cells as shown by the absence of caspase 3/7-like activity in BAMLET-treated cell lysates (Fig. 2B). Because MCF-7 cells do not express caspase-3 protein (30), we included MCF-7 cells transfected with caspase-3 (MCF-7-Casp3) into the analysis. In the presence of ectopic caspase-3, BAMLET induced a significant increase in the effector caspase activity (Fig. 2B). Ectopic caspase-3 failed, however, to sensitize the cells to BAMLET-induced cytotoxicity, and the inhibition of the caspase activity by 20 μmol/L zVAD-fmk had no protective effect in BAMLET-treated

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MCF-7-Casp3 cells (Fig. 2C and data not shown). Notably, a high concentration of zVAD-fmk (100 μmol/L), which in addition to caspases also inhibits other cysteine proteases such as cysteine cathepsins and calpains (29), effectively inhibited BAMLET-induced apoptotic cell death (Fig. 2D). However, inhibition of cysteine cathepsins by zFA-fmk failed to protect the cells, suggesting that a combination of caspases and cathepsins (possibly with other cysteine proteases and other effectors) are involved in the process.

BAMLET-Induced Cytotoxicity Does Not Depend on Autophagy

Macroautophagy (here referred to as autophagy) is a lysosomal catabolic pathway whereby cells recycle macromolecules and organelles. Although it serves a mainly cytoprotective function in cellular physiology, recent evidence suggests that in some cases it can also lead to an alternative caspase-independent cell death (6). Furthermore, autophagy has been suggested to contribute to the HAMLET-induced cytotoxicity (18). Thus, we investigated the involvement of autophagy in BAMLET-induced cytotoxicity in MCF-7 cells expressing the eGFP-LC3 fusion protein, a marker of autophagic vesicles (31). Treatment of MCF-7 cells with cytotoxic concentrations of BAMLET led to a rapid and transient translocation of eGFP-LC3 from a diffuse cytoplasmic and nuclear distribution to cytoplasmic dots and the accumulation of the lipidated autophagosome-associated form of eGFP-LC3 (LC3-II; Fig. 3A and B). The accumulation of autophagosomes was accompanied by a transient inhibition of the phosphorylation of $p70^{S6K1}$, a substrate of the major negative regulator of autophagy mTORC1 (Fig. 3B). The simultaneous increase in the phosphorylation of AMPK suggests that BAMLET-induced inhibition of mTORC1 is mediated by AMPK (Fig. 3B), which has previously been reported to inhibit mTORC1 and induce autophagy in response to various stresses (32). These data indicate that akin to HAMLET, BAMLET activates signaling pathways leading to a transient inhibition of mTORC1 activity and induction of autophagy. Inhibition of autophagy by 3-methyladenine or concanamycin A had, however, no effect on the viability of the BAMLET-treated cells as analyzed by the MTT assay (Fig. 3C and data not shown), and 3-methyladenine had a tendency to slightly sensitize the cells to the BAMLET-induced plasma membrane permabilization as analyzed by the lactate dehydrogenase release assay (data not shown). Thus, autophagy does not seem to be crucial for the BAMLET-induced cell death pathway. This conclusion was further supported by experiments comparing the BAMLET sensitivity of atg5- and atg7-deficient murine embryonic fibroblasts with their appropriate wild type or atg7+/− control cells, respectively (27, 28). The autophagy-defective cells showed slightly increased sensitivity to BAMLET as analyzed by

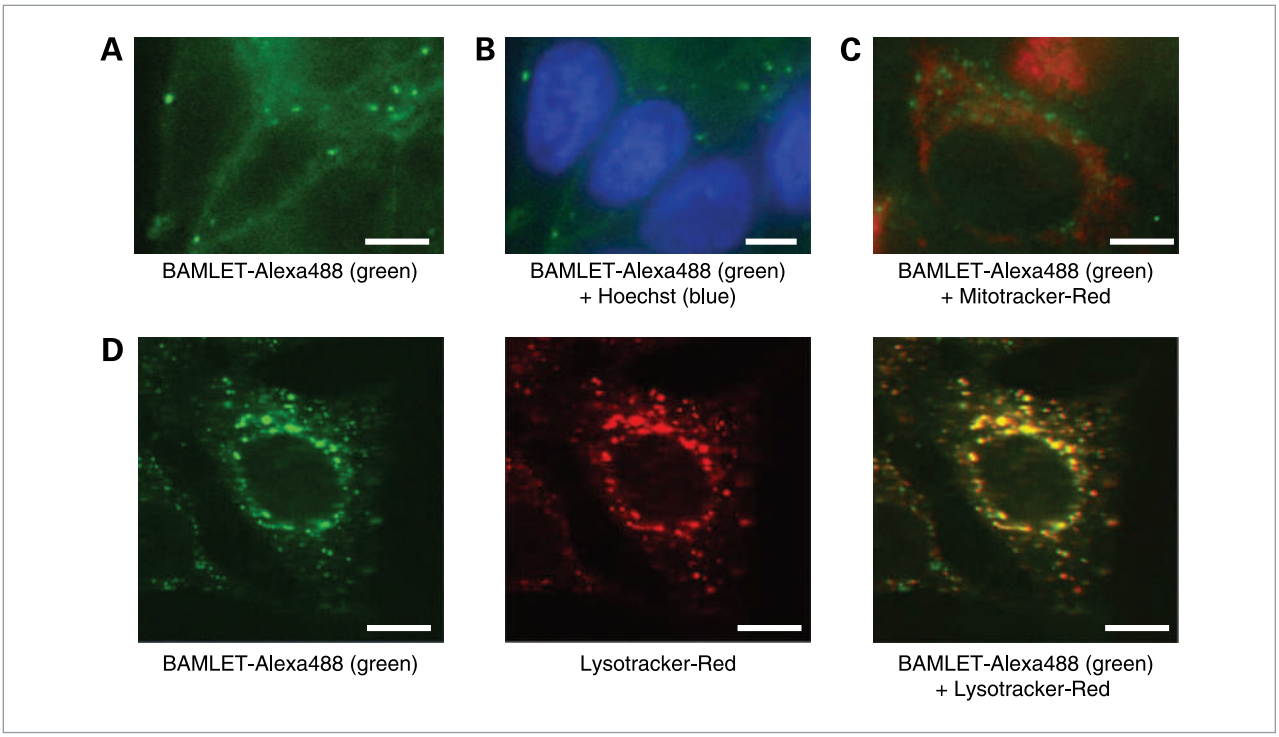

Figure 4. BAMLET translocates rapidly into the endolysosomal compartment. **A** to **C,** representative images of MCF-7 cells incubated for 1 h with 50 μg/mL BAMLET-Alexa488 alone (**A**) or in combination with Hoechst 33342 (**B**) or Mitotracker-Red (**C**) in serum-free medium. BAMLET-Alexa488 stains the plasma membrane and cytosolic dots that do not colocalize with mitochondria. No nuclear staining was detected. **D,** representative confocal images of MCF-7 cells incubated for 1 h with BAMLET-Alexa488 in serum-free medium followed by a 2-h chase in complete medium containing Lysotracker-Red. A clear colocalization of BAMLET-Alexa488 and Lysotracker-Red is shown. *Scale bars*, 10 μm.

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the MTT viability assay (Fig. 3D). Thus, autophagy may modulate the cellular response to BAMLET, but does not seem to be an essential part of the cytotoxic pathway.

BAMLET Activates a Lysosomal Cell Death Pathway

Having defined that the BAMLET-induced cytotoxicic activity was not brought about by classic apoptosis or autophagic cell death, we next investigated the subcellular localization of BAMLET to get clues to its mechanism of action. For this purpose, we first treated the MCF-7 cells with BAMLET coupled to Alexa488 for 1 hour. At this point, BAMLET-Alexa488 localized mainly to the plasma membrane and to cytosolic vesicle-like structures (Fig. 4A). No obvious costaining was detected with Hoechst-stained nuclei (Fig. 4B) or Mitotracker-Red–stained mitochondria (Fig. 4C), whereas Lysotracker-Red nicely colocalized with BAMLET-Alexa488 (data not shown). To get an even clearer picture of where BAMLET accumulates in the cell after the uptake, we treated the cells with BAMLET-Alexa488 for 1 hour followed by a 2-hour chase in a BAMLET-free medium to follow the fate of the membrane-bound BAMLET. The chase period resulted in the disappearance of the diffuse plasma membrane staining and a significant increase in the cytosolic vesicular staining (Fig. 4D). Costaining with Lysotracker-Red revealed a nearly perfect colocalization of BAMLET with the endolysosomal compartment (Fig. 4D). A similar staining pattern was observed in U-2-OS cells (data not shown). Diffuse cytoplasmic and nuclear staining was only observed after the cells had started to lose their infrastructure and die (6 hours and thereafter; data not shown), suggesting that BAMLET could trigger lysosomal membrane permeabilization.

Prompted by the clear colocalization of BAMLET with the Lysotracker-Red, we next tested whether BAMLET induced permeabilization of the lysosomal membranes. Indeed, cysteine cathepsin activity was readily detectable in the cytosolic extracts of cells treated with BAMLET for only 3 hours (Fig. 5A). At the same time point, detectable amounts of cathepsin L protein had leaked from the lysosomes into the cytosol in approximately 40% to 90% of the cells treated with 50 to 150 μg/mL BAMLET (Fig. 5B and C). Costaining with antibodies against cathepsin L and activated Bax revealed that BAMLET also induced the activation of Bax, a proapoptotic Bcl-2 family protein. A count of the changes in the individual cells revealed, however, that lysosomal leakage preceded the activation of Bax in BAMLET-treated cells: Approximately 15% to 35% of the cells had diffuse cathepsin L staining without active Bax, whereas practically no cells had active Bax in the absence of cytosolic cathepsin L (Fig. 5C).

The inhibition of the activity of lysosomal cathepsins by pharmaceutical protease inhibitors such as zFA-fmk significantly delays the death process in several lysosomal cell death models (4). This was not the case, however,

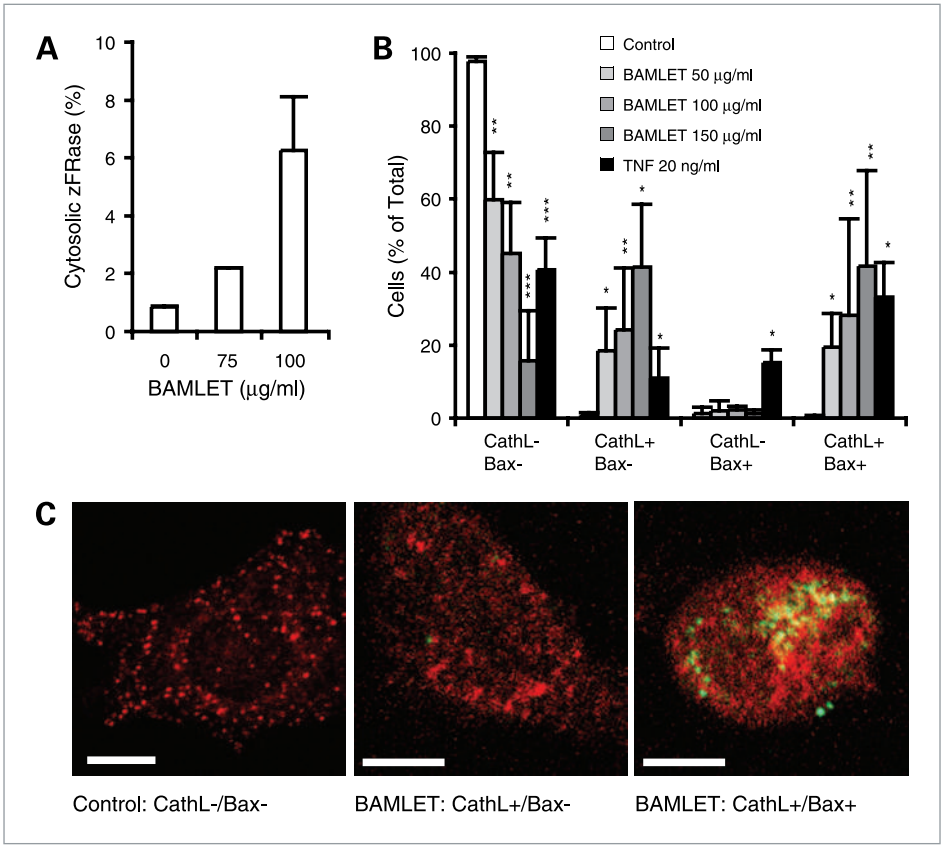

Figure 5. BAMLET induces lysosomal leakage followed by Bax activation. **A,** the cytosolic and total cysteine cathepsin activities were analyzed in MCF-7 cells treated with indicated concentrations of BAMLET for 3 h (1 h without serum followed by 2 h with serum). The values are means ± SD from a representative triplicate experiment. **B** and **C,** MCF-7 cells treated with indicated concentrations (**B**) or 100 μg/mL (**C**) of BAMLET for 3 h (1 h without serum followed by 2 h with serum) or with 20 ng/mL TNF for 24 h (positive control) were stained for cathepsin L (*red*) and activated Bax (*green*). Representative areas were counted for quantification of cells with cathepsin L released to cytosol and/or Bax activation. The values are means ± SD from five independent experiments with a minimum of 100 cells counted/experiment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared with untreated control cells. The images are representative of the indicated phenotypes. *Scale bars*, 10 μm.

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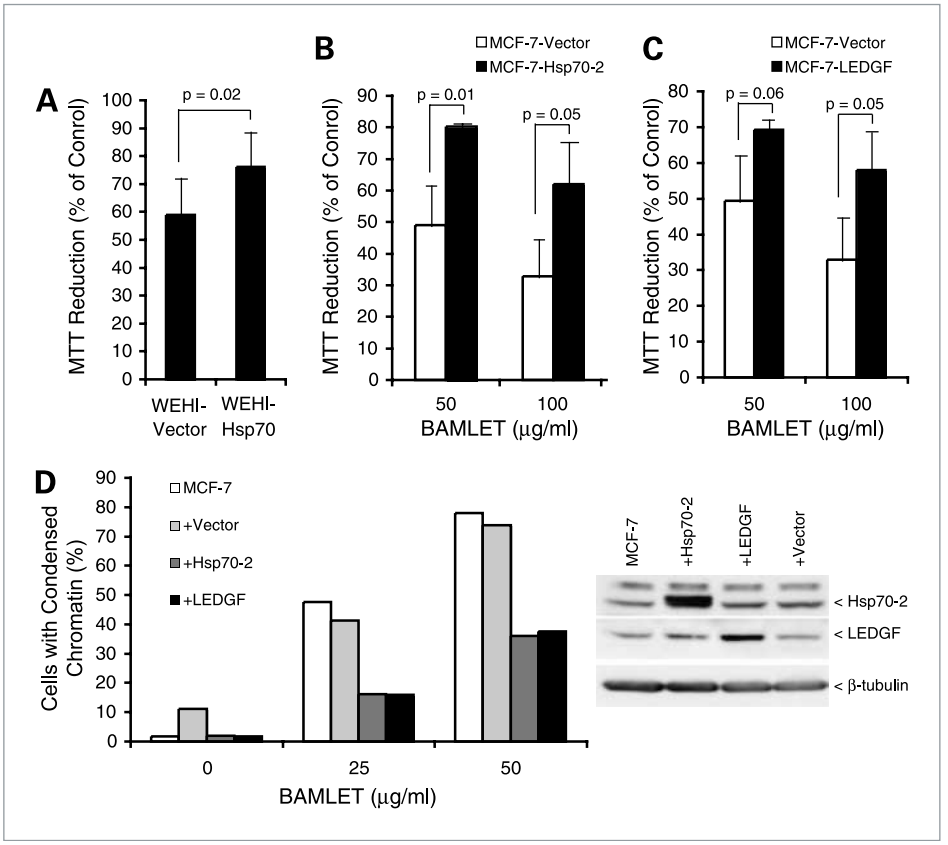

Figure 6. Hsp70, Hsp70-2, and LEDGF confer protection against BAMLET-induced cytotoxicity. **A** to **C,** the indicated cells were left untreated or treated with 600 μg/mL (**A**) or indicated concentrations (**B** and **C**) of BAMLET for 24 h (1 h without serum followed by 23 h with serum). The viability of the cells was assessed by the MTT assay and the values represent means of three independent triplicate measurements ± SD. The statistical significance was assessed by unpaired two-tailed *t*-test. **D,** parental MCF-7 cells and MCF-7 cells transfected with vector, Hsp70-2, or LEDGF were treated with indicated concentrations of BAMLET for 24 h (1 h without serum followed by 23 h with serum). Apoptotic cell death was assessed by counting the Hoechst-stained cells with condensed nuclei. The values represent percentages of apoptotic cells in a representative assay. *Left*, a minimum of 100 cells were counted/sample; *right*, representative immunoblot analysis of indicated proteins in parental MCF-7 cells and MCF-7 cells transfected with vector, Hsp70-2, or LEDGF.

in BAMLET-treated cells (Fig. 2D). Thus, lysosomal membrane permeabilization may simply be an epiphenomenon of the death process. Alternatively, the lysosomal permeabilization in BAMLET-treated cells may lead to the release of a deadly cocktail of lysosomal hydrolases, whose cytotoxic effect cannot be inhibited simply by blocking the activity of the cysteine cathepsins. To test the latter hypothesis, we analyzed the rescuing potential of three survival proteins that confer cytoprotection by inhibiting the lysosomal membrane permeabilization, i.e. the major stress inducible Hsp70, another Hsp70 family member (Hsp70-2), and LEDGF (25, 33). Remarkably, ectopic expression of Hsp70 in WEHI-S fibrosarcoma cells as well as that of Hsp70-2 or LEDGF in MCF-7 breast cancer cells conferred significant protection against BAMLET-induced cytotoxicity (Fig. 6). Thus, lysosomal membrane permeabilization is likely to contribute to the BAMLET-induced cell death process.

Concluding Remarks

New strategies to kill cancer cells by nonapoptotic mechanisms have flourished during the past decade, and many mediators of alternate cell death pathways have been identified (3–6). Among them are the lysosomes with their large arsenal of proteolytic and lipolytic hydrolases (4, 34, 35). Importantly, transformation is associated with an increase in the volume and activity of the lysosomal compartment and even highly apoptosis-resistant cancer cells are frequently prone to cell death pathways mediated by lysosomal cathepsins (36, 37). The data presented above add BAMLET to the growing list of cytotoxic agents that can induce a nonapoptotic lysosomal cell death pathway (35). In light of these data, combination therapies with agents further sensitizing cells to lysosomal destabilization (such as microtubule disturbing drugs vincristine and paclitaxel) as well as with those inducing lysosomal destabilization (such as siramesine) should be experimentally tested (38, 39). Given that many cancers have fundamental defects in both mitochondrial and death receptor signaling pathways, and that these apoptotic defects specifically characterize the most aggressive therapy resistant tumors, BAMLET alone or in combination therapies may provide new treatment options in aggressive cancers.

Disclosure of Potential Conflicts of Interest

M. Jäättelä: consultant, NatImmune. No other potential conflicts of interest were disclosed.

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