

HAMLET: functional properties and therapeutic potential

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Human α -lactalbumin made lethal to tumor cells (HAMLET) is the first member in a new family of protein–lipid complexes that kills tumor cells with high selectivity. The protein component of HAMLET is α -lactalbumin, which in its native state acts as a substrate specifier in the lactose synthase complex, thereby defining a function essential for the survival of lactating mammals. In addition, α -lactalbumin acquires tumoricidal activity after partial unfolding and binding to oleic acid. The lipid cofactor serves the dual role as a stabilizer of the altered fold of the protein and a coactivator of specific steps in tumor cell death. HAMLET is broadly tumoricidal, suggesting that the complex identifies conserved death pathways suitable for targeting by novel therapies. Sensitivity to HAMLET is defined by oncogene expression including *Ras* and *c-Myc* and by glycolytic enzymes. Cellular targets are located in the cytoplasmic membrane, cytoskeleton, mitochondria, proteasomes, lysosomes and nuclei, and specific signaling pathways are rapidly activated, first by interactions of HAMLET with the cell membrane and subsequently after HAMLET internalization. Therapeutic effects of HAMLET have been demonstrated in human skin papillomas and bladder cancers, and HAMLET limits the progression of human glioblastomas, with no evidence of toxicity for normal brain or bladder tissue. These findings open up new avenues for cancer therapy and the understanding of conserved death responses in tumor cells.

New, targeted cancer therapies are starting to appear but the lack of specificity for tumor cells remains a significant problem [1,2]. Few molecules destroy cancer cells without harming healthy tissues and the side effects of current cancer drugs are so common that they have become accepted as a necessary element of tumor therapy. Diverse technologies and conceptual approaches have therefore been explored to identify how tumor cells differ from healthy cells, to understand the mechanisms of tumorigenesis and to explore new targets for more tumor-selective therapies [3–8]. Promising candidates inducing tumor-specific cell death include chicken anemia virus-derived apoptosis-inducing protein (apoptin), adenovirus early region 4 open reading frame (E4orf4), parvovirus-H1-derived NS1, human cytokines MDA-7 and TNF-related apoptosis-inducing ligand [3,7].

Human α -lactalbumin made lethal to tumor cells (HAMLET) is a complex of partially unfolded α -lactalbumin and oleic acid that kills tumor cells and immature cells, but not fully differentiated healthy cells [9]. The activity of HAMLET was discovered by serendipity while using human milk fractions to block bacterial adherence to lung carcinoma cells. One milk fraction killed the tumor cells and the molecular

complex responsible for this effect was identified as a folding variant of α -lactalbumin bound to oleic acid [10]. Early *in vitro* experiments showed that HAMLET has broad antitumor activity with a high degree of tumor selectivity [9]. This is reviewed elsewhere [11]. Recently, HAMLET's broad antitumor activity has been explained by specific effects of oncogenic transformation and by targets in the metabolic machinery in tumor cells [12]. Furthermore, molecular and cellular targets have been extensively characterized.

The tumoricidal activity of HAMLET and the relative selectivity for tumor tissue is maintained *in vivo*, which has been shown in two human studies and several animal models. HAMLET treatment delayed the progression of human glioblastoma xenografts in nude rats and increased survival, triggering apoptotic changes in the tumor without evidence of cell death in healthy brain tissue [13]. In a placebo-controlled clinical study, topical administration of HAMLET removed skin papillomas without side effects [14] and in patients with bladder cancer, local instillations of HAMLET killed tumor cells but not healthy cells in surrounding tissues. In addition, HAMLET triggered rapid shedding of tumor cells into the urine and caused a reduction in tumor size in patients with bladder cancer [15].

Keywords

- clinical studies ■ conserved death pathways ■ HAMLET
- novel cancer therapies
- protein folding
- protein–lipid complex
- tumor cell death ■ tumor specificity

This review summarizes studies on the structure, function and therapeutic effects of the HAMLET complex and discusses HAMLET's potential as a tool in the development of new cancer therapeutics.

HAMLET: a tumoricidal complex of partially unfolded α -lactalbumin & oleic acid

The paradigm 'one gene, one protein and one function' was proposed by Tatum and Beadle [16]; however, since the completion of the human genome sequence in 2003, this paradigm has been widely debated [17–19]. Since the number of genes encoding proteins appears to be too small to fulfill the functional demands of complex organisms, additional mechanisms of functional diversification are being explored. While known structural modifications such as glycosylation and phosphorylation allow proteins to change their function and alternative splicing of mRNA transcripts may generate additional diversity, the HAMLET model suggests that partial unfolding enables proteins to generate new functional variants from a given polypeptide chain [10] (reviewed in [20]). When described, HAMLET was the first example of a protein that undergoes partial unfolding to attain a new function different from that of its native state. Importantly, HAMLET undergoes a partial unfolding and remains as such to fulfill its new biological function, clearly distinguishing proteins forming HAMLET and related protein–lipid complexes from the moonlighting proteins [21–23] and from the intrinsically unfolded proteins [24]. This mechanism also differs from amyloid formation, where unfolding is driven towards fibril formation, cytotoxicity for healthy tissues and the loss of function associated with numerous disease states [25].

HAMLET is a complex of partially unfolded α -lactalbumin and oleic acid (C18:1:9 *cis*) (FIGURE 1) [10]. The tumoricidal activity of HAMLET was discovered in casein, obtained after low pH precipitation of human milk [9] and isolated by ion exchange chromatography, when the active complex eluted as a sharp peak after high salt. Surprisingly, the major component of the eluate was the whey protein α -lactalbumin, which had not previously been identified in the casein fraction or associated with tumoricidal activity [9,26]. However, native α -lactalbumin was shown to lack tumoricidal activity and no post-translational modifications explaining the new activity were found. A loss of tertiary structure definition was detected by circular dichroism (CD) spectroscopy, however, this was accompanied

by increased binding of the fluorescent hydrophobic dye, 8-anilino-1-naphthalene-sulfonic acid, suggesting that the low pH used for casein precipitation was driving a conformational change required for HAMLET formation.

The α -lactalbumin protein has been extensively used as a model of protein folding [27–29], and partially unfolded states [27] have been studied by several techniques including hydrogen/deuterium exchange combined with NMR [30,31], photochemically induced dynamic nuclear polarization NMR [28], limited proteolysis [32] and mutational studies [33]. The crystal structure of human α -lactalbumin [34] reveals a large α -helical domain with three major α -helices and two short 3_{10} -helices. The small β -domain consists of a triple-stranded antiparallel β -sheet and a short 3_{10} -helix [34]. The protein is stabilized by four disulfide bonds [35] and a high-affinity calcium-binding site [36].

While partial unfolding of α -lactalbumin is an essential feature of HAMLET formation, the structure of the HAMLET complex is not fully understood. In the near-UV range for the HAMLET CD spectrum a loss of tertiary structure definition has been detected [10], and increased exposure of hydrophobic domains was suggested by a blue shift compared with the native protein, with increased 8-anilino-1-naphthalene-sulfonic acid binding in fluorescence spectroscopy. The $^1\text{H-NMR}$ spectrum of HAMLET showed broader peaks as well as lower intensities of the upfield methyl proton peaks, indicating a less-ordered protein, confirming the near-UV CD result. Through hydrogen/deuterium exchange coupled with peptic digestion, a conformational state distinct from both the native and the Ca^{2+} -depleted states was suggested [37]. Two constitutively unfolded α -lactalbumin mutants were used to prove this point. First, functional HAMLET complexes were produced from the D87 calcium-site mutant developed by Brooks and Berliner [38]. As this mutant does not bind calcium, it remains partially unfolded at solvent conditions used for the cell death assays. Second, a fully functional tumoricidal complex was readily formed from the Cys–Ala all-Ala mutant of α -lactalbumin [39]. This mutant lacks disulphide bonds with substitutions of all cysteines by alanines and remains non-native under all conditions. The studies of α -lactalbumin in HAMLET and of additional proteins (see below) illustrate how partial unfolding of proteins may be used as a successful route for functional diversification of proteins (FIGURE 2). Importantly, the constitutively unfolded mutants did not directly

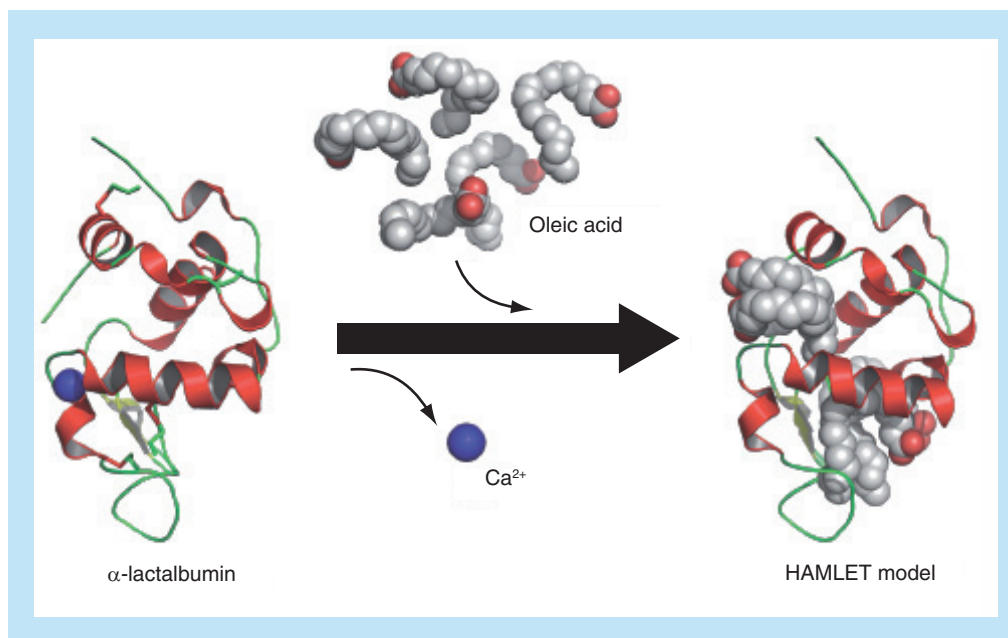


Figure 1. The two components of HAMLET with representation of the HAMLET model.

Native α -lactalbumin (Protein Data Bank ID: 1B9O) undergoes a depletion of calcium and binds to oleic acids to form HAMLET. A stoichiometric ratio of 1:5 (protein:fatty acid) is used for representation of the final HAMLET model. The structure is color coded, depicting a helix in red, the β -sheet in yellow and the random coil in green. Disulphide bonds are shown by stick representation. The calcium ion is the blue sphere. Oleic acid structure is derived from Protein Data Bank ID: 1GNI.

kill tumor cells, emphasizing the need for cofactors to form a functional HAMLET complex. The work has also been extended to address if peptides, rather than the entire protein, may possess tumoricidal activity. Tolin *et al.* have formed peptide–lipid complexes from peptic digests of α -lactalbumin, and have suggested that such peptides have tumoricidal activity, but only when bound to proper fatty acid cofactors [40].

Fatty acids are essential for the formation & function of the HAMLET complex

Early studies identified oleic acid as a cofactor required for the formation of an active HAMLET complex. NMR spectroscopy suggested that oleic acid is integrated into the complex, as the signal was broader than for oleic acid alone [10], and the lipid cofactor has been proposed to stabilize the partially unfolded state of α -lactalbumin, preventing the protein from reverting to the native state at physiologic solvent conditions [10].

To address whether HAMLET complex formation is specific for oleic acid, a fatty acid screening study was performed. Ion exchange matrices were preconditioned with fatty acids differing in chain length, saturation and configuration of the double bond(s). Interestingly, oleic acid (C18:1:9 *cis*) and vaccenic acid (C18:1:11 *cis*) were identified as the most efficient cofactors in

HAMLET complex formation. In addition, C16 or C20 *cis* unsaturated fatty acids readily formed complexes with apo α -lactalbumin [41], but *trans* fatty acids of similar carbon chain length were unable to form complexes, as were saturated fatty acids. In addition, deprotonated oleic acid (oleate) has been used successfully as a cofactor in HAMLET or BAMLET (the bovine counterpart of HAMLET), as expected from the alkaline pH conditions used in HAMLET formation [42]. The results suggest that specific fatty acid may be suitable for complex formation, by binding to domains or epitopes in the partially unfolded protein.

C18 *cis* fatty acid complexes showed enhanced tumoricidal activity, compared with other complexes, suggesting that fatty acids also participate in the interaction of target cells with HAMLET. However, whether the fatty acids act as direct, independent tumor cell agonists has been debated. Studies of complexes with high lipid content have recently suggested that unfolded proteins may function solely as ‘lipid carriers’, and that the lipids constitute the tumoricidal entity [43]. At the concentrations present in HAMLET, oleic acid/oleate cause cellular responses including a change in morphology, although there is no evidence that oleic acid or oleate alone trigger the entire tumoricidal response seen with HAMLET [44].

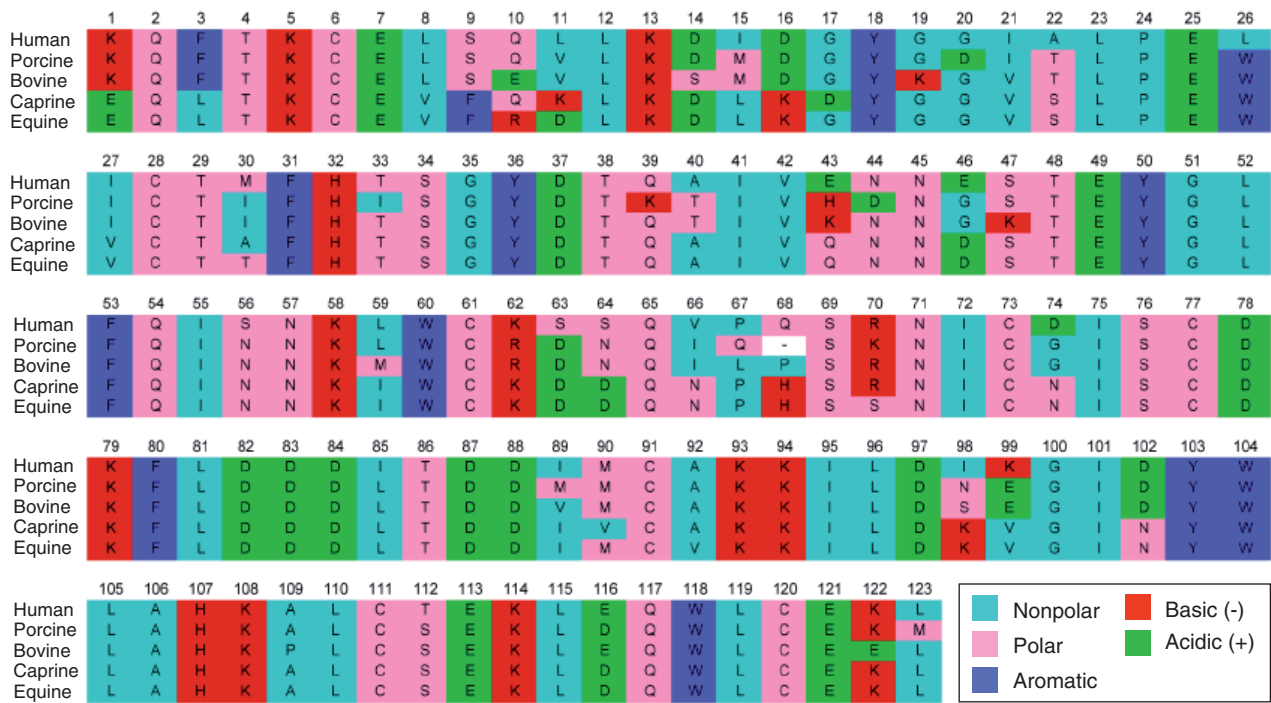


Figure 2. Sequence variation among α -lactalbumins from different species. Purified α -lactalbumins from these species readily form HAMLET-like complexes. However, acidification of milk samples from these species, analogous with casein precipitation of human milk, does not generate HAMLET-like complexes. Reproduced with permission from [66].

Cell death in response to HAMLET

Susceptibility of tumor cells to HAMLET

HAMLET appears to identify and exploit conserved features of cancer cells for its tumoricidal activity. To date, more than 40 different tumor cell lines have been exposed to HAMLET *in vitro* and shown to be sensitive, regardless of tumor type, species and tissue origin [9]. Arguably, such conserved features may either represent general features of tumor cells that also render them susceptible to HAMLET or may reflect the presence of specific, conserved targets critical for the cell death response. To identify properties that render tumor cells susceptible to HAMLET, a combination of shRNA inhibition, proteomic and metabolomic technology were used. Elevated *c-MYC* expression was shown to create a HAMLET-sensitive phenotype and the *c-MYC* and *RAS* oncogenes were identified as essential determinants of HAMLET sensitivity [12]. Furthermore, the shRNA screen identified Hexokinase 1, PFKFB1 and HIF1 α as determinants of HAMLET sensitivity. Hexokinase 1 was also shown to bind HAMLET in a protein array containing approximately 8000 targets [12]. Importantly, glucose deprivation sensitized tumor cells to HAMLET-induced cell death and

HAMLET triggered rapid metabolic paralysis in carcinoma cells. By mass spectrometry, the glycolytic machinery was shown to be modified by HAMLET and glycolysis was shifted towards the pentose phosphate pathway. These findings link the HAMLET sensitivity of tumor cells to conserved features defined by oncogenic transformation and the metabolic state (the Warburg effect).

The plasma membrane: the first barrier

HAMLET discriminates tumor cells from normal differentiated cells, and interactions at the plasma membrane level determine the difference in sensitivity. The key molecular interactions are not fully understood, but in early studies, HAMLET was shown to trigger ion fluxes across tumor cell membranes [9]. Recently, in lipid membrane models, HAMLET has been shown to interact with phospholipid bilayers in the absence of specific tumor cell membrane constituents [45]. HAMLET, bound to egg yolk and soybean membranes at physiological pH, perturbed membrane integrity and caused leakage of vesicular contents to the exterior. Vesicles composed of natural lipid mixtures showed drastically altered morphology in response to HAMLET, although

the natively folded protein or oleic acid did not have these effects [45,46]. Interestingly, the membrane elongation and a change in fluidity further emphasize the potential for ion channel activation by HAMLET through mechanosensing or, alternatively, the formation of HAMLET-specific ion channels [47]. The results suggest that the membrane is perturbed in concert by the partially unfolded protein and the fatty acid by a mechanism requiring both the protein and the fatty acid.

Relevance of these membrane perturbations and resulting ion fluxes for tumor cell death has also been documented [47]. HAMLET was shown to trigger rapid cation fluxes and the inhibition of these fluxes prevented many aspects of the cell death response to HAMLET. Furthermore, tumor specificity was suggested, as HAMLET was shown to alter membrane properties in plasma membrane vesicles from tumor cells while plasma membrane vesicles from normal, differentiated cells were not affected [45]. This may reflect differences in membrane composition, structural organization and functional properties between tumor cells and normal, differentiated cells, including membrane fluidity and the fatty acid composition of the phospholipids. Membranes of tumor cells have altered lipid composition and fluidity, which may alter the properties of membrane-bound receptors, enzymes and endocytic pathways and thereby the activation of cell death [48]. Thus, tumor cell membranes may favor HAMLET binding and facilitate HAMLET-induced membrane perturbations activating the cell death response.

An unfolded protein response: endoplasmic reticulum stress & proteasome fragmentation in HAMLET response

After perturbing the membranes of tumor cells, HAMLET enters the cytoplasm and translocates to the nuclei [10,13,49,50]. The authors have hypothesized that the internalization of HAMLET creates an unfolded protein-overload scenario that triggers endoplasmic reticulum stress and targets HAMLET to the proteasomes for degradation. Proteasomes normally control the level of endogenous unfolded proteins by degrading them in the proteolytic core, proteins that resist degradation or proteasome inhibition may cause cell death. Endogenous, unfolded proteins are degraded by 26S and 20S proteasomes but unfolded α -lactalbumin interacts mainly with the 20S proteasomes *in vitro* [51]. The authors have shown that HAMLET is targeted to 20S proteasomes in

tumor cells and triggers a change in proteasomes structure, with modifications of catalytic (β 1 and β 5) and structural subunits [52]. Evidence for a direct interaction of HAMLET with intact proteasomes and proteasome subunits was obtained *in vitro* [52]. Interestingly, HAMLET resisted degradation by proteasomal enzymes and inhibited proteasome activity. Thus, targeting of internalized HAMLET to the proteasomes and perturbations of proteasome structure might contribute to the cytotoxic effects of unfolded protein complexes that invade host cells.

Nuclear receptors & chromatin interactions of HAMLET

The nuclear translocation of HAMLET is rapid, with 75% of the complex reaching the nuclei within 1 h at 35 μ M concentrations. Healthy cells, by contrast, only take up small amounts of HAMLET and there is no evidence that HAMLET reaches the nuclei of healthy cells [13,49]. The nuclear accumulation of HAMLET

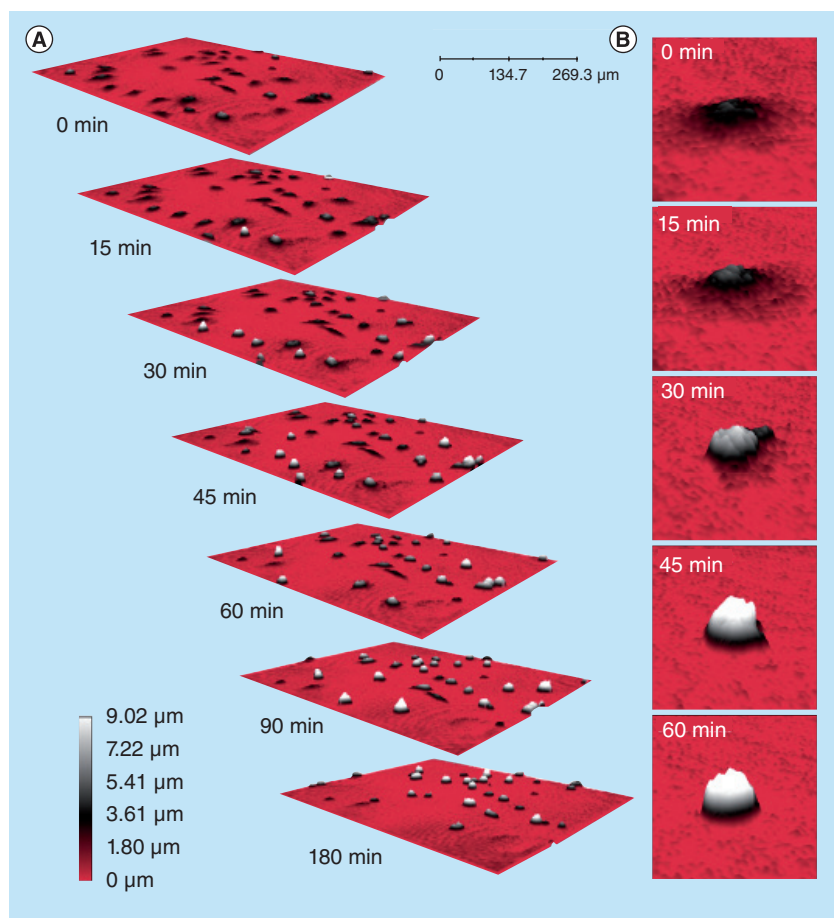


Figure 3. Cellular response to HAMLET visualized by holographic live-cell imaging. (A) Kinetics of the morphological change in lung carcinoma cells exposed to HAMLET (35 μ M). Cells start to round up after approximately 30 min, and, at 60 min, a reduction in cell number is observed. (B) Enlargement of a single cell.

has been proposed to reflect the role of histones as nuclear receptors for HAMLET, and the affinity binding, mainly to histones H3 and H4, creates virtually insoluble complexes [49]. In nuclear extracts, HAMLET, histones and DNA form virtually insoluble complexes. As chromatin accessibility is controlled by the acetylation state of the histone tail, histone deacetylase inhibitors are used to modify the chromatin accessibility of tumoricidal agents. Histone deacetylase inhibitors enhance the tumoricidal effects of HAMLET, in part by enhancing the hyperacetylation response [53]. Future studies in tumor models will be of interest to investigate whether the combination of HAMLET and histone deacetylase inhibitors may be used to increase the therapeutic efficiency *in vivo*.

Apoptosis & macroautophagy

HAMLET-treated cells show signs of apoptosis such as cell shrinkage, nuclear condensation, caspase activation and DNA fragmentation [9]. However, inhibition of apoptosis by the pan-caspase inhibitor zVAD-FMK does not prevent cells from dying and overexpression of the anti-apoptotic BCL-2 and BCL-XL proteins does not change the sensitivity of tumor cells towards HAMLET. HAMLET-induced cell death is also p53-independent [54].

BAMLET has been shown to induce caspase activation in several tumor cell lines [55]. In addition, BAMLET colocalizes with lysosomes in tumor cells and causes lysosomal membrane permeabilization followed by leakage of cathepsin L from the lysosome into the cytoplasm [55].

In parallel with apoptosis, HAMLET triggers macroautophagy [56]. Extreme responses may cause so-called autophagic/type II cell death [57–59]. Evidence of macroautophagy was first obtained by electron microscopy, when double membrane vesicles were observed after HAMLET treatment. Subsequently, other changes typical of macroautophagy were also observed, including LC3 translocation and accumulation [56]. Inhibition by Beclin 1 and *Atg5* siRNAs reduced HAMLET-induced macroautophagy and showed marginal effects on cell death, suggesting that autophagy is one of several responses occurring in cells that die in response to HAMLET.

Cell detachment induced by HAMLET

Adherent tumor cells detach in response to HAMLET *in vitro* (FIGURE 3) and, in patients with bladder cancer, local HAMLET instillations have been shown to trigger rapid tumor cell

detachment *in vivo* [15,60]. To identify molecules involved in cell detachment, cellular extracts were screened for HAMLET targets. One such screen identified α -actinin-1 and -4 as binding partners for HAMLET [60]. α -actinins are the major F-actin-binding and crosslinking proteins in human cells. Two α -actinin molecules form a functional antiparallel homodimer [61] and the cellular localization of α -actinin differs depending on the cell type [62]. α -actinin-1 has been shown to localize at focal adhesion plaques or adherence junctions and α -actinin-4 at points of cell–cell contact. However, α -actinin-4 has also been shown to interact with focal adhesion constituents, including vinculin and the cytoplasmic domain of β -integrins [63–65].

The α -actinin-4 domains interacting with HAMLET were identified using a synthetic peptide library mapped onto a cryoelectron microscopy reconstruction representation of smooth muscle α -actinin [60]. Four peptides facing the cavity between the actin-binding domain and the central rod domain were identified, potentially forming a combined binding site for HAMLET. In addition, a potential β -integrin-binding site was identified and HAMLET bound to two peptides in the α -actinin-C-terminal. HAMLET triggered a rapid disruption of focal adhesion complexes and cytoskeletal structure and focal adhesion kinase phosphorylation and signaling. The effect was only observed in carcinoma cells, as normal differentiated cells retained their morphology in the presence of HAMLET.

HAMLET-like complexes

Following the discovery of HAMLET, several HAMLET-like complexes have been produced. The properties of HAMLET-like complexes using α -lactalbumin derived from different species suggested that the conversion of α -lactalbumin to its tumoricidal form tolerates a certain degree of sequence variation. Bovine, equine, porcine and caprine α -lactalbumin differed greatly in the efficiency of complex formation, although the complexes with oleic acid showed similar tumoricidal activities [66]. Lysozyme, which is the closest structural homolog of α -lactalbumin, also forms cytotoxic complexes with oleic acid called ELOA [67]. In contrast with conventional noncalcium-binding c-type lysozyme, equine lysozyme binds calcium and exhibits enzymatic activity. Similar to α -lactalbumin, equine lysozyme forms a range of partially folded states under destabilizing conditions and also populates various kinetic intermediates, while retaining its native-like core conformation.

A few significant differences between ELOA and HAMLET should be discussed. ELOA exists as an oligomer, binding as many as 48 oleic acid molecules in a single complex, compared with four to eight oleic acids bound to the HAMLET monomers. This 'lipid overload' is likely to change the interaction with tumor cell membranes from a programmed cell death response to cell lysis, as high levels of oleic acid are cytotoxic [68–71].

Other HAMLET-like complexes have been produced by alternative methods, including mixing procedures under acidic [72] or alkaline [42] pH conditions or by heat denaturation [73]. By using proteolytic fragments of bovine α -lactalbumin mixed with oleic acid, Tolin *et al.* suggested that the protein component mainly acts as a binding partner for the fatty acid [40]. However, the peptide preparations contained two- to threefold more lipid compared with HAMLET, and target analysis in tumor cells has not yet been performed. The α -isoform of pike parvalbumin or bovine β -lactoglobulin [43] were both shown to form complexes with oleic acid, suggesting that proteins other than α -lactalbumin may act as binding partners for oleic acid. This was confirmed for β -lactoglobulin [74]. These complexes are cytotoxic for different cell lines, including human lung carcinoma cells A549 [72], laryngeal carcinoma (HEp-2) cells [70] and human monocytic cells (U937) [43]. Mechanistic characterizations of the implicated cell death pathways are needed to define if the activity of the complexes is associated with the cytotoxicity of oleic acid.

Cell death in response to HAMLET-like complexes

BAMLET has been the focus of several interesting studies addressing the interaction with tumor cells [55,66]. BAMLET reproduces many aspects of the tumoricidal response to HAMLET including the morphologic changes, internalization and loss of viability. Rammer *et al.* have shown that BAMLET activates caspase-dependent apoptosis-like cell death and autophagy, but does not kill cells by these mechanisms. In addition, the BAMLET complex has been shown to activate the lysosomal cell death pathway [55]. The ELOA complexes trigger cell death, accompanied with combined staining of acridine orange and ethidium bromide, following the accumulation of ELOA in the vicinity of the cell membrane [67]. Several other complexes derived from β -lactoglobulin and parvalbumin have also been shown to trigger cytotoxic responses involving membrane depolarization and membrane damage [43].

In vivo effects of HAMLET

Most current therapies lack tumor specificity and toxicity for healthy tissues is therefore a major concern. A variety of approaches are being explored to identify new drugs that selectively kill tumor cells. Significant progress is being made and new, targeted therapies include inhibitors of growth factors and their receptors, blockers of angiogenesis and proapoptotic drugs [4–6,8]. A high degree of selectivity for tumor cells *in vitro* and *in vivo* has identified HAMLET as an interesting candidate drug with tumor specificity in animal models and clinical studies [13–15,75].

HAMLET limits the progression of human glioblastoma xenografts

Malignant tumors of the brain may arise from cells in the brain tissue *per se* or from metastatic spread of tumor cells from peripheral organs. In both cases, therapeutic options are limited. Due to infiltrating growth, glioblastomas are not amenable to selective surgical removal and are resistant to irradiation and most, if not all, chemotherapy. Experimental therapies such as gene therapy and antisense, engineered and defective viruses may be efficient in brain tumor models, but those few that have made it to clinical trials have not been promising [76]. For example, regional infusion of a transferrin-diphtheria toxin complex has been shown to decrease tumor volume [77], but complications included necrosis, brain edema and destruction of brain capillary endothelial cells. There remains, therefore, a great unmet need for novel therapeutic approaches that selectively eliminate the tumor cells without damaging functional brain tissue.

Xenotransplantation of human glioblastoma cells into nude rats has been used extensively to screen novel therapeutic agents [78]. The human tumor is cultured *in vitro* and approximately 5–10 μ l of phosphate-buffered saline containing five biopsy spheroids is injected into the striatum. Rats are monitored daily and sacrificed when they develop symptoms such as passivity, clumsiness and paresis, and the tumor mass is quantified by MRI scans.

To administer HAMLET, the authors chose to use convection-enhanced delivery, where the region of the tumor was infused with HAMLET or α -lactalbumin through a cannula connected to an osmotic mini pump [13]. A 24-h infusion of HAMLET was sufficient to dramatically delay tumor development (FIGURE 4A) and the onset of pressure symptoms. By immunohistochemistry, the protein complex was shown to trigger apoptosis in tumor tissue and HAMLET penetrated

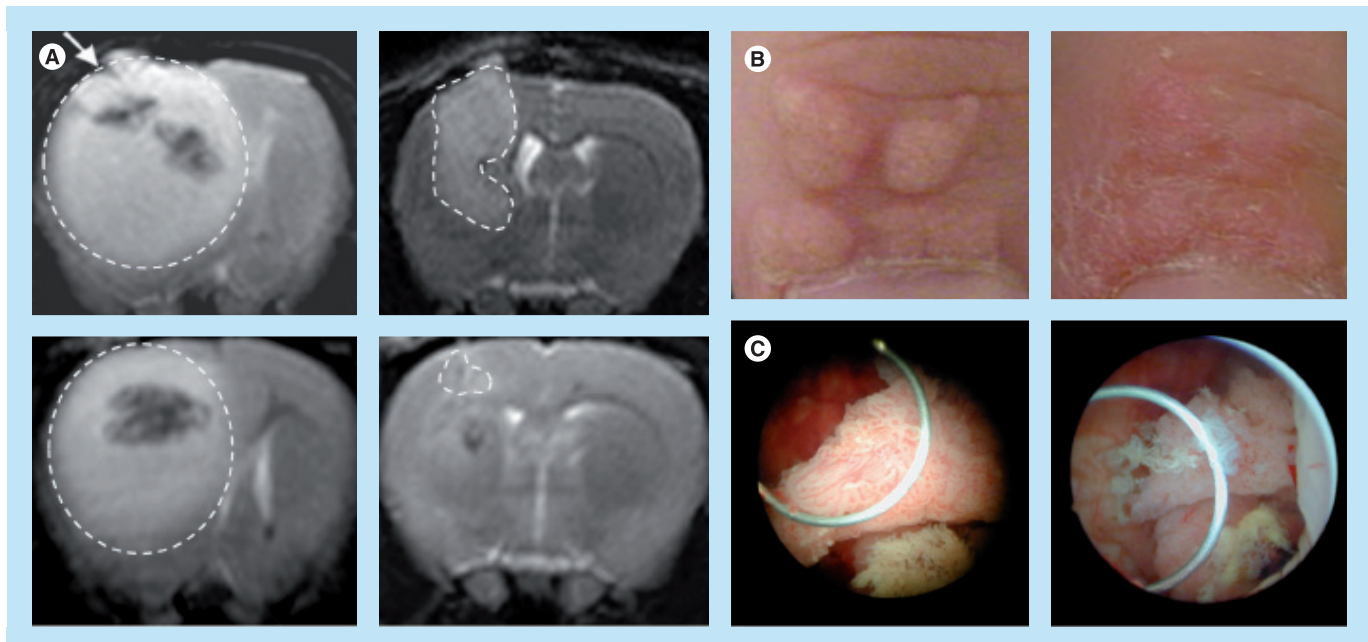


Figure 4. Therapeutic effects of HAMLET. (A) Delayed brain tumor development after convection-enhanced delivery of HAMLET into the brain of nude rats bearing human glioblastoma xenografts, compared with α -lactalbumin controls. (B) Human skin papillomas showing a reduction in lesion size after topical HAMLET administration. (C) Reduction in bladder cancer size after 1 week of HAMLET instillation into the bladder.

(A) Reproduced with permission from [13].

(B) Reproduced with permission from [14].

(C) Reproduced with permission from [15].

throughout the hemisphere that was injected. However, HAMLET infusions did not appear to harm the normal brain or cause neurological symptoms. It appears from these studies that regional therapy of HAMLET may be proposed as a possible novel approach to control the progression of malignant and invasive brain tumors and possibly of metastases of tumors from distant sites. Use of convection-enhanced delivery into the CNS is essential for future studies, as HAMLET is inactivated in human serum.

Effects of HAMLET on human skin papillomas: a placebo-controlled study

The authors' group selected skin papillomas for the first human study of HAMLET's effect as a topical therapeutic [14]. Human papillomavirus (HPV)-transformed keratinocytes proliferate and form warts, and most of the skin lesions remain benign. Cutaneous papillomas are caused by one or more of approximately 130 different HPV types [79]. Current treatments include cryotherapy, curettage, cautery, topical virucidal agents [80], lasers [81,82], anti-mitotic agents [83] and immunoactivators [84–86]. Immunosuppressed patients run an increased risk of developing papillomas and often carry multiple HPV types [87].

The authors tested topical treatment of human skin papillomas with HAMLET in a placebo-controlled, double-blind study. HAMLET (0.7 mM in 0.9% NaCl) or placebo (0.9% NaCl) was applied topically once a day for 3 weeks and the volume change was recorded. After the first trial, both the placebo and the treatment group were offered a 3-week course of HAMLET in an open arm of the study. The lesion volume was reduced by $\geq 75\%$ in the HAMLET group compared with 15% in the placebo group ($p < 0.001$). Complete resolution of all lesions had occurred in 90% of all HAMLET-treated patients after 5 months, and the time to resolution was shorter in the group receiving HAMLET from the start compared with the placebo group (FIGURE 4B). No adverse reactions were reported, and there was no difference in treatment outcome between immunocompetent and immunosuppressed patients. It was concluded that topical HAMLET treatment has a beneficial and lasting effect on skin papillomas.

Intravesical instillation of HAMLET in patients with bladder cancer

Bladder cancers are common, differing in severity and accessibility to therapy. Surgery alone or in combination with cytostatic drugs is

used successfully, but therapy-resistant tumors still cause significant morbidity and mortality [88]. After removal by transurethral resection of superficial papillary tumors the short-term prognosis is excellent. Intravesical instillation of the Bacille Calmette–Guerin vaccine results in a recurrence-free interval of at least 2 years in approximately 70% of the treated patients [89]. Still, the recurrence rate is high and due to the risk of dedifferentiation, patients require life-long follow-up. Invasive tumors are removed by radical cystectomy and some patients receive adjuvant systemic chemotherapy but may still have a poor prognosis [90]. Furthermore, Bacille Calmette–Guerin treatment has significant side effects and, especially in immunosuppressed patients, systemic antituberculous therapy may be required.

The authors' group therefore studied whether intravesical HAMLET instillations may be used to kill cancer cells *in vivo* [15]. In patients with superficial, exophytic bladder cancer or cancer *in situ*, an image of the tumor was obtained through the cystoscope at the time of diagnosis. After five daily intravesical instillations of HAMLET during the week before scheduled surgery, the tumor was again photographed and biopsies were obtained to examine tissue integrity and to compare the apoptotic response of the tumor and surrounding healthy tissues. By cystoscopy, a reduction in tumor size was detected (FIGURE 4C) and in biopsy specimens, apoptotic cells were seen in the remaining tumor. The patients with cancer *in situ* showed a reduction in the number of tumor-positive biopsies. By contrast, there was no TUNEL response in healthy tissue biopsies adjacent to the tumor and no cell exfoliation after intravesical NaCl instillations in the patients. In addition, each HAMLET instillation triggered the exfoliation of large numbers of tumor cells.

The results show that HAMLET exerts a direct and selective effect on bladder cancer tissue *in vivo*, and that local administration of HAMLET may cause a rapid reduction in tumor mass.

HAMLET treatment delays bladder cancer development in a mouse model

The human bladder cancer study was not designed as a controlled, therapeutic study. To evaluate whether HAMLET is efficient as a topical therapeutic agent in bladder cancer, the authors therefore used the mouse MB49 bladder carcinoma model [75]. Rapidly growing tumors were established by intravesical inoculation of tumor cells and HAMLET was applied topically.

Using tumor size as the therapeutic end point, a significant therapeutic effect of HAMLET was detected. A reduction in tumor development was observed in HAMLET-treated mice compared with the control group. Furthermore, by *in vivo* imaging of Alexa–Fluor-labeled HAMLET, accumulation was detected in tumor tissue for at least 24 h, but not in the healthy tissues surrounding the tumor. The results show that HAMLET is active as a tumoricidal agent with selective uptake in bladder tumor tissue and suggest that topical HAMLET administration should be further tested in patients with bladder cancer.

Effects of HAMLET on bacteria

The tumoricidal activity of HAMLET was discovered as a result of attempts to define the anti-adhesive effect of human milk casein. The authors had previously observed that casein reduced the attachment of *Streptococcus pneumoniae* to human respiratory tract cells and therefore used different casein fractions as a tool to further understand the molecular specificity involved in host cell recognition by pneumococci [91]. In addition to the lethal effects on tumor cells, HAMLET was shown to also kill the bacteria. Analysis of the antibacterial spectrum showed sensitivity mainly of streptococci; most Gram-negative and other Gram-positive bacteria were resistant. Thus, in addition to its tumoricidal effect, HAMLET shows significant antimicrobial activity.

Further studies showed that an apoptosis-like response is also activated by HAMLET in bacterial cells. Striking similarities were observed, including DNA fragmentation and a change in morphology [92]. HAMLET induced a calcium-dependent membrane depolarization in both host cells [9] and bacteria [92], possibly reflecting the shared evolutionary origin of mitochondria and bacteria. In addition, a bovine α -lactalbumin-OA complex prepared under alkaline conditions (bLA-OA-45) was shown to depolarize and damage the plasma membrane in *S. pneumoniae* D39 [42]. Downstream degradation pathways involved protease and endonuclease activity. These findings suggest that pathways associated with apoptosis are present in prokaryotes and that mechanisms of bacterial cell death may be explored to further our understanding of key activation mechanisms leading to cell death in eukaryote cells. Furthermore, the bacterial responses may prove essential to identify novel targets for future antimicrobial therapy. The similarities between mitochondrial and bacterial responses to HAMLET may be essential in this context.

Conclusion

HAMLET is the first member of a new, expanding family of unfolded protein–lipid complexes that kill a broad range of cancer cells while sparing normal, differentiated cells. HAMLET thus identifies targets that are highly conserved among cancer cells and whose activation tilts the balance from exaggerated cell survival towards death. Here, aspects on the structure, cellular targets and *in vivo* effects of HAMLET are reviewed. The results suggest that HAMLET offers a two-tiered therapeutic approach, killing cancer cells while stimulating an innate immune response in surrounding healthy tissues. The HAMLET-induced tumor-selective death is particularly significant in view of HAMLET's already documented tumoricidal effect in patients and animal models.

Future perspective

To further elucidate the HAMLET phenomenon, a combination of structural studies, cell biology, animal models and clinical trials will be needed. It is encouraging that several international groups actively pursue this field of research [12,42,45,46,55,75,92]. New data on the structure of HAMLET, BAMLET, ELMLET and other similar complexes are rapidly being generated. Cellular targets that initiate the cell death process are being identified in the cytoplasmic membrane and it should be possible in the near future to characterize targets that distinguish tumor cells from healthy, differentiated cells in order to understand the relative tumor selectivity of HAMLET. Finally, the range of therapeutic targets for HAMLET is being expanded, hopefully serving as a source of inspiration for continued development of HAMLET into a fully available therapeutic agent.

Specifically, we propose that HAMLET should be further explored as a novel therapeutic agent against HPV-induced tumors. HPV infection is an important cause of cervical cancer [93],

making effects on skin papillomas of potential interest for patients with cervical dysplasia or HPV-induced genital warts. HPV vaccines are efficient new tools to prevent cervical papillomas, although the morbidity in unvaccinated subjects remains a major health issue. A study of topical HAMLET administration in women with cervical dysplasia would be of great interest, as removal of cervical tissue by conization remains the only therapeutic option at present.

We also propose that the therapeutic value of HAMLET should be explored in controlled trials of bladder cancer and brain tumors, where experimental and human data are available. Further characterization of basic mechanisms of cell death regulation may also be useful to design future disease therapies involving both eukaryotic and prokaryotic cells.

Financial & competing interests disclosure

This study was supported by the Sharon D Lund foundation grant and the American Cancer Society, the Swedish Cancer Society, the Medical Faculty (Lund University), the Söderberg Foundation, the Segerfalk Foundation, the Anna-Lisa and Sven-Erik Lundgren Foundation for Medical Research, the Knut and Alice Wallenberg Foundation, the Lund City Jubileumsfond, the John and Augusta Persson Foundation for Medical Research, the Maggie Stephens Foundation, the Gunnar Nilsson Cancer Foundation, the Inga-Britt and Arne Lundberg Foundation, the HJ Forssman Foundation for Medical Research and the Royal Physiographic Society. HAMLET patents are held by HAMLET Pharma – currently an inactive company. The studies described in this manuscript were not supported by commercial sources/partnerships. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- The HAMLET model suggests that partial unfolding enables proteins to generate new functional variants from a given polypeptide chain.
- The lipid cofactor serves the dual role as a stabilizer of the altered fold and a coactivator of specific steps in tumour cell death.
- HAMLET is broadly tumoricidal *in vitro*, but spares healthy, differentiated cells.
- The tumoricidal response is initiated at the membrane by mechanisms involving membrane perturbations and ion fluxes. Subsequent responses include death signaling pathways and direct interactions of HAMLET with intracellular compartments such as mitochondria, proteasomes, endoplasmic reticulum stress and binding to histones/chromatin in tumor cell nuclei.
- Therapeutic studies include a placebo-controlled study of human skin papillomas and an explorative study of human bladder cancer. Therapeutic efficacy has also been documented in animal models of glioblastoma, bladder cancer and colon cancer.
- These properties identify HAMLET as a new type of tumoricidal compound with great potential as a tumoricidal agent and as a tool to reveal new, conserved death pathways in tumor cells.

References

Papers of special note have been highlighted as:

- of interest
- of considerable interest

1. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 100(1), 57–70 (2000).
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 144(5), 646–674 (2011).
- **Proposes an additional four hallmarks of cancer to aid our understanding on cancer.**
3. Argiris K, Panethymitaki C, Tavassoli M. Naturally occurring, tumor-specific, therapeutic proteins. *Exp. Biol. Med. (Maywood)* 236(5), 524–536 (2011).
- **Gives a comprehensive overview of other tumor-specific killer proteins.**
4. Berge E, Thompson C, Messersmith W. Development of novel targeted agents in the treatment of metastatic colorectal cancer. *Clin. Colorectal Cancer* 10(4), 266–278 (2011).
5. Flaherty KT, Hodi FS, Fisher DE. From genes to drugs: targeted strategies for melanoma. *Nat. Rev. Cancer* 12(5), 349–361 (2012).
6. Fu Y, Zheng S, An N *et al.* β -catenin as a potential key target for tumor suppression. *Int. J. Cancer* 129(7), 1541–1551 (2011).
7. Los M, Panigrahi S, Rashedi I *et al.* Apoptin, a tumor-selective killer. *Biochim. Biophys. Acta* 1793(8), 1335–1342 (2009).
8. Sethi N, Kang Y. Unravelling the complexity of metastasis – molecular understanding and targeted therapies. *Nat. Rev. Cancer* 11(10), 735–748 (2011).
9. Hakansson A, Zhivotovsky B, Orrenius S, Sabharwal H, Svanborg C. Apoptosis induced by a human milk protein. *Proc. Natl Acad. Sci. USA* 92(17), 8064–8068 (1995).
- **Marks the first observation of tumoricidal effects of HAMLET.**
10. Svensson M, Hakansson A, Mossberg AK, Linse S, Svanborg C. Conversion of α -lactalbumin to a protein inducing apoptosis. *Proc. Natl Acad. Sci. USA* 97(8), 4221–4226 (2000).
11. Svanborg C, Agerstam H, Aronson A *et al.* HAMLET kills tumor cells by an apoptosis-like mechanism – cellular, molecular, and therapeutic aspects. *Adv. Cancer Res.* 88, 1–29 (2003).
12. Storm P, Aits S, Puthia MK *et al.* Conserved features of cancer cells define their sensitivity to HAMLET-induced death; c-Myc and glycolysis. *Oncogene* 30(48), 4765–4779 (2011).
- **Cytotoxic specificity of HAMLET towards cancer cells is discussed and HAMLET is**

shown to target c-Myc and glycolytic machinery.

13. Fischer W, Gustafsson L, Mossberg AK *et al.* Human α -lactalbumin made lethal to tumor cells (HAMLET) kills human glioblastoma cells in brain xenografts by an apoptosis-like mechanism and prolongs survival. *Cancer Res.* 64(6), 2105–2112 (2004).
14. Gustafsson L, Leijonhufvud I, Aronsson A, Mossberg AK, Svanborg C. Treatment of skin papillomas with topical α -lactalbumin-oleic acid. *N. Engl. J. Med.* 350(26), 2663–2672 (2004).
- **In vivo efficacy of HAMLET on treating skin papillomas is shown.**
15. Mossberg AK, Wullt B, Gustafsson L, Mansson W, Ljunggren E, Svanborg C. Bladder cancers respond to intravesical instillation of HAMLET (human α -lactalbumin made lethal to tumor cells). *Int. J. Cancer* 121(6), 1352–1359 (2007).
16. Beadle GW, Tatum EL. Genetic control of biochemical reactions in neurospora. *Proc. Natl Acad. Sci. USA* 27(11), 499–506 (1941).
17. Valdivia HH. One gene, many proteins: alternative splicing of the ryanodine receptor gene adds novel functions to an already complex channel protein. *Circ. Res.* 100(6), 761–763 (2007).
18. Venter JC. Genome-sequencing anniversary. The human genome at 10: successes and challenges. *Science* 331(6017), 546–547 (2011).
19. Venter JC, Adams MD, Myers EW *et al.* The sequence of the human genome. *Science* 291(5507), 1304–1351 (2001).
20. Pettersson-Kastberg J, Aits S, Gustafsson L *et al.* Can misfolded proteins be beneficial? The HAMLET case. *Ann. Med.* 41(3), 162–176 (2009).
21. Jeffery CJ. Moonlighting proteins. *Trends Biochem. Sci.* 24(1), 8–11 (1999).
22. Jeffery CJ. Multifunctional proteins: examples of gene sharing. *Ann. Med.* 35(1), 28–35 (2003).
23. Jeffery CJ. Moonlighting proteins – an update. *Mol. Biosyst.* 5(4), 345–350 (2009).
24. Dyson HJ, Wright PE. Intrinsically unstructured proteins and their functions. *Nat. Rev. Mol. Cell Biol.* 6(3), 197–208 (2005).
25. Soto C. Transmissible proteins: expanding the prion heresy. *Cell* 149(5), 968–977 (2012).
26. Svensson M, Sabharwal H, Hakansson A *et al.* Molecular characterization of α -lactalbumin folding variants that induce apoptosis in tumor cells. *J. Biol. Chem.* 274(10), 6388–6396 (1999).
27. Kuwajima K. The molten globule state of α -lactalbumin. *FASEB J.* 10(1), 102–109 (1996).
28. Mok KH, Nagashima T, Day IJ, Hore PJ, Dobson CM. Multiple subsets of side-chain packing in partially folded states of α -lactalbumins. *Proc. Natl Acad. Sci. USA* 102(25), 8899–8904 (2005).
29. Peng ZY, Kim PS. A protein dissection study of a molten globule. *Biochemistry* 33(8), 2136–2141 (1994).
30. Chyan CL, Wormald C, Dobson CM, Evans PA, Baum J. Structure and stability of the molten globule state of guinea-pig α -lactalbumin: a hydrogen exchange study. *Biochemistry* 32(21), 5681–5691 (1993).
31. Schulman BA, Redfield C, Peng ZY, Dobson CM, Kim PS. Different subdomains are most protected from hydrogen exchange in the molten globule and native states of human α -lactalbumin. *J. Mol. Biol.* 253(5), 651–657 (1995).
32. Polverino De Lauro P, Frare E, Gottardo R, Fontana A. Molten globule of bovine α -lactalbumin at neutral pH induced by heat, trifluoroethanol, and oleic acid: a comparative analysis by circular dichroism spectroscopy and limited proteolysis. *Proteins* 49(3), 385–397 (2002).
33. Wu LC, Peng ZY, Kim PS. Bipartite structure of the α -lactalbumin molten globule. *Nat. Struct. Biol.* 2(4), 281–286 (1995).
34. Acharya KR, Ren JS, Stuart DI, Phillips DC, Fenna RE. Crystal structure of human α -lactalbumin at 1.7 Å resolution. *J. Mol. Biol.* 221(2), 571–581 (1991).
35. Vanaman TC, Brew K, Hill RL. The disulfide bonds of bovine α -lactalbumin. *J. Biol. Chem.* 245(17), 4583–4590 (1970).
36. Hiraoka Y, Segawa T, Kuwajima K, Sugai S, Murai N. α -lactalbumin: a calcium metalloprotein. *Biochem. Biophys. Res. Commun.* 95(3), 1098–1104 (1980).
37. Casbarra A, Birolo L, Infusini G *et al.* Conformational analysis of HAMLET, the folding variant of human α -lactalbumin associated with apoptosis. *Protein Sci.* 13(5), 1322–1330 (2004).
38. Svensson M, Fast J, Mossberg AK *et al.* α -lactalbumin unfolding is not sufficient to cause apoptosis, but is required for the conversion to HAMLET (human α -lactalbumin made lethal to tumor cells). *Protein Sci.* 12(12), 2794–2804 (2003).
39. Pettersson-Kastberg J, Mossberg AK, Trulsson M *et al.* α -lactalbumin, engineered to be nonnative and inactive, kills tumor cells when in complex with oleic acid: a new biological function resulting from partial unfolding. *J. Mol. Biol.* 394(5), 994–1010 (2009).

40. Tolin S, De Franceschi G, Spolaore B *et al.* The oleic acid complexes of proteolytic fragments of α -lactalbumin display apoptotic activity. *FEBS J.* 277(1), 163–173 (2010).
41. Svensson M, Mossberg AK, Pettersson J, Linse S, Svanborg C. Lipids as cofactors in protein folding: stereo-specific lipid-protein interactions are required to form HAMLET (human α -lactalbumin made lethal to tumor cells). *Protein Sci.* 12(12), 2805–2814 (2003).
42. Permyakov SE, Knyazeva EL, Leonteva MV *et al.* A novel method for preparation of HAMLET-like protein complexes. *Biochimie* 93(9), 1495–1501 (2011).
43. Permyakov SE, Knyazeva EL, Khasanova LM *et al.* Oleic acid is a key cytotoxic component of HAMLET-like complexes. *Biol Chem.* 393(1–2), 85–92 (2012).
44. James Ho CS, Petter Storm, Anna Rydström *et al.* Lipids as tumoricidal components of HAMLET; unique and shared effects on signaling and death. In: *The Mechanism of HAMLET-Induced Cell Death – Cellular Signalling, Oncogenes and Clinical Perspectives.* Lunds Universitet, Sweden (2012).
45. Mossberg AK, Puchades M, Halskau O *et al.* HAMLET interacts with lipid membranes and perturbs their structure and integrity. *PLoS ONE* 5(2), e9384 (2010).
46. Baumann A, Gjerde AU, Ying M *et al.* HAMLET forms annular oligomers when deposited with phospholipid monolayers. *J. Mol. Biol.* 418(1–2), 90–102 (2012).
47. Storm P, Klausen TJ, Trulsson M *et al.* A unifying mechanism for tumor cell death by ion channel activation. *PLoS One* (In Press).
48. Baritaki S, Apostolakis S, Kanellou P, Dimanche-Boitrel MT, Spandidos DA, Bonavida B. Reversal of tumor resistance to apoptotic stimuli by alteration of membrane fluidity: therapeutic implications. *Adv. Cancer Res.* 98, 149–190 (2007).
49. Durringer C, Hamiche A, Gustafsson L, Kimura H, Svanborg C. HAMLET interacts with histones and chromatin in tumor cell nuclei. *J. Biol. Chem.* 278(43), 42131–42135 (2003).
50. Kohler C, Hakansson A, Svanborg C, Orrenius S, Zhivotovsky B. Protease activation in apoptosis induced by MAL. *Exp. Cell Res.* 249(2), 260–268 (1999).
51. Wenzel T, Baumeister W. Conformational constraints in protein degradation by the 20S proteasome. *Nat. Struct. Biol.* 2(3), 199–204 (1995).
52. Gustafsson L, Aits S, Onnerfjord P, Trulsson M, Storm P, Svanborg C. Changes in proteasome structure and function caused by HAMLET in tumor cells. *PLoS ONE* 4(4), e5229 (2009).
53. Brest P, Gustafsson M, Mossberg AK *et al.* Histone deacetylase inhibitors promote the tumoricidal effect of HAMLET. *Cancer Res.* 67(23), 11327–11334 (2007).
54. Hallgren O, Gustafsson L, Irjala H, Selivanova G, Orrenius S, Svanborg C. HAMLET triggers apoptosis but tumor cell death is independent of caspases, Bcl-2 and p53. *Apoptosis* 11(2), 221–233 (2006).
55. Rammer P, Groth-Pedersen L, Kirkegaard T *et al.* BAMLET activates a lysosomal cell death program in cancer cells. *Mol. Cancer Ther.* 9(1), 24–32 (2010).
56. Aits S, Gustafsson L, Hallgren O *et al.* HAMLET (human α -lactalbumin made lethal to tumor cells) triggers autophagic tumor cell death. *Int. J. Cancer* 124(5), 1008–1019 (2009).
57. Baehrecke EH. Autophagy: dual roles in life and death? *Nat. Rev. Mol. Cell Biol.* 6(6), 505–510 (2005).
58. Debnath J, Baehrecke EH, Kroemer G. Does autophagy contribute to cell death? *Autophagy* 1(2), 66–74 (2005).
59. Codogno P, Meijer AJ. Autophagy and signaling: their role in cell survival and cell death. *Cell Death Differ.* 12(Suppl. 2), S1509–S1518 (2005).
60. Trulsson M, Yu H, Gisselsson L *et al.* HAMLET binding to α -actinin facilitates tumor cell detachment. *PLoS ONE* 6(3), e17179 (2011).
61. Menez J, Le Maux Chansac B, Dorothee G *et al.* Mutant α -actinin-4 promotes tumorigenicity and regulates cell motility of a human lung carcinoma. *Oncogene* 23(15), 2630–2639 (2004).
62. Gonzalez AM, Otey C, Edlund M, Jones JC. Interactions of a hemidesmosome component and actinin family members. *J. Cell Sci.* 114(Pt 23), 4197–4206 (2001).
63. Franzot G, Sjoblom B, Gautel M, Djinic Carugo K. The crystal structure of the actin binding domain from α -actinin in its closed conformation: structural insight into phospholipid regulation of α -actinin. *J. Mol. Biol.* 348(1), 151–165 (2005).
64. Otey CA, Vasquez GB, Burr ridge K, Erickson BW. Mapping of the α -actinin binding site within the β 1 integrin cytoplasmic domain. *J. Biol. Chem.* 268(28), 21193–21197 (1993).
65. Zaidel-Bar R, Itzkovitz S, Ma'ayan A, Iyengar R, Geiger B. Functional atlas of the integrin adhesome. *Nat. Cell Biol.* 9(8), 858–867 (2007).
66. Pettersson J, Mossberg AK, Svanborg C. α -lactalbumin species variation, HAMLET formation, and tumor cell death. *Biochem. Biophys. Res. Commun.* 345(1), 260–270 (2006).
67. Wilhelm K, Darinskas A, Noppe W *et al.* Protein oligomerization induced by oleic acid at the solid-liquid interface – equine lysozyme cytotoxic complexes. *FEBS J.* 276(15), 3975–3989 (2009).
68. Brinkmann CR, Heegaard CW, Petersen TE, Jensenius JC, Thiel S. The toxicity of bovine α -lactalbumin made lethal to tumor cells is highly dependent on oleic acid and induces killing in cancer cell lines and noncancer-derived primary cells. *FEBS J.* 278(11), 1955–1967 (2011).
69. Cury-Boaventura MF, Pompeia C, Curi R. Comparative toxicity of oleic acid and linoleic acid on Jurkat cells. *Clin. Nutr.* 23(4), 721–732 (2004).
70. Knyazeva EL, Grishchenko VM, Fadeev RS, Akatov VS, Permyakov SE, Permyakov EA. Who is Mr. HAMLET? Interaction of human α -lactalbumin with monomeric oleic acid. *Biochemistry* 47(49), 13127–13137 (2008).
71. Zhu Y, Schwarz S, Ahlemeyer B, Grzeschik S, Klumpp S, Krieglstein J. Oleic acid causes apoptosis and dephosphorylates Bad. *Neurochem. Int.* 46(2), 127–135 (2005).
72. Yang F Jr, Zhang M, Chen J, Liang Y. Structural changes of α -lactalbumin induced by low pH and oleic acid. *Biochim. Biophys. Acta* 1764(8), 1389–1396 (2006).
73. Liskova K, Kelly AL, O'Brien N, Brodtkorb A. Effect of denaturation of α -lactalbumin on the formation of BAMLET (bovine α -lactalbumin made lethal to tumor cells). *J. Agric. Food Chem.* 58(7), 4421–4427 (2010).
74. Liskova K, Auty MaE, Chaurin V *et al.* Cytotoxic complexes of sodium oleate with β -lactoglobulin. *Eur. J. Lipid. Sci. Tech.* 113(10), 1207–1218 (2011).
75. Mossberg AK, Hou Y, Svensson M, Holmqvist B, Svanborg C. HAMLET treatment delays bladder cancer development. *J. Urol.* 183(4), 1590–1597 (2010).
76. Rainov NG, Kramm CM. Vector delivery methods and targeting strategies for gene therapy of brain tumors. *Curr. Gene Ther.* 1(4), 367–383 (2001).
77. Laske DW, Youle RJ, Oldfield EH. Tumor regression with regional distribution of the targeted toxin TF-CRM107 in patients with malignant brain tumors. *Nat. Med.* 3(12), 1362–1368 (1997).
78. Mahesparan R, Tysnes BB, Read TA, Enger PO, Bjerkvig R, Lund-Johansen M. Extracellular matrix-induced cell migration from glioblastoma biopsy specimens *in vitro*. *Acta Neuropathol. (Berl.)* 97(3), 231–239 (1999).
79. de Villiers EM. Papillomavirus and HPV typing. *Clin. Dermatol.* 15(2), 199–206 (1997).

80. Bunney MH, Nolan MW, Williams DA. An assessment of methods of treating viral warts by comparative treatment trials based on a standard design. *Br. J. Dermatol.* 94(6), 667–679 (1976).
81. Mancuso JE, Abramow SP, Dimichino BR, Landsman MJ. Carbon dioxide laser management of plantar verruca: a 6-year follow-up survey. *J. Foot Surg.* 30(3), 238–243 (1991).
82. Stender IM, Na R, Fogh H, Gluud C, Wulf HC. Photodynamic therapy with 5-aminolaevulinic acid or placebo for recalcitrant foot and hand warts: randomised double-blind trial. *Lancet* 355(9208), 963–966 (2000).
83. Hursthouse MW. A controlled trial on the use of topical 5-fluorouracil on viral warts. *Br. J. Dermatol.* 92(1), 93–96 (1975).
84. Yilmaz E, Alpsoy E, Basaran E. Cimetidine therapy for warts: a placebo-controlled, double-blind study. *J. Am. Acad. Dermatol.* 34(6), 1005–1007 (1996).
85. Leman JA, Benton EC. Verrucas. Guidelines for management. *Am. J. Clin. Dermatol.* 1(3), 143–149 (2000).
86. Gibbs S, Harvey I, Sterling J, Stark R. Local treatments for cutaneous warts: systematic review. *BMJ* 325(7362), 461 (2002).
87. Harwood CA, Suretheran T, McGregor JM *et al.* Human papillomavirus infection and nonmelanoma skin cancer in immunosuppressed and immunocompetent individuals. *J. Med. Virol.* 61(3), 289–297 (2000).
88. Sengupta N, Siddiqui E, Mumtaz FH. Cancers of the bladder. *J. R. Soc. Health* 124(5), 228–229 (2004).
89. Hudson MA, Herr HW. Carcinoma *in situ* of the bladder. *J. Urol.* 153(3 Pt 1), 564–572 (1995).
90. Stein JP, Lieskovsky G, Cote R *et al.* Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. *J. Clin. Oncol.* 19(3), 666–675 (2001).
91. Hakansson A, Svensson M, Mossberg AK *et al.* A folding variant of α -lactalbumin with bactericidal activity against *Streptococcus pneumoniae*. *Mol. Microbiol.* 35(3), 589–600 (2000).
92. Hakansson AP, Roche-Hakansson H, Mossberg AK, Svanborg C. Apoptosis-like death in bacteria induced by HAMLET, a human milk lipid-protein complex. *PLoS ONE* 6(3), e17717 (2011).
93. Bosch FX, Manos MM, Munoz N *et al.* Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *J. Natl Cancer Inst.* 87(11), 796–802 (1995).