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Mini review

HAMLET, protein folding, and tumor cell death

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HAMLET (Human α-lactalbumin made lethal to tumor cells) was discovered by serendipity. The first publication in 1995 described the discovery and the unusual properties of what later became the HAMLET complex [1]. A fraction of human milk was able to kill tumor cells by a mechanism resembling apoptosis, and many different types of tumor cells were susceptible to this effect while healthy differentiated cells were resistant. The activity was shown to reside in a complex between the human milk protein α-lactalbumin and oleic acid, which is the most abundant fatty acid in human milk [2]. Since then, the HAMLET complex has been characterized in detail in order to determine the structural basis and mechanism(s) of the tumoricidal activity, and HAMLET has been used to treat tumors in animals and patients [3,4]. In 2006, researchers working on HAMLET and related topics met in Lund in Sweden for the First International HAMLET Symposium. This review summarizes the current state of knowledge in this area based on the presentations made at the conference.

Protein folding and the structural properties of HAMLET

Many unfolded proteins represent a threat to tissue homeostasis [5,6] and protein unfolding has often been associated with tissue destruction and disease. Misfolding accompanies normal protein synthesis, and a portion of newly synthesized peptides does not fold properly and must be removed by the proteasomes. Protein misfolding is also caused by mutations that permanently disturb the conformation, and such misfolded species accumulate in the tissues of patients with amyloid disorders [7a,b]. Alpha-lactalbumin is the major protein constituent of human milk. The three dimensional structure of this globular 14 kDa protein has been elucidated, revealing four α-helices, a triple-stranded β-sheet, and a Ca^{2+}-binding site [8,9]. The native protein serves as a co-enzyme in lactose synthesis, but does not cause tumor cell death. To become tumoricidal, the protein must undergo partial unfolding and bind the fatty acid cofactor (Fig. 1A), which allows α-lactalbumin to remain partially unfolded under physiological conditions (Fig. 1B). In the absence of the fatty acid the unfolded state is unstable, and at physiological solvent conditions the protein reverts to the native state. HAMLET exemplifies how a change in three-dimensional structure may allow a protein to alter its function in response to environmental signals. In addition, HAMLET is of potential interest as a model of unfolded protein cytotoxicity, particularly in view of its relative selectivity for tumor cells.

C.M. Dobson (University of Cambridge, UK) reviewed current knowledge of protein folding and amyloidogenesis. In 1998, Dobson and colleagues first discovered and characterized amyloid fibrils in proteins that do not have any association with disease, subsequently leading to his hypothesis about "the formation amyloid fibrils as a

Abbreviations: AFM, atomic force microscopy; ATP, adenosine triphosphate; BAMLET, bovine α-lactalbumin made lethal to tumor cells; CIDNP, chemically induced dynamic nuclear polarization; ER, endoplasmic reticulum; H/D, hydrogen/deuterium; HAMLET, human α-lactalbumin made lethal to tumor cells; HDAC, histone deacetylase; kDa, kilo Dalton; LAMPA, α-lactalbumin modified by poly-amino acid; MAL, multimeric α-lactalbumin; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PrP, prion protein; TEM, transmission electron microscopy; TUNEL, TdT-mediated dUTP nick end labelling.

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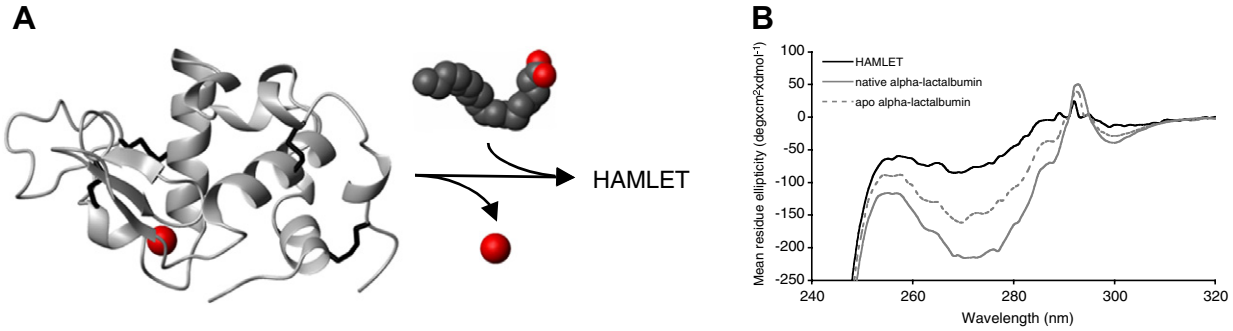

Fig. 1. HAMLET consists of partially unfolded α-lactalbumin and oleic acid. (A) HAMLET is formed by a two-step procedure. First, α-lactalbumin is partially unfolded by removing the calcium ion (red) with EDTA or acid. Second, oleic acid is bound to the protein and HAMLET is formed. Protein and oleic acid structure are from pdbID 1HML [51] and 1LID [52], respectively, and modified with MOLMOL [53]. (B) Native α-lactalbumin shows distinct signals in near UV CD spectroscopy, characterizing a well folded protein. The HAMLET spectrum shows a decrease in signal compared to the native and apo protein suggesting a partially unfolded state.

generic property of protein folding" [10,11]. Currently, more than 60 proteins have been shown to form amyloid-like fibrils by a variety of techniques. The simple principles extracted from aggregation rates for proteins based on extensive mutational analyses are now being implemented in *in vivo* studies, in which the expression of human amyloid β1-42 peptide variants in *Drosophila melanogaster* was shown to cause major behavioural changes (unpublished observation). Dobson also presented computational data from M. Vendruscolo et al., suggesting that highly accurate three-dimensional structures can be determined with sole information from heteronuclear chemical shifts. Obtaining high-resolution details of amyloid fibrils has been challenging, as protein aggregates in general do not usually form well-diffracting crystals. L.C. Serpell from the University of Sussex, UK described an elegant model system, where the atomic level structure of a designed 12-mer peptide has been solved with cryo-electron microscopy and electron and X-ray diffraction [12]. The 12-mer peptide sequence (KFFEAAAKKFFE) is notable for its high content of Phe side chains, and there is extensive π–π stacking of the aromatic rings for the purpose of inter-sheet stabilization (a "Phe zipper"). The results agree with the high occurrence of aromatic side chains in proteins involved in amyloidogenesis, such as PrP (the prion protein), Sup35p (yeast prion protein), amyloid β, human calcitonin, and others. Owing to these and many other significant developments, the field is now at the point of actively designing drug candidates to intervene with the formation of pre-fibrils and mature fibrils *in vivo*.

The formation of HAMLET from its constituent components (calcium-depleted α-lactalbumin and oleic acid) through chromatographic methods has always intrigued workers in this area. HAMLET is formed during ion-exchange chromatography, when the unfolded protein interacts with a matrix, preconditioned with oleic acid, and is eluted with high salt [2,13,14]. The structural prerequisites for complex formation were discussed by A.-K. Mossberg and J. Pettersson (Lund University, Sweden). An experiment using an α-lactalbumin mutant showed that unfolding of the protein alone does not cause cytotoxicity; rather, it was the resulting three-dimensional structure of the protein that was responsible for the activity. The calcium binding site mutant was stable in a molten globule like state but was not cytotoxic, unless in complex with oleic acid [15]. When a large range of different fatty acids were tested for the generation of the active complex, it was found that fatty acid stereospecificity may be a significant factor in the conversion of HAMLET and for its biological activity [13].

A structural description of HAMLET and BAMLET (the bovine α-lactalbumin complex analogue) was presented by K.H. Mok (University of Oxford, UK, and presently Trinity College, Dublin, Ireland). By probing the various classical molten globular forms of α-lactalbumin using a battery of biophysical techniques including NMR, TEM (transmission electron microscopy), AFM (atomic force microscopy), and pulse-labelled CIDNP (chemically-induced dynamic nuclear polarization) NMR [16], it was shown that the partially denatured form of the protein could be created and stabilized by many different combinations of internalized amino acid side chains, and that the hydrophobic cores of HAMLET and BAMLET appear to be distinct from these classical forms. In addition, diffusion NMR and small angle X-ray scattering results were presented to characterize the hydrodynamic properties, and Mok showed fascinating AFM images of the surface-deposited complex, suggesting, perhaps, a new structural paradigm where protein is encapsulating lipid molecules and not *vice versa*—as would be the case for micelles or vesicles. These structural features may account for the *in vivo* activity of the complex.

The conformational differences between HAMLET, apo-α-lactalbumin, and holo-(native)-α-lactalbumin have also been characterized by mass spectrometry, H/D exchange, and limited proteolysis by L. Birolo (University of Naples, Italy) [17]. Whereas the number of exchanged deuterated amide NH-sites was greater in HAMLET than in the apo-protein, analysis of proteolytic fragments suggested that the oleic acid moiety may be altering the con-

formation in different and subtle ways unseen in the calcium-depleted, molten globule form, rendering the β-domain in HAMLET relatively less susceptible to proteolytic enzymes. The results suggested that the oleic acid binding site may reside in the β-domain, and that HAMLET may experience protein breathing on a time scale consistent with H/D exchange, but much faster than that of the time scale of proteolytic enzyme-substrate recognition.

L.A. Morozova-Roche (Umeå University, Sweden) presented for the first time HAMLET-like cytotoxic properties from a protein other than α-lactalbumin, in this case a complex prepared from equine lysozyme and oleic acid. Based on her extensive studies on hen and equine lysozyme protein oligomers, Morozova-Roche was able to show that cytotoxic effects appeared only when oligomers reached the size of octamers (and up to 20-mers) and developed cross-β-sheet structure [18]. On the other hand, non-cross-β-sheet protein oligomers were found to be non-toxic. This was found to be true even with a highly soluble, highly charged *de novo* designed protein called albebetin, where there appeared to be multiple pathways of amyloid assembly [19]. In addition, it was suggested that the presence of a core of secondary structure, as found in the cross-β-sheets, may be important in stabilizing the oligomers and preventing them from dissociation upon penetration through the cell membrane.

Prior to the purification and identification of HAMLET as the bi-molecular species with cell death-inducing activity [2], the early ion-exchange chromatographic fractionations of casein produced an active fraction called MAL (multimeric α-lactalbumin), which consisted of monomeric and oligomeric components of HAMLET [1]. B. Spolaore (University of Padua, Italy) was able to show that MAL could be generated from physical mixing of purified protein and oleic acid—not necessarily requiring the aforementioned chromatographic methods—and presented experimental results on its physical properties through utilization of chemical cross-linking, dynamic light scattering, and limited proteolysis. In particular, limited proteolysis experiments identified highly protected regions of the protein, namely in helices A, B, D, and portions of helix C of α-lactalbumin, suggesting that these regions may be interacting strongly with oleic acid.

The malleability of α-lactalbumin to transform into alternative molten globule-like states when interacting with other biomolecules was further discussed by E.A. Permyakov (Russian Academy of Sciences, Pushchino, Russia), who together with L.J. Berliner (University of Denver, USA), showed that co-incubation of monomeric α-lactalbumin with poly-Lys and poly-Arg generated a molten-globule-like structure with substantially decreased affinity for calcium ions [20]. Permyakov and Berliner named this state LAMPA (α-lactalbumin modified by poly-amino acid) and described its properties using fluorescence, circular dichroism, and differential scanning calorimetry. Permyakov, Berliner, and colleagues have also previously shown that oleic acid was not necessary in the α-lactalbumin binding of histones HI, HIIB, and HIII, where the binding process is driven by electrostatic interactions with a stoichiometry of four [21]. Hence α-lactalbumin appears to be potentially useful for disorganizing chromatin, while Permyakov and colleagues consider the presence of oleic acid in HAMLET as more important for the complex's transport into tumor cells.

Mechanisms of tumor cell death

Like non-malignant cells, tumor cells can undergo various types of cell death, including apoptosis, necrosis, autophagic cell death, and mitotic catastrophe. However, ionizing radiation and most chemotherapeutic agents kill tumor cells by apoptosis, which most often is triggered by activation of the mitochondrial signalling pathway, leading to caspase activation, cleavage of cellular proteins, cell death, and phagocytosis. To avoid death, tumor cells have developed various mechanisms of resistance, including gene amplification, deletions and mutations. Hence, evasion of apoptosis is regarded as one of the major characteristics of malignant growth. The mechanisms of apoptotic cell death were reviewed by S. Orrenius (Karolinska Institute, Stockholm, Sweden) [22–24].

HAMLET represents a new type of tumoricidal molecule. It can activate cell death pathways in tumor cells, which might be resistant to both chemotherapy and ionizing radiation. In addition, HAMLET appears to trigger a similar death response in tumor cells of very different origins, while healthy, differentiated cells are resistant. Hence, it is important to identify the mechanism(s) responsible for HAMLET-induced cell death, as such information may help design more specific tumor therapies in the future.

The current knowledge about HAMLET and tumor cell death was reviewed by C. Svanborg (Lund University, Sweden), who proposed the Lernaean Hydra from Greek mythology as a metaphor for HAMLET (Fig. 2A). This serpent-like animal was said to have used its many heads to attack intruders and hence was known to be virtually impossible to destroy, as new heads would emerge when one was cut off. HAMLET resembles an animal with many heads, as it attacks tumor cells by direct invasion and interacts independently with several critical organelles (Fig. 2B). Hence, the lethal effect is not due to a single surface receptor, or signal transduction pathway, but rather to a multifacetted attack on the tumor cell integrity. Healthy cells survive, either because they are not properly attacked by the Hydra, or because they respond like the Greek hero Hercules, who succeeded to cut off all the heads.

Membrane interactions

HAMLET starts the attack on tumor cells by binding to the cell surface, and thereafter rapidly invades the tumor cell. The mechanism is not fully understood, but invasion requires both the unfolding of α-lactalbumin and the presence of the fatty acid. The native protein does not invade

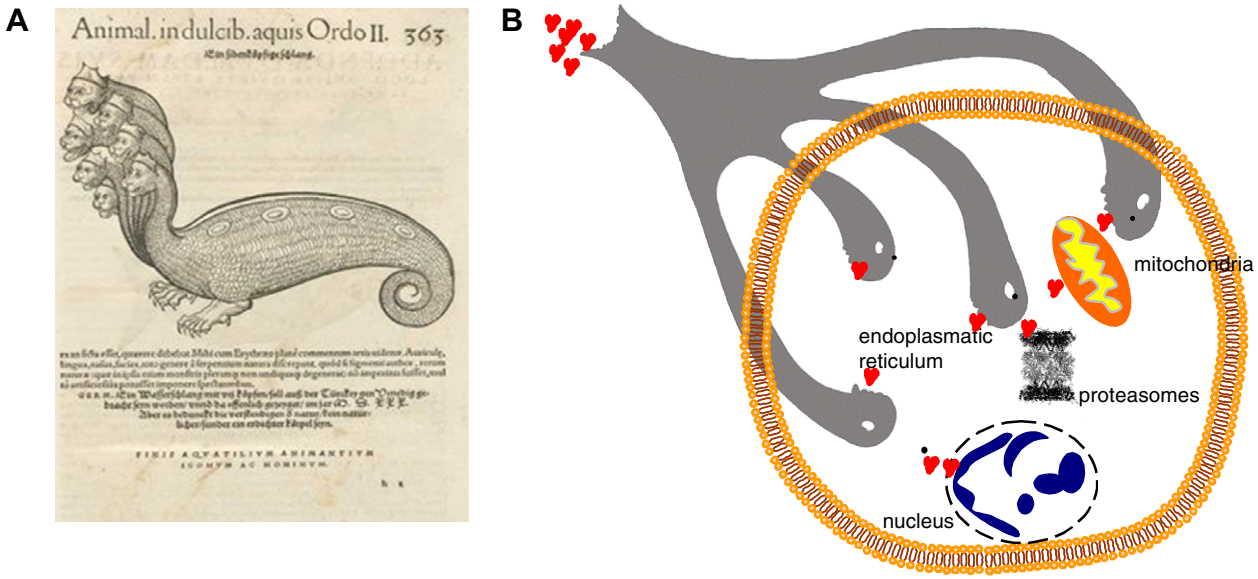

Fig. 2. HAMLET—the biological Hydra. (A) The Lernaean Hydra is proposed as a metaphor for HAMLET. The mythological animal was said to use its many heads to attack enemies. If one head was cut off, new heads would soon emerge. The antique depiction showing the Hydra was by a 16th-century German illustrator (www.wikipedia.org, origin unknown). (B) So far, HAMLET has been shown to have several targets in the tumor cells: (i) the mitochondria, (ii) the proteasomes, (iii) the endoplasmatic reticulum and (iv) the histones in the cell nuclei. Image drawn by Lotta Gustafsson.

cells efficiently, nor kills them; neither does stably unfolded α-lactalbumin mutants. The uptake of HAMLET by tumor cells is activated by the fatty acid and unfolded protein in combination. Invasion by HAMLET is likely to be an important determinant of cell sensitivity, as large amounts of the complex invade tumor cells whereas differentiated cells take up only small amounts of the complex. The interaction between phospholipid membranes and α-lactalbumin has been studied at the University of Bergen, Norway and was discussed by Ø. Halskau. Bovine α-lactalbumin binds to negatively charged lipid vesicles in a pH-dependent manner, as observed with fluorescent methods and H/D exchange NMR [25–27]. Particularly with NMR, Halskau and colleagues were able to show that the amide NH-exchange patterns were in general similar to the molten globule state, but in the regions of helices C and A the protection of certain backbone amides was even greater than that of the native state. Furthermore, the protection patterns were found to differ depending on the lipids, suggesting that membrane fluidity may be important for the interaction of α-lactalbumin with membranes. Within the context of HAMLET's putative interaction with cancer cells, Halskau hypothesized that the role of the bound oleic acid is to stabilize the protein in a conformation suitable for interacting with membranes, and that the abnormal amount of phosphatidylserine, or the *O*-glycosylated mucine in the outer layer of the cell membrane, may be sufficient to allow HAMLET to preferentially bind to the membranes of cancer cells.

Apoptosis

Early studies revealed apoptotic features in tumor cells that die after treatment with HAMLET. Mitochondrial damage and cytochrome *c* release were detected in both intact tumor cells and isolated mitochondria, and there was a weak caspase response, including activation of effector caspases-3 and -9, and of the DNA damage-related, nuclear caspase-2 [28,29]. The apoptotic response was not the cause of cell death, however, as caspase inhibitors did not rescue the cells from dying. Further, there was no protective effect of Bcl-2 overexpression, and the sensitivity of tumor cells to HAMLET was independent of *p*53 [29].

The mitochondria are only one of several targets for HAMLET in tumor cells. Additional targets include the proteasomes, which normally degrade endogenous unfolded or ubiquitinylated proteins [30–32]. The effect of HAMLET on proteasomes and the involvement of proteasomes in cell death were discussed by L. Gustafsson (Lund University, Sweden). The invasion by HAMLET exposes the proteasomes to large quantities of unfolded protein, leading to activation of the 20s proteasomes [33]. The degradation of HAMLET by proteasomal enzymes is inefficient compared to that of the unfolded protein alone, however, and the elimination of HAMLET from the cell interior is slow. We have observed that activated proteasomes change their structure in a manner suggesting degradation of structural and catalytic subunits, and that a reduction in proteasomal activity occurs in response to HAMLET. To our knowledge, this type of proteasome response has not previously been reported. However, inhibition of proteasome activity is not responsible for the cytotoxic effect of HAMLET, since traditional proteasome inhibitors reduce, rather than potentiate, HAMLET toxicity. Finally, HAMLET triggers also other features of an unfolded protein response, with classical signs of ER stress.

HAMLET also interacts with tumor cell nuclei. Upon exposure of tumor cells to the LD50 concentration of

HAMLET, the bulk of the complex is found within the nuclei after about one hour. This suggests a rapid translocation process and passage of HAMLET across the nuclear membrane. In the nuclei, HAMLET binds with high affinity to histones H3 and H4, and with lower affinity to histones H2a and H2b [34]. HAMLET is also able to bind intact nucleosomes with very high affinity, causing the formation of virtually insoluble chromatin complexes in the nuclei of tumor cells. As a consequence, transcription is impaired and cell death becomes irreversible. The combined effect of HAMLET and HDAC (histone deacetylase) inhibitors [35] was discussed by P. Brest (University of Nice, France and Lund University, Sweden). He showed that HDAC inhibitors increased the cell death response to HAMLET in a dose- and time-dependent manner. As indicated by flow-cytometry, there was an increase in the sub-G1 population, and a decrease in cell viability was seen, using ATP levels and trypan blue exclusion as end points. HAMLET was also shown to increase histone acetylation when combined with HDAC inhibitors, but not alone. The HDAC inhibitors had no effect on the overall chromatin structure, but HAMLET caused chromatin condensation with shrinkage of the nuclei. The HAMLET-induced DNA damage was associated with an increase in the expression of DNA damage sensor proteins, such as p53, p21waf1, and gadd153. These results suggest that HDAC inhibitors potentiate the cytotoxic effects of HAMLET, including chromatin shrinkage, DNA damage and DNA fragmentation.

HAMLET induces macroautophagy in tumor cells, and this appears to be partly responsible for HAMLET-induced cell death. This was discussed by S. Aits (Lund University, Sweden). Autophagy is a cellular process used for the degradation of long-lived cytosolic proteins and organelles [36]. During macroautophagy, portions of the cytoplasm and organelles are enwrapped in membrane sacs, forming double-membrane-enclosed vesicles, termed autophagosomes, which are detectable by electron microscopy. Autophagosomes subsequently fuse with lysosomes, and lysosomal enzymes degrade their contents for reutilization [37]. Macroautophagy occurs at basal levels in most cells, but it is increased in response to cellular stress such as starvation [38]. It also plays a role in development and differentiation [38,39] and in immune defense [40]. In addition, it has been proposed that macroautophagy is involved in a non-apoptotic form of programmed cell death, called autophagic cell death or type II cell death. However, the exact role of macroautophagy in cell death is still a matter of intense debate [41,42]. Electron microscopy of HAMLET-treated cells has revealed extensive cytoplasmic vacuolisation, damaged mitochondria and double-membranes, suggestive of macroautophagy. Furthermore, inhibition of macroautophagy by RNA interference against Beclin-1, which is involved in autophagosome formation, protected cells from loss of viability in response to HAMLET. The results suggest that HAMLET triggers macroautophagy, and that this response might contribute to cell death.

M. Stefani (University of Florence, Italy) guided the audience into his extensive and fascinating research field of cell surface interactions with aggregated proteins, particularly with regards to the HypF N-terminal domain, Ure2p and amyloid β peptides [43–46]. Amyloid aggregates of these proteins were found capable of permeabilizing the membrane, resulting in the increase of free calcium, oxidative stress, and mitochondrial dysfunction, culminating into apoptotic, or sometimes necrotic cell death. In addition, there appeared to be cell culture-specific differences in the vulnerability towards the protein aggregates, as best demonstrated by the current mystery into why some neuronal cell types are killed by amyloid β, whereas other exposed cells remain perfectly healthy. Stefani also showed some of his group's work into testing different phases of the cell cycle against protein aggregates, and hypothesized that S-phase cells may be most vulnerable because of the significantly reduced content in membrane cholesterol. His insightful contrast between the highly specific and functional roles possessed by a native protein and the generic cytotoxic effects possessed in the hidden, dark world of aggregate proteins [47] was a very suitable characterization of what all protein chemists are witnessing today.

In vivo effects of HAMLET in tumor cell models

Three types of *in vivo* models have been employed to investigate if HAMLET can be used to treat tumors *in vivo*: (a) human glioblastoma xenografts in nude rats, (b) topical treatment of skin papillomas in patients, and (c) intravesical inoculation of HAMLET in patients with bladder cancer.

The results from the human glioblastoma xenograft model and the possibilities for the future treatment of malignant brain tumors with HAMLET were discussed by W. Fischer (University Hospital of Bergen, Norway) [4]. Malignant brain tumors represent a major therapeutic challenge in that no selective or efficient treatment is available. Intra-tumoral administration of HAMLET prolongs survival in rats with human glioblastomas, however. Invasively growing human glioblastomas were established in nude rats by xeno-transplantation of human biopsy spheroids [48], and the therapeutic effect of HAMLET was compared with the folded, native protein. Intra-cerebral, convection-enhanced delivery of HAMLET dramatically reduced the intra-cranial tumor volume and delayed the onset of pressure symptoms in the tumor bearing rats. HAMLET triggered apoptosis in the tumor, but failed to induce apoptosis in adjacent healthy brain tissue. Neither did it cause toxic side effects after infusion of therapeutic concentrations into the brains of healthy rats. The results identify HAMLET as a potential new tool in cancer therapy, and suggest that HAMLET should be further explored as a novel approach to controlling glioblastoma progression.

The results of HAMLET treatment in patients with skin papillomas were summarized by L. Gustafsson (Lund Uni-

versity, Sweden) [3]. Forty-two patients were enrolled in a placebo-controlled, double-blind study. The majority of the patients were resistant to conventional therapy. Either HAMLET or placebo was applied to the papillomas topically for three weeks. Within a month after the completion of treatment, the volume of the papillomas had decreased by $\geqslant 75\%$ in the HAMLET-treated group (20/20 patients, 88/92 papillomas), whereas in the placebo group a similar effect appeared in only 3/20 patients, or 15/74 papillomas ($p < 0.001$). After HAMLET treatment of the placebo group, an 82% reduction in papilloma volume was recorded. Complete resolution of all the papillomas occurred in 83% (29/35) of the HAMLET-treated patients. The time to resolution was shorter in the group that had received HAMLET from the start as compared to the group that had received placebo from the start (1.8 vs. 6.6 months). No adverse reactions were recorded, and there was no difference in outcome for those patients who were immuno-suppressed. It was concluded that HAMLET has a therapeutic potential for papillomas.

The response of bladder cancers to intra-vesical HAMLET instillations was discussed by B. Wullt (Lund University Hospital, Sweden). The results showed that HAMLET exerts a direct and selective effect on bladder cancer tissue *in vivo*. Nine patients with superficial transitional cell carcinomas received five daily intra-vesical instillations of HAMLET (1.7 mM) during the week before scheduled surgery. Controls received α-lactalbumin, PBS or NaCl. HAMLET stimulated rapid shedding of tumour cells and aggregates thereof into the urine daily, during the five days of instillation. The effect was specific, as NaCl, PBS or native α-lactalbumin did not cause cell shedding. A reduction in tumor size, or change in tumour character, was detected by endoscopic photography in 8/9 patients. Most of the shed cells were dead, as defined by the trypan blue exclusion test, and there was no difference relating to the type of bladder cancer. An apoptotic response in shed cells was detected by the TUNEL assay in 6/9 patients, and in sections of their remaining tumors. Benign adjacent tissue biopsies from six patients showed no evidence of apoptosis and no toxic response. Local HAMLET administration might thus be of value in the future treatment of bladder cancers.

Why has a molecule like HAMLET evolved and for what purpose?

Alpha-lactalbumin is the most abundant protein in human milk, and oleic acid is the most abundant fatty acid. HAMLET is not present in newly expressed milk, however, as α-lactalbumin is in the native state and the fatty acids are bound in triglycerides. It is not known whether HAMLET is formed *in vivo*, but it may be argued that this is likely to occur since the acidic conditions in the stomach are favourable for HAMLET formation. Low pH is known to cause α-lactalbumin to partially unfold, due to the release of the strongly bound calcium ion [9]. A pH sensitive lipase hydrolyzes milk triglycerides, releasing oleic acid [49]. The components needed to form HAMLET are thus present in the stomach of breast-fed babies, and it is tempting to speculate that the complex may be formed there. The gastrointestinal tract of the newborn individual undergoes very rapid maturation, and it is possible that there is a risk for cells to de-differentiate and form tumor progenitor cells. The presence of a substance like HAMLET might help by removing these cells, and such a mechanism would be of obvious benefit to the organism. It is also interesting that the gut-associated lymphoid tissue develops after birth and in response to microbial challenge. Case control studies show that breast-fed children have a reduced frequency of lymphoid malignancies, suggesting that substances in milk may aid to protect against tumor development [50]. A local effect of HAMLET on lymphoid cells in the gut might be useful in this regard. Finally, the papilloma studies suggest that HAMLET is active against virus-infected cells. A locally active substance, like HAMLET, might aid in limiting the viral load in the gut of the newborn child, by removing rapidly proliferating, virus-infected cells. However, these speculations need to be addressed experimentally.

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