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Research Article

HUMAN MILK: EXCELLENT ANTICANCER ALTERNATIVE

Shashi Singh*, Avinash Suryawanshi, YM Joshi, Dnyanesh Limaye and Vilasrao Kadam

Department of Pharmacology, Bharati Vidyapeeth's College of Pharmacy, Sector-8, C.B.D. Belapur, Navi Mumbai, India.

ABSTRACT

In human milk α-lactalbumin is one of the main proteins which harbors tumor-selective capabilities, named human α-lactalbumin made lethal to tumor cells (HAMLET). HAMLET is formed by binding of α-lactalbumin with oleic acid resulting in release of its Ca2+ ion and a folding change.

It induces apoptosis in tumor cells but leaves normal differentiated cells unaffected. It has the capability to enter efficiently tumor cells and to accumulate in their nuclei, mitochondria, endoplasmic reticulum and proteasomes. HAMLET activates the caspase pathways by release of cytochrome c. In the tumor nuclei, HAMLET associates with histones resulting in an irreversible disruption of the chromatin organization which is crucial for its tumor-selective apoptosis induction. It also activates 20S proteasomes, which also contributes to cell death. HAMLET induces tumor-selective apoptosis in a p53 independent manner. HAMLET kills a wide range of malignant cells in vitro and maintains this activity in vivo in patients with skin papillomas. In addition, HAMLET has striking effects on human glioblastomas in a rat xenograft model. HAMLET thus shows great promise as a new therapeutic with the advantage of selectivity for tumor cells and lack of toxicity.

Keywords: Human milk; α- lactalbumin; Structure of hamlet; Cellular targets in tumor cells.

INTRODUCTION

In human milk α-lactalbumin is one of the main proteins. A structural derivative of α-lactalbumin harbors tumor-selective capabilities, named human α-lactalbumin made lethal to tumor cells (HAMLET)[1].

HAMLET (human α lactalbumin made lethal to tumor cells) is a molecular complex of α -lactalbumin and oleic acid[2,3].(**Fig.1**). Conversion of isolated α-lactalbumin to HAMLET is achieved due to binding with oleic acid resulting in release of its $Ca2^+$ ion and a folding change[4].

The activity of HAMLET was discovered by serendipity, while using human milk fractions to investigate bacteria adherence to lung carcinoma cell lines. In addition to blocking adherence, one milk fraction actually killed the cells by inducing apoptosis [1]. Two clinical trials have been carried out successfully; HAMLET was proven to be active against skin papillomas and bladder cancers, whereas no side effects to adjacent healthy tissue could be observed[5]. Almost all treated skin papillomas disappeared after HAMLET treatment. Intravesical HAMLET delivery resulted in a significant reduction in bladder tumor in 8 out of 9 treated patients. These first clinical results show that local HAMLET might be of huge value in the future treatment of cancers. The first publication in 1995 described the discovery and the unusual properties of what later became the HAMLET complex[6]. 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[7, 8]. In 2006, researchers working on HAMLET and

related topics met in Lund in Sweden for the First International HAMLET Symposium.
It induces apoptosis in tumor cells, but normal differentiated cells are resistant to its effect[9]. Cell death was accompanied by changes in morphology, nuclear condensation, cytoplasmic blebbing, and formation of apoptotic bodies, similar to cells that undergo classical apoptosis according to Kerr et al.[10]. HAMLET has several unique features. HAMLET kills tumor cells and immature cells but not normal differentiated cells. HAMLET is active against a broad range of tumor cell lines. Because HAMLET kills by an apoptosis-like mechanism, it is unlikely to provoke harmful side effects. HAMLET may contribute to the natural defense against cancer[11].
Three main aspects of the HAMLET project are being investigated:

1. The HAMLET structure and the molecular basis of the novel function.
2. The cellular targets of HAMLET in tumor cells.
3. The in vivo effects of HAMLET on experimental and established tumors.

1. HAMLET
structural aspects

α lactalbumin is the major protein constituent of human milk. The three dimensional structure of this globular 14 kDa proteins has been elucidated, revealing four a-helices, a triple-stranded b-sheet, and a $Ca2^{+}$-binding site[12, 13]. 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. 2A), which allows α -lactalbumin to remain partially unfolded under physiological conditions (Fig. 2B). 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[14].

Structural differences between HAMLET and native α -Lactalbumin

NMR spectroscopic studies of isotope labeled samples (the ligand and/or the protein is labeled) are used to elucidate the structural differences between α -lactalbumin and HAMLET.

HAMLET consists of partially unfolded α-lactalbumin and oleic acid.
(A) HAMLET is formed by a two-step procedure.
1. First, α-lactalbumin is partially unfolded by removing the calcium ion (red) with EDTA or acid.
2. Second, oleic acid is bound to the protein and HAMLET is formed.
(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.(Fig2)The formation of HAMLET from its constituent component (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. An experiment using a 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. When a large range of different fatty acids were tested for the generation of the active complex, it was found that fatty acid stereo specificity may be a significant factor in the conversion of HAMLET and for its biological activity.

α- Lactalbumin can be converted to an apoptosis-inducing complex only in the presence of a lipid cofactor

The complex is formed from pure components (α-lactalbumin and oleic acid), each of which is inactive in the apoptosis assays. The folding change and the lipid cofactor were both necessary to attain this new function. The specificity of the lipid cofactor was investigated using fatty acids differing in carbon-chain length, saturation, or *cis/trans* conformation. We identified unsaturated C18 fatty acids in the *cis* conformation as the cofactors that interact with partially unfolded α -lactalbumin and form HAMLET. The interaction between protein and fatty acid was specific, because saturated C18 fatty acids or unsaturated C18:1 *Trans* conformers were unable to form complexes with partially unfolded α-lactalbumin, as were fatty acids with shorter or longer carbon chains. Unsaturated *cis* fatty acids other than C18:1, 9 *cis* were able to form

stable complexes, but these were not active in the apoptosis assay [15].

Partially unfolded α -lactalbumin does not induce apoptosis in the absence of the lipid cofactor

Mutations in the $Ca2^+$ binding site of bovine α -lactalbumin were used to create a α-lactalbumin conformer that maintained the unfolded conformation at physiologic pH and in the presence of $Ca2^+$. A point mutation at position D87A inactivated the $Ca2^+$binding site and caused a change in tertiary structure, locking the protein in the partially unfolded conformation [16]. This mutant did not induce apoptosis, however, demonstrating that a conformational change in α -lactalbumin is not sufficient to trigger apoptosis. The mutant bovine protein could still be converted to a HAMLET-like complex in the presence of oleic acid, demonstrating that the biological properties of HAMLET are defined both by the protein and the lipid cofactor. Furthermore, the activity of the converted mutant protein demonstrated that a functional calcium-binding site is not required for the apoptotic function of HAMLET [17].

In conclusion, the α-lactalbumin structure can be adjusted by shifting environments and functional diversity can be created by changes in tertiary structure. Also, lipid cofactors enable proteins to adopt stable novel conformations and thus to act as partners in protein folding. In this way, a single polypeptide chain can vary its structure and function, thereby participating in different biological processes in distinct environments.

II. Cellular targets of HAMLET in tumor cells

HAMLET has unique biological properties, because it selectively purges malignant cells by an apoptosis-like mechanism but leaves normal cells unharmed [2,9]. This suggests that HAMLET bypasses the different blocks of apoptosis in many tumor cells and that HAMLET activates other cell death pathways that remain active in tumor cells.

Cellular trafficking of HAMLET

The subcellular localization of HAMLET is a potential key to distinguish the cellular responses of sensitive tumor cells from responses by the resistant normal cells. The trafficking of HAMLET in tumor cells and normal differentiated cells was compared by confocal microscopy. The availability of surface receptors is not the limiting step, nor the critical factor determining sensitivity, because both cell types showed rapid surface binding of HAMLET. Translocation of HAMLET to the cytoplasm was detected in both cell types but with different efficiency. Large amounts of HAMLET reached the cytoplasm of the tumor cells and formed cytoplasmic aggregates. Uptake was not blocked by cycloheximide, showing that this step does not require protein synthesis. There was some cytoplasmic accumulation of HAMLET in normal cells. These observations suggested that the translocation into the cytoplasm per se does not distinguish the more sensitive from the less sensitive cells but that massive cytoplasmic accumulation of HAMLET characterizes the tumor cells.

The subsequent redistribution of HAMLET from the cytoplasm to the perinuclear region occurred only in the tumor cells. Despite the entry of HAMLET into the cytoplasm of normal cells, no trafficking to the perinuclear region was observed. In tumor cells, this effect was abrogated by cycloheximide, demonstrating that the perinuclear translocation of HAMLET required cellular metabolism. The translocation to the perinuclear region was accompanied by the movement of mitochondria, as shown by co-staining with mitochondria specific markers. Finally, HAMLET was shown to accumulate in tumor-cell nuclei and the apoptotic bodies stained positive for HAMLET[18].

HAMLET interacts with histones and chromatin in tumor cell nuclei

The accumulation of HAMLET in tumor cell nuclei encouraged us to identify molecular targets for HAMLET. The initial studies, using crude cellular fractions, showed that HAMLET binds to histone H3 in nuclear fractions from tumor cells. Using purified histones, HAMLET was shown to interact with histones H2B, H3, and H4. To fold properly, histones need to be present as dimers of H2A–H2B and tetramers of H3–H4. Such natively folded and biologically functional histones were purified from cells and were used to further study the interactions with HAMLET. In affinity chromatography, HAMLET bound all 4 histones, and Biacore assays showed a high affinity binding with very slow dissociation.

Mixing histones with HAMLET in solution resulted in precipitation of the proteins, further illustrating the high affinity of the interaction. Both denatured and native histones were precipitated by HAMLET, with a preference for H3 and H4. The relevance of these interactions in vivo was demonstrated in HeLa tumor cells expressing GFP-tagged histones. HAMLET colocalized with histones in cell nuclei and induced changes in the global chromatin structure. The chromatin was

condensed to the nuclear periphery or to large, spherical structures. HAMLET was present in both of these chromatin patterns [19].

HAMLET interacts with all structural and functional conformations of histones, from denatured proteins to natively folded, soluble histones and histones in nucleosomes. This suggests a number of potential functional consequences for the cell. When HAMLET enters the tumor cell, it may interact with newly synthesized histones, compete with chaperones for the histones, and prevent their transport to the nucleus. This would inhibit the chromatin assembly machinery of histones and induce chromatin damage. In the nucleus, HAMLET could bind histones in chromatin and either removes them from DNA or directly binds nucleosomes and impairs their function. Alternatively, the binding of HAMLET to chromatin could induce DNA damage. It has been observed that defects in chromatin assembly can lead to double-strand DNA breaks and activation of the S-phase checkpoint [20]. However, it is not clear how HAMLET damages the DNA.

In conclusion, HAMLET binds to histones in the nuclei of tumor cells dying after HAMLET treatment. This interaction may disturb the structure and function of the chromatin and could be an important feature of HAMLET-induced cell death.

HAMLET-induced cell death is independent of p53.

The strong interaction between HAMLET and histones in tumor cell nuclei suggest that the nuclear effects of HAMLET may be the trigger of cell death. The *p53* tumor suppressor serves as a guardian of DNA integrity, and *p53*-dependent cell death mechanisms are activated after irreparable DNA damage [21, 22]. We therefore investigated HAMLET sensitivity of tumor cells as a function of their *p53* status. Surprisingly, there was no difference between cells with mutated or wild type *p53*, suggesting that HAMLET-induced cell death does not require p53 activity.

HAMLET interacts mitochondria and the caspase cascade.

HAMLET interacts with mitochondria, as shown by colocalization in living cells and by studies of isolated mitochondria. Furthermore, HAMLET triggers membrane depolarization, release of cytochrome C and activates pro-apoptotic caspases [23, 24]. However, HAMLET-induced cell death does not rely on caspases, as the pan-caspase inhibitor ZVAD did not prevent cell death[9]. HAMLET-induced cell death differs from most classical apoptotic systems in that caspase inhibitors do not rescue cells. We conclude from these and other studies that HAMLET induces apoptosis-like death by a novel mechanism involving trafficking to the perinuclear region and translocation to the cell nuclei. The nuclear accumulation of HAMLET disrupts the chromatin and marks the irreversible stage of tumor cell apoptosis. In parallel, HAMLET activates known effectors of apoptosis, including the caspase cascade.

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[25-27].

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. 3A). 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. 3B). Hence, the lethal effect is not due to a single surface receptor, or signal transduction pathway, but rather to a multifaceted 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 a-lactalbumin and the presence of the fatty acid. The native protein does not invade cells efficiently, nor kills them; neither does stably unfolded a-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 a-lactalbumin. Bovine a-lactalbumin binds to negatively charged lipid vesicles in a pH-dependent manner, as observed with fluorescent methods[28-30]. 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 a-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.

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 [31, 32]. The apoptotic response was not the cause of cell death, however, as caspase inhibitors did not rescue the cells from dying[32].The mitochondria are only one of several targets for HAMLET in tumor cells. The effect of HAMLET on proteasomes and the involvement of proteasomes in cell death[33-35].

The invasion by HAMLET exposes the proteasomes to large quantities of unfolded protein, leading to activation of the 20s proteasomes [36]. 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. 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.

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 [37]. 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[38]. He showed that HDAC inhibitors increased the cell death response to HAMLET in a dose- and time-dependent manner. 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.

Autophagy is a cellular process used for the degradation of long-lived cytosolic proteins and organelles [39]. 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[40]. Macroautophagy occurs at basal levels in most cells, but it is increased in response to cellular stress such as starvation [41]. It also plays a role in development and differentiation [41, 42] and in immune defense [43]. 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 [44, 45]. 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.

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)[8]. 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 [46], 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 University, Sweden) [7]. 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 P75% 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 a-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 a-lactalbumin did not cause cell shedding. A reduction in tumor size, or change in tumor 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 6/9SSS 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.

CONCLUSION

HAMLET (Human α-lactalbumin made lethal to tumor cells) was discovered by serendipity. 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 favorable for HAMLET formation. Low pH is known to cause α-lactalbumin to partially unfold, due to the release of the strongly bound calcium ion. A pH sensitive lipase hydrolyzes milk triglycerides, releasing oleic acid. 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. 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. A fraction of human milk was able to kill tumor cells were susceptible to this effect while healthy differentiated cells were resistant. Since then, the HAMLET complex has been characterized in detail in order to determine the structural basis and mechanisms of the tumoricidal activity and HAMLET has been used to treat tumors in animals and patients.

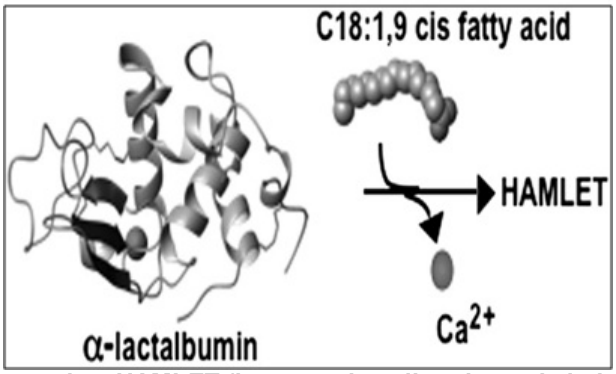

Fig. 1: The HAMLET complex. HAMLET (human α-lactalbumin made lethal to tumor cells) is a molecular complex of α-lactalbumin and oleic acid (C18:1, 9 *cis*) from human milk. Native α-lactalbumin can be converted to HAMLET by treatment with EDTA, which removes $Ca2^{+}$ and by the addition of the fatty acid C18:1, 9 *cis*. The figure is based on the α-lactalbumin crystal structure.

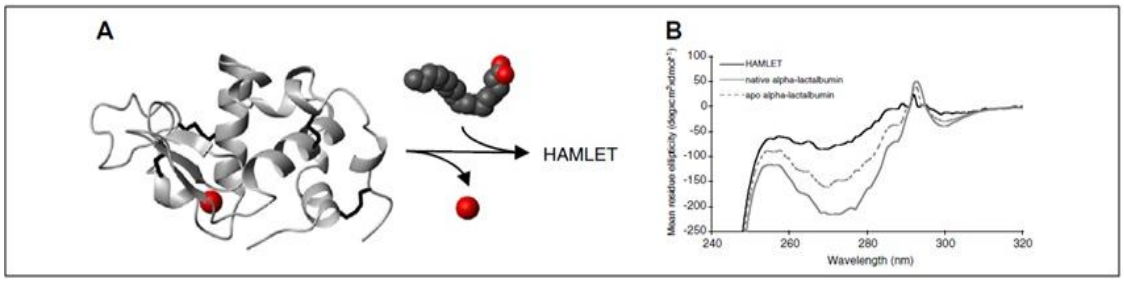

Fig. 2: HAMLET consists of partially unfolded a-lactalbumin and oleic acid. (A) HAMLET is formed by a two-step procedure. First, a-lactalbumin is partially unfolded by removing the calcium ion (red) with EDTA or acid. Second, oleic acid is bound to the protein and HAMLET is formed. (B) Native a-lactalbumin 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.

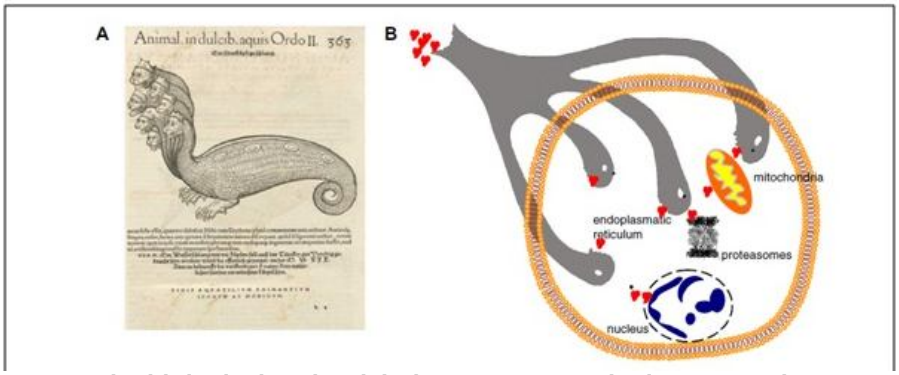

Fig. 3: HAMLET—the biological Hydra. (A) The Lernaean Hydra is proposed as a metaphor for HAMLET. The mythological animal was said to use its many heads to attack enemies. If one head was cut off, new heads would soon emerge. The antique depiction showing the Hydra was by a 16th-century German illustrator (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.

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