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The Alpha-Lactalbumin/Oleic Acid Complex and Its Cytotoxic Activity

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<http://dx.doi.org/10.5772/47433>

1. Introduction

The protein alpha-lactalbumin (α -LA) is present in the milk of nearly all mammals. Here it has a well-described function of controlling the specificity of a galactosyltransferase towards the formation of lactose, the common sugar present in milk. In recent years an entirely different activity has been associated with the protein. By forming a complex with oleic acid (OA) it shows cytotoxic activity against cells with an apparent selectivity for cancer cells. The complex is called HAMLET (Human α -lactalbumin Made LEthal to Tumor cells) and was originally discovered by serendipity. The complex was revealed during studies of the effect of anti-adhesive molecules from human milk on bacterial attachment to alveolar lung carcinoma cells [1]. A casein fraction, obtained after low pH precipitation of human milk at pH 4.3, was shown to exhibit cytotoxic activity. The dying cells displayed changes in morphology, cytoplasmic blebbing, nuclear condensation, and formed apoptotic bodies, comparable to cells that undergo classical apoptosis [2]. The tumoricidal component of the casein was retained in a DEAE-Trisacryl M column upon ion exchange chromatography, but eluted at high salt concentration (1M).

The active component was identified as multimeric α -LA (MAL) by its oligomeric appearance in SDS-PAGE analysis with bands at 14, 28, and 100 kDa and was subsequently confirmed by Edman degradation and mass spectrometry [1,3]. The MAL was shown to contain a molten globule-like structure and cause apoptosis-induction in a variety of transformed and immature cells, but not in healthy differentiated cells [1,2,4]. The affected cells were carcinomas of lung, kidney, throat, bladder, colon, ovaries and the prostate, glioblastomas in the brain, melanomas, and leukemias. In contrast, native monomeric α -LA isolated from human whey showed no apoptosis-inducing activity.

The difference in cytotoxic activity between the native-state α -LA and α -LA in MAL was not a consequence of post-translational modifications, but rather a conformational change

altering the tertiary structure while leaving the secondary structure intact, resulting in the partial unfolding and molten globule-like conformation of the protein in MAL [3].

The link between the altered folding of α -LA and apoptosis induction was subsequently identified by deliberate *in vitro* conversion of apo-state α -LA deprived of Ca^{2+} , to an apoptosis-inducing complex on an anion-exchange chromatography column previously exposed to human milk casein [5]. Additional studies showed the requirement of a lipid cofactor in the stabilization of the partially unfolded state of α -LA, by the finding that α -LA in the complex was preserved in a molten globule-like conformation even at physiological pH and in the presence of Ca^{2+} at 25-37°C [2]. The lipid cofactor was identified as oleic acid (C18:1 cis Δ 9) by chemical extraction of casein-conditioned column matrices and GC-MS analysis [5,6]. The name MAL was subsequently changed to HAMLET. A similar complex with bovine α -lactalbumin has been called BAMLET.

2. Structure of α -LA

Human α -LA is an acidic globular protein composed of 123 amino acids with a molecular mass of 14.2 kDa and homologous with the lysozyme protein family. It is secreted by the epithelia cells of the mammary gland during lactation, and this is the only tissue which expresses the protein [7-9]. The crystal structure of human and bovine α -LA has been resolved by X-ray crystallography [10,11]. The protein consists of two domains, a large one composed mainly of α -helical structures and a smaller β -sheet domain. The overall three dimensional structure is stabilized by a Ca^{2+} binding loop [12,13] (Figure 1).

The large domain contains four α -helices corresponding to amino acids 5-11 (A, dark blue), 23-34 (B, light blue), 86-98 (C, yellow) and 105-109 (D, orange) and three short 3_{10} -helical domains corresponding to amino acids 12-16, 101-104 and 115-119. The smaller β -sheet domain contains a triple-stranded antiparallel β -sheet (amino acids 40-50, green) and a short 3_{10} -helical domain (amino acids 76-82) [14]. The two domains are connected through a cysteine bridge (amino acids 73,91) creating the Ca^{2+} binding loop region. Additionally, two cysteine bridges are located in the α -helical structures (amino acids 6,120 and 28,111) and finally one bridge in the β -sheet domain (amino acids 61,77), aiding in the stabilization of the native conformation of α -LA [10,12]. The binding of Ca^{2+} to α -LA is required for correct formation of the disulfide bonds during protein folding [15].

3. Calcium-binding properties of α -LA

The native human α -LA is a metalloprotein containing a high-affinity primary Ca^{2+} binding site and Ca^{2+} -binding is required for the stabilization and structural integrity of the native fold [13,16-19]. The strong Ca^{2+} binding site, with an apparent association constant of $3 \times 10^8 \text{ M}^{-1}$ at 20°C, is located in a loop between the two domains, and is coordinated by the side chain carboxylates of Asp82, Asp87, Asp88 and carbonyl oxygens of Lys79 and Asp84 [10,20,21]. In Figure 1 only the side chains Asp82, Asp87 and Asp88 are shown. Furthermore, two water molecules also participate in the coordination of Ca^{2+} at the site,

creating a distorted pentagonal bipyramidal structure [10-13]. A secondary surface-located and low-affinity Ca^{2+} binding site was discovered by X-ray crystallography, which only binds Ca^{2+} at high concentrations. However, this binding site is believed to play no structural role in human α -LA [22].

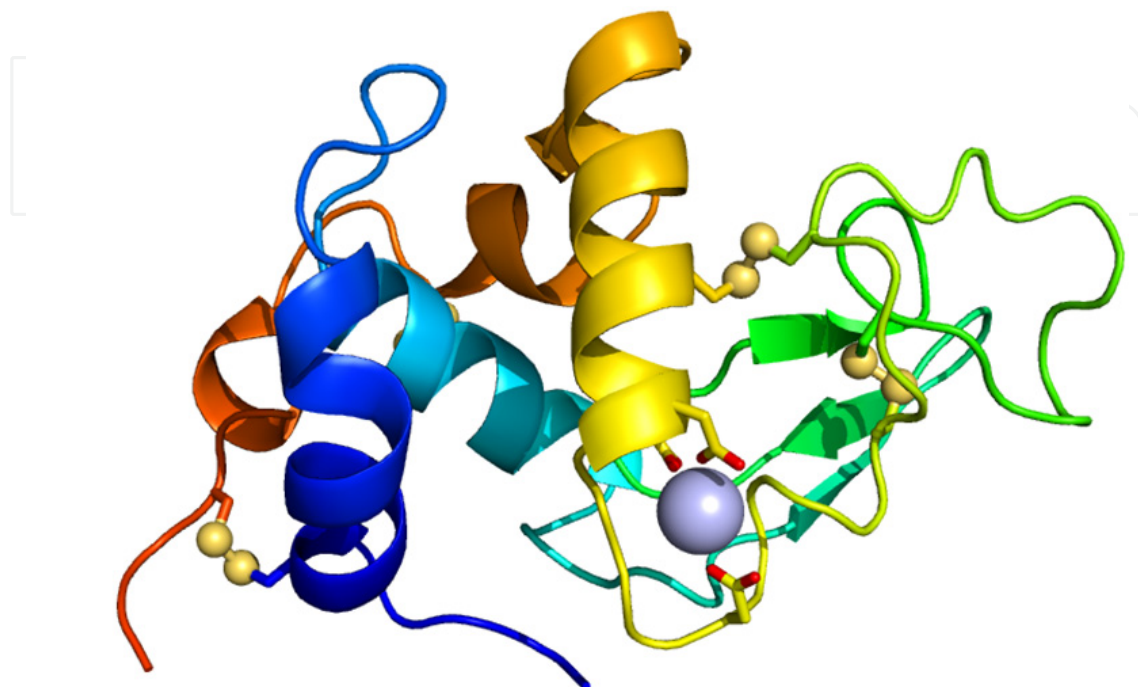


Figure 1. Model of human α -lactalbumin drawn by PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC using the coordinates from [10]. The Ca^{2+} is shown as a ball in purple, helix A in dark blue, helix B in light blue, helix C in yellow, helix D in orange, while the β -sheet is in green. The sulfur atoms in the four disulfide bonds are indicated by small yellow balls.

Other divalent cations, such as Mn^{2+} , Mg^{2+} , Na^+ and K^+ can also bind α -LA, competing with Ca^{2+} for the same binding site [13,23,24]. A distinct zinc binding site different from the calcium binding site has also been localized [25]. However, binding of these cations causes only minor structural changes and does not play a major structural role in α -LA, compared to the binding of Ca^{2+} [13].

The binding of Ca^{2+} to α -LA through re-normalization of the solvent Ca^{2+} , temperature or pH conditions, and the release of Ca^{2+} through low pH, EDTA or heat treatment, have been shown in both circular dichroism spectroscopy and fluorescence spectroscopy data analysis, to cause structural and functional changes mainly in the tertiary structure, while leaving a native-like secondary structure [13,18,20,24,26]. The native folded Ca^{2+} -bound α -LA has higher structural stability against increased temperatures, pressures or denaturant concentrations [13,24,27-30].

4. Biological function of α -LA

Alpha-lactalbumin folding occurs in the lumen of the endoplasmic reticulum and the protein is subsequently transported to the membrane surface of the Golgi apparatus, where

it interacts with galactosyltransferase creating the lactose synthase complex [13,31]. In the absence of α -lactalbumin, the enzyme is a non-specific galactosyltransferase involved in transferring galactosyl groups from UDP-galactose to a range of different substrates [13,31]. Alpha-lactalbumin functions as a substrate modifier, increasing specificity and affinity of the galactosyltransferase for glucose, when catalyzing the final step of lactose production in a lactating mammary gland [7,32].

5. Folding of α -LA

A noteworthy property of α -LA is the ability of the protein to adopt relatively stable partially unfolded intermediates under various conditions. Accordingly, it has been intensely used as a protein folding model in many studies. The classical molten globule of α -LA was defined as the acid denatured compact state of α -LA at pH 2.0 with fluctuating tertiary structure [13,33,34]. However, α -LA can adopt molten globule-like states during Ca^{2+} -free (apo-state) conditions, during high temperatures at 90°C and in the presence of denaturants [14,17,35,36]. Similar conformational states can also be formed by reduction of the disulfide bonds in α -LA [37,38].

The molten globule-like conformational states of α -LA are not significantly populated at physiological conditions [26]. In addition, stable unfolded states of α -LA only persist at low pH or in the absence of metal ions, since the protein instantly returns to the native conformation, if solution conditions are re-normalized [19]. In the molten globule conformation, α -LA has a native-like secondary structure, a less well-defined tertiary structure and a larger Stokes radius compared to the native protein [14,34,39].

The apo-state of α -LA, a molten globular-like conformation, is required for the formation of HAMLET. This conformation has high instability and sensitivity towards the ionic strength of the solution, compared to the native state of the protein [19]. However, the apo-state α -LA is more hydrophobic than the native state of the protein, rendering it prone to fatty acid binding [2]. The apo-state of α -LA is thus stabilized by binding to specific fatty acids in the HAMLET complex [6]. The proposed requirement of a special molten globular-like conformation of the proteinaceous component in HAMLET-like complexes has been challenged, as bovine β -lactoglobulin in complex with OA had cytotoxic activity exceeding that of HAMLET without retaining such a structure [40].

6. Fatty acid binding profile of α -LA

Alpha-lactalbumin possesses several fatty acid binding sites, and has been shown to bind stearic acid, palmitic acid and oleic acid spin-labelled fatty acid analogs [6,41]. The release of the Ca^{2+} ion and subsequent unfolding of the protein triggers α -LA to achieve a new fatty acid binding profile, with a higher affinity for cis-unsaturated fatty acids than for the corresponding trans conformations.

The interaction between apo-state α -LA and distinctive fatty acids has been studied, with the observation that certain fatty acids interact with apo-state α -LA in a stereo-specific manner [6]. It has been found, that saturated C18 fatty acids, unsaturated C18:1 *trans* fatty

acids and fatty acids with longer or shorter carbon chains only form insignificant amounts of complexes with apo-state α -LA [6]. All unsaturated *cis* fatty acids are able to form stable complexes with apo-state α -LA, with oleic acid showing the highest conversion efficiency [6]. It was found, however, that only oleic acid (C18:1 *cis* Δ 9 – in HAMLET) and vaccenic acid (C18:1 *cis* Δ 11) in complex with apo-state α -LA showed biological activity in apoptosis assay, suggesting that unsaturated C18:1 *cis* fatty acids have the correct stereo-specificity to bind apo-state α -LA in the formation and stabilize the HAMLET complex [6]. Another study has found that vaccenic acid, palmitoleic acid linoleic acid had similar activity as oleic acid while elaidic acid and stearic acid showed a weaker activity [42]. The fatty acid profile of α -LA thus seems to be rather broad and not as specific as initially anticipated.

Based on the three-dimensional structures of native and apo-state α -LA, two tentative hydrophobic regions and possible fatty acid binding sites within α -LA have been proposed. One is suggested in the interface between the two sub-domains including the C- and D-helix and the β -sheet domain [6,43,44]. The other is anticipated to be formed by residues within the A, B and 3₁₀-helices.

The binding of oleic acid to bovine apo-state α -LA has previously been shown to induce changes in the secondary structure of the protein, resulting in a typical molten globule state of the protein [45]. Far-UV CD of oleic acid binding to human apo-state α -LA showed similar results, with an increase in α -helical structure, largely independent of the temperature conditions [30].

Upon Ca^{2+} release structural changes in α -LA also occur in the cleft between the two domains, with the slight expansion of the Ca^{2+} -binding loop tilting the 3₁₀ helix toward the C helix, causing disruption of the aromatic cluster composed of Trp60, Trp104, Phe53 and Tyr103 in the interface between the two domains [11]. X-ray crystallography and NMR spectroscopy studies have revealed that apo-state bovine α -LA contains a largely intact α -helical domain with native side chain packing and an unstructured β -sheet domain [11,46]. Similar findings have been made for the molten globule-like conformation of human apo-state α -LA [47]. Therefore, it has been proposed that oleic acid binds in the area between the two domains, thus stabilizing the molten globule-like state of HAMLET and allowing Ca^{2+} binding without any effect on the tumoricidal activity [2,6]. Nevertheless, it is still unclear exactly how oleic acid binds to apo-state α -LA. Although the apo-state of α -LA is negatively charged, it may be hydrophobic interactions mediating the binding of the oleic acid, considering the increased hydrophobicity of apo-state α -LA compared to native α -LA [11,46,48,49]. Furthermore, even though the apo-state is negatively charged, it may also possess positively charged residues that are able to bind to the negatively charged polar head-groups of the oleic acids through electrostatic interactions [48,49]. Accordingly, it has been suggested that hydrophobic amino acids in the interface between the two domains may bind the fatty acid tail, while positively charged amino acids such as Arg70, Lys94 and Lys99 are plausible coordination residues for the fatty acid head group [2].

In contrast to these above-mentioned suggestions, one of the same studies showed that several proteolytically generated fragments of α -LA were able to incorporate oleic acid,

implying that there is not only one single fatty acid binding site in HAMLET [48]. The exact mechanism of fatty acid binding to α -LA remains uncertain.

7. Structure of HAMLET

HAMLET is defined as a conversion complex between apo-state α -LA and a C18:1 *cis* fatty acid co-factor, which has the ability to induce apoptosis selectively in tumor cells [6]. HAMLET has been described as the first discovered example of a protein that exhibits a well-defined function in the native structure, but additionally acquires a potentially beneficial function following partial unfolding [1,5,38].

Studies of the HAMLET structure by tryptophan fluorescence spectroscopy, near-UV CD spectroscopy and far-UV CD spectroscopy have revealed that the complex retains a stable, partially unfolded, molten globule-like conformation even at physiological conditions, in contrast to partially unfolded α -LA, which reverts to the native conformation, if solution conditions are re-normalized [3,5,14,19,29,45]. In this molten globule-like conformation, α -LA in the HAMLET complex showed retention of the secondary structure, near-complete loss of the tertiary structure, a larger stokes radius, and increased exposure of hydrophobic surfaces, compared to native α -LA [3,5,14,34,39,50].

8. Characteristics of HAMLET

The formation, stabilization and cytotoxic activity of the HAMLET complex have been shown to require both partial unfolding of α -LA and the binding of a fatty acid co-factor [5,6,19,51]. The partial unfolding of α -LA, e.g. through metal ion depletion, results in destabilization of the β -sheet domain, while leaving the α -helix domain largely unchanged, paving the way for fatty acid binding and subsequent HAMLET formation [19,50]. In accordance with this, it has been shown that the tumoricidal activity of HAMLET is largely independent of the β -sheet domain and C-terminal portion of human α -LA, since the isolated α -helix domain of α -LA was able to form a cytotoxic complex with OA [52].

Additional changes in the α -LA structure can induce the formation of HAMLET or HAMLET analogs. It has been proposed that the entire 123-residue sequence of α -LA is not required for cytotoxic activity, as fragments of α -LA in a complex with OA were able to induce apoptosis in Jurkat tumor cells [48]. Furthermore, it has been shown that a recombinant variant of human α -LA, where all cysteine residues were substituted with alanine residues, had the ability to form cytotoxic HAMLET complex analogs [38]. In addition, an α -LA mutant where Asp87 was shifted to an alanine with no Ca^{2+} binding activity was also able to form cytotoxic complexes with oleic acids, implying that a functional Ca^{2+} site is not required for the conversion of α -LA to the active complex or to cause cell death [19]. This being the case despite the fact that HAMLET has a high Ca^{2+} -affinity, with a Ca^{2+} association constant of $5.3 \times 10^6 \text{ M}^{-1}$ at physiological salt conditions [19]. However, the structural changes induced upon Ca^{2+} binding have no effect on the cytotoxic activity of the complex [19,30].

Interestingly, human α -LA is not the only variant of the protein able to form cytotoxic complexes with OA. Alpha-lactalbumin of bovine, equine, caprine, and porcine origin also have the ability to form complexes with OA, showing HAMLET-like cytotoxicity, while natural HAMLET formation in acid precipitates of casein was unique to human milk [9]. Comparison of highly purified human and bovine α -LA's ability to form complexes with OA showed that the two proteins behaved very similarly and that the cytotoxic activity was comparable [53].

The revelations that neither native-state, apo-state nor otherwise partially unfolded α -LA alone shows significant cytotoxic activity, in addition to the above-mentioned findings, have caused researchers to focus on the possibility that the cytotoxic component of HAMLET may be the fatty acid and not the α -LA protein [3,5,19,50]. As a consequence, it has been suggested that α -LA is merely a carrier or synergist of the tumor-killing activity of the associated oleic acid [48,49]. In agreement with this, studies have found that the cytotoxicity of OA alone is very similar to that of BAMLET, the bovine ortholog of HAMLET, and that of HAMLET-like complexes composed of bovine β -lactoglobulin and pike parvalbumin in complex with OA [40,54]. The cytotoxic activity of the β -lactoglobulin and paralbumin complexes even exceeded that of HAMLET, indicating that the proteinaceous component of the complexes is of less importance than the OA component in relation to the cytotoxic activity [40]. The potent toxicity of free oleic acid against Jurkat and HL-60 tumor cells has been shown, supporting the proposal that the fatty acid could be the cytotoxic component of the HAMLET complex [54,55]. However, in contrast with this proposal, α -LA has previously been found to be cytotoxic in the absence of oleic acid [56-60].

9. Stoichiometry of the protein and fatty acid in HAMLET and HAMLET-like complexes

The stoichiometry of protein and fatty acid in HAMLET or HAMLET-like complexes remains a topic of debate. Initial gas chromatography and mass spectrometry data suggested that the average number of oleic acid molecules bound in the HAMLET complex was 0.9, with some batch variation [6]. The assumption of a 1:1 protein/fatty acid ratio was also reported in the preparation of BAMLET by chromatography [61]. By gas chromatography and mass spectrometry a later study estimated an α -LA to OA ratio of 1:5.4 in a HAMLET complex prepared by the OA-preconditioned anion exchange chromatography method [38]. An α -LA to OA ratio of 1:8.2 in HAMLET determined by LC-MS has also been reported [52]. A ratio of 1:10-1:13 was found for BAMLET and HAMLET complexes formed by a new alternative preparation method, using two consecutive DEAE-Sepharose ion exchange columns for the purification of α -LA and subsequent complex formation, but high variation in stoichiometry was common within different batches [53].

The average number of oleic acid molecules bound per α -LA in LA/OA complexes has been spectrofluorimetrically estimated to be 2.9 for LA/OA formed at 17°C and 9 for LA/OA formed at 45°C [30]. Another study has reported that the bovine LA/OA complex, prepared

by direct mixing of the constituents, consists of 4-5 protein molecules with 68-85 bound molecules of OA, thus indicating that every α -LA binds on average 17 OA molecules [49].

A possible explanation for the variation in the observed α -LA to OA ratios might be that the different preparation techniques and experimental conditions (e.g. changes in temperature or altered α -LA to OA ratios) could have a major impact on the observed stoichiometry. In addition, it has been proposed that some of the OA might be present in an unbound form resulting in a higher OA to α -LA ratio [30].

10. Methods of HAMLET or HAMLET-analog preparation *in vitro*

Several different methods have been utilized for the *in vitro* preparation of HAMLET or its orthologs from α -LA and OA. These include OA-conditioned anion exchange chromatography using either a DEAE-Trisacryl M in the conventional method [3,5,6,19] or a DEAE-Sepharose column in an alternative method [53]. Direct mixing under heat denaturation [30,36,52], heat denaturation of α -LA and OA-conditioned anion exchange chromatography [62], direct mixing in solution at room temperature with alternated pH conditions [45,48,49,63], direct mixing followed by anion exchange chromatography [61] and alkaline conditions [64] have also been used.

The conventional method of HAMLET preparation is OA-preconditioned anion exchange chromatography [5]. This method is initiated with the purification of native-state α -LA by hydrophobic interaction chromatography and subsequent EDTA-treatment, resulting in the loss of Ca^{2+} and partial unfolding of the protein. The apo-state α -LA is subjected to a DEAE-Trisacryl M ion exchange chromatographic column previously exposed to human milk or pre-loaded with fatty acids. The apo-state α -LA interacts with the OA pre-loaded column, forming the HAMLET complex, which elutes at a salt concentration of 1M. The eluted complex is desalted by dialysis and lyophilized to obtain HAMLET dry powder.

Regardless of the preparation method used in the different experiments, it has been shown that the cytotoxicity of the resulting complexes was similar to that of conventionally prepared HAMLET or BAMLET [30,36,48,49,54,61-65].

11. Cellular trafficking of HAMLET

In order to clarify the subcellular localization of HAMLET and the interactions of the complex with different tumor cell compartments, researchers have used biotinylated [5,66], I^{125} radioactive labeled [66,67] and Alexa Fluor 568-labeled HAMLET complexes in confocal microscopy and subcellular fractionation experiments [67-69]. Site-specific labeling of HAMLET and HAMLET-like complexes with aminooxy-Alexa Fluor 488 or biotin molecules has also been used [52].

It has previously been described that α -LA interacts with cell membranes and lipid bilayers, as well as fatty acids [41,70-74]. In addition, it has been observed that α -LA is able to rapidly insert itself into the lipid bilayer at pH 2 [75]. However, native human α -LA is only

inefficiently bound to and internalized by tumor cells, and does not translocate to the nucleus, nor influence tumor cell viability [3,5,68,69,76]. Even partial unfolding of human α -LA has been shown to be insufficient for increased protein internalization into tumor cells [38]. In addition, human α -LA and oleic acid alone were unable to alter membrane structures, further indicating the necessity of interaction between unfolded α -LA and an associated fatty acid for efficient internalization of the HAMLET complex in tumor cells [76].

Even though a number of studies have focused on investigating the mechanism of HAMLET uptake by tumor cells, it still remains unclear. HAMLET has been shown to interact with the cell surface of tumor cells, be internalized and subsequently accumulate in the nucleus [3,5,67,68]. In the case of healthy cells, HAMLET interacted with the cell membrane and was internalized, but did not accumulate in the nuclei of the cells [4,67].

A study of the interaction between HAMLET and natural or artificially generated, negatively charged, model lipid membranes has revealed that the interaction results in loss of cell membrane integrity, leakage of vesicular contents and morphological changes of the membranes [76]. Interestingly, HAMLET disturbs the integrity of the tested membranes, even under physiological conditions, as opposed to native-state α -LA that only disrupts liposomes at acidic pH [77]. Similarly, at physiological conditions HAMLET readily distorts lipid monolayers at low concentrations, while forming pore-like oligomeric structures resembling annular oligomers at higher concentrations [78]. The ability to form oligomers is suggested to be a property of the α -LA polypeptide chain, enhanced by the fatty acid component, which might be important for the cytotoxic activity of HAMLET [78]. It has been suggested that the degree to which HAMLET and other LA-OA-complexes are able to disturb the membrane integrity, depends on the lipid composition and physical characteristics of the membrane [74,76].

HAMLET binds to the cell surface of intact tumor cells in a patchy distribution, indicating the possible targeting (e.g. receptor-binding) of the complex to specific membrane regions [76]. Another study found that HAMLET and an α -LA/OA complex formed at 17°C, interacts with and alters trans-membrane integral currents of artificial vesicles and natural plasma membranes of the green alga *C. coralline* [65]. The binding of the OA-complexes caused suppression of Ca^{2+} current and Ca^{2+} -activated Cl^- current, as well as increased nonspecific K^+ leakage, indicating nonselective permeability of the depolarized plasma membrane [65]. Furthermore, all the OA-bound states of α -LA had a higher affinity for interaction with the membranes compared to α -LA alone, indicating a Ca^{2+} -independent association. Similar results were obtained regarding the bactericidal activity and membrane depolarization effect of HAMLET, bovine β -lactoglobulin and pike parvalbumin in complex with OA on *S. pneumoniae* bacteria [40,64]. Accordingly, it has been proposed that once the complex has adsorbed to the plasma membrane, it is most likely internalized by nonspecific pinocytosis, while other mechanisms of endocytosis, such as receptor-mediated endocytosis have not been ruled out [65].

Translocation of HAMLET from the membrane to the cytoplasm has been followed by confocal microscopy, showing the formation of intracellular aggregates in both normal cells

and in tumor cells, although accumulation of HAMLET was higher in tumor cells [4]. The mechanism of HAMLET redistribution from the cytoplasm to different organelles of the cell, especially the nucleus, remains to be clarified. Initial studies found that the nuclear uptake of α -LA/OA occurred through the nuclear pore complex in a Ca^{2+} -independent manner, and that the accumulation of α -LA in the nucleus resulted in Ca^{2+} -dependent DNA fragmentation in the tumor cells [66]. Later, it was suggested that the interaction between HAMLET and ribosomal proteins could initiate nuclear translocation and targeting of the complex [2].

12. Tumor cell death mechanisms of HAMLET

A key unresolved issue regarding HAMLET is by what biological mechanisms the complex induces tumor cell death. A broad variety of transformed and immature tumor cells, but not healthy differentiated cells, have been found to be affected by the complex and even antibiotic-resistant strains of *S. pneumoniae* bacteria seem to be prone to the cytotoxic activity, showing apoptosis-like morphological and mechanistic changes [4,79,80]. HAMLET is believed to have multiple intracellular targets and has metaphorically been called a “Lernaean Hydra”, a mythological creature that uses numerous heads to attack enemies [51]. It has been shown that HAMLET induces at least three major responses, namely an apoptotic pathway, an autophagic pathway and chromatin structure disorder [1,67,81]. All the suggested tumor cell death mechanisms of HAMLET or HAMLET-like complexes are listed in Table 1.

Besides the three major responses, it has been shown that BAMLET activates a caspase-independent lysosomal cell death pathway mainly in tumor cells, causing lysosomal membrane permeabilization possibly contributing to BAMLET-induced cell death [61]. In a study of HAMLET binding to α -actinin, it has been indicated that the apoptosis-promoting p38 pathway is the top-scoring pathway in HAMLET-induced cell death, with p38 inhibition delaying death of HAMLET-treated tumor cells [82].

An elevated expression level of c-Myc, an oncogene that has the ability to bind a significant fraction of all known gene promoters, has been shown to increase HAMLET-sensitivity of various tumor cells, suggesting that the level of c-Myc expression could be a direct determinant of HAMLET susceptibility [83]. Furthermore, a reduction in the extracellular glucose levels or degree of glycolysis, predominantly the aerobic glycolysis of tumor cells known as the Warburg Effect, of A549 lung carcinoma cells resulted in an enhanced HAMLET-sensitivity [83]. HAMLET was shown to bind and reduce the activity of the glycolytic enzyme hexokinase 1 in the A549 carcinoma cells, which could partly explain the inhibitory effect of HAMLET on glycolysis [83].

HAMLET has been shown to interact directly with 20S proteasome subunits *in vitro* as well as *in vivo*, resulting in structural modifications and partial inhibition of 20S proteasome activity, possibly leading to accumulation of incorrectly folded proteins contributing to cell death [69]. Moreover, HAMLET was found by N-terminal sequencing and MALDI-TOF-MS to bind intact ribosomes, as well as individual ribosomal proteins L4, L6, L15, L13a, L30,

L35a, S12 and L21 [2]. The plausible effects of the interaction are protein translational blocking and ribosome-initiated nuclear targeting of HAMLET [2].

Responses	Effects
Major responses:	
Apoptotic pathway [2,54,84,87,90,91]	MAL co-localizes with mitochondria and causes release of cytochrome <i>c</i> from the IMM to the cytosol MAL causes loss of mitochondrial membrane potential ($\Delta\Psi_m$) and abnormal MPT HAMLET causes p53-independent apoptotic or apoptotic-like cell death in tumor cells BAMLET-induced cell death varies according to cell type
Autophagic pathway [81,91]	HAMLET induces an macroautophagic response in tumor cells
Chomatin structure disorder [67,90,100]	HAMLET accumulates in the nucleus of tumor cells HAMLET binds to histones, independent of the histone tail, resulting in chromatin structure disorder in tumor cells HAMLET causes caspase-dependent and caspase-independent chromatin condensation and acts in synergy with histone deacetylase inhibitors
Other responses:	
Anti-adhesion [1,82,102]	MAL and HAMLET facilitates tumor cell detachment
c-Myc oncogene status [83]	Elevated expression of c-Myc oncogene sensitizes tumor cells to HAMLET-induced tumor cell death
Inhibition of 20S proteasome activity [69]	HAMLET binds to 20S proteasome subunits causing structural modifications and partial activity inhibition
Inhibition of glycolysis [83]	HAMLET binds to and reduces the activity of HK1
Lysosomal pathway [61]	BAMLET activates a caspase-independent lysosomal pathway in tumor cells leading to lysosomal membrane permeabilization
p38 pathway [82]	Indicated to be the top-scoring cell death pathway of HAMLET
Ribosome interactions [2]	HAMLET binds to intact ribosomes and individual ribosome proteins

Table 1. The tumor cell death mechanisms of HAMLET and HAMLET-like complexes.

For elaboration of the specific tumor cells prone to the mentioned effect, the reader is encouraged to see the references. HK1, hexokinase 1; IMM, inner mitochondrial membrane; MAL, multimeric α -lactalbumin; MPT, mitochondrial permeability transition.

13. HAMLET and apoptosis

Several studies have focused on clarifying the precise role of apoptosis in the HAMLET-induced cell death of tumor cells. Initially, it was shown by the use of an anti-CD95 antibody that the CD95 receptor-mediated apoptotic pathway had no effect on the apoptosis-inducing activity of MAL [84]. It was also found that MAL co-localized with mitochondria and caused release of cytochrome *c* from the inner mitochondrial membrane space to the cytosol, resulting in activation of caspase-3-like enzymes and, to a lesser degree, caspase-6-like enzymes of the caspase cascade [84]. The activation of cytosolic caspases by apogenetic factors released from the inner mitochondrial membrane (e.g. cytochrome *c*), and subsequent Apaf-1, procaspase-9 and dATP association, resulting in formation of the apoptosome complex, are normal events of the classical apoptotic pathway [85-87]. It was later revealed that MAL induced a loss of the mitochondrial membrane potential ($\Delta\Psi_m$) and abnormal mitochondrial permeability transition (MPT) in isolated mitochondria through opening of the MPT pore, resulting in Ca^{2+} -dependent release of cytochrome *c* to the cytosol [87].

HAMLET-treated Jurkat leukemia cells and A549 lung carcinoma tumor cells exhibited classical apoptotic changes, as well as apoptosis-like changes, such as proapoptotic caspase activation, phosphatidyl serine externalization, DNA fragmentation, apoptotic body formation and compacted chromatin condensation [88-90]. However, classical apoptosis was not the cause of death, as a pan-caspase inhibitor zVAD-fmk was unable to rescue HAMLET-treated cells from dying [84,90]. This was confirmed by the finding that HAMLET-induced cell death is independent of the anti-apoptotic Bcl-2 and Bcl-xL proteins in Jurkat leukemia, K562 promyelocytic leukaemia and FL5.12 murine prolymphocytic tumor cells, as over-expression of Bcl-2 failed to prevent cell death [90]. In addition, the tumoricidal activity of HAMLET is independent of the tumor suppressor protein p53, as there was no difference in HAMLET susceptibility of tumor cells with wild-type, deleted or mutated p53 gene [2,90,91]. Analyzing the effect of HAMLET on Jurkat and THP1 cells by flow cytometry indicated that the death of Jurkat cells looked more apoptotic than the death of THP1 cells which were more necrotic, showing that the actual mechanism of cell death apparently varies between different types of cells [53].

14. HAMLET and macroautophagy

Autophagy has been a topic of comprehensive debate concerning its function as an alternative caspase-independent type II programmed cell death pathway, in addition to serving as a survival mechanism during cellular stresses [92-95]. Macroautophagy, the only autophagic response included in type II programmed cell death, is an adaptive stress response observed in cells exposed to cellular stresses (e.g. starvation), which triggers the

recycle of organelles and long-lived proteins as a nutritional source utilized to prolong cell survival [91,92, 96].

HAMLET induces a macroautophagic response in treated tumor cells, with the appearance of cytoplasmic vacuoles and double-membrane enclosed vesicles [81,91]. Furthermore, HAMLET was shown to alter the staining-pattern of LC3-GFP-transfected cells from uniform (LC3-I) to granular (LC3-II), reflecting the translocation of LC3 to autophagosomes during macroautophagy [81]. HAMLET also induced LC3-II accumulation, detected by a Western Blot, when lysosomal degradation was inhibited, which is a clear indicative of macroautophagy [81].

The inhibition of macroautophagy in HAMLET-treated tumor cells by RNA interference of Beclin-1 synthesis resulted in reduced cell death and inhibition of the increase in granular LC3-GFP staining, suggesting that macroautophagy is an important response pathway in HAMLET-induced cell death [81,91]. In accordance with this finding, it was shown that mTOR, an inhibitor of macroautophagy, is inactivated in tumor cells in response to HAMLET [81,97]. An important note is that the mitochondrial damage observed in the different tumor cells of the mentioned experiments also has the potential to trigger macroautophagy in the cells [81,98,99].

15. HAMLET cell nuclei interactions

A striking feature of HAMLET is the ability to move through the cytoplasm and accumulate in the nucleus of tumor cells, as initially elucidated by the study of the active human milk fraction [3,66]. Healthy, differentiated cells did not accumulate biotinylated MAL or Alexa-labeled HAMLET in the nucleus, although uptake in the cytoplasm was observed [66,67].

Through combination of *in vitro* and *in vivo* experiments, HAMLET was found to co-localize with histones and bind strongly to histone H3, as well as histones H4 and H2B to a lesser degree, resulting in perturbation of the chromatin structure and chromatin assembly in tumor cells, possibly impairing transcription, replication and recombination [67,100]. The binding of HAMLET to the histones was independent of the histone tail, and the binding impairs histone deposition on DNA.

In a different study it has also been shown that monomeric Ca^{2+} -loaded α -LA and apo-state α -LA alone can bind histone H3 *in vitro*, possibly through electrostatic interactions with several α -LA molecules bound per histone protein [101]. However, the finding should have no major implications on HAMLET studies, as native monomeric α -LA is unable to reach the cell nuclei of intact tumor cells [3,5,31].

HAMLET has been shown to act in synergy with histone deacetylase inhibitors, as pre-treatment of Jurkat tumor cells with these resulted in enhanced lethal effect of HAMLET and an increased hyperacetylation response [100]. Caspase-independent DNA damage and DNA fragmentation was observed after treatment, suggesting that apoptosis was not the key pathway involved in the combined effect [100]. In addition, HAMLET caused chromatin condensation, as HAMLET-treatment of stably transfected HeLa cells resulted in a decrease

of nuclear size. The ability of HAMLET to induce both caspase-dependent and caspase-independent chromatin condensation pattern was found in another study, indicating that the response to HAMLET involves both classical apoptosis and caspase-independent cell death pathways [90].

16. Anti-adhesive properties of HAMLET

The ability of HAMLET to facilitate tumor cell detachment has been described both in early and recent literature. Initially, MAL was found to possess anti-adhesive properties against bacterial attachment to alveolar type II lung carcinoma cells [1]. Subsequently, an *in vivo* experiment studying the therapeutic effects of HAMLET on bladder cancer showed that HAMLET triggers massive shedding of dead tumor cells into the urine, further indicating the anti-adhesive properties of HAMLET towards tumor cells [102]. Later, *in vitro* studies revealed that HAMLET binds to α -actinin-4, in addition to interacting with α -actinin-1, and causes detachment of A549 lung carcinoma cells [82]. Furthermore, a reduction in β 1 integrin staining, as well as in FAK and ERK1/2 phosphorylation was observed, suggesting that HAMLET-treatment induced disruption of integrin-dependent cell adhesion signaling.

17. Therapeutic applications of HAMLET

A major reason for studying the cytotoxic activity of HAMLET is the apparent specificity towards cancer cells. Although this specificity of cytotoxic HAMLET-induced cell death remains unclear, there have been continuous indications in many *in vitro* studies that HAMLET could have a therapeutic potential. As a consequence, researchers started conducting *in vivo* studies of the possible applications of HAMLET [68,102-105]. The proposed therapeutic applications of HAMLET and the observed effects are listed in Table 2. One problem in using HAMLET as a potential cancer agent is its interaction with albumin, which through binding of free fatty acids could neutralize the cytotoxic activity of HAMLET or BAMLET [53,87,102]. As albumin is present in nearly all physiologic fluids, it will in many cases be difficult for HAMLET to reach the target cancer cells in an active form.

The observed effects mentioned are *in vivo* unless otherwise noted. GBM: glioblastoma multiforme.

18. HAMLET as a skin papilloma treatment

Papillomas are characterized as premalignant lesions of the skin and mucosal surfaces, caused by human papillomavirus transformation of keratinocytes [4,104]. The therapeutic treatment options are ineffective and include cryotherapy, immuno modulators, curettage, cautery, salicylic acid, CO₂ laser, antimitotic agents, or photo-dynamic therapy [106,107].

Skin papillomas were the first model selected for the examination of the *in vivo* effects of HAMLET [104]. Patients resistant to conventional treatments were included in this randomized, placebo-controlled, double-blinded study. HAMLET was shown to reduce the papilloma volume in 100% (20/20) of the patients and 96% of their lesions (88/92), compared

to 15% in the placebo group patients (3/20) and 20% (15/74) of their lesions. By studying the long-term effects of HAMLET-treatment against skin papillomas, it was found that all lesions had completely resolved in 83% of the patients after 2 years. Furthermore, there were no observed differences between immunocompetent and immunosuppressed patients, indicating the potential of HAMLET-treatment for immunosuppressed skin papilloma patients, instead of the currently used invasive methods [104].

Applications	Effects
Bladder cancer treatment [2,102,105]	Induces bladder carcinoma cell death (<i>in vitro</i>) Increases daily shedding of dead tumor cells into the urine Reduces tumor size or area and changes tumor character Delays progression of bladder cancer
Glioblastoma (multiforme) treatment in a rat model [68]	Induces apoptosis in GBM biopsy spheroids (<i>in vitro</i>) Reduces intracranial tumor volume Delays onset of pressure symptoms
Skin papilloma treatment [104]	Reduces the volume of papillomas Leads to long-term resolution of lesions Similar effects of treatment in immunosuppressed patients as in immunocompetent patients

Table 2. Possible therapeutic applications of HAMLET.

19. HAMLET as a bladder cancer treatment

The development of bladder cancers is common and remains a massive worldwide challenge, despite continuous advances in the therapeutic options available. The prevalence of bladder cancer is 1 in 4000 people, accounting for about 5% of all cancers, making it the fourth most common malignancy in the United States and the fifth most common in Europe [108]. The current treatments of bladder cancer typically involve intravesical instillation of antitumor agents such as *Bacillus Calmette-Guerin*, thiotepa, epirubicin and mitomycin C [109].

Early *in vitro* studies found that HAMLET induced rapid cell death in numerous cell lines, including bladder carcinoma cells [2]. The direct *in vivo* effect of HAMLET on bladder cancers was initially shown, when 9 male superficial bladder cancer patients received intravesical HAMLET-treatment during the week before scheduled transurethral surgery [102]. HAMLET stimulated a rapid increase in the daily shedding of dead tumor cells into the urine in 8 of 9 patients and an apoptotic response was detected by the TUNEL DNA fragmentation assay in 6 of 9 patients. In addition, a reduction in tumor size or a change in tumor character was observed at surgery in 8 of 9 patients.

The therapeutic potential of HAMLET was further studied in a mouse bladder carcinoma model, with MB49 carcinoma cells installed via a catheter into the bladder of anesthetized mice, followed by five intravesical installations of HAMLET [105]. The treatment resulted in significantly decreased tumor areas and delayed the progression of bladder cancer compared to controls. Furthermore, whole body imaging of Alexa Fluor 568-labeled HAMLET revealed that the uptake and retention of the complex were tumor tissue-specific.

20. HAMLET in treatment of glioblastoma

Gliomas are a heterogeneous group of intracranial neoplasms originating from neuroglial cells, which accounts for over 60% of all primary brain tumors and have an unfavorable prognosis [110-113]. Glioblastoma multiforme (GBM), grade IV on the current WHO grading-scale for tumors in the central nervous system, is the most malignant of the gliomas, showing a mean survival time of less than a year [113,114]. The current treatment is only palliative, involving surgery, radiotherapy and chemotherapy [115].

HAMLET has been shown to induce apoptosis in GBM biopsy spheroids *in vitro*, as detected by the TUNEL DNA fragmentation assay [68]. Similarly, *in vivo* studies of the effect of HAMLET-treatment on GBM's, established by xenotransplantation of human glioblastoma biopsy spheroids into nude rat brains, showed that HAMLET reduced the intracranial tumor volume and delayed the onset of pressure symptoms in tumor-xenografted rats [68,116].

21. HAMLET and involution of the mammary gland

The development of the mammary gland through puberty, pregnancy and lactation has been intensively studied and much is known about the factors responsible for regulating the processes. When suckling stops, the gland goes into involution, and despite much research little is known regarding the factors involved in this remodeling of the gland.

Milk synthesis occurs in mammary epithelial organized in small hollow "balls" called alveoli. It is known that the accumulation of milk in the alveoli results in down regulation of protein synthesis as well as reduction in the number of secretory cells by apoptosis [117]. It is also believed that local factors secreted into the milk are responsible for the effect, but molecular details of such compounds are lacking. A protein called FIL (Feedback Inhibitor of Lactation) from milk has been isolated and partly characterized [118], but at present its existence is unconfirmed.

Some sea animals have a special lactation cycle due to long periods of foraging trips away from the offspring. Remarkably, despite the absence of suckling for many days no apoptosis occurs in the mammary gland of e.g. Cape fur seals, and this phenotype has been correlated with lack of α -LA in their milk [119]. The possibility that α -LA either alone or in a complex with fatty acids is involved in the regulation of involution has been suggested as apoptotic cell death in the mammary gland was enhanced by the introducing of HAMLET in the gland of lactating mice [103].

22. Conclusion

The milk protein alpha-lactalbumin (α -LA) is one of the most studied proteins with respect to structure and function and has been a model for investigating intermediate folding conformations generally called molten globule states. It has a well-described function as part of the enzyme lactose synthetase, where α -LA is one component of the two-polypeptide enzyme. The other part is a non-specific galactosyl transferase, which in complex with α -LA becomes highly specific for forming lactose from UDP-galactose and glucose.

In the late nineties an entirely different activity of α -LA was discovered. When α -LA is in a partly unfolded conformation it can form a complex with oleic acid (OA) where the complex shows cell-killing activity specific for cancer cells. This partly unfolded conformation of α -LA can be obtained by removing a tightly bound calcium ion, leaving the protein in the apo-state capable of interacting directly with OA and thereby forming the cytotoxic complex. The complex between human α -LA and OA has been called HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cells) and similar complexes can be formed with α -LA from other species.

In cell culture systems HAMLET shows cytotoxic activity in μ M concentrations but the activity varies greatly between cell types. The exact mechanism of cell-killing is not clear, but HAMLET has been shown to interact with numerous cell organelles including the nucleus, lysozymes, mitochondria, proteasomes and ribosomes. Apoptosis, autophagy and chromatin structure disorder are three different cytotoxic pathways described when HAMLET is added to cell cultures. It is therefore likely that different cells will show different death mechanisms dependent on the experimental conditions.

When the cytotoxic activity of α -LA/OA complexes are measured in a dose-response manner, it is characteristic that very steep curves are obtained where as little as a four-fold dilution can result in no activity compared to 100% cell death in the undiluted solution. This underlines the importance of defining the experimental conditions exactly when measuring cytotoxic activity as small changes in concentration might lead to contradictory conclusions. This aspect is especially important when the activity of HAMLET is compared between healthy differentiated normal cells and tumor cells. According to numerous reports HAMLET kills tumor cells as well as undifferentiated cells but leave normal cells untouched. This has recently been questioned as it was found that both normal white blood cells and fully developed erythrocytes were highly sensitive to the cell-killing activity.

The potential of HAMLET as a therapeutic agent has been investigated in three different models. First, skin papillomas were treated in a double-blinded study showing positive results compared to the controls, secondly, patients with bladder cancer indicated reduction in tumor size when the bladder was flushed with a solution of HAMLET, and finally, in a glioblastoma rat model HAMLET treatment resulted in a decrease of the intracranial tumor volume. This suggests that tumor cells in some cases might be more sensitive to HAMLET when compared with the healthy cells from which the tumors originate.

It has been theorized that HAMLET could be naturally formed in the digestive system of breast-fed children, due to low pH conditions of the stomach, serving the function as a

natural scavenger and selective cytotoxic killer of cancerous cells in early infancy. The development of normal epithelia cells in the digestive system could also be affected. Obviously, many dairy products contains α -LA in fairly high concentrations and numerous production techniques, such as acid pH and heat treatment, facilitate the formation of partly unfolded α -LA making the protein ready to bind fatty acids and other similar compounds. To what degree such complexes in fact are formed in dairy products remains to be seen.

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23. References

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