

Alternatively folded proteins with unexpected beneficial functions

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Abstract

HAMLET (human α -lactalbumin made lethal to tumour cells) and its related partially unfolded protein–fatty acid complexes are novel biomolecular nanoparticles that possess relatively selective cytotoxic activities towards tumour cells. One of the key characteristics is the requirement for the protein to be partially unfolded, hence endowing native proteins with additional functions in the alternatively folded states. Beginning with the history of its discovery and development, the cellular targets that appear to be strongly correlated with tumour cell death are introduced in the present article.

Introduction

It is well accepted that a nascent polypeptide chain released from the ribosome folds to its global free energy minimum where the native three-dimensional structure is defined and where its native, and almost always beneficial, biological function is displayed [1]. In contrast, partially folded intermediates and/or their misfolded aggregates are usually considered functionally inconsequential forms, with the exception of a growing list which includes the Pmel17 protein in melanosomes [2] and the *Saccharomyces cerevisiae* Sup35 prions [3]. Apart from these ‘functional amyloids’, the biological activity attributed to the misfolded species, for example, upon formation of oligomeric amyloid pre-fibrils, has almost always been shown to be detrimental to the host cell [4], and an extensive body of work has been accumulated to characterize the relevant structures and the fibrillogenesis assembly mechanisms [5]. Separately classified, but nonetheless biologically important, are the intrinsically (or natively) unfolded proteins, where loose polypeptide chains that are in rapid conformational exchange experience folding upon binding endogenous target(s) and elicit biological function [6]. Nature has therefore appeared to have successfully found myriad ways to diversify its ‘protein toolbox’, particularly in the light of the limited number of distinct genes transcribed in humans. In the present paper, we attempt to show that, in addition to the above examples,

further possibilities exist for Nature to endow partially folded proteins with distinct and beneficial functions quite unlike the native protein.

The discovery and elucidation of HAMLET (human α -lactalbumin made lethal to tumour cells)

HAMLET is a protein–fatty acid complex that is formed from partially unfolded α -lactalbumin and oleic acid. α -Lactalbumin is the most abundant protein in human milk, and its three-dimensional structure consists of four α -helices, a triple-stranded β -sheet, a calcium-binding site and four disulfide bonds which stabilize the protein in its native conformation [7] (Figure 1). Ever since its original identification and characterization, considerable interest in this protein–fatty acid complex has been shown due to its properties of relatively preferring to cause cell death of tumour cells while leaving healthy differentiated cells unharmed [8–10]. HAMLET is the first example of a protein that exhibits a well-defined function in its native state, but which also acquires a new and beneficial function after partial unfolding (forming a molten globule) [11]. Other complexes similar to HAMLET are found as well, which consist of protein and fatty acid components that when combined, exhibit cytotoxic activities [12]. Examples of these complexes include BAMLET (bovine α -lactalbumin made lethal to tumour cells) [13–18], ELOA (equine lysozyme with oleic acid) [19,20] and other oleic acid complexes with camel α -lactalbumin [21], β -lactoglobulin [22] or pike parvalbumin [23]. The production methodology of these complexes have since diversified and evolved, initially from the original ion-exchange chromatography pre-conditioned with oleic acid [9]; however, it has been shown that moderate heat treatment

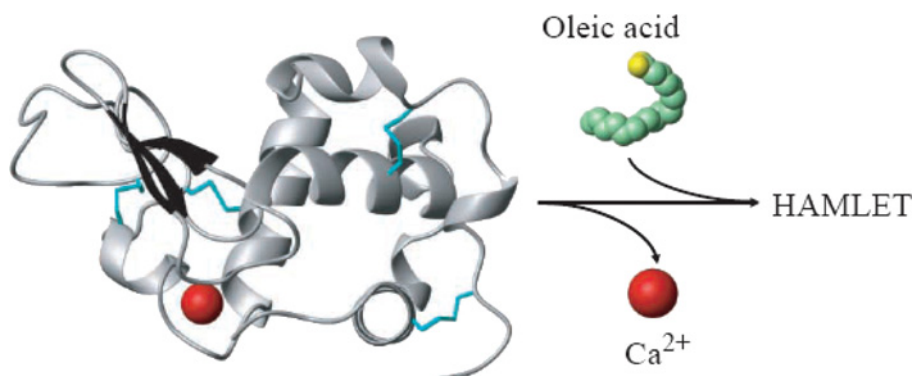
Key words: alternatively folded protein, bovine α -lactalbumin made lethal to tumour cells (BAMLET), equine lysozyme with oleic acid (ELOA), human α -lactalbumin made lethal to tumour cells (HAMLET), multimeric α -lactalbumin (MAL).

Abbreviations used: BAMLET, bovine α -lactalbumin made lethal to tumour cells; ELOA, equine lysozyme with oleic acid; HAMLET, human α -lactalbumin made lethal to tumour cells; HDI, histone deacetylase inhibitor; MAL, multimeric α -lactalbumin; PT, permeability transition.

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Figure 1 | Schematic diagram of HAMLET formation from α -lactalbumin and oleic acid

Partial unfolding is achieved by the removal of calcium, and the unfolded protein binds oleic acid. From Pettersson-Kastberg, J., Aits, S., Gustafsson, L., Mossberg, A.-K., Storm, P., Trullsson, M., Persson, F., Mok, K.H. and Svanborg, C., *Ann. Med.* 2009; **41**, 162–176, © 2009, Informa Healthcare. Reproduced with permission of Informa Healthcare.



with incubation with oleic acid and/or oleate appears to generate comparable, but not necessarily identical, complexes [16,24], and studies are actively ongoing to distinguish any structural and functional differences.

The unearthing of HAMLET was a rather fortunate discovery. Catharina Svanborg and co-workers discovered the effects of what would later become known as HAMLET while studying the effect of human milk on bacterial adherence to a human lung cancer cell line [8]. They observed that the cancer cells underwent apoptosis-like death when treated with human milk, whereas the differentiated cells were unharmed. The active component of milk that was triggering the cancer cell death and cytotoxic activity precipitated with the casein fraction of human milk. Upon elution of the casein fraction through ion-exchange chromatography and isolating the apoptosis-inducing fraction, it was found that the fraction was made up of both monomeric and multimeric α -lactalbumin. The active fraction was believed to be the multimeric form of α -lactalbumin because the monomeric form had no effect on cell viability; the active component purified from casein was therefore called MAL (multimeric α -lactalbumin) [8]. Through other experiments, it was later recognized that the formation of an oligomeric complex was not important for toxicity [9] (note that the formation of oligomers appears to be important for cytotoxicity in other cases, such as ELOA [12]).

α -Lactalbumin's main functions include the synthesis of lactose as a component of lactose synthase [7] and serving as an important source of nutrition for infants [25]. When the α -lactalbumin that had the cytotoxic properties was analysed, it was found that it did not have any post-translational modifications which may have changed its function, and neither was there any change in the secondary structure of the protein usually found in human milk. It was deduced that the reason for the activity of the cytotoxic α -lactalbumin was due to a change in tertiary structure towards a molten-globule-like state (the term molten globule

describes a protein that has a native-like secondary structure but a less well-defined tertiary structure) [26,27]. The change in tertiary structure was confirmed through near-UV CD spectroscopy, the binding of the hydrophobic dye ANS (8-anilino-naphthalene-1-sulfonic acid) and ^1H -NMR spectroscopy. These methods also confirmed that the secondary structure of the cytotoxic α -lactalbumin did not change in comparison with native α -lactalbumin. It was proposed that α -lactalbumin was an example of a protein that could acquire various functions depending on its folding state [28].

Shortly after this, two key elements that define the formation of HAMLET were identified [9]. (i) The partial unfolding of α -lactalbumin was required to form a folding variant. Partial unfolding alone, however, does not make α -lactalbumin tumoricidal [12]. (ii) A co-factor, known as oleic acid, was also needed to convert native α -lactalbumin into its cytotoxic variant [29] (see Figure 1).

The next study [30] compared structural variants of oleic acid (along with other fatty acids differing in their carbon chain length, degree of saturation and *cis/trans* conformation) and their effects on the formation of HAMLET. Clean columns were conditioned with the individual fatty acids and the degree of binding of apo- α -lactalbumin and its cytotoxic activity were measured. The optimal co-factors were identified as $\text{C}_{18:1}$ fatty acids (fatty acids with 18 carbons and one double bond) with a double bond in the *cis* conformation at position 9 or 11. Saturated C_{18} fatty acids, unsaturated fatty acids in the *trans* conformation and fatty acids with shorter carbon chains were shown not to form HAMLET. The study showed that lipid co-factors can enable proteins to adopt stable and novel conformations and in this way can act as partners in protein folding.

Following this, an important study into the minimal structural requirement of the protein moiety was performed [31]. A protein–fatty acid complex prepared with an α -lactalbumin variant where all four disulfide bridges were

eliminated (hence rendering the protein non-native and biologically inactive under all conditions) nevertheless displayed equivalent tumoricidal activity. As a result, it became clear that there was no need for the protein to recover to its native state upon uptake into the tumour cells, hinting further that, potentially, the role of the protein may be to serve as cargo carrier (a 'mule').

Mechanisms of cell death in response to HAMLET

HAMLET differs from other apoptosis-inducing agents in that it attacks multiple cellular targets (such as the proteasome, the mitochondria and the chromatin). The 'Lernaean Hydra' metaphor has been used to explain HAMLET's multiplicity of targets [10] and activates multiple signalling cascades in tumour cells. This multiplicity of targets ensures that cell death can be reached in many different types of tumour cells [32]. HAMLET has been shown to have activity in over 40 different cell lines; it can induce apoptosis in carcinomas of the lung, colon, throat, kidney, bladder, prostate and ovaries, in melanomas, in leukaemias and in glioblastomas of the brain [33]. The effects of HAMLET are not just specific to human tumours either; HAMLET has been shown to kill tumour cell lines of primate, bovine, murine and canine origin [33]. The proteins that HAMLET interacts with in the cell to cause cell death are numerous and still being investigated.

Apoptosis

In early studies, HAMLET was shown to trigger apoptotic changes in tumour cells [8]. As apoptosis involves mitochondria and depends on initiator and effector caspases, experiments were carried out to assess the impact of HAMLET on these cellular components, and to see whether apoptosis really was the main cause of cell death in tumour cells.

One of the first experiments carried out examined caspase activation in cells treated with MAL [34]; they found that when MAL was added to cell cultures there was an increase in activation of caspase-3-like and caspase-6-like enzymes, enzymes that are associated with apoptosis. In addition, through the co-localization of biotin-labelled MAL and labelled mitochondria, the interaction of MAL with the mitochondria and the release of cytochrome *c* was also detected in cells treated with MAL.

Further experiments showed that treatment with HAMLET led to the formation of a PT (permeability transition) pore, which caused the release of cytochrome *c* into the cytosol and the possible activation of caspases [35]. The mitochondrial membrane potential was also measured showing that HAMLET induced a biphasic decrease in the mitochondrial membrane potential; one phase was due to the formation of the PT pore due to the action of HAMLET, whereas the other phase was as a result of the action of oleic acid which caused the uncoupling of the mitochondria (the dissipation of the proton gradient before it can be used to provide energy for oxidative phosphorylation).

A subsequent experiment was carried out which re-examined the contribution of apoptosis to cell death in response to HAMLET [32]. This experiment compared the effects of an anticancer agent etoposide (which leads to the production of caspases and causes death through apoptosis) and HAMLET on undifferentiated cells. Phosphatidylserine exposure and the fragmentation of DNA were seen to be caused by HAMLET and to be caspase-dependent processes, but the cells treated with HAMLET still died in the presence of caspase inhibitors. Only a small increase in the concentration of HAMLET was necessary in the cells that had been treated with caspase inhibitors to achieve the same percentage of cell death as when no caspase inhibitors were used, illustrating the fact that although caspases had a part to play in the death of the cells, they were not essential, i.e. tumour cell death in response to HAMLET relied on caspase-independent mechanisms. This theory was reinforced when the effects of HAMLET on tumour cells that overexpressed Bcl-2 and Bcl-xL (anti-apoptotic proteins that prevent the formation of the PT pore and therefore the release of cytochrome *c* which activates the caspases) were studied; the cells that overexpressed Bcl-2 and Bcl-xL which were treated with etoposide did not undergo apoptosis (owing to the protective effects of Bcl-2 and Bcl-xL which prevented the formation of caspases), whereas those same cells, when treated with HAMLET, died even with the extra protection of Bcl-2 and Bcl-xL. Cell death was therefore shown to be independent of Bcl-2 and Bcl-xL [32]. The conclusions drawn from the experiment were that apoptosis, although having a part to play in tumour cell death, was not the cause of cell death. Parallel work on the effects of HAMLET on bacteria cell death has shown that such morphological changes and biochemical responses can also be seen in prokaryotes [36].

Nuclear invasion

The interaction of HAMLET with the nucleus of tumour cells was recognized quite early in the experiments conducted with HAMLET [28]. It was seen that the interaction of HAMLET and the nucleus was crucial for the induction of DNA fragmentation since inhibition of nuclear uptake saved cells from DNA fragmentation. The nuclear target molecules for HAMLET in cancer cells were identified as the histones H3, H4, H2A and H2B [37]. HAMLET, in binding to the histones, disrupts the chromatin and prevents transcription, translation and replication from occurring leading to cell death. On the basis of confocal fluorescence, this transport into the nucleus only occurs in tumour cells [28], however, and, because of this, healthy differentiated cells are unharmed. That HAMLET cell death is not mediated entirely by apoptosis was shown further from the p53-independent response despite the changes in chromatin structure that would activate a p53-dependent apoptotic response [32].

The accessibility of the chromatin for HAMLET can be controlled by the acetylation and deacetylation of the

histone tails [37]. An investigation into how the combination of HAMLET and HDIs (histone deacetylase inhibitors) influence histone acetylation, DNA integrity and cell viability was conducted [38]. HDACs (histone deacetylases) regulate the acetylation/deacetylation of lysine residues in histone tails, and they are generally overexpressed in tumour cells, leading to the blocking of transcription of anti-tumoral genes [38]. The results of the experiment showed that HDI pre-treatment caused a significant increase in the death response to HAMLET, and there was also a further increase in histone acetylation response to HDIs (due to the presence of HAMLET). HAMLET was the first compound found that increased the hyperacetylation response to HDIs; when HDIs have been combined with other anti-tumoral agents such as cisplatin, no further increase in acetylation is observed [38]. HDIs are already being used for the treatment of breast and prostate cancer, and they can ameliorate the effects of other anti-tumoral drugs in cancer therapy. It was suggested in the study that the use of HDIs and HAMLET together in the treatment of cancer may be of some value.

Disruption of proteasome function

Studies suggest that HAMLET enters cells and interacts directly with 20S proteasomes, but resists degradation, causes the modification of the proteasome structure and leads to the inhibition of their activity [39]. However, this activity is only seen in tumour cells. The proteasome is essential for the degradation of misfolded proteins in the cell, and proteasome inhibition and the failure to eradicate misfolded proteins is a known cause of amyloid formation and disease. Although HAMLET and the proteins that lead to amyloid disease have similar properties and effects, HAMLET 'appears to have struck a balance between beneficial and destructive mechanisms, which may be a key to tumour cell death' [39]. Because of the interaction, there is a large accumulation of HAMLET within the cells. The inhibition of proteasome activity was found not to be responsible for the cytotoxic effect of HAMLET, however, as traditional proteasome inhibitors reduced, rather than potentiated, HAMLET activity.

Macroautophagy

Macroautophagy, usually associated with the degradation and the reutilization of long-lived proteins and organelles especially in response to starvation, has been put forward as a form of cell death called autophagic/type 2 cell death [11]. It may be a mechanism through which early tumorigenesis is suppressed and macroautophagy-inducing drugs are in development for the treatment of cancer [40]. The appearance of double-membrane-enclosed vesicles in HAMLET-treated cells suggested to scientists that the cause of tumour cell death may be related to macroautophagy [40]; however, in a separate study, HAMLET has been shown to induce lysosomal death involving the permeabilization of the lysosomal membrane [41].

HAMLET sensitivity and the 'hallmarks of cancer'

In addition to the multiple targets above, HAMLET has been demonstrated to bind with α -actinins, which are important for cell adhesion [42]. A more surprisingly relevant area of targets deals with HAMLET's sensitivity to tumour cells that have oncogenic c-Myc expression. Moreover, HAMLET's sensitivity was shown to be modulated by the glycolytic state of the tumour cells. Using an shRNA (small hairpin RNA) screen, several key enzymes in the glycolytic machinery (such as hexokinase 1,6-phosphofructo-2-kinase/fructose biphosphatase and hypoxia-inducible factor 1α) were found to be involved, thereby suggesting that it may also be exploiting the common features observed in cancer cells such as the Warburg effect [43].

HAMLET as a therapeutic agent

HAMLET has been tested on over 40 different cell lines, and the effects of HAMLET are not just specific to human tumours; HAMLET has been shown to have activity in tests on tumour cell lines of primate, murine, bovine and canine origin [33]. It was thought that toxic side effects due to the treatment of HAMLET were unlikely because HAMLET consists of molecules from human milk and is ingested by newborn infants [33].

The *in vivo* effects of HAMLET in a glioblastoma model were examined in one of the experiments on the therapeutic actions of HAMLET [44]. Glioblastomas are extremely hard to treat, and any treatment that is given to patients concentrates on reducing the severity of the disease symptoms rather than trying to halt/reverse the progression of the disease or provide a cure [33]. In the experiment, glioblastoma tumours were xenografted in nude rats and either HAMLET or native α -lactalbumin (which acted as the control) was injected by CED (convection-enhanced delivery) into the area of the brain with the glioblastoma. HAMLET caused a reduction in the intracranial tumour volume and also delayed the onset of pressure symptoms that usually accompany glioblastomas. The rats treated with the native α -lactalbumin control, however, showed no such improvements. The LD₅₀ values of the non-transformed cells were much higher than that of the transformed cells, suggesting that the therapeutic concentrations of HAMLET were not toxic for intact brain tissue.

The therapeutic effects of HAMLET in the treatment of skin papillomas was later conducted as the first model to examine HAMLET treatment in humans [45]. Papillomas are pre-malignant lesions of the mucosal surfaces and the skin and therapeutic options are often very limited [33]. HAMLET treatment saw a 75 % reduction in the volume of the papillomas after 2 years, whereas the placebo had no such efficacy. No adverse effects were reported from the treatment.

In addition, an investigation into the therapeutic effects of HAMLET as applied to human bladder cancers using intravesical instillations (introduction within the urinary bladder) has been conducted [46]. HAMLET caused a rapid increase in the shedding of tumour cells into the urine and

a reduction in the tumour volume, whereas the intravesical instillation of NaCl, PBS or native α -lactalbumin (all of which acted as controls) did not lead to either of these results. There was no evidence of any harm to the differentiated cells surrounding the treatment area, which presented further evidence in support of the HAMLET selectivity for tumour cells. A more detailed study using a mouse bladder cancer model provided further evidence that HAMLET possessed strong therapeutic potential [47].

HAMLET and comparisons with amyloid

Equine lysozyme is the closest structural equivalent to α -lactalbumin and has widely been used as a model in protein folding and amyloid studies over the last two decades [48]. It is similar to α -lactalbumin in that it forms a range of partially folded states under equilibrium destabilizing conditions, and it can be coupled with oleic acid to produce a compound known as ELOA. Equine lysozyme also forms oligomeric and fibrillar amyloid structures (oligomers are likely to have a larger role in the pathogenesis of protein deposition diseases than amyloid fibrils) under acidic conditions, where it is in its partially folded state. The fact that equine lysozyme, considered to be an evolutionary bridge between the C-type lysozymes and α -lactalbumin, can form such oligomeric and fibrillar structures is quite important because this suggests that HAMLET may also form such amyloid structures.

To see whether HAMLET formed amyloid fibrils, solutions of HAMLET were left at various temperatures for various durations, ranging from days to months, and the presence of aggregates was examined [11]. No fibril formation was seen and further experiments to see whether the mechanisms of cytotoxicity of HAMLET are substantially different from those of amyloid fibrils are actively underway.

Conclusion

HAMLET is a complex that has much potential as a therapeutic agent in the treatment of cancer and has been shown to cause tumour cell death in many different tumour cell lines [33]. Further studies on the structure of HAMLET and its analogues, along with their cellular targets, will shed light on the functions of partially unfolded proteins as interesting neurobiological entities.

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