Structure and Potential Cellular Targets of HAMLET-like Anti-Cancer Compounds made from Milk Components

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ABSTRACT - The HAMLET family of compounds (Human Alpha-lactalbumin Made Lethal to Tumours) was discovered during studies on the properties of human milk, and is a class of protein-lipid complexes having broad spectrum anti-cancer, and some specific anti-bacterial properties. The structure of HAMLET-like compounds consists of an aggregation of partially unfolded protein making up the majority of the compound's mass, with fatty acid molecules bound in the hydrophobic core. This is a novel protein-lipid structure and has only recently been derived by small-angle X-ray scattering analysis. The structure is the basis of a novel cytotoxicity mechanism responsible for anti-cancer activity to all of the around 50 different cancer cell types for which the HAMLET family has been trialled. Multiple cytotoxic mechanisms have been hypothesised for the HAMLET-like compounds, but it is not yet clear which of those are the initiating cytotoxic mechanism(s) and which are subsequent activities triggered by the initiating mechanism(s). In addition to the studies into the structure of these compounds, this review presents the state of knowledge of the anti-cancer aspects of HAMLET-like compounds, the HAMLET-induced cytotoxic activities to cancer and non-cancer cells, and the several prospective cell membrane and intracellular targets of the HAMLET family. The emerging picture is that HAMLET-like compounds initiate their cytotoxic effects on what may be a cancer-specific target in the cell membrane that has yet to be identified.

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INTRODUCTION

HAMLET-like compounds characterised to date consist of partially-unfolded protein, usually a milk protein, complexed with oleic acid (1), a similar fatty acid (2,3), or sodium oleate (4,5). The protein is partially unfolded and the available structural evidence suggests that the protein polypeptide, referred to in this review simply as protein, is partially folded around the oleate molecules that are aggregated in the centre of the complex (5,6). The fatty acid is in the free monomeric form, which may make membranes and membrane proteins particularly sensitive to it (Figure 1), and is not in the triglyceride form of the oleic acid in olive oil. The first discovered member of this family is HAMLET - Human Alpha-lactalbumin Made LEthal to Tumours. During studies into the anti-bacterial properties of human milk, it was observed that one of the milk fractions induced cell death in the cancer cells used to investigate host bacterial infection (7). The active ingredient, HAMLET, was identified as composed of the α-lactalbumin protein and oleic acid from the human milk (1). The column chromatography fractionation process had unexpectedly and serendipitously created the HAMLET compound having novel anti-cancer properties that the milk protein alone does not possess. When cow’s milk protein α-lactalbumin is used instead of the human version of the protein to create the complex with oleate, the complex is known as BAMLET - Bovine Alpha-lactalbumin Made LEthal to Tumours (8).
HAMLET-like compounds can be created from a range of proteins and a few fatty acid variants, and the structural characteristics of this class of compound are reviewed. Methods other than column chromatography, involving heating or high pH conditions to unfold the protein and the mixing of a carefully controlled amount of oleic acid or fatty acid anion (9-12), are also used to prepare HAMLET-like compounds.

HAMLET-like compounds have generated interest in several research groups for two main reasons:

1) *In vitro*, HAMLET and BAMLET compounds show cytotoxicity to over 50 cancer cell lines (7,13-20) including cancer cells resistant to cell death (21) while most primary cell cultures investigated, with the exception of blood cells, show resistance to toxicity from HAMLET-like compounds (7,13,14). Embryonic cells (7) and immortalised non-cancer cells (7,14,16,22) are sensitive to HAMLET. A summary of results of HAMLET-like compounds on cell lines and primary cells is provided in Figure 2 and Supplementary Table 1, and is informative for the analyses of the mechanistic studies into HAMLET's modes of action and potential targets.

2) *In vivo* experiments demonstrated HAMLET and BAMLET anti-cancer activity and showed no toxicity. The researchers who discovered HAMLET have shown HAMLET to reduce tumours without toxic side effects *in vivo* in human and mouse orthotopic bladder cancer (23,24), in orthotopic glioblastoma tumours in nude rats (25), and in mice predisposed to colon cancer (26). HAMLET treatment resulted in a reduction of warts and no signs of toxicity in both immunocompetent and immunosuppressed patients (27). An independent group of researchers showed that BAMLET administered by catheter instillation reduced orthotopic bladder tumours without toxicity in rats (14).

The structure of HAMLET-like compounds and how the biological effects are a result of this structure are becoming matters of consensus by researchers in the field, and form an important basis for this review.

Twenty years of research have revealed numerous intracellular targets for the HAMLET-like compounds and have shown HAMLET-induced effects on the cell membrane and its ion fluxes. It is not yet clear which one initiates the cascade of effects that leads to cell death. Recent studies are focussed on the membrane protein effects as possible initiators of the subsequent intracellular effects and differing modes of cell death in different cell types (28-33). In addition to presenting a comprehensive survey of the constellation of multiple cell membrane and intracellular mechanisms associated with HAMLET-induced cell death, this review synthesises the voluminous and sometimes contradictory information and offers speculative but plausible explanations for HAMLET cell death mechanisms. An example of confounding results is that HAMLET appears to be deactivated by extracellular calcium (4,29,34), and yet intracellular calcium may be necessary for the HAMLET cytotoxic effect to occur (7,28).

An open question remains as to whether the complex would be formed by conditions in the stomach when milk containing the HAMLET ingredients is ingested. HAMLET-like compounds also possess promising anti-bacterial potential, not reviewed in this paper, killing *Streptococcus pneumoniae* (35) and resensitising methicillin-resistant *Staphylococcus aureus* (MRSA) as well as other drug-resistant bacterial organisms to antibiotics so that these bacteria are killed when treated with a combination of HAMLET and methicillin or other antibiotics (36,280).

**CYTOTOXICITY TO CANCER AND NON-CANCER CELLS**

*In vitro cytotoxicity*

Cytotoxicity of HAMLET-like compounds has been reported by 12 different research groups in 23 publications on a wide range of cancer cell lines *in vitro* and found to be cytotoxic at doses that the controls, often the α-lactalbumin protein without oleic acid, were not cytotoxic, or at doses that were not cytotoxic to non-cancer cells. Supplementary Table 1 lists the 50% lethal doses (LD50) and viability assays for cancer and non-cancer cell lines and for non-cancer primary cells treated with HAMLET-like compounds. In some cases, different research groups have reported different LD50 values for the same cell lines, and this is due to differences in the amount of active oleate ingredient in the compounds in addition to differences in experimental conditions and protocols. This highlights that cytotoxicity for cancer and non-cancer cells can only be readily compared for experiments using the same HAMLET-like compound under the same conditions. These comparisons are shown in
Figure 2 and show the trend that cancer cell lines are more sensitive to HAMLET-induced cell death than non-cancer primary cells. Håkansson et al. (Figure 2A (7)) found that the human epithelial cancer cell lines A498 kidney, A549 lung, NCI-H292 bronchial, HT29 intestine, CaCO2 intestine, J82 bladder, and HTB9 bladder are more sensitive to HAMLET than primary human epithelial cells from kidney, nasopharynx, bladder, and urinary tract. Håkansson et al. (Figure 2B (37)) also found that the human epithelial cancer cell line A549 lung is more sensitive to HAMLET than primary human renal epithelial cells. Both Gustafsson et al. (Figure 2D (18)) and Zhang et al. (Figure 2G (38)) found that brain-derived glioma cancers are more sensitive to HAMLET than brain-derived non-cancer astrocytes. However, for non-blood cells, there are 2 outliers to this trend. Non-cancer primary keratinocytes are as sensitive to HAMLET as cancer cells, and colorectal cancer cell line CaCO2 is as resistant to HAMLET as non-cancer primary human umbilical vein endothelial cells (Figure 2M (13)). All the primary blood cells assayed are more sensitive to HAMLET than cancer cells (Figure 2M (13)) and so don't conform to this trend either. Thus, there is no dose at which HAMLET kills all cancer cells and spares all non-cancer cells. Another trend shown by Figure 2 is that leukaemias and lymphomas are generally more sensitive to HAMLET-induced death than carcinomas (Figure 2J (39), Figure 2L (16) and Figure 2M (13)), and primary non-cancer blood cells are more sensitive than primary non-cancer epithelial cells (Figure 2A (7) and Figure 2M (13)). However, although HAMLET-like compounds have been assayed on 50 different cancer cell lines, the literature contains only 11 types of primary cell cultures, and thus it remains to be seen whether these trends will hold when more types of primary cells are investigated. The 6 immortalised non-cancer cells lines investigated have sensitivities to HAMLET that are in the same range as the cancer cell lines (7,14,16,22). It is not yet clear whether this is due to a general toxicity of HAMLET to healthy cells, or whether HAMLET is targeting a fundamental cancer-like characteristic that is possessed by immortalised non-cancer cell lines. It is also not clear how other factors present in the in vivo environment may provide an increased difference in sensitivity of immature and cancer cells compared to healthy differentiated cells.

In addition to the 50 different cancer cell lines, HAMLET was found to be cytotoxic to 2 embryonic primary cell types (7). These results are the basis for the reports that HAMLET is selectively cytotoxic to cells with an immature phenotype (7). Generally, the more immature a tumour is, the more aggressive it is (40-42). It is a goal of cancer research to find compounds that are effective in killing undifferentiated aggressive cancers (41,43), and interest in HAMLET stems from HAMLET's ability to kill immature cells such as cancer cells and the possibility that this cytotoxicity is selective for immature cells over healthy differentiated cells.

**In vivo cytotoxicity and efficacy**

HAMLET and BAMLET reduced bladder cancer tumours without toxicity to healthy tissue in humans (23), rats (14), and mice (24). Nine human patients awaiting transurethral surgery for bladder cancer received HAMLET instillations via urinary catheter for 5 consecutive days (23). Massive shedding of tumour cells into the urine and a reduction in tumour size occurred, and no cell death was detected in biopsies of healthy tissue adjacent to the tumours (23). In an orthotopic bladder cancer model of immunocompetent rats, BAMLET treatment resulted in tumour growth inhibition and long term survivor numbers that were comparable to the control treatment that consisted of what is currently the most effective treatment for non-invasive bladder cancer (Bacillus Calmette-Guérin), and found no evident BAMLET side-effects to bladder walls, rat body-weight change or general well-being (14). In an orthotopic bladder cancer model of immunocompetent mouse, HAMLET treatment resulted in delayed tumour development and reduced tumour volumes (24). TUNEL staining showed that tumour tissue underwent apoptosis and adjacent healthy tissue did not (24). HAMLET delivered by a 24 hours intra-cranial infusion reduced orthotopic glioblastoma tumours in rats and prolonged their survival without signs of toxicity (25). MRI showed no oedema or tissue damage in the surrounding healthy brain, and histopathology showed no significant signs of toxicity in surrounding brain parenchyma compared with the control group (25). HAMLET treatment taken orally resulted in a reduction of the number of colon tumours and longer survival of tumour-bearing mice predisposed to colon cancer (26).
Figure 1. The cellular membrane showing HAMLET (green and yellow aggregates, adapted from (6)) on the extracellular side, and a kinked oleic acid (yellow and red) in the free fatty acid form, spanning the outer bilayer leaflet (grey and pink). The oleic acid is shown to scale as being a similar length to the lipids making up the bilayer leaflet. Identification of the HAMLET targets remains unresolved, and some potential membrane targets (sodium channel, sodium exchangers, and ectopic ATP synthase with 2 yellow and red oleates placed in the centre of the c-ring where they are hypothesised to have similar affinity as cardiolipin) are shown in grey with a question mark. Calcium ions (green spheres) are shown stabilising the extracellular side of the plasma membrane, and may have a fundamental role in HAMLET-induced cell death.

Analysis of intestinal segments found that HAMLET-treated mice had normal villi whereas untreated mice had intestinal villus hyperplasia (26). HAMLET was used as a topical cream treatment for skin warts of immunocompetent and immunosuppressed human patients, as warts are induced by papillomaviruses and therefore immune responsive (27). Most of the warts lost volume during HAMLET treatment and most warts had disappeared at the 2 year follow-up in both populations (27). Visual inspection and interview with patients reported no symptoms to healthy skin adjacent to the treated warts (27).

HAMLET compounds are partially deactivated in blood (4,13), and thus these in vivo studies avoided contact with blood. BAMLET is toxic to primary blood cells in vitro (13,29,44) (Figures 2A, 2H, and 2M) and the lack of toxicity of HAMLET and BAMLET to blood cells in vivo may be explained by the fact that the in vivo studies avoided contact with blood and may also be due to HAMLET being deactivated by components in blood upon contact.
Figure 2.
Figure 2. Comparisons of levels of toxicity induced in vitro by HAMLET, BAMLET and other HAMLET-like compounds to cancer and non-cancer cells taken from A (7), B (37), C (25), D (18), E (16), F (28), G (38), H (44), I (22), J (39), K (25), L (16), M (13), N (14), and O (5,44). For each graph, cancer cells are shown on the left and non-cancer cells on the right, and are categorised into carcinomas and papillomas (orange), pheochromocytomas, osteosarcomas, and melanoma (magenta), gliomas (dark red), lymphomas and leukaemias (bright red), non-cancerous immortalised cell lines (purple), non-cancerous embryonic cells (green), non-cancerous primary non-blood cells (light blue), and non-cancerous primary blood cells (dark blue). The graph regions are coloured by cell type and the width of a region is proportional to the number of cell types for which assays have been performed. Results for viability assays are presented (graphs A-I), followed by LD₅₀ results (graphs J-O). For cells that are resistant to HAMLET, the LD₅₀ values were not determined, and those cells appear in viability assay graphs only. For cells that are sensitive to HAMLET cytotoxicity, LD₅₀ values were determined and the cells appear in both the viability and LD₅₀ graphs. In graph L, where two values appear for one cell line, one value is for HAMLET and the other for BAMLET. In graph O, the same cell type appears more than once because it was examined with several HAMLET-like species. The descriptive names of cell lines appear in Supplementary Table 1.
Figure 2 Continued…

J

K

L

M

N

O

- Carcinomas and papillomas
- Pheochromocytomas, osteosarcomas, and melanoma
- Gliomas
- Lymphomas and leukemias
- Non-cancerous immortalised cell lines
- Non-cancerous embryonic cells
- Non-cancerous primary non-blood cells
- Non-cancerous primary blood cells
**STRUCTURE**

**Preparation from several proteins**

The most studied HAMLET-like compounds are HAMLET and BAMLET, prepared from oleic acid and either human or bovine α-lactalbumin protein, respectively. Other proteins from which cytotoxic HAMLET-like compounds can be made include α-lactalbumin of goat (referred to as GAMLET), horse, pig, and camel (19,46,47), and other proteins altogether, such as bovine β-lactoglobulin (3,4,17), bovine lactoferrin (48), bovine serum albumin (5), pike fish parvalbumin (17), horse lysozyme (referred to as ELOA) (49), recombinant dog milk lysozyme mutant (47), horse heart apo-myoglobin (47,50), chicken ovalbumin (5), recombinant β2-microglobulin (47), and human immunoglobulin G (5).

Structural changes have been made to α-lactalbumin, and it continues to be able to make cytotoxic compounds when complexed with oleic acid. These include chemically cross-linking residues before, during or after adding oleic acid (44,51); binding the protein to relatively large Sepharose 4B beads (44); replacing all the cysteines with alanines so as to remove all disulphide bonds (52); clipping the protein into peptides by proteolytic enzymes (53); or use of synthesised peptides from the various domains having cysteines modified to alanines (54).

Cytotoxic HAMLET-like compounds can be made from many different types of proteins indicating that the HAMLET activity is not due to a specifically folded protein structure.

**Oleic acid is the main active component**

It is generally accepted that the main active component of HAMLET-like compounds is the oleate, whether it comes from oleic acid or sodium oleate, as few substitutions of the lipid allow for HAMLET-like cytotoxicity (4,17,51,53,54).

Some studies of HAMLET-like compounds have found that the complex is as cytotoxic to cells as is oleic acid alone when the same amounts of oleic acid are applied as the amounts present in the complexes (13). Other studies found that the protein-lipid complex is more cytotoxic than similar amounts of oleic acid or sodium oleate alone (3-5,15,17,38,44,50,54). It is not yet clear whether these are true differences in cytotoxicity for the different protein carriers of oleate, or whether these different results reflect difficulties in solubilising oleic acid alone for delivery in water-based cellular systems, or are a result of accuracy and precision in measuring the oleate amounts present in the HAMLET-like compounds. It is also not clear whether the mechanisms of cell death induced by HAMLET-like compounds or oleic acid alone are the same.

In a study that further supports this observation, BAMLET protein was linked to Sepharose 4B beads – which are unlikely to enter cells – and the BAMLET beads nonetheless exhibited cytotoxicity to red blood cells. With each reuse, the cytotoxicity was reduced (44). These results suggest that the cytotoxic component of BAMLET is oleic acid, which dissociates from the protein to exert the cytotoxic effect, so that less oleate is available next time the BAMLET beads are used.

Increasing the oleate content of a HAMLET-like compound increases its cytotoxicity. The amount of oleate that can be added to a partially-unfolded protein to form a cytotoxic HAMLET-like compound is approximately 1 oleate per 1 kDa molecular weight of protein (5). Adding less oleate during preparation than the maximum amount results in less oleate being complexed with protein and approximately correspondingly less cytotoxicity (higher LD<sub>50</sub>) (4).

Supplementary Table 2 lists the reported stoichiometries of protein and fatty acid in HAMLET-like compounds made from several proteins by various methods.

Unsaturated cis fatty acids of carbon chain length from 16 to 20 are able to bind α-lactalbumin by column chromatography and form HAMLET, while saturated fatty acids or unsaturated trans fatty acids are not (55). The fatty acids that produce the most cytotoxic HAMLETs are C18:1:9 cis (oleic acid) and C18:1:11 cis (vaccenic acid), and others that produced HAMLETs of lesser cytotoxicity are C18:3 cis (α-linolenic acid and γ-linolenic acid), C18:1:6 cis (petroselinic acid), C18:2 cis (linoleic acid), and C16:1:9 cis (palmitoleic acid) (55). When complexed with bovine β-lactoglobulin, oleic acid and linoleic acid have similar cytotoxicities (3).

In vitro cell death assays of HAMLET and BAMLET frequently use the α-lactalbumin protein without oleic acid as the control, and find that the apo-protein is not cytotoxic (3,13-16,21,24,38,44). Calcium depleted α-lactalbumin takes on the same folded structure as the native
calcium-bound form (66) and the apo-protein used in cell death assays can be assumed to be folded. However, an in vivo experiment treating bladder cancer in rats by instilling BAMLET into the bladder by catheter found that a high dose of α-lactalbumin showed similar tumour-reducing activity as the low dose of BAMLET (14). In this particular case, the apo-protein may have been unfolded by the urine in the bladder, and this result suggests that the unfolded protein may have cytotoxic effects on cancer cells even without oleic acid. If the oleate in HAMLET separates from the protein to exert its cytotoxic effect, then the protein component is probably left in a state of unfoldedness, and in this state it may also exert toxic effects.

The α-lactalbumin protein component of HAMLET enters cells and associates with intracellular components (18,37,56-59), as does the β-lactoglobulin protein component when HAMLET is prepared from that protein (4). It is not known whether the HAMLET protein is folded when it is inside the cell, and unfolded protein inside the cell may fold spontaneously or with the assistance of chaperone proteins (60,61). HAMLET protein inside the cell, whether folded or not, may exert detrimental effects. There is evidence that α-lactalbumin triggers apoptosis of mammary cells during weaning, as the regulatory mechanism for breast involution when milk is no longer needed (62). Alpha-lactalbumin protein triggers cell death in normal rat intestinal epithelial cells (63) and virus-transformed mouse leukaemia cells (64). These results and HAMLET results showing HAMLET cytotoxicity superior to that of the oleic acid alone (3-5,15,17,38,44,50,54), indicate that α-lactalbumin protein is also playing role in HAMLET-induced cell death.

**Partially unfolded**

Far-UV circular dichroism (CD) studies show that the protein component of HAMLET-like compounds is in a partially-unfolded molten globule-like state (1,5,19,51). Most methods for associating oleate with protein to make a HAMLET-like compound involve unfolding the protein by heating or by extreme pH in the presence of the fatty acid anion so that when conditions are returned to physiological and the protein folds again, it folds around the fatty acid anion (9-11,65). The hydrophobic fatty acid is thus associated with the hydrophobic surfaces of the interior of the partially refolded protein, and the protein is inhibited from folding back to its native conformation by the presence of the fatty acid in its interior. As the native structure of α-lactalbumin strongly binds a calcium ion (66), some HAMLET preparation methods involve removing calcium with EDTA so as to facilitate the unfolding of the native structure of the polypeptide (1,9,11) and the resulting HAMLET is anticipated to not contain calcium, whereas other methods do not remove calcium (7,10,12). Repeated heating of BAMLET or bovine α-lactalbumin without oleate, to temperatures that allow the formation of BAMLET from its protein and fatty acid components, show that the unfolding is a gradual, continuous process (6). The thermal unfolding of ELOA is continuous even though the native equine lysozyme without oleic acid exhibits the expected specific transition points of unfolding (49). Small-angle X-ray scattering (SAXS) studies of BAMLET and other HAMLET-like compounds at the high pH that allow the formation of HAMLET from its components indicate that the protein's native tertiary structure is altered (5,6,54).

As HAMLET-like compounds can be made by chromatography (1) or from mixing the components without further steps to partially unfold the protein (51), the oleate may associate with the hydrophobic protein interior by entering and exiting the protein during the normal protein breathing that is facilitated at lower dilutions and is suppressed in conditions of molecular crowding (67). HAMLET preparation methods involving sustained partial unfolding by heating or extremes of pH result in higher amount of incorporated oleate (5,9).

**Aggregation**

Chemical cross-linking experiments, gel electrophoresis, particle sizing measurements and turbidity observations show that HAMLET-like compounds aggregate at physiological pH (5,7,50,51). Supplementary Table 2 lists the measured amounts of protein and oleic acid per aggregate for several HAMLET-like compounds.

 Pulse field-gradient NMR studies show that a solution of HAMLET at pH 7.5 contains some native protein (without bound oleic acid), the monomeric molten-globule state protein (bound with oleic acid), and oligomers (bound with oleic acid) (47). Gel filtration column chromatography of BAMLET at pH 7.4 at 15 to 25 mg/ml
produces non-cytotoxic monomers and cytotoxic oligomers of ~110 kDa (51). Increasing the ratio of oleic acid to protein results in higher molecular weight oligomers and the almost disappearance of monomers (51). Decreasing BAMLET concentration to ~2 mg/ml results in monomers eluting, but dynamic light scattering (DLS) shows that the complex is oligomeric in solution (51). Cross-linking 0.1 mg/ml of BAMLET with glutaraldehyde produces oligomers in SDS-PAGE (51). These results suggest that the amount of oleic acid bound to protein in HAMLET-like compounds and how much protein remains unbound are functions of how much oleic acid is available during preparation of the complex. These results also suggest that the oligomeric BAMLET is a dynamic aggregate in rapid exchange with the monomeric form and the aggregation can be interrupted by the gel filtration column (51).

At pH 7.4, the diameter of the main species of BAMLET aggregates is 7.13±0.98 nm as determined by DLS (51). The pH 7.5 solution of 4.2 mg/ml HAMLET contains three species, which are the native protein of diameter 4 nm, monomeric HAMLET of diameter 4.8 nm, and HAMLET oligomers of diameter 11.4 to 12.2 nm, as determined by pulse field-gradient NMR (47). Modelling of SAXS data at pH 9.0 and RP-HPLC measurements give diameters of 1.8 to 3.6 nm depending on the BAMLET preparation method and diameters of 1.8 to 2.8 nm for HAMLET-like compounds made from β-lactoglobulin, lysozyme, parvalbumin, ovalbumin, albumin or immunoglobulin G (5). In filtered water, BAMLET has diameters ranging from 227 to 292 nm, as measured by DLS, depending on the BAMLET preparation method (22). Figure 3 shows a plausible model for a BAMLET oligomer at physiological pH.

On a surface, ELOA oligomerises into ring formations (49). HAMLET is able to form rings on negatively-charged membranes at neutral pH (68). This phenomenon is not thought to be involved with the compound's cytotoxic activity (68).

**Novel lipid-binding**

SAXS can provide valuable low-resolution structural information on biological molecules (69,70). When a biological compound in solution contains monodispersed, identical particles, analysis of scattering data allows the modelling of the compound's individual unit (70). However, many different models can produce the same scattering data and thus a given SAXS-derived model is not guaranteed by the scattering data alone to be the actual 3D structure of a given compound (69,71). SAXS studies of BAMLET and other HAMLET-like compounds indicate that the protein tertiary structure is dramatically different to the native fold (5,6). The SAXS data at the high pH of complex formation has a characteristic bump at high scattering vector (q) values, consisting of a non-sharp first minimum followed by a well-developed secondary maximum, not seen for native proteins, that indicates that the protein is on the outside of the complex (5,6). The characteristic bump occurs at q = 0.11 to 0.18 Å⁻¹, which corresponds to distances of 35 to 57 Å between the centres of mass of the opposing sides of the shell. The oleate is assumed to be mediating the peripheral protein positioning by being located on the inside (5). The bump is similar to what is produced by core-shell models suggesting that BAMLET has a core-shell-like structure. However, regular, symmetrical core-shell models having traditional homogeneous or gradient density shells cannot be fitted to the SAXS curves of HAMLET-like compounds. At pH 9 to 11, SAXS data shows that the complex is aggregated and the oleate is proposed to be a spherical micelle formation in the centre decorated with protein on the periphery having a flexible, partially unfolded, irregular conformation (5). Increasing the pH to pH 12 inhibits the aggregation of BAMLET, revealing the protein as having the form of a winding, irregular, thick, cylindrical thread (6) that is well fitted by an alpha-helical secondary structure and a small amount of protein tertiary structure domains. Figure 3 is a representation of a winding, irregular, thick, cylindrical thread binding fatty acid in its core. This newly reported type of lipid-protein structure (5,6) has been named the “liprotide” model (to denote lipids and partially denatured proteins) (5). Albumin, a protein that has high affinity binding sites for oleic acid (72,73), is one of the proteins from which a HAMLET-like compound can be made. The SAXS-determined high pH structure of the HAMLET form of albumin having HAMLET-like cytotoxicity to erythrocytes is characteristic of liprotide structures (5) and not of the native protein at high pH (74) nor of the native protein binding oleate at physiological pH (75). This
indicates that the oleate is loosely bound in the HAMLET form of albumin, and does not have the high affinity binding as found in the native form. These structures can be interpreted as showing that the function of the protein of HAMLET-like compounds is that of a carrier of the fatty acid, keeping the fatty acid watersolubilised and inhibiting it from self-associating to form a fatty, unreactive micelle. When this liprotide structure comes into contact with cell membranes, the fatty acid would have higher affinity for the cell membrane and leave the protein complex. The mechanism of the cell membrane sequestering the fatty acid from BAMLET has been named as the “cargo off-loading mechanism” (5,76). If the fatty acid was incorporated into a fatty acid micelle, instead of inside a partially unfolded protein, it could be expected that the majority of the fatty acid would exhibit higher affinity for staying in the micelle. The common structure of HAMLET-like compounds is thought to explain the similar effects exerted on cells by compounds made from different proteins (5).

Secondary structure
The protein secondary structure of α-lactalbumin, β-lactoglobulin and lysozyme in HAMLET-like compounds is similar to the molten globule forms of those proteins (4,49,77). It has also been observed that the molten-globule state of HAMLET is similar to the state of α-lactalbumin interacting with liposomes or micelles (51). HAMLET protein contains more alpha-helical and less beta-sheet secondary structure than does the unbound molten globule structure of the α-lactalbumin protein (6,77,78). The proteolytic fragments of α-lactalbumin become more ordered and increase in alpha-helical secondary structure content upon binding oleic acid to form HAMLET-like compounds (53). The HAMLET-like compound formed from horse heart apomyoglobin maintains its highly helical conformation (50). The BAMLET-like compound formed from bovine β-lactoglobulin and sodium oleate shows an increase in the protein's mainly beta-sheet structure and less tendency to form intermolecular beta-sheet aggregation upon heating than does the native protein (4). HAMLET-like compounds made of bovine serum albumin, chicken ovalbumin and human immunoglobulin G, proteins that have different secondary structures to each other, show that on forming the complex with oleate, all exhibit a change in secondary structure compared to the native proteins and that the change is different for the different proteins (5).

Proteolysis experiments show that the beta domain of bovine α-lactalbumin is amenable to proteolysis and thus must be a flexible region, and the N- and C-terminal regions are resistant (51). These characteristics remain the same for BAMLET (51).

These results indicate that although the liprotide complexes have a global structural similarity of partially unfolded peripheral protein (5,6), at the detailed level of protein secondary structure, the structure is dependent on the protein in the complex and there is not a universal HAMLET-like protein secondary structure.

Bonding interactions between protein and fatty acid
The oleate is assumed to bind the protein of HAMLET-like compounds due to both hydrophobic and electrostatic interactions (51). The partial unfolding of the protein is necessary to expose hydrophobic surfaces (53) that are mainly in the core of folded soluble protein. Isothermal titration calorimetry experiments at pH 7.0 show that the reaction of complexing oleic acid and bovine α-lactalbumin is entropy driven, indicating that the formation of complexes occurs as a result of hydrophobic interaction rather than as a result of electrostatic interactions (79). NMR of ELOA shows resonance signals for oleate protons with aromatic residues of the lysozyme protein (49), which indicates that the hydrophobic oleate carbon chain is associating with those residues by hydrophobic interaction.

At physiological pH, free oleic acid is near half-ionisation (80), and thus deprotonated negatively charged oleates are available for electrostatic interaction with positively charged protein residues of the HAMLET protein, as occurs for oleate bound to albumin (75,81). Chemically removing the positive charge of α-lactalbumin's lysine side chains removes the ability of the protein to form BAMLET (78). Xie et al. suggest that the negatively charged oleate in BAMLET binds to the positively charged lysine side chains of the protein (78). Alternatively, changing the positive charges of lysine side chains to neutral or negative charges may result in the protein no longer folding, and thus no longer folding around the oleate. The chromatography
results indicate that the protein having lysine side chains modified to negative charges is no longer folded, and that the protein having lysine side chains modified to neutral remains folded before addition of oleic acid but is no longer folded when oleic acid is added (78). Thus, these results don't unequivocally show that the oleate in HAMLET is binding the protein by electrostatic attraction.

NMR studies show that the oleic acid in HAMLET is not associated with the protein in the same position as the oleic acid in GAMLET, even though the human and goat α-lactalbumin proteins are very similar (47). The human and goat α-lactalbumin sequences have 10 of the 14 lysines in the same place (82,83). These NMR results are consistent with general hydrophobic interactions rather than specific binding domains for the oleates in the conserved α-lactalbumin protein.

Incubating BAMLET with CaCl₂ results in the formation of a calcium oleate precipitate making the solution turbid, and further incubation with the calcium chelator EDTA results in the solution becoming clear again (51). The clear solution indicates that EDTA has sequestered the calcium from the calcium oleate precipitation. The oleate has not formed aggregates with itself because a solution containing oleic acid micelles appears turbid, not clear (51). These results indicate that the binding of oleic acid to BAMLET protein is dynamic.

Incubating the proteins ovalbumin, bovine serum albumin (BSA), and immunoglobulin G by themselves at high temperature results in a turbid solution, which indicates that the proteins have partially unfolded and their exposed hydrophobic surfaces are interacting, and adding oleic acid makes the solution clear again, indicating that oleic acid has bound the hydrophobic surfaces of the partially unfolded proteins and thus reduced the aggregation (5).

The general hydrophobic and electrostatic interactions of oleate with HAMLET-like proteins are thought to not involve specific binding cavities in the protein, and this interaction is referred to as “solubilising” the oleic acid (11,13,51,53,76). As when water solubilises salts, the specific water molecules associated with specific salt ions is dynamic and is not generally identified.

Deactivation by blood, albumin, and calcium
BAMLET is deactivated in BSA and by fetal bovine serum (FBS) that contains albumin (34). When BSA or FBS is present, the LD₅₀ of BAMLET for HL60 human promyelocytic leukaemia cells is an order of magnitude higher (eg. the LD₅₀ of around 0.5 mg/ml increases to around 2 mg/ml in the presence of 20% FBS or 5 mg/ml BSA) (34). Preincubation of isolated mitochondria with BSA partially inhibits HAMLET's and oleic acid's effects on those mitochondria (84). Albumin binds oleic acid with strong affinity (72,73) and it is assumed the albumin sequesters the oleic acid from BAMLET thus deactivating BAMLET (23). The transfer of oleate from BAMLET to the albumin that has higher affinity can be considered another example of the cargo off-loading mechanism.

Calcium in the cell culture medium can drastically reduce BAMLET's cytotoxicity to red blood cells (29). The oleate in HAMLET-like compounds appears to be precipitated out of the complex by calcium. Incubating BAMLET with CaCl₂ results in the solution becoming turbid, attributed to the formation of rather insoluble calcium oleate, and the protein secondary structure changes from that of BAMLET to that of native unbound protein (51). Adding more EDTA than calcium ions so that the calcium ions will be sequestered by EDTA results in the CD-determined protein structure changing back to that of BAMLET (51). When BAMLET is exposed to human blood serum but without contact with the albumin in blood, a calcium-oleate precipitate forms and the remaining BAMLET protein is no longer cytotoxic to cancer cells (4). A high salt concentration also appears to precipitate the oleate out of BAMLET as an oleate salt (4). At low pH (pH 3 to 5), both oleate and protein precipitate out of BAMLET solution, and when solubilised again in water, form the cytotoxic BAMLET compound (4). A plausible explanation for this result is that the low pH causes the protein to unfold enough for the oleate to dissociate from the protein, and the resulting hydrophobic components (unfolded protein and oleic acid) form precipitated aggregates, and the unfolded state of the protein permits the reassociation of the oleate when resolubilised in the higher pH of water. All together these results demonstrate that HAMLET-like compounds are deactivated by high concentrations of calcium.

Dynamic association of protein and oleate
The oleate in HAMLET-like compounds appears
to be loosely bound and its association with the protein is in a dynamic state. NMR of ELOA shows that there is an equilibrium between free and bound oleic acid. As ELOA is progressively diluted, more oleic acid dissociates from the protein (76) and the size of ELOA complexes decreases (85). Surface plasmon resonance experiments at pH 4.0 indicate that the amount of sodium oleate that binds partially unfolded bovine α-lactalbumin is proportional to the concentration of sodium oleate in the solution, and increases over time until it reaches a plateau limit at a given concentration of oleate (79), indicating that the binding of oleate to BAMLET protein is an equilibrium between bound and unbound oleate. The reversible deactivation of HAMLET-like compounds by calcium described above is a result of this dynamic association of the oleate with the protein. Several residues of α-lactalbumin appear to be involved in the binding of this protein to negatively charged membranes because the residues are more protected from NMR-monitored hydrogen-deuterium exchange when the protein is bound versus when not bound (86). These results suggest that binding of BAMLET to a membrane will involve release of its bound oleic acid.

**Stability**

HAMLET prepared by the heating method was able to exert its disruptive effects on membrane ion channels even after one month in solution at 4°C (31). This suggests that the oleate remains bound to the protein during storage. The biological effect of HAMLET may be due to the instability of the association of oleate with protein (when the complex is in the vicinity of lipid cell membranes for which the oleate has a higher affinity), in which case it is conceivable that over storage time, a decrease in affinity of the oleate for the protein will result in an apparent increase in biological activity, until such storage time is reached that the oleate dissociates from the protein and thus loses its biological HAMLET-like effect. Lyophilised BAMLET looks like protein powder and is how HAMLET-like compounds are usually stored. Should the oleate dissociate from the lyophilised protein over time, it is expected to look oily instead.

When incubated with trypsin or chymotrypsin proteases, native calcium-bound α-lactalbumin remains intact after 54 hours of trypsin and 29 hours of chymotrypsin (18). HAMLET starts to degrade after ½ hour of trypsin and 1 hour of chymotrypsin and is completely degraded after 2 hours of chymotrypsin and 6 hours of trypsin (18). Partially-unfolded α-lactalbumin not bound to either calcium or oleic acid is completely degraded after only 5 minutes of trypsin or chymotrypsin (18).

**Figure 3.** A model of BAMLET showing the protein monomers of Rath et al. (6) (shades of green), that were determined by SAXS at pH 12, with the hypothesised positions of oleic acid oil drops (yellow), as an aggregation of 8 monomers so as to produce the protein molecular weight of ~110 kDa at pH 7.4 of Spolaore et al. (51). Each protein monomer is partially folded around an oleate oil drop which is associated to the hydrophobic core of the protein by hydrophobic interaction. The partially unfolded monomers associate with each other, to create the aggregated complex, by hydrophobic interaction of the exposed hydrophobic protein surfaces that are usually found in the core of the folded protein.
CELL MEMBRANE TARGETS

Interactions with ion channels and transporters

Sodium fluxes in human cells

Introduction of amiloride significantly reduces HAMLET-induced cell death in A549 lung carcinoma, HeLa cervical carcinoma, and Jurkat lymphoma cells (28). Amiloride inhibits three types of Na⁺ channels and transporters - Na⁺/H⁺ exchangers (NHE), Na⁺/Ca²⁺ exchangers (NCX), and epithelial sodium channels (EnaC) (Figure 4), and the blockage is dependent on voltage (87,88). In all types of cells, when intracellular pH decreases, the NHE is activated to maintain pH at around 7.2 by extruding the excess H⁺, and is driven by the extracellular [Na⁺] gradient maintained by the cell (89). In eukaryote cells, the NCX ensures that cytosolic [Ca²⁺] is low by pumping out excess Ca²⁺ so that the cell can use the [Ca²⁺] gradient to respond quickly to extracellular signals, and is driven by the extracellular [Na⁺] gradient. The NCX can also work in reverse to bring Ca²⁺ back into the cell (90). In polarised epithelial cells, Na⁺ diffuses into the cell through influx via the ENaC, which provides the electrochemical driving force for outward K⁺ flux via K⁺ channels (91). There are also reports that amiloride can inhibit L-type calcium channels (92), the low threshold (T) calcium channel (93), and mechanosensitive channels (94). HAMLET triggers a rapid increase in intracellular [Na⁺] in Jurkat lymphoma cells (28), and this change in [Na⁺] is inhibited by amiloride and also by BaCl₂ which is a K⁺ channel inhibitor and oleate precipitator. The α-lactalbumin protein alone (that has not had its calcium removed) or oleic acid alone do not trigger changes in [Na⁺] (28). Introduction of amiloride effectively inhibits BAMLET-induced lysis of human erythrocytes and of human erythrocyte ghosts that do not contain haemoglobin and do contain spectrin and other cytoskeletal structure (29).

The significant reduction of HAMLET-induced cell death by blockade of epithelial [Na⁺] channels and exchangers with amiloride indicates that HAMLET-induced cell death is a result of Na⁺ influxes. These results may also indicate that HAMLET-induced cell death cannot take place when cytosolic pH is low or when there is an excess of [Ca²⁺] in the cytosol, because those are the abnormal cell conditions that may occur when amiloride blocks sodium exchangers.

Alternatively, the reduction of HAMLET-induced cell death by amiloride may be due to amiloride deactivating HAMLET. Our own unpublished experiments of incubating 2 mg/ml BAMLET or bovine α-lactalbumin alone or 57 μM oleic acid alone in 50 mM HEPES and 10 mM of amiloride at pH 7.4 produced precipitation. When BAMLET or protein alone was 0.5 mg/ml or oleic acid alone was 15 μM, and amiloride was 1 mM, which are similar concentrations to those used in (28), the solutions become cloudy. These observations indicate that amiloride causes precipitation of protein alone, oleic acid alone, and of BAMLET, and that amiloride may deactivate BAMLET.

Potassium fluxes in human cells

Introduction of BaCl₂ reduces HAMLET-induced cell death in A549, HeLa, and Jurkat cells (28). Introduction of both BaCl₂ and amiloride inhibits HAMLET-induced cell death even more than either agent alone (28). Ba²⁺ blocks the wide variety of K⁺ channels that enable both inward and outward K⁺ flux, including Ca²⁺-activated K⁺ channels and voltage-dependent K⁺ channels, and the extent of blocking shows dependence on the [K⁺] on both sides of the pore (95). HAMLET triggers an increase in intracellular [K⁺] in A549 lung carcinoma cells and this change in [K⁺] is inhibited by BaCl₂ (28). Introduction of tetrandrine (an inhibitor of large conductance Ca²⁺-activated K⁺ channels) or gadolinium chloride (GdCl₃) (a general inhibitor of mechanosensitive channels) does not affect HAMLET-induced cell death or ion fluxes (28). Introduction of BaCl₂ significantly inhibits BAMLET-induced lysis of human erythrocytes and of human erythrocyte ghosts (29), and erythrocyte membranes possess Ca²⁺-activated K⁺ channels that have been implicated in erythrocyte cell death (96).

An initial interpretation of these results suggests that HAMLET-induced K⁺ effluxes, or the fluxes of another ion such as Ca²⁺ that are managed by the K⁺ effluxes, contribute to HAMLET-induced cell death (28, 29). The efflux of ions and of potassium ions in particular, plays a significant role in apoptosis (97). Alternatively, the reduction by BaCl₂ of HAMLET-induced cell death is likely due to barium binding to the oleate in HAMLET and
thus causing partial deactivation, as the divalent barium cation has similar ability to the divalent calcium cation in binding to and precipitating fatty acid anions (98) which have negative charge delocalised over the two oxygen atoms of the carboxyl head. Our own unpublished experiments of incubating 2 mg/ml BAMLET in 50 mM HEPES and 100 mM of CaCl$_2$ or BaCl$_2$ at pH 7.4 produced a precipitate. In the same unpublished experiments, when BAMLET was 0.5 mg/ml and BaCl$_2$ was 1 mM, which are similar concentrations to those used in (28), a very small amount of precipitation was observed. The protein remained in the solution and the protein concentration of the precipitation was close to zero. Incubating CaCl$_2$ or BaCl$_2$ with amounts of oleic acid alone that were similar to the amounts present in BAMLET produced similar precipitations as for BAMLET. The precipitation is assumed to be calcium or barium oleate. Incubating CaCl$_2$ or BaCl$_2$ with amounts of bovine α-lactalbumin alone that were similar to the amounts present in BAMLET did not produce any cloudiness or precipitation.

**Calcium fluxes in human cells**

Introduction of thapsigargin inhibits the HAMLET-induced DNA fragmentation that is characteristic of HAMLET-induced cell death in A549 lung carcinoma cells (7). Thapsigargin causes an influx of calcium into the cytosol via plasma membrane store operated Ca$^{2+}$ (SOC) channels when calcium diffuses out of the endoplasmic reticulum (99) due to inhibition by thapsigargin of the sarco/endoplasmic reticulum calcium ATPase (SERCA) transporters (100). HAMLET-induced cell death in the non-cancerous immortalised MDCK cell line is inhibited by incubating with HAMLET in a calcium-free medium instead of a calcium-containing medium (7). HAMLET triggers a rapid and sustained increase in intracellular [Ca$^{2+}$] in A549 cells, and the α-lactalbumin protein alone or oleic acid alone does not (28). Co-incubation of HAMLET with phospholipase C inhibitor U73122 leads to cell death but does not increase intracellular [Ca$^{2+}$] (28). When phospholipase C is not inhibited it produces a signal that leads to the release of waves of calcium into the cytosol from the internal calcium stores of endoplasmic and sarcoplasmic reticulum. The suppression of HAMLET-induced cytosolic Ca$^{2+}$ flux by U73122 indicates that the Ca$^{2+}$ flux came from the internal stores of endoplasmic and sarcoplasmic reticulum.

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A first interpretation of these results suggests that the ability of HAMLET to induce cell death is not correlated with the status or flux of calcium in the cell. Cell death occurs for HAMLET alone and for HAMLET plus U73122 and a cytosolic Ca$^{2+}$ flux is observed for the former but not for the latter. The Ca$^{2+}$ flux is observed for HAMLET plus thapsigargin treatment for which cell death is inhibited, and thus intracellular Ca$^{2+}$ flux does not seem to be correlated with HAMLET-induced cell death or lack thereof.

Alternatively, interpreting these results with the generally accepted assumption that U73122 inhibits calcium store release into the cytosol, these results indicate that HAMLET-induced cell death activity requires the internal stores to contain calcium, as store depletion (due to thapsigargin or elimination of calcium from the medium) protects cells from HAMLET-induced cell death.

**Non-selective cation fluxes in human cells**

The multiple cation fluxes (Na$^+$, K$^+$, and Ca$^{2+}$) triggered by HAMLET (28) suggest that HAMLET may trigger a non-selective cation channel that allows the extracellular cations to flow down their gradient into the cell. Such a cation leak is expected to be cytotoxic to the cell.

**Calcium fluxes in plant cells**

The study model of ion channels in green algae *Chara coralina* cells is a convenient tool for the study of ion channels in humans because the Ca$^{2+}$, K$^+$, and Ca$^{2+}$-dependent Cl$^-$ channels are analogous in structure and control mechanisms to those of many animal cells, and *C. coralina* cells...
conveniently are large and can grow in laboratory conditions (31). In *C. coralina* cells, HAMLET and oleic acid irreversibly decrease plasma membrane inward Ca\(^{2+}\) channel current, as does α-lactalbumin alone but to a lesser extent (31). Extracellular oleic acid initially produces an increase in the Ca\(^{2+}\) current for around 5 minutes followed by a significant and reversible decrease (30). Increasing the oleic acid concentration induces an almost complete and irreversible suppression of the inward Ca\(^{2+}\) current (30). Introducing oleic acid intracellularly by perfusion reversibly suppresses the Ca\(^{2+}\) current, and increasing the oleic acid concentration then causes a reversible shift in the threshold voltage value at which a Ca\(^{2+}\) current appears (30). The changes in Ca\(^{2+}\) currents caused by HAMLET and oleic acid are thought to be due to effects on the Ca\(^{2+}\) ion channels, and the irreversible suppression of Ca\(^{2+}\) currents by higher concentrations of HAMLET and oleic acid may be due to structural damage to Ca\(^{2+}\) ion channels (30). Changes to Ca\(^{2+}\) channel threshold and inhibition of Ca\(^{2+}\)-activated Cl\(^{-}\) current, as is observed for HAMLET and oleic acid treatment, can also be caused by a Ca\(^{2+}\)-chelating agent such as EGTA intracellularly (30,105). Although they are not referred to as calcium chelators, the components of HAMLET are both known to associate with Ca\(^{2+}\) (106,107). These calcium current results obtained from treating green algae cells with HAMLET, do not follow the same pattern as the results from treating human cancer cells with HAMLET, but do show that HAMLET and its components affect and may even physically disrupt Ca\(^{2+}\) ion channels in the membrane.

**Chloride fluxes in plant cells**

The Ca\(^{2+}\)-activated Cl\(^{-}\) channel current results obtained from treating *C. coralina* cells with HAMLET or oleic acid, are basically the same for the Ca\(^{2+}\) ion channels, and may result from the HAMLET-induced events on Ca\(^{2+}\) ion channel currents making available Ca\(^{2+}\) ions to the Ca\(^{2+}\)-activated Cl\(^{-}\) channel rather than physical effects on the Ca\(^{2+}\)-activated Cl\(^{-}\) channel itself. HAMLET and oleic acid irreversibly decrease plasma membrane Ca\(^{2+}\)-activated Cl\(^{-}\) channel current, as does α-lactalbumin alone but to a lesser extent (31). Extracellular oleic acid induces a reversible decrease in the Ca\(^{2+}\)-activated Cl\(^{-}\) current, and increasing the oleic acid concentration induces an almost complete and irreversible suppression of the current (30).

**Potassium fluxes in plant cells**

In *C. coralina* cells, HAMLET and oleic acid increase outward K\(^{+}\) leakage current, and α-lactalbumin alone does not (31). A high extracellular oleic acid concentration causes an increase in K\(^{+}\) leakage (30). Perfusion of oleic acid into the cell does not affect the K\(^{+}\) leakage (30). In this model, K\(^{+}\) leakage, such as that observed for extracellular HAMLET, oleic acid, and α-lactalbumin and not for intracellular oleic acid, is indicative of membrane lipid disruption (30).

**Calcium and sodium fluxes in bacterial cells**

The study of bacterial systems can yield insights into human systems because ion channels and transporters exist in bacteria that are homologous with human varieties. Bacterial genomes have significantly fewer genes and do not have the complexity of isoforms and regulation by non-coding DNA that is found in human genomes, making interpretation of observations more straightforward. A further motive for reasoning that HAMLET bacterial studies may shed light on HAMLET mechanisms in human cells is that human mitochondria are descendants of, and share similarities with, bacteria (33), and HAMLET exerts effects on isolated mitochondria (84).

HAMLET-like compounds are bactericidal to *Streptococcus pneumoniae* (17,32,35). Introduction of the Ca\(^{2+}\) transport inhibitor ruthenium red inhibits bacterial death, inhibits the intracellular [Ca\(^{2+}\)] increase, and inhibits the membrane depolarisation that HAMLET or ELOA normally induce in *S. pneumoniae* (32,33). Introduction of Na\(^{+}\)-channel inhibitors amiloride or 3',4'-dichlorobenzamil (DCB) inhibits membrane depolarisation, inhibits the intracellular [Ca\(^{2+}\)] increase, and reduces the bacterial death induced by HAMLET or ELOA treatment, and the effects are stronger when DCB (a more specific Na\(^{+}\)/Ca\(^{2+}\) exchanger inhibitor) is used instead of amiloride (32,33). A consistently observed plateau in the middle of an otherwise steady linear increase of intracellular [Ca\(^{2+}\)] (32,33) suggests that active transport of calcium is involved, as the plateau can be caused by
precipitation of calcium with the inorganic phosphate resulting from ATP hydrolysis (108,109). Decreasing the amount of extracellular Na⁺ available increases intracellular [Ca²⁺] and increases HAMLET-induced cell death (33). HAMLET also induces a decrease in intracellular [K⁺] in *S. pneumoniae* which is not considered significant because inhibiting this decrease in flux does not impact cell death (33). In the bacterium *Staphylococcus aureus* HAMLET dissipates the proton gradient, and this dissipation is not fully inhibited by the calcium channel inhibitor ruthenium red nor by the sodium channel inhibitor amiloride, indicating that another type of channel is involved in HAMLET-induced membrane depolarisation in this bacterium (36).

One interpretation of the *S. pneumoniae* results is that HAMLET-induced cell death in bacteria does not require membrane depolarisation and does require an increase in intracellular [Ca²⁺] that is fed by Na⁺ efflux. Another interpretation is that depolarisation is downstream of the initial dissipation of the proton gradient and inhibition of calcium fluxes does not block the upstream proton gradient dissipation.

**Disruption of membrane lipid organisation**

**Binding to lipid membranes**

HAMLET binds zwitterionic small unilamellar vesicles (SUV) at pH 7.4 and the amount of HAMLET that binds is nearly twice the amount of native α-lactalbumin that binds the SUVs (31). When calcium is present, the amount of HAMLET that binds decreases to the same level as for α-lactalbumin only (31). In light of the HAMLET-deactivating properties of calcium, this decrease is due to calcium stripping out the oleic acid from HAMLET and leaving protein only to bind the vesicle bilayer. At pH 7.0, HAMLET binds to the single bilayer of zwitterionic SUVs (110) and binds throughout the multiple layers of lipid membrane of zwitterionic multilamellar vesicles (110). HAMLET, and not α-lactalbumin alone, binds negatively charged large unilamellar vesicles (LUV) at physiological pH 7.4 (110,111). HAMLET binds these LUVs regardless of variations in their fluidity and 18:1 acyl chain content (110) and thus membrane 18:1 acyl content or fluidity are not the factors that determine the binding of HAMLET. ELOA, and not equine lysozyme alone, binds negatively-charged giant unilamellar vesicles (GUV) and makes them less rigid (76). In light of the HAMLET cargo offloading hypothesis, these membrane binding results suggest that when the oleic acid in HAMLET leaves HAMLET to associate with the cell membrane, the exposed positively charged α-lactalbumin residues associating with the negatively charged membrane lipids augment the binding of the partially unfolded HAMLET protein and its exposed hydrophobic surfaces to the mainly hydrophobic lipid cell membrane.

When ELOA is first incubated with fat-free bovine serum albumin, which is expected to sequester all the oleate in ELOA, ELOA's interaction with membranes is abolished, indicating that the oleate in ELOA mediates the protein's interaction with membranes (76).

**Changes to morphology of lipid membranes**

BAMLET's effect on negatively-charged GUVs is membrane blebbing, with the blebs moving from the membrane to inside the vesicle, until the GUVs finally disappear (111). Oleic acid alone or α-lactalbumin protein alone did not cause membrane blebbing (111). HAMLET, and not oleic acid alone, nor α-lactalbumin alone nor an α-lactalbumin mutant containing no cysteines, causes zwitterionic vesicles to change morphology from spherical to an elongated morphology deformed by greater flexibility of the thinner membrane at pH 7 (110).

**Leakage of lipid membranes**

BAMLET causes leakage through the membrane of negatively-charged LUVs at pH 7.5 (12,111). The amount of leakage increases when pH is 4.5, and the amount of leakage decreases when pH is 9.1 (111). The membrane leakage is a function of the amount of oleate in the BAMLET compounds, with a high concentration of low-oleate BAMLET causing as much leakage as a lower concentration of high-oleate BAMLET (111). Oleic acid alone or α-lactalbumin protein alone do not cause membrane leakage of those GUVs (111). HAMLET, and not oleic acid alone, nor α-lactalbumin alone nor an α-lactalbumin mutant containing no cysteines, causes leakage from negatively-charged LUVs at pH 7.4 (110). HAMLET does not cause leakage from zwitterionic LUVs at pH 7.4, but does cause leakage at pH 5.0 (110). However, ELOA does not cause leakage from negatively-charged GUVs and LUVs (76).
Binding at cell membranes

Both HAMLET and α-lactalbumin alone bind the HAMLET-sensitive A549 human lung carcinoma and L1210 mouse leukaemia cells and HAMLET-insensitive human renal tubular epithelial cells (HRTEC) cells, and this binding is visible by microscopy after 10 minutes and is maximal by 30 minutes (37). The binding is distributed in patches separated by areas of membrane where HAMLET does not bind (37). More total HAMLET binds than total α-lactalbumin alone (37). ELOA accumulates in high concentration at the cell membrane of PC12 (rat pheochromocytoma) cancer cell membranes in a non-uniform distribution, until the cell membrane ruptures and ELOA swiftly flows into the cell, filling the interior within half a minute (49,85).

The non-uniform distribution of HAMLET and ELOA bound to cell membranes suggests that they are associating with specific regions in the cell membrane, such as membrane proteins, lipid rafts or non-lipid raft domains.

Leakage through cancer cell membranes

Plasma membrane vesicles (PMV) can be prepared from cancer cells and contain the membrane lipids and membrane proteins of the cancer cells and are significantly smaller than the cancer cell (112), with an increased membrane curvature. HAMLET binds PMVs from PC12 (rat pheochromocytoma) and A549 cancer cells (110) in a non-uniform manner, indicating that certain regions of the membrane have higher affinity for HAMLET (110). The PMV morphology changes from round to elongated shapes in response to HAMLET (110). Oleic acid alone also binds in a non-uniform manner, and does not induce conformational change of the PMV, indicating that oleic acid incorporates into PMV membranes without disrupting the structure (110).

Passes through cellular membranes

The protein component of HAMLET translocates across cellular membranes into the cytoplasm (4,16,18,37,49,59) and later accumulates in the nucleus (37). One hypothesis is that the oleate, having a higher affinity for the cell membrane, dissociates from the protein. After the oleate has dissociated from the protein, the protein is left in an unfolded state with exposed hydrophobic surfaces that have high affinity for cell membranes and thus the unfolded state of the protein facilitates its translocation across the lipid membrane. Contributing to this hypothesis is the evidence reviewed above concerning HAMLET binding the multiple lipid membrane layers of multilamellar vesicles (110) and causing blebbing of homogenous phospholipid membranes (111). Also contributing to this hypothesis is the evidence that oleic acid increases the flexibility of membranes and conformational freedom of the lipids in the membrane (113,114). Unfortunately, the enlightening experiment of tracking oleic acid introduced to a cell, either alone or in a HAMLET-like compound, to determine whether it remains in the membrane or enters the cell with or separately from the protein, has not yet been conducted.

ELOA, however, does not cross PC12 rat pheochromocytoma cell membranes, and instead accumulates on the cell membrane until it lyse the cell membrane and ELOA then flows into the cell (49). Upon incubation with bacteria, ELOA regains some of the native enzymatic activity of the protein component lysozyme (76). This is attributed to some of the lysozyme protein component refolding to a native-like bactericidal conformation after it has released the oleate cargo to the bacterial membrane (76). These results of ELOA lysozyme protein appearing to refold and observed to not enter the cell, support the hypothesis that it is the unfolded state of HAMLET proteins that facilitates their entry into cells by hydrophobic interaction with the cell membrane.

INTRACELLULAR TARGETS

DNA fragmentation and caspases activation of classical apoptosis

After HAMLET treatment, classic apoptosis cell death characteristics are observed: nuclear condensation, cell shrinkage, cytoplasmic blebbing, and DNA fragmentation (7,16,37,57). The loss of cell viability of A549 human lung carcinoma and L1210 mouse leukaemia cells upon exposure to HAMLET is accompanied by apoptosis-associated DNA fragmentation (37).

In human leukaemia Jurkat cells, caspase-3 activity increases 10-fold 3 hours after HAMLET treatment, and in A549 it increases 7-fold 2 hours after treatment (56). Caspase-6 transiently increases 40% at 2 hours in Jurkat and increases to 55% at 4 hours in A549 after HAMLET treatment (56).
Figure 4. The hypothesised interactions of HAMLET components with the cell membrane and indirectly with some key membrane proteins. The oleic acid (yellow with red balls) has dissociated from the protein of HAMLET (dark green) (6) and is associating with the lipid membrane (grey and pink) potentially making the membrane more fluid, and also affecting lipid packing around the membrane protein channels. The membrane proteins are an epithelial sodium channel (PDBID:4NTX (115), left), a Na+/H+ exchanger (PDBID:4CZA (116) and PDB ID:2BEC (117), central), and a Na+/Ca2+ exchanger (PDB ID:3V5U (118), PDB ID:1H6G (119), PDB ID:2FWS (120), and PDB ID:2FWU, arranged according to (121), right).

Co-treatment with the caspase inhibitor zVAD-fmk increases cell viability and inhibits the DNA fragmentation (56). The PARP, lamin B, and α-fodrin cleavage targets of caspsases are cleaved in Jurkat, and PARP and lamin B are cleaved in A549, in response to HAMLET treatment, and zVAD-fmk inhibited some of this cleavage (56). The Fas cell surface death receptor is not activated by HAMLET, as an antagonistic anti-CD95 receptor antibody, ZB4, does not affect the HAMLET-induced caspase-3 activity (56). The activation of caspase-3 coincides with the release of cytochrome c from the mitochondria into the cytosol (56). However, adding HAMLET to cytosolic extracts does not activate caspase-3 (56). This suggests that HAMLET does not directly activate caspsases.

In human leukaemia Jurkat cells and A549 human lung adenocarcinoma cells, HAMLET treatment induces an increase in caspase-2, caspase-3 and caspase-9, but the levels are significantly lower than the response of these cells to the chemotherapy compound etoposide (21). HAMLET induces a 4.8-fold increase in phosphatidyl serine (PS) exposure, which is reduced by the pan-caspase inhibitor zVAD-fmk, whereas etoposide induces an 11.2-fold increase (21). In Jurkat human leukaemia cells, use of the pan-caspase inhibitor zVAD-fmk reduces HAMLET-induced cell death LD50 from 20 μM to
17 μM (21). In A549 human lung adenocarcinoma cells, use of the pan-caspase inhibitor zVAD-fmk reduces HAMLET-induced cell death LD50 from 46 μM to 41 μM (21). These results indicate that apoptosis-like cell death is triggered in response to HAMLET and may be enough to kill the cell. However, this mechanism does not explain all aspects of cell death that are observed, and thus other mechanisms are driving the HAMLET-induced cell death.

In U87MG human glioma cells, HAMLET treatment leads to activation of caspase-8 followed by apoptotic cell death as indicated by TUNEL assay, regulated mostly by caspase-8 and caspase-9 (38). The caspase-8 inhibitor reduces HAMLET-induced cell death by the same amount as does the pan-caspase inhibitor zVAD-fmk, which suggests that caspase-8 is the main type of caspase regulating apoptosis in U87MG cells (38).

BAMLET induces dose-dependent condensation of chromatin and shrinkage of cells, hallmarks of apoptosis-like cell death, in MCF7 human breast adenocarcinoma, even though these cells do not express caspase-3 (16). When transfected with caspase-3, these cells exhibited caspase-3 activity in response to BAMLET, but the sensitivity to BAMLET did not change and inhibition of caspases by zVAD-fmk did not change the sensitivity (16). Again, these results indicate that apoptosis-like cell death is triggered in response to HAMLET, but that other mechanisms are ultimately driving the cell death.

Jurkat cells treated with HAMLET exhibit chromatin condensation into small spheres, large spheres and crescents, which are chromatin changes associated with apoptosis (21). HAMLET treatment also induces marginalisation of chromatin to the nuclear periphery (21). Co-treatment with the caspases inhibitor zVAD-fmk leads to a decrease in the apoptosis-like chromatin changes and an increase in the chromatin marginalisation changes (21). These results show that HAMLET induces chromatin condensation in a caspase-dependent and a caspase-independent manner, and blocking caspases shifts the chromatin response to the caspase-independent pathway (21).

These results suggest that HAMLET triggers a low level of apoptosis-like cell death, but the cell death mechanisms do not rely solely on activation of caspases.

Cells having classic apoptosis-resistance mechanisms

The p53 tumour-suppressor gene is defective in over 50% of cancers, particularly at relapse (122). There is no difference in or inhibition of HAMLET-induced cell death in HCT116 human colorectal carcinoma cells having a homozygous p53 deletion compared to those having the wild type p53 gene (21). HAMLET is equally effective in killing H1299 human lung carcinoma cells having homozygous p53 deletion and H1299 dominant-negative His175 gain of function mutant that is even more resistant to apoptosis (21).

The Bcl-2 family of proteins regulates cellular commitment to apoptosis and overexpression of pro-survival Bcl-2 is associated with cancers (123). HAMLET treatment of K562 human myelogenous leukaemia cells transfected such that they overexpress Bcl-2 does not affect Bcl-2 expression levels and does not affect or inhibit cell death (21). HAMLET treatment of Jurkat human leukaemia cells transfected such that they overexpress Bcl-2 does not affect caspase-3 response and does not affect or inhibit cell death (21).

The Bcl-xl protein is one of the pro-survival Bcl-2 family members (123). HAMLET treatment of Fl5.12 immortalised non-cancerous mouse pro-B-cell lymphocytic cells transfected such that they overexpress Bcl-xl does not affect or inhibit cell death (21). HAMLET treatment of A498 human kidney carcinoma cells transfected such that they overexpress Bcl-xl does not affect or inhibit cell death (21).

These results show that HAMLET is effective in killing cancer cells that possess the anti-apoptosis mechanisms of decreased expression of the p53 tumour-suppressor gene, increased Bcl-2 expression, or increased Bcl-xl expression.

Autophagy

Autophagy, the mechanism of degradation of cellular proteins and components through the action of autophagosomes and lysosomes, is involved with cell survival during times of starvation and is considered to be part of orderly autophagic cell death programs (124-127).

In A549 human lung carcinoma cells, knockdown by interference RNA of Beclin-1, a protein involved in autophagosome formation,
reduces HAMLET-induced cell death to 45% whereas it is 70% for control cells (128). Knockdown of Atg5, another protein involved in autophagosome formation, reduces HAMLET-induced cell death to 46% compared to 70% for controls (128). Various indicators of the occurrence of autophagy are observed in HAMLET-treated A549 cells, including a granular cytosolic pattern of LC3-II, LC3-II flux visible when its degradation is inhibited; a decrease in the phosphorylation of the autophagy inhibitor mTOR and of an mTOR substrate p70 S6K; an increase in protein levels of Beclin-1; and increases in mRNA levels of Beclin-1, Atg5 and Atg7 (128). When the apoptosis caspase inhibitor zVAD-fmk is used in conjunction with Beclin-1 knockdown, there is no additional reduction in HAMLET-induced cell death (128). These results are evidence that A549 human lung carcinoma cells undergo autophagy before dying in response to HAMLET treatment. As is usually the case with autophagic cell death (126,127), these results demonstrate that autophagy is present in HAMLET-treated A549 cells but do not demonstrate that the causative mechanism of cell death is autophagy.

In U87MG human glioma cells treated with HAMLET, a granular cytosolic LC3-II flux is seen along with an increase in p62, a protein involved with autophagosome development (and also involved in cross-talk with the proteasome system (129)), and the p62 protein colocalises with the LC3-II, suggesting that the cells are carrying out autophagy in response to HAMLET (38). HAMLET treatment plus activation of autophagy by rapamycin treatment leads to an increase in cell viability and decrease in p62 levels (38). HAMLET treatment plus activation of autophagy with starvation leads to a decrease in cell viability and increase in p62 levels (38). HAMLET treatment plus knockdown of autophagy protein Atg5 leads to a decrease in cell viability and increase in p62 levels (38). These results suggest that autophagy has a protective role in stress conditions and HAMLET-induced cell death, and when the stress is too much for survival then the autophagy process becomes exhausted and autophagy proteins accumulate because they are no longer being processed (38).

Knockdown of p62 reduces the sensitivity of U87MG cells to HAMLET treatment, and overexpression of p62 increases the HAMLET cytotoxicity (38). When the C-terminus of p62 is truncated, U87MG cells are more resistant to HAMLET-induced cell death, with cell viability increasing to 80% from the 65% for controls (38). These results suggest that HAMLET-induced autophagy processes are contributing to cell death in U87MG cells because disabling a part of the autophagy system reduces HAMLET-induced cell death.

Lysosomal cell death program
HAMLET treatment of MCF7 human breast adenocarcinoma cells and U2OS human bone osteosarcoma cells results in BAMLET co-localising with acidic endolysosomal organelles, permeabilisation of the lysosomal membrane, release of cathepsin L into the cytosol, then activation of the pro-apoptotic Bax protein (16). This cell death program is not delayed by the protease inhibitor zFA-fmk (16). In MCF7 cells, expression of proteins that stabilise lysosomes, HSP70, HSP70-2 and lens epithelium-derived growth factor, decrease the BAMLET-induced cell death. It is thought that BAMLET-induced cell death involves lysosomal membrane permeabilisation that leads to the release of many cytotoxic lysosomal hydrolases whose effects cannot be inhibited by inhibiting cysteine cathepsin activity (16).

Necrotic cell death
In A549 human lung carcinoma cells, HAMLET treatment causes up to 10% of the cells to undergo necrosis, with the cells showing ruptured plasma membranes, loss of cellular material, and merging of nuclear and cytoplasmic content (128). THP1 human monocytic leukaemia cells undergo a necrotic-like cell death in response to HAMLET treatment (13).

Localisation with nuclei and binding of histones
After entering A549 human lung carcinoma cells, L1210 mouse leukaemia cells, or non-cancerous human renal tubular epithelial cells, 125I-labelled, biotinylated or fluorescently-labelled HAMLET protein is first seen evenly distributed throughout the cytoplasm (37). In the cancer cells, the bulk of labeled HAMLET protein localises to the nuclei, which does not occur for the non-cancerous cells (37,57). The uptake of HAMLET protein into the nucleus is inhibited by wheat-germ agglutinin.
(WGA), which inhibits transport through the nuclear pore (37). HAMLET protein binds strongly to histone H3, and weakly to histones H2B and H4, and binds both natively folded and denatured forms of histones (57). Bovine α-lactalbumin protein, without oleic acid, binds isolated histones (130), demonstrating that it is not necessary for oleic acid to be bound to the HAMLET or BAMLET protein component for that protein to bind histones.

Mitochondrial association and mitochondrial permeability transition in isolated mitochondria

In Jurkat human leukaemia cells, cytoplasmic HAMLET co-localises with mitochondria (56). In A549 human lung carcinoma cells, HAMLET treatment causes mitochondria to become swollen with disrupted membranes and loss of cristae organisation (128). Adding HAMLET to Ca\(^{2+}\)-loaded isolated rat liver mitochondria induces swelling and the release from the mitochondrial intermembrane space of adenylate kinase-2 and cytochrome c (84), whose release is an event in classic apoptosis upstream of caspases activation. The calcium chelator EGTA prevents the HAMLET-induced swelling and protein release, as does ruthenium red (84), which interacts with various proteins including the mitochondrial Ca\(^{2+}\) uniporter (131) and thus blocks uptake of calcium into mitochondria, which inhibits permeability transition of the mitochondria which is an apoptosis activation pathway. This indicates that calcium is required for the HAMLET-induced swelling effect. The HAMLET-induced swelling and protein release is inhibited by the mitochondrial permeability transition (MPT) pore inhibitors cyclosporin A or ubiquinone 0 (84). This indicates that HAMLET-induced swelling and release of apoptosis-associated protein in isolated mitochondria involves opening of the MPT pore. Adding HAMLET to Ca\(^{2+}\)-loaded isolated mitochondria causes a biphasic decrease in mitochondrial membrane potential – a first and fast phase followed by a longer phase that ends in complete loss of mitochondrial membrane potential (84). Adding cyclosporin A or ATP, which can also inhibit the MPT pore, does not block the first phase and does block the second phase (84). This suggests that the second phase of mitochondrial membrane potential loss is due to the MPT, and the first phase is due to HAMLET inducing a proton leak by some other means. Adding oleic acid, dissolved and thus solubilised in ethanol, to the Ca\(^{2+}\)-loaded isolated mitochondria induces the first phase mitochondrial membrane potential decrease but does not induce the second phase of mitochondrial membrane potential collapse (84). This indicates that the HAMLET-induced second phase mitochondrial membrane potential collapse is not due to the oleate in HAMLET and that oleic acid does not open the MPT pore. Adding bovine serum albumin delays the mitochondrial swelling and delays the dissipation of the mitochondrial potential (84). This indicates either that it is not the oleic acid in HAMLET that causes these effects, or that it is the oleic acid and BSA only partially deactivates HAMLET.

Mild proton leak in isolated mitochondria

When isolated mitochondria are in state 4 respiration due to the depletion of ADP, increasing HAMLET or increasing oleic acid leads to an increase in state 4 respiration and a decrease in the respiratory control ratio, and this mild mitochondrial uncoupling occurs even in the absence of Ca\(^{2+}\) and in the presence of cyclosporin A (84). This indicates that the oleic acid in HAMLET enhances the proton permeability of the inner mitochondrial membrane by a mechanism other than the MPT.

Interaction with proteasomes

Confocal microscopy of A549 human lung carcinoma cells shows that in the cell membrane region, there is 65% co-localisation of HAMLET with the 20S core proteasomes, and in the cytosolic region the co-localisation is 64% (18). Incubation of HAMLET with isolated 20S proteasomes induces an initial 10 minutes burst of proteasome activity followed by a partial inhibition that results in the proteasomes processing at approximately half their maximal rate (18). In A549 cells treated with HAMLET, proteasome activity steadily decreases to around half its initial rate by 6 hours (18). After 1 hour incubation with HAMLET in A549 cells, an increase in proteasome antibody staining is observed, suggesting that some proteasomes have undergone degradation (18). SDS-PAGE provides evidence that after 20 minutes incubation with HAMLET, isolated proteasomes undergo some autoproteolysis of the proteasome (18). These
results suggest that HAMLET is recognised by the 20S proteasomes and either resists degradation (18); is activated by a low concentration of HAMLET's oleic acid (132) leading to self-proteolysis of the proteasome (18); is deactivated by higher concentrations of HAMLET's oleic acid (132); or else orderly degradation of HAMLET protein by the proteasome is interrupted by HAMLET-triggered cell death program events elsewhere in the cell that affect the proteasome system.

Interaction with α-actinin
HAMLET treatment of A549 human lung carcinoma cells results in HAMLET binding α-actinin proteins (58), which are spectrin proteins involved in cytoskeletal scaffolding (133). HAMLET binds several places on the α-actinin proteins (58). Knockdown of α-actinin gene expression does not change HAMLET-induced cell death of suspended A549 cells and results in more pronounced HAMLET-induced cell morphology changes as the cells undergo cell death (58). This indicates that the binding of HAMLET to actinins is not the initiating cell death mechanism, and may be an example of unfolded protein binding cellular proteins indiscriminately.

Binding to nucleotide-binding proteins
Co-immunoprecipitation studies show that HAMLET protein binds to over 400 out of 8,000 human proteins on a protein microarray, and 35% of the proteins that the protein component of HAMLET binds are nucleotide-binding ATPases and GTPase, including kinases that are involved with cellular signalling via phosphorylation of their targets (134).

Interaction with ATP synthase
ATP synthase in the mitochondria, also known as complex V, is the molecular complex responsible for generating most of the ATP energy molecules in healthy cells (using the proton gradient produced by mitochondrial complexes I, III, and IV to drive a molecular motor that creates ATP from ADP and inorganic phosphate). HAMLET protein interacts with the soluble F1 catalytic head of ATP synthase (59), and after HAMLET treatment, cellular ATP levels decrease and rotation of the ATP synthase motor is no longer detected (59). In the HAMLET ATP synthase study, adherent A549 human lung carcinoma cells manifest a punctate pattern of ATP synthase subunits alpha and beta throughout the cytosol (59) which is the part of the cell where mitochondria are found. After HAMLET treatment and the cell has started dying, subunit beta co-localises with HAMLET protein in the cytosol, and subunit alpha co-localises with HAMLET protein in a few large aggregates in the cytosol (59).

Subunits alpha and beta are bound together in the intact F1 component of ATP synthase, and their divergent paths upon HAMLET treatment that results in cell death indicates that the ATP synthase subunits are no longer bound together in an intact ATP synthase F1 component. Their divergent paths after HAMLET treatment that initiates cell death may also indicate that these subunits have roles in cell death regulation. Indeed, ATP synthase subunit beta has been linked to Bcl-xl which is involved in apoptosis regulation (135). Subunit beta is also involved in calcium signalling at the plasma and intracellular membranes of developing muscle cells (136). As the main cytotoxic component of HAMLET is the oleic acid rather than the protein, the binding of HAMLET protein to ATP synthase protein subunits is likely a result of the unfolded HAMLET protein, no longer partially folded around its oleic acid cargo, binding to the ATP synthase subunits that are in proximity to HAMLET and are themselves now dissociated. Mutations in ATP synthase subunits are associated with some cancers (137-139).

ATP synthase can also be found on the plasma membrane in some cell types, located in lipid rafts (140), with the F1 soluble head extracellular (141). Plasma membrane ATP synthase subunit beta is involved in stimulating angiogenesis in endothelial cells, and inhibiting it via binding of angiostatin or anti-beta-subunit antibodies inhibits endothelial cell proliferation (142-144). Plasma membrane ATP synthase generates ATP as part of shear stress management in pulmonary artery endothelial cells (145,146), umbilical vein endothelial cells (147), and lymphatic endothelial cells (148). Ectopic ATP synthase is involved in HDL uptake and transport in aortic endothelial cells (149). In hepatocytes, plasma membrane ATP synthase subunit beta is involved in endocytosis of HDL as part of cholesterol management (150). In keratinocytes, ectopic ATP
synthase releases ATP as part of wound healing (151). Ectopic ATP synthase is present in antigen-presenting cells (152), neurons (153), colon cells (154), rat tonsils (155), and in the immortalised mouse embryonic fibroblast cell line NIH-3T3 (140). Ectopic ATP synthase is not found in erythrocytes and lymphocytes (156).

Ectopic ATP synthase on the plasma membrane has been reported for some cancer cell types: K562 leukaemia (156), A549 lung carcinoma (156,157), Raji Burkitt lymphoma (156), HepG2 hepatocellular carcinoma (150), Jurkat leukaemia (158), osteosarcoma (159), neuroblastoma (153), CaCo2 colorectal adenocarcinoma (154), HeLa ovarian carcinoma (160), breast cancer specimens (161), pheochromocytoma (162), and paraganglioma (162). Ectopic ATP synthase on tumour cells is involved in a mechanism by which a class of T lymphocytes recognises the tumour cells (163,164). Inhibition of ATP generation by plasma membrane ATP synthase is currently under investigation as a target of anti-cancer drugs for lung cancer (157,165,166), breast cancer (161,167-169), colon cancer (154), urinary bladder carcinoma (170), pheochromocytoma (162), and paraganglioma (162). In a lung adenocarcinoma study, the ATP synthase complex and the beta subunit were found to be present in the plasma membrane of A549 and CL1-0 lung cancer cells in flow cytometry experiments (157).

The other members of the oxidative phosphorylation electron transport chain – complexes I, II, III, and IV – in addition to complex V, were also found to be present in the plasma membrane in Western blot experiments (157). Treatment with the ATP synthase inhibitor citreoviridin leads to accumulation of reactive oxygen species and cell death (157). This indicates that plasma membrane ATP synthase is involved in managing the intracellular acidification caused by glycolysis in those cancer cells by allowing the protons generated by glycolysis to run down their gradient and out of the cell so that the cytosol does not remain acidic. As the protons run down their gradient, the ATP synthase molecular motor is operated and ATP is generated.

It is not yet understood how ATP synthase arrives at the plasma membrane (171,172). In hepatocytes (160) and HepG2 hepatocellular carcinoma (173), the plasma membrane ATP synthase appears to be assembled in and transported from the mitochondria. In HeLa ovarian carcinoma cells, cholesterol loading causes translocation of subunit beta from the mitochondria to the cell surface (174). In neurons, deficiency of palmitoyl protein thioesterase 1 is linked to an increase in ectopic F1 (175).

### Lysing of red blood cells

BAMLET exposure of 10 minutes induces a change in morphology of erythrocytes, with the disc shaped cells becoming cup shaped or displaying blebs or angular projections on the surface (29). Longer incubations of BAMLET or other HAMLET-like compounds causes haemolysis of erythrocytes (5,13,29,44) and this type of erythrocyte cell death is called eryptosis (29). At 4°C BAMLET does not lyse erythrocytes (29). Although BAMLET-exposed erythrocytes display the classic apoptosis characteristic of phosphatidyl serine exposure on the outside of the cell membrane, caspases inhibitors and thiol proteases do not restrict the BAMLET-induced haemolysis (29). Amiloride, BaCl2, or the calcium ionophore A23187, reduce the BAMLET-induced haemolysis (29), which suggests that ion fluxes or calcium are required for BAMLET-induced haemolysis of red blood cells. The intracellular calcium levels of erythrocytes do not rise after BAMLET treatment and pre-loading the cells with calcium does not change the level of BAMLET induced lysis (29), which leads to the interpretation that calcium is not involved in the BAMLET-induced lysis of erythrocytes. Inclusion of CaCl2 during incubation of erythrocytes with BAMLET inhibits the lysis (29), and given that calcium deactivates BAMLET, this is due to the deactivation of BAMLET by calcium rather than due to the involvement of calcium fluxes in erythrocyte cell death.

The BAMLET-induced cup shape morphology of erythrocytes looks similar to their cup shape form when travelling through very small blood vessels (176). The cup shaped morphology is caused by shear forces (176,177). Reorganisation of cytoskeletal spectrin is involved in the formation of the cup shape morphology when erythrocytes are subject to shear forces (178). As HAMLET has been shown to interact with α-actinin, a type of spectrin protein, the HAMLET-induced deformation of erythrocytes may be due to HAMLET protein.
interaction with α-actinin in the cytoskeleton.

Oleic acid causes reversible morphological changes to red blood cells, from an irregularly contoured disk, to a flat cell with spicules on the surface, to a spherical cell with spicules on the surface, as oleic acid concentration is increased (179). At higher concentrations, oleic acid and fatty acids cause haemolysis (179-181). At low concentrations oleic acid actually protects red blood cells from osmotic rupture and this is thought to be due to binding of the fatty acid to the membrane or a membrane protein (180). Oleic acid and other amphipathic anions mimic the action of calmodulin in activating the red blood cell plasma membrane Ca\(^{2+}\) pump ATPase (182). This activated efflux of calcium may be how low concentrations of oleic acid protect red blood cells from osmotic rupture. The red blood cell plasma membrane Ca\(^{2+}\) pump ATPase contains a central pore similar to the calcium pump of sarcoplasmic reticulum (183,184). This pump is very sensitive to the phospholipid environment (185-187) due to its autoinhibition domain binding being disrupted by particular lipids, thus activating the pump (187). Removal of autoinhibition of the Ca\(^{2+}\) pump ATPase leading to outflow of calcium may be part of the mechanism of how higher concentrations of oleic acid lead to haemolysis. The Na\(^+\)/Ca\(^{2+}\) exchanger is a more powerful Ca\(^{2+}\) extrusion system and thus has a more significant effect on global cytosolic calcium levels than does the Ca\(^{2+}\) pump ATPase (187). However, current thinking is that the Ca\(^{2+}\) pump ATPase may have significant effects on calcium levels and signalling in microdomains of the red blood cell that in turn affect nearby proteins that have the ability to make larger global effects (187). Given that oleic acid can bind and activate the Ca\(^{2+}\) pump ATPase (182), this pump may be involved in BAMLET’s oleic acid induced haemolysis in the presence of ATP, even though global erythrocyte calcium levels do not change in response to BAMLET (29).

That calcium is able to delay or prevent the haemolysis that occurs in red blood cells when they are placed in a hypotonic solution of non-electrolytes such as dextrose or lactose has long been known (188). Membranes are less leaky in the presence of calcium because calcium exerts stabilising effects on the structural and functional properties of biomembranes (189). Calcium is adsorbed onto the phospholipid cell membrane and stabilises the membrane into an ordered and less fluid state (189,190). The oleic acid induced lysis of red blood cells may be due to an increase in membrane fluidity caused by insertion of oleic acid into the membrane (191,192). The oleic acid-induced lysis of red blood cells may be due to an interaction between oleic acid and calcium that results in less calcium available to bind and stabilise the membrane. Figure 2O shows that erythrocytes are significantly more sensitive to BAMLET when incubated in a calcium-free medium (5) than when calcium is present (44). These results may indicate that calcium is an important factor in BAMLET-induced and oleic acid-induced lysis of erythrocytes. Erythrocytes do not have the internal mechanisms for sequestering and controlling intracellular levels of calcium possessed by other types of cells (189) which simplifies the analysis of the BAMLET-induced cell death. If disruption of calcium membrane binding is the mechanism by which BAMLET induces cell death in erythrocytes, this may indicate that the same mechanism is the root cause of BAMLET-induced cell death in other types of cells that possess more sophisticated mechanisms for controlling the fluctuations of calcium in its environment. Epithelial cells lose their integrity and become detached from one another in calcium-deficient media (189).

**Inhibition of BAMLET by ectopic protein chaperones**

Ectopic expression of heat shock protein HSP70-2 protein in MCF7 human breast adenocarcinoma cells or of HSP70 protein in mouse fibrosarcoma inhibits around 20 to 40% of the BAMLET-induced cell death normally experienced by these cells (16). In MCF7 cells, ectopic expression of lens epithelium-derived growth factor (LEDGF), a protein that may upregulate heat shock proteins (193), inhibits around 50% of the BAMLET-induced cell death (16). The HSP70 family of protein chaperones bind the exposed hydrophobic surfaces of soluble proteins such as proteins that are being translated and thus not yet folded (194,195), and are also able to bind fatty acids (196-198). It is plausible that HSP70 disables BAMLET by binding the oleic acid.
Figure 5. Potential intracellular targets of HAMLET, not drawn to scale. BAMLET protein (dark green) (6) is shown binding histones (cyan protein with orange DNA wrapped around it) (PDBID:1AOI (278)) in the nucleus (light blue), proteasomes (dark blue) (PDBID:1FNT (202)), lysosome membranes (brown), α-actinin (the purple cross-bars connecting purple spectrin rods) (modified from PDBID:4D1E (203)), and ATP synthase (pink) (PDBID:2XOK (204)) in the mitochondrion (red).

This indicates that the anticipated resistance mechanisms that cancer cells would evolve in response to treatment with HAMLET-like compounds is upregulation of heat shock proteins outside the cell membrane, to bind the active fatty acid ingredient and deactivate HAMLET.

Phagocytosis-like particles
Although not explicitly reported, published images show that HAMLET, presumably unfolded after having offloaded its oleic acid cargo, forms concentrated round particles inside the cell (Figure 8B in (4), Figure 3C in (28), Figure 2B in (59), Figure 6B in (134)) that are reminiscent of the particles of phagocytosis, such as when the epithelial cells A549 human lung carcinoma carry out phagocytosis of *Escherichia coli* bacteria (Figure 4 in (199)). Phagocytosis is the ingestion by cells of large particles ≥ 0.5 μm diameter, such as bacteria, apoptotic bodies and debris (200). The 227 to 292 nm diameter of BAMLET (22) is of the same order of magnitude as the 0.7–1.0 μm diameter of *E. coli* (201).

ARE HAMLET-LIKE COMPOUNDS FORMED IN NATURE?
The components of HAMLET (free fatty acid and undigested and digested protein) and the conditions for creating HAMLET (mixing of components at low pH or even at higher pH) exist in the digestive tract of adults and infants when they drink milk. α-Lactalbumin is generally the
most abundant protein in human milk, making up 39 to 60% of the whey protein depending on weeks post-partum (205-207). Oleic acid is the most common fatty acid in human milk and 98% is in triglyceride form (208). In bovine milk, around 73% of whey protein and 10% of total protein is β-lactoglobulin, and around 27% of whey protein and 5% of total protein is α-lactalbumin (209). Around 23% of the fatty acids in bovine milk fat triacylglycerols is oleic acid (210). In the stomach and small intestine, the triacylglycerols in milk globules undergo sequential lipolysis by lipases to release the free fatty acids such as oleic acid (208,211). HAMLET-like compounds can be made from the intact α-lactalbumin and β-lactoglobulin proteins or from digested protein fragments (1,7,8,53,54). HAMLET-like compounds can be made by mixing the components, and lowering pH to 3 promotes unfolding of the protein which assists in the creation of HAMLET-like compounds (53,65). The gastric pH of adults before drinking or eating is around pH 2 and rises to pH 5 to 7 after drinking a protein drink (212). The gastric pH of infants before drinking is around pH 2, and frequent feeding means that the gastric pH is often at pH 4 and above (213).

A study using a mixture of calcium and bovine α-lactalbumin (both found in milk) and oleic acid dissolved in ethanol, subjected to simulated gastric conditions of pH 2.5 then raised to pH 7, demonstrated that a cytotoxic compound with the same structural characteristics as BAMLET is formed (214). A capsule endoscopy study sampling the gastric contents of adults drinking a water-based mixture containing bovine α-lactalbumin, sucrose, and oleic acid dissolved in ethanol, found that the protein unfolds but the gastric contents after drinking this drink are not able to kill cancer cells in vitro (212). A cytotoxic HAMLET-like compound was not produced in the stomach from drinking this mixture and it is concluded that the mixing of components that took place in the stomach was not adequate for the formation of BAMLET (212). However, unlike the drink used in the capsule endoscopy study, milk is a complicated system where milk fats and stomach bile salts interact to form longer lasting fat globules and other structures (211). The structures may even provide a surface where α-lactalbumin may interact with oleic acid as it comes off the triglycerides similar to the formation of HAMLET on a chromatography column. Thus it is not possible to exclude that HAMLET-like complexes are formed in the stomach when milk is ingested.

A qualitative retrospective study of 20 cancer patients who drank human milk as a complementary or alternative therapy reported that patients or their proxies found that human milk consumption did not change their disease outcome (215). The patients appear to have received a psychological benefit from the possibility of hope from this unproven therapy (215). Some patients drank raw human milk. However, other patients drank human milk that was pasteurised rather than fresh, which may affect milk lipase activity in hydrolysing triglycerides to release free fatty acid and may affect α-lactalbumin function.

A study in mice predisposed to colon cancer, who received HAMLET orally (26) suggests that HAMLET, or oleic acid alone in solubilised form, may survive into the colon to exert an anti-tumour effect.

Studies and meta-analyses of studies looking for links between being breastfed as an infant and incidence of childhood cancers find a very weak decrease in occurrence of leukaemia, Hodgkin's disease, neuroblastoma, and non-haematological cancers, and wonder whether the weak association is due to confounding factors rather than being a true association (216-219). A 65-year follow-up study found no correlation between being breastfed as an infant and occurrence of cancer in adulthood (217).

DISCUSSION

It is often pointed out that cancer is really over 200 different diseases, with each cancer displaying unique characteristics and adaptations to selective pressure. HAMLET has broad-spectrum activity against over 50 cancer cells lines. As 16 possible membrane and intracellular targets (Figure 5) have been identified by distinct experiments and over 400 protein targets have been identified by microarray experiments, it is possible that HAMLET targets different cancer types by different mechanisms. Another explanation for HAMLET's broad-spectrum anti-cancer activity is that the oleic acid of HAMLET, with its potential effects on membrane lipids, membrane protein lipid packing, and signalling, is toxic to all cells without there being a cancer-
specific HAMLET target. However, the resistance to HAMLET of 9 of the 10 non-blood non-cancerous primary cell cultures treated at levels that killed cancer cell lines argues for extensive specificity for cancer cells over broad-spectrum toxicity to all cells. The simplest explanation for HAMLET's ability to kill cancer cells, though certainly not yet proven, is that HAMLET targets features present in all cancer cells that are representative of cancer cells. If this is the case, then the fact that HAMLET kills immortalised cell lines that generally form benign or short-lived tumours in vivo (220-223) and are generally classified as non-cancerous and neoplastic, at the same range of doses as it kills cancer cell lines (Figure 2), indicates that the proposed HAMLET target is one of the early characteristics that a cell acquires in the process of becoming cancerous. Just as the over 200 different types of cancers all have the characteristic of more frequent cell division with the consequent exposure of DNA, the characteristic exploited by the most successful DNA-intercalation chemotherapies of the last 40 years, pin-pointing the HAMLET target may be tantamount to pin-pointing a new cancer target that is shared by the over 200 different types of cancers. And just as there are non-cancerous cells that divide frequently, leading to toxicities induced by the DNA-intercalation chemotherapies, the HAMLET target will be a feature of some non-cancerous cells and thus HAMLET treatment may involve some HAMLET-related toxicities that will need to be managed. The in vivo HAMLET experiments and trials to date, however, have demonstrated no toxic side effects (14,23-27).

The mechanism employed by HAMLET-like compounds to initiate cell death of cancer cells is not yet clear. Inhibition of ion fluxes over the cell membrane appears to significantly inhibit HAMLET's cytotoxicity, whereas inhibition of intracellular HAMLET targets does not lead to strong inhibition of HAMLET-induced cell death. It is likely that the initiating HAMLET-induced cell death mechanism involves changes in permeability to ions through cancer cell membrane proteins. If the active ingredient of HAMLET-like compounds is the oleic acid lipid, as is now generally accepted, it is quite plausible that the initiating damage by HAMLET to cancer cells is a biophysical effect involving membrane lipids or lipids associated with membrane embedded protein rather than an effect involving highly evolutionarily-crafted biological components consisting of specific binding of signalling molecules to receptors. A more general biophysical interaction between lipid species is expected to be a more robustly enduring interaction in the face of the constant and creative evolution of cancer cells, and would explain how HAMLET's mode of action is broad spectrum. Many intracellular targets of HAMLET invoking multiple types of cell death have been identified, and these may be downstream effects from a cell death mechanism actually initiated earlier at the plasma membrane. Once HAMLET has damaged a cell's permeability to ions and therefore control of ion trafficking, programmed cell death would be triggered in those cancer cells that retain functioning cell death mechanisms, and necrosis would be triggered in those having diminished or damaged cell death mechanisms, leading to cell death nonetheless. Based on the process by which the HAMLET complexes are formed, more or less oleic acid lipid is present and the level of oleic acid present may determine which primary activation mechanism is triggered by the cell.

Once HAMLET has offloaded its oleate cargo at a cell membrane, what remains is the unfolded protein polypeptide component of HAMLET that passes to the inside of the cell. Once in the cytosol, the unfolded protein of HAMLET has been shown to bind to many other intracellular components, and this may additionally adversely affect cellular health and result in supplementation of the other cell death activities already underway. An excess of unfolded protein inside the cell activates the unfolded protein response (UPR), and when the presence of unfolded protein causes persistent endoplasmic reticulum stress, the UPR induces apoptotic cell death (279). Unfolded HAMLET protein inside the cell may trigger cell death by this UPR mechanism.

The picture that has emerged from structural studies is that HAMLET-like compounds consist of a novel structure of partially-unfolded protein having free fatty acid associated in non-specific binding sites in the protein's hydrophobic interior. The biological anti-cancer activity may be due to the semi-stability of such a structure. When the compound encounters a cancer cell membrane, the oleate may leave the complex and preferentially associate with the lipid membrane due to a higher biophysical attraction between a free lipid, not associated with a lipid micelle, and
a lipid membrane, than that between a lipid and a mainly hydrophobic protein interior.

Most studies on cancer cell membrane fluidity find that cancer cells are significantly softer than normal cells, which facilitates the cancer cell's invasion of surrounding tissue, intravascular extravasation into lymphatic and blood vessels, and metastasis (224-232). Membrane elasticity may be the selective cancer cell and red blood cell characteristic that HAMLET targets. The oleic acid of HAMLET may increase the fluidity (191,192) to a level that compromises the membrane's impermeability to ions. The softer cancer cell membrane may be the characteristic that allows entry of HAMLET protein into the cell.

One of the possible HAMLET targets under important consideration because it may represent a specific characteristic of all cancer cells, is the ATP synthase complex that was recently reported to be involved in HAMLET-induced cell death (59). ATP synthase has recently been identified as likely to be the elusive main component of the mitochondrial permeability transition pore (MPTP) (233-239) that is an upstream initiator of programmed cell death (234). One of the proposals is that the centre of the c-subunit ring of the F0 membrane component forms the pore (233). Cancer cells would need to reduce or eliminate ATP synthase from the mitochondria, so that the deregulated state of the mitochondria would not trigger programmed cell death via opening of the MPTP, leading to a need for cancer cells to produce ATP by alternative means. ATP synthase activity is downregulated in cancer (240,241) and its inhibitory factor is upregulated (242). Two alternative means of generating ATP are the upregulation of glycolysis, which is a well-known feature of cancers (243-245), and rerouting ATP synthase to the plasma cell membrane and nuclear membrane to generate ATP there instead of in the mitochondrial inner membrane. The oleate from HAMLET may be localising within a lipid plug in the centre of the ATP synthase c-ring, and then being precipitated by calcium, carrying the lipid plug with it, and evacuating a hole - the MPTP - in the plasma cell membrane. This hypothesised mechanism would require intracellular calcium to be present, and the original HAMLET study found an indication that the presence of intracellular calcium is required for the HAMLET anti-cancer effect (7). The lipid plug may consist of cardiolipin, as cardiolipin is associated with ATP synthase (246) and is localised in the c-ring centre “hole” of the V-type ATPase molecular motor (247) that is structurally similar to ATP synthase. Precipitation of the plug by excess calcium and phosphate may be the usual MPTP trigger in mitochondria of non-cancer cells. The ease with which oleate could approach the ATP synthase c-ring centre extracellularly may depend on the state of the regulation of the connection/disconnection of the F1 soluble head to the F0 membrane component, as the F1 soluble head is found extracellularly in ectopic ATP synthase (141). This working hypothesis can indicate which non-cancer cells will be sensitive to HAMLET (endothelial cells, keratinocytes, and embryonic fibroblast NIH-3T3 have ectopic ATP synthase and were sensitive to HAMLET with LD50 doses determined in the same range as LD50 doses for cancer cell lines, Figure 2). This hypothesis relative to the ATP synthase does not explain the sensitivity of erythrocytes to HAMLET, which are particularly sensitive to oleic acid due to other factors discussed in the section on haemolysis of red blood cells. That the HAMLET anti-cancer target may be a mitochondrial cell death triggering pore, in the form of ATP synthase, located in the plasma membrane of cancer cells, is a highly speculative but intriguing working hypothesis for HAMLET's broad spectrum anti-cancer activity.

Alternatively, oleic acid may be generally toxic to all cells rather than being more specifically toxic to only cancer cells. Membrane proteins are sensitive to the fluidity of the membrane (248) and oleic acid increases membrane fluidity (191,192). The signalling of oleic acid and free fatty acids has been extensively reported (249-258) and oleic acid signalling could be involved in oleic acid induced cell death. Initial deleterious effects of HAMLET might involve specific signals of evolutionarily-crafted biological components. Oleic acid alone (179-181) and HAMLET (5,13,29,44) are toxic to erythrocytes, causing haemolysis, and the synthesis of available evidence is that the erythrocyte plasma membrane Ca²⁺ pump ATPase is the target, as the signalling emanating from this pump is known to be sensitive to oleic acid (185). These pumps are ubiquitous in eukaryote cells (259) and may prove to be a significant HAMLET and oleate target in all cells, cancerous and non-cancerous.

The mainstay of current anti-cancer
chemotherapies is small molecule drugs. A common chemotherapy-resistance mechanism employed by cancers is the multi-drug efflux pumps that recognise many different molecules as being foreign and pumps them out of the cancer cell (260). It is thought that resistance to chemotherapy causes treatment failure in over 90% of patients with metastatic cancer (261). HAMLET compounds are protein-based and their size dictates that they will not be pumped out of cancer cells by multi-drug efflux pumps. At ~110 kDa (51), BAMLET is of a similar size to drug efflux pumps themselves, such as the 170 kDa P-glycoprotein (262), whereas the pump removes small molecule drugs from 330 Da to 4 kDa (263). Specifically, one of the most potent anticancer therapies is the 300 Da cisplatin, and drug efflux pumping is one of the main mechanisms for cancer resistance to cisplatin (264). Although the mechanism employed by HAMLET to kill cells is not well-understood, it is already clear that HAMLET's mechanism for killing cancer cells is a new type of anti-cancer mechanism not employed by currently available anti-cancer chemotherapies. Although the cell death mechanisms of HAMLET-like compounds are not yet unambiguously understood, the evidence is that HAMLET-like compounds represent a novel type of protein-lipid structure and make use of a new type of cell death mechanism having broad-spectrum anti-cancer activity and a significant although not exclusive selectivity for cancer cells versus healthy, differentiated cells. Thus, we anticipate that HAMLET will be effective in killing cancer cells that have developed resistance to currently available chemotherapies. The cytotoxicity to cancer cells of HAMLET-like compounds may prove to be synergistic with existing anti-cancer therapies, enabling the use of lower doses of the latter, resulting in lower toxicities, and ultimately making it more difficult for a cancer cell to simultaneously possess resistance to the two different cell death mechanisms of both HAMLET and existing anti-cancer therapies. Indeed, preliminary studies indicate that treatment of cancer cells with fatty acid is synergistic with radiation therapy (265,266), and it is very clear that BAMLET is a fatty acid treatment (4,17,51,53). Thus, BAMLET treatment in combination with radiation therapy may prove to be more effective in treating tumours than radiation therapy alone.

Immunotherapies are currently emerging as successful anti-cancer therapies (267). HAMLET appears to work equally well as an anti-cancer therapy in immunocompromised patients and animals as for immunocompetent subjects (18,25,27). Cancer chemotherapy treatments and cancer itself suppress parts of the immune system (268-271). Immunocompromised transplant recipient patients, who have a higher incidence of cancer than the rest of the immunocompetent population (272), might not be able to benefit from the emerging immunotherapies due to immunotherapy-triggered increased risk of organ rejection (273) or of acquiring a deadly infection (274,275), or due to decreased immunotherapy effectiveness resulting from the immunosuppression (276). HAMLET's ability to be effective without requiring immunocompetence may turn out to be an important factor in clinical success.

One immediate task for the HAMLET field is to continue developing delivery systems that avoid HAMLET-like compounds being deactivated in blood and enhance delivery directly to cancer cells. As the HAMLET mode of action involves the oleate having higher affinity for cell membranes once it comes into contact, HAMLET will probably not transit intact through healthy tissue in order to reach cancer cells. The in vivo experiments already carried out involved direct delivery of HAMLET to the bladder (by urinary tract catheter) (14,23,24), brain (by intracranial infusion) (25), skin (as a topical cream for warts) (27), and upper respiratory tract instillation (32). These are all creative examples of direct delivery of HAMLET-like compounds to tumours without HAMLET exposure to blood. Now that we have good understanding of the structure of HAMLET-like compounds (5,6), it is possible to carry out rational drug design to develop delivery systems for the HAMLET-like compounds that conserve their structure, which is crucial as it is the basis for their anti-cancer activity, whilst avoiding problem deactivation by blood. The understanding of structure will also guide the choice of compatible delivery vehicles that can attach, incorporate, and accommodate a semi-stable compound of the size of HAMLET protein. A goal of HAMLET delivery vehicles would be to enable HAMLET to be injected into the blood stream, or to be ingested as a pill such that HAMLET survives digestion in the stomach to end up in the blood stream, thereby reaching the
tumour sites around the body. Although HAMLET is toxic to blood cells and to a lesser extent endothelial cells in vitro, it is anticipated that any small amounts of HAMLET that might inadvertently find its way into the blood stream will be deactivated by the calcium and albumin in blood. A large amount of HAMLET in the blood or in proximity to sensitive endothelial cells would overcome the blood's capacity to deactivate HAMLET. If there is a risk of this leading to toxicities, then HAMLET-like compounds will need to be administered in many small doses or administered slowly, so that the blood's protective effects against HAMLET toxicity is not overwhelmed, similar to how widely used cisplatin chemotherapy is administered to cancer patients over hours so as to avoid cisplatin toxicity caused by overwhelming the kidneys (277).

The other immediate continuing task for HAMLET researchers is to determine the HAMLET target, because the available in vitro and in vivo evidence is that this target is a characteristic of all cancers and could be the basis of a new generation of broad-spectrum anti-cancer therapies. Identifying the HAMLET target would permit rational drug design efforts to design new compounds having the same target, perhaps small soluble molecules that do not have HAMLET's problem of being deactivated in blood.

We look forward to the continuing research developments – developments in identifying the HAMLET cell death mechanism(s) and developments in clinical anti-cancer applications – concerning this intriguing family of compounds composed of various proteins, including the most common human milk protein, and the most common human milk fatty acid.

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Supplementary information for Structure and potential cellular targets of HAMLET-like anti-cancer compounds made from milk components

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**Supplementary Table 1.** Cytotoxicity of HAMLET-like compounds to various cell lines and cell types. The 50% lethal dose (LD₅₀) values (without standard error) from the references are quoted where they were published or were otherwise visually read from the published graphs. The differences in values from different papers, for the same cells, are due to differences in oleate content of the HAMLET-like species prepared by different research groups and due to differences in experimental parameters. The experiments generally involved removing serum during the period of incubation of the cells with the HAMLET-like compound. Concentrations are for the protein component and not the fatty acid anion component. Interconversion calculations from mg/ml to μM used molecular weights of 14,078 Da for HAMLET, 14,186 Da for BAMLET, and 18,281 Da for bovine β-lactoglobulin BAMLET.

| Cells                        | Type            | Compound   | LD₅₀ or effect (will be referenced by number in final draft) |
|------------------------------|-----------------|------------|-------------------------------------------------------------|
| Carcinomas and papillomas    |                 |            |                                                             |
| A549, human lung carcinoma   | cancerous       | HAMLET     | 1.25 mg/ml, 89 μM (Svanborg et al. 2003, Gustafsson et al. 2009) |
|                              |                 | HAMLET     | 0.3 mg/ml, 20 μM (Hallgren et al. 2006)                     |
|                              |                 | recombinant His-tagged HAMLET | 0.2 mg/ml, 14 μM (Zhang et al. 2013) |
|                              |                 | HAMLET     | 0.5 mg/ml, 3 μM (Storm et al. 2013, Ho et al. 2013)         |
|                              |                 | HAMLET     | 1.5 mg/ml, 107 μM (Håkansson et al. 1999)                    |
|                              |                 | HAMLET     | 0% viable at 10 mg/ml (Håkansson et al. 1995)                |
|                              |                 | BAMLET     | 7% viable at 1.7 mg/ml, 117 μM (Delgado et al. 2015)         |
| NCI-H292, human bronchial carcinoma | cancerous | HAMLET     | 1 mg/ml, 71 μM (Svanborg et al. 2003)                       |
|                              |                 | HAMLET     | 0% viable at 10 mg/ml (Håkansson et al. 1995)                |
| A498, human kidney carcinoma | cancerous       | HAMLET     | 1 mg/ml, 71 μM (Svanborg et al. 2003, Gustafsson et al. 2009) |
|                              |                 | HAMLET     | 5% viable at 10 mg/ml (Håkansson et al. 1995)                |
|                              |                 | HAMLET     | 0.2 mg/ml, 14 μM (Hallgren et al. 2006)                     |
| A498, human kidney carcinoma, transfected to upregulate Bcl-xl | cancerous | HAMLET | 0.2 mg/ml, 14 μM (Hallgren et al. 2006) |
| CaCO₂, human intestine carcinoma | cancerous       | HAMLET     | 0.55 mg/ml, 39 μM (Svanborg et al. 2003)                     |
|                              |                 | BAMLET     | 1 mg/ml, 70 μM (Brinkmann et al. 2011)                       |
|                              |                 | BAMLET     | <0.4 mg/ml, <30 μM (Fang et al. 2012)                        |
| HT29, human intestine carcinoma | cancerous | HAMLET     | 1.25 mg/ml, 89 μM (Svanborg et al. 2003)                     |
| Cell Line                          | Type            | Drug | Concentration | Reference                  |
|-----------------------------------|-----------------|------|---------------|----------------------------|
| BAMLET                            |                | <0.4 mg/ml, <30 μM | (Fang et al. 2012)         |
| HAMLET                            |                | 6% viable at 10 mg/ml | (Håkansson et al. 1995)    |
| MCF7, human breast adenocarcinoma | cancerous       | HAMLET | 1.25 mg/ml, 89 μM | (Svanborg et al. 2003)     |
|                                   |                | BAMLET | <0.4 mg/ml, <30 μM | (Fang et al. 2012)         |
|                                   |                | BAMLET | 0.09 mg/ml, 6 μM | (Rammer et al. 2010)       |
| β-lactoglobulin BAMLET            |                | 1.6 mg/ml, 90 μM | (Fang et al. 2015)         |
| β-lactoglobulin BAMLET with linoleic acid | | 1 mg/ml, 60 μM | (Fang et al. 2015)         |
| SK-BR-3, human breast adenocarcinoma | cancerous       | HAMLET | 1.25 mg/ml, 89 μM | (Svanborg et al. 2003)     |
| MBD-231, human breast adenocarcinoma | cancerous       | HAMLET | 1.25 mg/ml, 89 μM | (Svanborg et al. 2003)     |
| T47d, human breast ductal carcinoma | cancerous       | HAMLET | 1.25 mg/ml, 89 μM | (Svanborg et al. 2003)     |
| MBD-175VII, human breast carcinoma | cancerous       | HAMLET | 1.5 mg/ml, 107 μM | (Svanborg et al. 2003)     |
| PC3, human prostate adenocarcinoma | cancerous       | HAMLET | 1 mg/ml, 71 μM | (Svanborg et al. 2003)     |
|                                   |                | HAMLET | 0.17 mg/ml, 12 μM | (Rammer et al. 2010)       |
|                                   |                | BAMLET | 0.23 mg/ml, 16 μM | (Rammer et al. 2010)       |
| DU145, human prostate carcinoma    | cancerous       | HAMLET | 1.25 mg/ml, 89 μM | (Svanborg et al. 2003)     |
|                                   |                | camel HAMLET | 0.6 mg/ml, 4 μM | (Atri et al. 2011)         |
| WEHI-164, mouse fibrosarcoma       | cancerous       | HAMLET | 0.75 mg/ml, 53 μM | (Svanborg et al. 2003)     |
| HCT116, human colon carcinoma      | cancerous       | HAMLET | 0.6 mg/ml, 40 μM | (Svanborg et al. 2003)     |
| HCT116, human colon carcinoma, p53 wildtype | cancerous | HAMLET | 0.7 mg/ml, 48 μM | (Hallgren et al. 2006)     |
| HCT116, human colon carcinoma, p53 deletion | cancerous | HAMLET | 0.7 mg/ml, 44 μM | (Hallgren et al. 2006)     |
| H1299, human lung carcinoma        | cancerous       | HAMLET | 0.7 mg/ml, 50 μM | (Svanborg et al. 2003)     |
|                                   |                | recombinant His-tagged HAMLET | 0.07 mg/ml, 5 μM | (Zhang et al. 2013)         |
| H1299, human lung carcinoma, p53 deletion | cancerous | HAMLET | 0.6 mg/ml, 42 μM | (Hallgren et al. 2006)     |
| H1299, human lung carcinoma, p53 His-175 gain of function | cancerous | HAMLET | 0.6 mg/ml, 42 μM | (Hallgren et al. 2006)     |
| Skov3, human ovarian carcinoma     | cancerous       | BAMLET | 0.2 mg/ml, 14 μM | (Brinkmann et al. 2011)    |
| MB49, mouse bladder carcinoma      | cancerous       | HAMLET | 0.2 mg/ml, 14 μM | (Mossberg 2010)            |
| HeLa, human cervical epithelial adenocarcinoma | cancerous | HAMLET | 0.2 mg/ml, 14 μM | (Rammer et al. 2010)       |
|                                   |                | BAMLET | 0.12 mg/ml, 8 μM | (Rammer et al. 2010)       |
| Cell Line | Condition | BAMLET | β-lactoglobulin BAMLET | β-lactoglobulin BAMLET with linoleic acid |
|-----------|-----------|--------|------------------------|---------------------------------|
| HEP2      | cancerous | 0.028 mg/ml, 2 μM | 2 mg/ml, 110 μM | 1 mg/ml, 60 μM |
| J82, human bladder transitional cell carcinoma | cancerous | 0.75 mg/ml, 53 μM | 0.028 mg/ml, 2 μM | 0.022 mg/ml, 2 μM |
| HTB9, human bladder carcinoma | cancerous | 0.15 mg/ml, 11 μM | 0.27 mg/ml, 19 μM | 0.15 mg/ml, 11 μM |
| UMUC3, human bladder urothelial cancer | cancerous | 0.13 mg/ml, 9 μM | 0.3 mg/ml, 18 μM | 0.34 mg/ml, 24 μM |
| UMUC6, human bladder urothelial cancer | cancerous | 0.15 mg/ml, 11 μM | 0.15 mg/ml, 11 μM | 0.15 mg/ml, 11 μM |
| UMUC9, human bladder urothelial cancer | cancerous | 0.8 mg/ml, 56 μM | 0.8 mg/ml, 56 μM | 0.8 mg/ml, 56 μM |
| T24, human bladder urothelial cancer | cancerous | 0.35 mg/ml, 25 μM | 0.35 mg/ml, 25 μM | 0.35 mg/ml, 25 μM |
| 253J, human metastatic bladder urothelial transitional cell carcinoma | cancerous | 0.15 mg/ml, 11 μM | 0.15 mg/ml, 11 μM | 0.15 mg/ml, 11 μM |
| MGH-U3, human bladder urinary tract carcinoma | cancerous | 0.35 mg/ml, 25 μM | 0.35 mg/ml, 25 μM | 0.35 mg/ml, 25 μM |
| RT112, human differentiated urinary bladder transitional cell carcinoma | cancerous | 0.4 mg/ml, 28 μM | 0.4 mg/ml, 28 μM | 0.4 mg/ml, 28 μM |
| TRAIL-resistant UMUC14, human bladder epithelial carcinoma | cancerous | 0.28 mg/ml, 20 μM | 0.28 mg/ml, 20 μM | 0.28 mg/ml, 20 μM |
| TRAIL-resistant HT1376, human urinary bladder epithelial carcinoma | cancerous | 0.47 mg/ml, 33 μM | 0.47 mg/ml, 33 μM | 0.47 mg/ml, 33 μM |
| AY27, rat bladder urothelial cancer | cancerous | 0.2 mg/ml, 14 μM | 0.2 mg/ml, 14 μM | 0.2 mg/ml, 14 μM |
| RT4, human bladder transitional cell papilloma | cancerous | 0.44 mg/ml, 31 μM | 0.44 mg/ml, 31 μM | 0.44 mg/ml, 31 μM |
| Pheochromocytomas, osteosarcomas, and melanoma |
| PC12, rat adrenal pheochromocytoma, | cancerous | 0.09-0.16 mg/ml, 6-11 μM | 0.09-0.16 mg/ml, 6-11 μM | 0.09-0.16 mg/ml, 6-11 μM |

Notes: BAMLET = Bacterial Lipid Exocomplex; HAMLET = Human Anti-Microbial Lipocystein; TRAIL = Tumor Necrosis Factor-Related Acidic Ligand.
| Tissue Type | Cancerous Cell Line | Concentration |
|-------------|---------------------|---------------|
| Undifferentiated State | PC12, rat adrenal pheochromocytoma, differentiated state | BAMLET 0.2-1 mg/ml, 10-55 μM (depending on oleate content) |
| | | β-lactoglobulin 0.2-0.1 mg/ml, 10-55 μM (depending on oleate content) |
| | PC12, rat adrenal pheochromocytoma | ELOA visualised cell death at 0.0035 mg/ml, 0.24 μM, 61 mins |
| Gliomas | U2OS, human bone osteosarcoma | BAMLET 0.06 mg/ml, 4 μM |
| | | HAMLET 0.075 mg/ml, 5 μM |
| Lymphomas and leukaemias | Jurkat, human acute T-cell lymphocytic leukaemia | HAMLET 0.4 mg/ml, 28 μM |

This table summarizes the concentrations of β-lactoglobulin and BAMLET in different cancerous cell lines. The concentrations are given in mg/ml and μM, depending on the specific oleate content. The table includes concentrations for different tissue types and cancerous cell lines, with some data from Brinkmann et al. (2011), Lišková et al. (2011), and others.
| Cell Line Description                                                                 | Concentration | Reference                          |
|-------------------------------------------------------------------------------------|---------------|------------------------------------|
| **HAMLET**                                                                          | 0.6 mg/ml, 46 μM (Hallgren et al. 2006) |
| **BAMLET**                                                                          | 0.09 mg/ml, 6 μM (Brinkmann et al. 2011, Brinkmann 2013) |
| Jurkat, human acute T-cell lymphocytic leukaemia, transfected to upregulate Bcl-2    | 0.3 mg/ml, 18 μM (Hallgren et al. 2006) |
| **HAMLET**                                                                          | 0.5 mg/ml, 36 μM (Håkansson et al. 1999) |
| L1210, mouse prolymphocytic leukaemia                                               | 0.3 mg/ml, 21 μM (Svanborg et al. 2003) |
| **HAMLET**                                                                          | 0.05 mg/ml, 4 μM (Rammer et al. 2010) |
| B9, mouse lymphocytic leukaemia                                                     | 0.3 mg/ml, 21 μM (Svanborg et al. 2003) |
| **HAMLET**                                                                          | 0.5 mg/ml, 36 μM (Svanborg et al. 2003) |
| **HAMLET**                                                                          | 0.03 mg/ml, 2 μM (Brinkmann et al. 2011, Brinkmann 2013) |
| **BAMLET**                                                                          | 0.4 mg/ml, <30 μM (Fang et al. 2012) |
| **HAMLET**                                                                          | 0.3 mg/ml, 18 μM (Hallgren et al. 2006) |
| K562, human myelogenous leukaemia                                                   | 0.1 mg/ml, 9 μM (Hallgren et al. 2006) |
| **HAMLET**                                                                          | 0.5 mg/ml, 36 μM (Svanborg et al. 2003) |
| **HAMLET**                                                                          | 0.14 mg/ml, 10 μM (Svanborg et al. 2003) |
| **BAMLET**                                                                          | 0.1 mg/ml, 9 μM (Håkansson et al. 1999) |
| **BAMLET**                                                                          | 0.1 mg/ml, 9 μM (Hallgren et al. 2006) |
| **Non-cancerous immortalised cell lines**                                          |                |
| GMK, monkey kidney                                                                  | 1 mg/ml, 71 μM (Svanborg et al. 2003) |
| Vero, monkey kidney                                                                 | 1.75 mg/ml, 124 μM (Svanborg et al. 2003) |
| MDCK, dog kidney                                                                    | 0.75 mg/ml, 53 μM (Svanborg et al. 2003) |
| NIH-3T3, mouse embryonic fibroblast                                                 | >0.4 mg/ml, >30 μM (75% viable at 0.4 mg/ml) (Rammer et al. 2010) |
| **BAMLET**                                                                          | 15% viability at 1.7 mg/ml, 117 μM (Delgado et al. 2015) |
| Cho-K1, Chinese hamster ovary epithelial                                            | 30% viability at 1.7 mg/ml, 117 μM (Delgado et al. 2015) |
| Cell Type                                      | Condition                          | Protein   | Concentration | Notes                                                                 |
|-----------------------------------------------|------------------------------------|-----------|---------------|-----------------------------------------------------------------------|
| F2P6, human benign fibroblast                 | non-cancerous, immortalised        | BAMLET    | >0.8 mg/ml, 56 μM (resistant at dose of 0.8 mg/ml that killed AY27 cancer cells in same spheroid) (Xiao et al. 2013) |
| Human non-transformed astrocytes from manufacturer CC 2565, Cambrex, La Jolla, CA, USA | non-cancerous                        | HAMLET    | >0.48 mg/ml, >34 μM (resistant at 34 μM although some HAMLET entered cells) (Gustafsson et al. 2009) |
| Human non-transformed astrocytes from manufacturer CC 2565, Cambrex, La Jolla, CA, USA | non-cancerous                        | HAMLET    | >0.48 mg/ml, >34 μM (resistant at 34 μM although some HAMLET entered cells) (Gustafsson et al. 2009) |
| human embryonic lung                          | non-cancerous, embryonic           | HAMLET    | 8% viable at 10 mg/ml (Håkansson et al. 1995)                           |
| human embryonic foreskin                      | non-cancerous, embryonic           | HAMLET    | 8% viable at 10 mg/ml (Håkansson et al. 1995)                           |
| non-cancerous primary non-blood cells          |                                    |           |               |                                                                       |
| Normal adult human keratinocytes              | non-cancerous, primary             | BAMLET    | 0.1 mg/ml, 7 μM (Brinkmann et al. 2011)                                 |
| Human umbilical vein endothelial cells (HUVEC) | non-cancerous, primary             | BAMLET    | 1 mg/ml, 70 μM (Brinkmann et al. 2011)                                 |
| primary human renal tubular epithelial cells  | non-cancerous, primary             | HAMLET    | >0.1 mg/ml, >7 μM (resistant at 0.1 mg/ml) (Düringer et al. 2003)        |
| human renal epithelial cells (HRTEC)          | non-cancerous, primary             | HAMLET    | >0.48 mg/ml, >34 μM (resistant at 34 μM) (Gustafsson et al. 2009)       |
| human healthy renal proximal tubule cells, semi-differentiated (HRPTEC) | non-cancerous, primary             | HAMLET    | >0.3 mg/ml, >21 μM (viable after 24 h exposure to 21 μM) (Storm et al. 2011) |
| primary mouse brain cells                     | non-cancerous, primary             | HAMLET    | >0.07 mg/ml, >4 μM (100% viable at 0.07 mg/ml) (Fischer et al. 2004)     |
| primary mouse astrocytes                      | non-cancerous, primary             | recombinant His-tagged HAMLET | >0.2 mg/ml, >14 μM (90% viable at 0.2 mg/ml) (Zhang et al. 2013) |
| nontransformed human nasopharynx epithelium   | non-cancerous, primary             | HAMLET    | 45% viable at 10 mg/ml (Håkansson et al. 1995)                          |
| nontransformed human urinary epithelium       | non-cancerous, primary             | HAMLET    | 75% viable at 10 mg/ml (Håkansson et al. 1995)                          |
| nontransformed mouse kidney epithelial        | non-cancerous, primary             | HAMLET    | 62% viable at 10 mg/ml (Håkansson et al. 1995)                          |
| nontransformed mouse bladder epithelial       | non-cancerous, primary             | HAMLET    | 48% viable at 10 mg/ml (Håkansson et al. 1995)                          |
| mouse adipocytes                              | non-cancerous, primary             | BAMLET    | >0.05 mg/ml, 282 μM (resistant at 4 mg/ml dose that killed cancerous MCF-7 and Jurkat cell lines) (Hoque et al. 2013) |
| non-cancerous primary blood cells             |                                    |           |               |                                                                       |
| Peripheral blood mononuclear cells (PBMC)     | non-cancerous, primary             | BAMLET    | 0.02 mg/ml, 1 μM (Brinkmann et al. 2011)                                 |
| human erythrocytes                            | non-cancerous, primary             | BAMLET, BLAGLET, ELOA parvalbumin HAMLET | 0.02 mg/ml, 1.4 μM, (20 μM oleate) (Kaspersen et al. 2014) |
|                                                |                                    | ovalbumin HAMLET     | 0.02 mg/ml, 0.44 μM, (20 μM oleate) (Kaspersen et al. 2014) |
immunoglobulin G HAMLET 0.02 mg/ml, 0.13 μM, (20 μM oleate) (Kaspersen et al. 2014)
albumin BAMLET 0.028 mg/ml, 0.4 μM, (28 μM oleate) (Kaspersen et al. 2014)

| Species            | Protein  | Fatty acid anion | Preparation method       | Ref.                                      |
|--------------------|----------|------------------|--------------------------|------------------------------------------|
| mouse erythrocytes | BAMLET   | oleic acid       | column chromatography    | (Svensson et al. 2000)                   |

Supplementary Table 2. Stoichiometry of protein to fatty acid in various HAMLET-like compounds made by various methods and with different protein or fatty acid. Different method types can produce similar amounts of incorporated oleate, and small changes in the parameters of a method type can produce significant differences in the amounts of incorporation of the active ingredient oleate.

| Protein: oleate stoichiometry | Number of molecules per aggregate | Species  | Protein                     | Fatty acid anion | Preparation method       | Ref.                                      |
|--------------------------------|----------------------------------|----------|-----------------------------|------------------|--------------------------|------------------------------------------|
| 1 : 0.9±0.36                   |                                  | HAMLET   | human α-lactalbumin         | oleic acid       | column chromatography    | (Svensson et al. 2000)                   |
| 1 : 5.4±1.5                    |                                  | HAMLET   | human α-lactalbumin         | oleic acid       | column chromatography    | (Pettersson-Kastberg et al. 2009)        |
| 1 : 2.9±0.1                    |                                  | HAMLET   | human α-lactalbumin         | oleic acid       | heating to 17°C          | (Knyazeva et al. 2008)                   |
| 1 : 9                          |                                  | HAMLET   | human α-lactalbumin         | oleic acid       | heating to 45°C          | (Knyazeva et al. 2008)                   |
| 1 : 11.5                       | recombinant His-tagged HAMLET    | recombinant human α-lactalbumin | oleic acid       | heating method          | (Zhang et al. 2013)                  |
| 1 : 7.5                        | HAMLET                           | recombinant human α-lactalbumin | oleic acid       | heating method          | (Nakamura et al. 2013)               |
| 1 : 10±1.4                     | BAMLET                           | bovine α-lactalbumin           | oleic acid       | heating method          | (Hoque et al. 2013)                   |
| Ratio | Description | Amount (Concentration) | Method/Condition | Reference |
|-------|-------------|------------------------|-----------------|-----------|
| 1 : 11±1.52 | BAMLET bovine α-lactalbumin oleic acid cross-link protein then add oleate by heating method | | (Hoque et al. 2013) |
| 1 : 10±1.4 | BAMLET bovine α-lactalbumin oleic acid add oleate by heating method then cross-link protein | | (Hoque et al. 2013) |
| 1 : 11 | BAMLET bovine α-lactalbumin oleic acid high pH method | | (Permyakov et al. 2011) |
| 1 : 0.7 (1.4%) | BAMLET bovine α-lactalbumin oleic acid mixing at pH 2.0 | | (Fang et al. 2012) |
| 1 : 0.5 (1%) | BAMLET bovine α-lactalbumin oleic acid mixing at pH 3.0 | | (Fang et al. 2012) |
| 1 : 1.7 (3.3%) | BAMLET bovine α-lactalbumin oleic acid mixing at pH 4.0 | | (Fang et al. 2012) |
| 1 : 0.8 (1.5%) | BAMLET bovine α-lactalbumin oleic acid mixing at pH 5.0 | | (Fang et al. 2012) |
| 1 : 0.2 (2.2%) | BAMLET bovine α-lactalbumin oleic acid mixing at pH 6.0 | | (Fang et al. 2012) |
| 1 : 3 (5.8%) | BAMLET bovine α-lactalbumin oleic acid mixing at pH 7.0 | | (Fang et al. 2012) |
| 1 : 4 (7.5%) | BAMLET bovine α-lactalbumin oleic acid mixing at pH 8.0 | | (Fang et al. 2012) |
| 1 : 4.7 (8.5%) | BAMLET bovine α-lactalbumin oleic acid mixing at pH 9.0 | | (Fang et al. 2012) |
| 1 : 33.9±11 | BAMLET bovine α-lactalbumin oleic acid heating method | | (Rath et al. 2014) |
| 1 : 4.1±0.3 | 2.8±0.1 protein : 12±1 oleate | BAMLET bovine α-lactalbumin oleic acid column chromatography | (Kaspersen et al. 2014) |
| 1 : 7.4±0.7 | 3.4±0.2 protein : 25±2 oleate | BAMLET bovine α-lactalbumin oleic acid high pH method | (Kaspersen et al. 2014) |
| 1 : 11±1 | 3.0±0.1 protein : 33±1 oleate | BAMLET bovine α-lactalbumin sodium oleate mixing method | (Kaspersen et al. 2014) |
| 1 : 8.2±0.6 | 4.0±0.2 protein : 33±2 oleate | BAMLET bovine α-lactalbumin oleic acid heating to 80°C | (Kaspersen et al. 2014) |
| 1 : 8.1±0.6 | 2.3±0.1 protein : 19±1 oleate | BAMLET bovine α-lactalbumin oleic acid heating to 60°C | (Kaspersen et al. 2014) |
| 1 : 4 to 15 | BAMLET bovine α-lactalbumin oleic acid column chromatography | | (Brinkmann et al. 2011) |
| 1 : 17±0.6 | 4-5 protein : 68-85 oleate | BAMLET bovine α-lactalbumin oleic acid column chromatography | (Spolaore et al. 2010) |
| 1 : 9±2 | BAMLET bovine α-lactalbumin oleic acid gentle heating at high pH 12, then cooling at low pH | | (Delgado et al. 2015) |
| 1 : 11±3 | BAMLET bovine α-lactalbumin oleic acid heating at pH 8, then cooling at low pH | | (Delgado et al. 2015) |
| 1 : 4±1 | BAMLET bovine α-lactalbumin oleic acid high pH, then heating | | (Delgado et al. 2015) |
| Ratio | Protein to Oleic Acid | Source | Protein Type | Oleic Acid Type | Method | Reference |
|-------|-----------------------|--------|--------------|-----------------|--------|-----------|
| 6.9   | 1:12±1                | β-lactoglobulin | Bovine β-lactoglobulin | Oleic acid | Heating to 70-90°C | (Nakamura et al. 2013) |
| 9     | 2:2±0.1 protein : 26±1 oleate | β-lactoglobulin | Bovine β-lactoglobulin | Sodium oleate | Column chromatography | (Lišková et al. 2011) |
| 12±1  | β-lactoglobulin | Bovine β-lactoglobulin | Oleic acid | Heating to 70-90°C | (Kaspersen et al. 2014) |
| 17    | β-lactoglobulin | Bovine β-lactoglobulin | Oleic acid | High pH method | (Permyakov et al. 2012) |
| 9     | 44-1440 protein : 142-480 oleate | β-lactoglobulin | Bovine β-lactoglobulin | Sodium oleate | Heating to 60°C | (Lišková et al. 2011) |
| 11 to 48 | 4-30 protein : 144-44 oleate | ELOA | Horse lysozyme | Oleic acid | Column chromatography | (Wilhelm et al. 2009) |
| 7.1±0.4 | 4.9±0.2 protein : 35±1 oleate | Chicken ELOA | Chicken egg lysozyme | Oleic acid | Heating to 70-90°C | (Kaspersen et al. 2014) |
| 14±1  | 4.5±0.2 protein : 60±3 oleate | Chicken ELOA | Chicken egg lysozyme | Oleic acid | Heating to 70-90°C with extra oleic acid | (Kaspersen et al. 2014) |
| 13    | 1.9±0.2 protein : 18±2 oleate | Parvalbumin | Pike parvalbumin | Oleic acid | Heating to 70-90°C, 1 mg/ml | (Kaspersen et al. 2014) |
| 9.2±1.2 | 2.1±1.0 protein : 20±1 oleate | Parvalbumin | Pike parvalbumin | Oleic acid | Heating to 70-90°C, 3 mg/ml | (Kaspersen et al. 2014) |
| 9.4±0.8 | 0.45±0.04 protein : 24±2 oleate | Ovalbumin | Chicken ovalbumin | Oleic acid | Heating to 70-90°C | (Kaspersen et al. 2014) |
| 53±7  | 0.28±0.01 protein : 28±1 oleate | BSA BAMLET | Bovine serum albumin | Oleic acid | Heating to 70-90°C | (Kaspersen et al. 2014) |
| 100±6 | 0.086±0.008 protein : 12±1 oleate | Immunoglobulin G HAMLET | Human immunoglobulin G | Oleic acid | Heating to 70-90°C | (Kaspersen et al. 2014) |