Title: **HAMLET KILLS TUMOR CELLS BY APOPTOSIS - CELLULAR, MOLECULAR AND THERAPEUTIC ASPECTS**

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ABSTRACT

HAMLET (Human α-lactalbumin made lethal to tumor cells) is a protein-lipid complex that induces apoptosis in tumor cells, but leaves fully differentiated cells unaffected. This review summarizes the information on the *in vivo* effects of HAMLET in patients and tumor models, on the tumor cell biology and on the molecular characteristics of the complex. HAMLET limits the progression of human glioblastomas in a xenograft model and removes skin papillomas in patients. The broad anti-tumor activity includes >40 different lymphomas and carcinomas and apoptosis is independent of *p53* or *bcl-2*. In tumor cells, HAMLET enters the cytoplasm, translocates to the perinuclear area and enters the nuclei, where it accumulates. HAMLET binds strongly to histones and disrupts the chromatin organization. In the cytoplasm, HAMLET targets ribosomes and mitochondria. The formation of HAMLET relies on the propensity of α-lactalbumin to alter its conformation when the strongly bound Ca^{2+} ion is released and the protein adopts the so called apo conformation that exposes a new fatty acid binding site. Oleic acid (C18:1,9cis) fits this site with high specificity, and stabilizes the altered protein conformation. The results illustrate how protein folding variants may be beneficial, and how their formation in peripheral tissues may depend on the folding change and the availability of the lipid cofactor. One example is the acid pH in the stomach of the breast fed child that promotes the formation of HAMLET, which may contribute to the protective effect of breast feeding against childhood tumors. We propose that HAMLET should be explored as a novel approach to tumor therapy.

I. BACKGROUND

HAMLET (human α-lactalbumin made lethal to tumor cells) is a molecular complex formed by α-lactalbumin and oleic acid (Hakansson *et al.*, 1995; Svensson *et al.*, 2000) (Fig. 1). Remarkably, it induces apoptosis in tumor cells, but healthy differentiated cells are resistant to its effect.

The activity of HAMLET was discovered by serendipity, while using breast milk fractions to investigate how bacteria attach to lung carcinoma cell lines. In addition to blocking adherence, one milk fraction actually killed the cells, by forcing them to undergo apoptosis (Fig. 2 and Table. 1). Cell death was accompanied by the characteristic changes in morphology, with nuclear condensation, formation of apoptotic bodies, and cytoplasmic blebbing, as described in 1972 by Kerr, Wyllie and Currie, who named the phenomenon apoptosis (Kerr *et al.*, 1972).

The active molecular complex was obtained from the casein fraction of milk, after precipitation at low pH, and was shown to contain α-lactalbumin (Svensson *et al.*, 1999). α-Lactalbumin is the most abundant protein in human milk (Heine *et al.*, 1996), and is well-known as a coenzyme in lactose synthesis (Brew *et al.*, 1968; Musci and Berliner, 1985). This form of α-lactalbumin had no effect on the tumor cells, however, suggesting that a structural difference must exist between the two activity states of the molecule. Post-translational modifications were excluded by mass spectrometry (Svensson *et al.*, 1999), leaving differences in tertiary structure as a putative explanation of the novel biologic activity. As human α-lactalbumin is known to form stable folding intermediates at low pH, we investigated the conformation of the active complex. We

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showed that the active form of the protein had altered its fold to a molten globule like state. The link between apoptosis induction and the folding change was subsequently proven by deliberate unfolding of α-lactalbumin and by conversion to the apoptosis inducing form, in the presence of the lipid cofactor. The chemically defined active complex was named HAMLET (Fig. 1) (Hakansson *et al.*, 1995; Svensson *et al.*, 2000).

HAMLET has several unique features. 1) It kills cancer cells, but not healthy differentiated cells. 2) It is active against a broad range of tumor cell lines. 3) It kills by apoptosis, which is non-toxic to the tissues. 4) It is formed from human milk, at low pH, and is thus unlikely to provoke harmful side effects if given as therapy.

This review summarizes the work on HAMLET to date; the *in vivo* evidence for therapeutic effects in animal models and patients, the studies on the cellular targets of HAMLET in tumor cells, and the structural characterization of this protein folding variant.

II. EFFECTS OF HAMLET IN TUMOR MODELS

Cellular spectrum. The selectivity of the active complex for tumor cells is quite remarkable, as is the broad activity against very different tumor cell types (Table 1). To date, over 40 different cell lines have been tested. HAMLET induces apoptosis in carcinomas of the lung, throat, kidney, colon, bladder, prostate and ovaries, in melanomas, glioblastomas of the brain, and leukemias. The effect is not just specific for human tumors, but HAMLET kills tumor cell lines of primate, bovine, murine and canine origin (Table 1) (Hakansson *et al.*, 1995). The lymphoid tumor cells are the most

sensitive, requiring only 0.01 mM of the protein to kill 50% of the cells in 6 hours, but also the carcinoma cells undergo apoptosis, and about 0.04 mM of HAMLET kills 50% of the cells in 24 hours (Table 1).

The selectivity of HAMLET for tumor cells is unexpected, as apoptotic death programs are thought to have evolved to purge healthy cells from the tissues, as they need to be replaced during tissue growth and maturation. The broad activity against vastly different tumor cells is even more unexpected, as many cancer cells have inactivated the apoptosis pathways that operate in healthy cells. For cancer cells, suicide is an aberration, and short-circuited apoptosis pathways usually allow these cells to multiply undisturbed.

These properties make HAMLET a rather unique novel tool in cancer therapy. Due to its selectivity, HAMLET should be able to purge tumor cells from the tissues, by triggering them to undergo apoptosis. As HAMLET shows no effect on healthy cells the tumor cells should disappear without damage to the surrounding tissues. Finally, as HAMLET consists of molecules from human milk, which are ingested daily by premature and newborn infants, toxic side effects are unlikely.

In vivo *effects of HAMLET in a glioblastoma (GBM) model.* The majority of intra-cranial neoplasms originate from neuroglial cells, and form a heterogeneous group known as gliomas (Russel and Rubinstein, 1989). They account for more than 60% of all primary brain tumors, and have the most unfavorable prognosis. GBMs of WHO grade IV show a mean survival time of less than 1 year (Gundersen *et al.*, 1996), and they

constitute approximately one fourth of all intra-cranial tumors in neuro-surgical and neuro-pathological series.

In recent years, surgical treatment of gliomas has made significant technical advances. Microsurgery and neuro-navigation as well as new diagnostic high resolution imaging techniques have reduced surgical mortality and morbidity, but there has been no significant improvement in survival. The tumors are inaccessible to complete surgical removal, due to their invasive nature and diffuse infiltrating growth, and the current treatment of patients with malignant gliomas is palliative, involving surgery, radiotherapy and chemotherapy.

During our survey of tumor cell lines, we observed that GBM cells undergo apoptosis in response to HAMLET (Table 1). Native α-lactalbumin, which was used as a control throughout these studies, did not influence cell viability or cause DNA fragmentation. HAMLET did not induce apoptosis in differentiated brain cells. The healthy cells maintained their viability and showed intact DNA after 24 hours exposure to HAMLET.

The effect on HAMLET was investigated in a rat model of human glioblastoma (Goldbrunner *et al.*, 2000) (Fig. 3B). Xeno-transplantation of human glioma biopsies into the nude rat brain offers a unique model to study the human disease under experimental conditions as the xenografts show the infiltrative growth characteristic of human tumors (Engebraaten *et al.*, 1999). In this model, human tumor biopsies are allowed to form spheroids in vitro as an intermediate step to obtain standardized inocula of tumor cells. After xenotransplantation, the rats develop pressure symptoms

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after eight weeks with little variation, and large tumor masses can be detected by MRI scans.

To investigate the effect of HAMLET on tumor tissue rather than cell lines, human glioblastoma biopsy spheroids were exposed to HAMLET or α-lactalbumin *in vitro,* and apoptotic cells were detected by the TUNEL assay. HAMLET was shown to induce apoptosis throughout the tumor spheroids but α-lactalbumin had no effect, as compared to the medium control (Fig. 3A)

The therapeutic potential of HAMLET was investigated in this model (Fig. 3). The tumor cells were allowed one week to become integrated in the brain. HAMLET was then administered by convection-enhanced delivery for 24 hours with α-lactalbumin as a control, and the rats were surveilled for another seven weeks, resulting in a total experimental period of eight weeks. HAMLET was shown to inhibit tumor development (Fig. 3C) as shown by a delayed onset of pressure symptoms (Fig. 3D). Rats receiving α-lactalbumin developed symptoms significantly earlier than the HAMLET treated animals (p< 0.01).

Apoptosis induction *in vivo* was examined by subjecting sections from the treated rats to the TUNEL assay. There was extensive apoptosis in the tumor but the tissue surrounding the tumor did not show TUNEL-labeling (not shown). Furthermore, the infusion of HAMLET did not harm the normal brain and did not produce any neurological symptoms.

These effects of HAMLET on established tumors must be regarded as quite promising. We conclude that HAMLET has the potential to act as a selective inducer of apoptosis in patients with malignant gliomas.

Effects of HAMLET on human skin papillomas. Papillomas are premalignant lesions of the skin and mucosal surfaces (Tyring, 2000); (Majewski and Jablonska, 2002). The human papilloma virus (HPV) can cause condyloma acuminatum, laryngeal and genital papillomas, and are extremely common. Therapeutic options are limited and often ineffective or destructive (Gibbs *et al.*, 2001). They include cryotherapy, curettage, cautery, salicylic acid, CO_2 laser (Mancuso *et al.*, 1991), photodynamic therapy (Ammann *et al.*, 1995), antimitotic agents such as podophyllin (Simmons, 1981), bleomycin (Bunney *et al.*, 1984) and fluorouracil (Hursthouse, 1975), or immune modulators such as cimetidine (Yilmaz *et al.*, 1996), intrawartal interferon (Gibson, 1988; Condylomata International Collaborative Study Group, 1991) and imiquimod. Even distant healing has been tried (Harkness *et al.*, 2000). Currently, HPV vaccines are being developed to prevent HPV infection, but they are not available for use.

We selected skin papillomas as a first model to examine HAMLET treatment in humans (Fig. 4). Topical treatment was performed, according to a double-blind, placebo-controlled protocol. HAMLET or placebo was applied topically, once a day for three weeks. The lesions were measured and photographed once a week during the treatment period and at follow up visits, 1 and 2 months after completed treatment (Fig. 4A). The treatment was deemed successful if the patient showed a reduction in

papilloma volume by >75%. Indeed, HAMLET treatment reduced the papilloma volume in 100% (20/20) of the patients compared to 15% (3/20) in the placebo group (p<0.001) (Fig. 4C).

Based on these findings we propose that HAMLET should be tested on a larger scale as a treatment for skin papillomas.

Summary, **in vivo** *studies.* These *in vivo* trials of HAMLET are quite promising. HAMLET induces apoptosis *in vivo,* slows down tumor development in the brain and removes premalignant lesions in the skin. Continued studies will explore these effects further, and extend them to other tumor models. We propose that HAMLET should also be explored as a topical treatment for genital papillomas, and other accessible mucosal tumors such as bladder cancers, gastric cancers and possibly lung carcinomas.

III. CELLULAR TARGETS OF HAMLET IN TUMOR CELLS

Several approaches have been taken to identifying the mechanisms of apoptosis in response to HAMLET, and to understand the difference in sensitivity between tumor cells and healthy cells. Initially, we examined tumor cells known to resist apoptosis due to mutations in e.g. the *bcl-2* or *p53* genotype, but found no effect of these mutations (see below). We then examined the FAS-FAS ligand pathway, using specific inhibitors, and found that HAMLET did not depend on this pathway (Kohler *et al.*, 1999). A re-examination of HAMLET emphasized the broad anti-tumor spectrum, suggesting that HAMLET bypasses the different blocks of apoptosis in many tumors (Johnstone *et al.*,

2002). We conclude that HAMLET must be able to activate fundamental pathways of apoptosis common to all tumor cells tested.

In an approach to understanding the molecular basis of this activity, we have studied the interaction of HAMLET with different cellular compartments by real-time confocal microscopy, by isolation of cellular organelles, and by purifying molecular targets for HAMLET in these organelles (see below). Activated or suppressed effector pathways have also been studied by microarray technology.

HAMLET induced apoptosis is P53 independent. *p53* mutations are frequent in tumor cells, and offer one mechanisms of resistance to apoptosis (Johnstone *et al.*, 2002). The initial screening of tumor cell lines showed no apparent association with the *p53* genotype (Table 1), suggesting that apoptosis was P53 independent. The role of P53 for HAMLET-induced apoptosis was further investigated, using cellular models with defined *p53* genotypes.

The HCT116 human colon carcinoma cell line, with a tumor derived ARF deletion and wt *p53* (+/+), was compared to the clone HCT116 (-/-) that carries a *p53* deletion. Furthermore, the lung carcinoma H1299 carrying a *p53* deletion was compared to the V175A stable transfectant, expressing P53 under the control of tetracyclin (data not shown) (Bykov *et al.*, 2002). There was no difference in susceptibility to HAMLET between the *p53+, p53- or p53* mutant cell lines (Fig. 5A). The loss of cell viability was accompanied by DNA fragmentation. We conclude that HAMLET induces apoptosis regardless of *p53* status.

HAMLET induced apoptosis is bcl-2 independent. The bcl-2 proteins are major regulators of apoptosis. Localized at the mitochondrial outer membrane, bcl-2 interacts with the permeability transition pore of the mitochondria, blocking the release of apoptogenic factors from the inter-membrane space (Kroemer and Reed, 2000;Green and Reed, 1998;Gross *et al.*, 1999;Bossy-Wetzel *et al.*, 1998; Yang *et al.*, 1997; Kluck *et al.*, 1997). Over-expression of the anti-apoptotic bcl-2 family members is common in tumor cells, and increases their resistance to the apoptosis signals that kill healthy cells.

We exposed tumor cells, differing in bcl-2 expression, to HAMLET and related survival to their *bcl-2* status. No variation in sensitivity was observed. *Bcl-2* transfectants, over expressing the protein, remained fully sensitive to HAMLET (Fig. 5B). We conclude that HAMLET induced apoptosis is not controlled by *bcl-2*.

Cellular trafficking of HAMLET. The subcellular localization of HAMLET has been studied in search for a mechanism that may distinguish the sensitive tumor cells from the resistant healthy cells (Fig. 6). HAMLET was conjugated to the succinimidyl-ester Alexa Fluor 568 (Molecular Probes Inc.) for detection by real-time confocal microscopy in living cells (Gustafsson *et al.* manuscript).

Two carcinomas (A549 an A498), two lymphomas (Jurkat and L1210) and three gliomas (D53, U-251 and CRL 2356) have been examined by this technique. The Alexa-HAMLET complex was shown to retain the properties of HAMLET, killing tumor cells by apoptosis, with the development of cytoplasmic vesicles and cytoplasmic blebs,

cell shrinkage and apoptotic body formation (Fig. 6). The healthy cells, in contrast, remained viable and morphologically intact.

The trafficking of HAMLET was compared between tumor cells and healthy differentiated cells. Surface binding of Alexa-HAMLET was rapid for both tumor cells and healthy cells (Fig. 6). Alexa-HAMLET then entered the cytoplasm and formed cytoplasmic aggregates in both the tumor cells and the healthy cells. Uptake was not blocked by cycloheximide showing that this step does not require protein synthesis. These observations suggested that the availability of surface receptors is not the limiting step, or the critical factor determining sensitivity, and that the translocation into the cytoplasm did not distinguish the more from the less sensitive cells.

In tumor cells, HAMLET was redistributed from the cytoplasm to the perinuclear area. This effect was only observed in living tumor cells, and was abrogated by cycloheximide, demonstrating that it is an active process, requiring cellular metabolism. Despite the entry of Alexa-HAMLET into the cytoplasm of healthy cells, no further trafficking was observed. As HAMLET did not activate the apoptotic pathway in healthy cells, the redistribution appeared to be a key to apoptotic death in the tumor cells. The translocation to the perinuclear area was accompanied by the movement of mitochondria, as shown by co-staining with the mitochondria-specific dye Mitotracker.

Finally, HAMLET was shown to accumulate in tumor cell nuclei. With time, > 75% of all cells showed nuclear staining for HAMLET (Hakansson *et al.*, 1999) and the apoptotic bodies stained positive for Alexa-HAMLET (Gustafsson *et al*. manuscript).

The mechanisms of nuclear targeting are not well understood, as α-lactalbumin does not contain known nuclear targeting sequences, and the native protein does not enter into tumor cell nuclei. The relative role of the lipid and the protein for nuclear targeting and the fate of the complex in the cells, remain to be examined.

These experiments demonstrate that the subcellular localization of HAMLET reflects differences in susceptibility. We conclude that critical molecular targets allow HAMLET to reach the perinuclear and nuclear compartments in tumor cells. The restriction of HAMLET movement in healthy cells suggested that they failed to sense the presence of HAMLET in the cytoplasm or that inhibitors of cellular trafficking were active in healthy cells. The redistribution of HAMLET to the perinuclear region and the nuclear accumulation marked the irreversible stage of tumor cell apoptosis.

Histone specific interactions of HAMLET in tumor cell nuclei. The molecular basis for the nuclear accumulation of HAMLET in tumor cells was examined. Sensitive cells were fractionated, the cytoplasmic membrane, the cytoplasm and nuclear fractions were saved. HAMLET was allowed to interact with the nuclear fraction of A549 cell homogenates in an overlay assay (Fig. 7A). The nuclear target molecules were identified as histones by MALDI-TOF or N-terminal sequencing. HAMLET showed high affinity for histone H3, intermediate affinity for H4, lower affinity for H2A and H2B and no affinity for H1 (Fig. 7B). The high affinity interactions were confirmed by several techniques, including BIAcore and affinity chromatography.

HAMLET was then tested for interactions with chromatin, and specifically with native histones in preformed nucleosomes. Nucleosomes are formed from histones and DNA (Stein, 1979), and consist of a core histone octamer wrapped with approximately 146 bp of DNA (Arents and Moudrianakis, 1993) (Fig 7C). The core histone octamer consists of one $(H3-H4)_2$ tetramer, and two H2A-H2B dimers positioned on each side of the tetramer and adjacent nucleosomes are connected by linker DNA and the linker histone H1. HAMLET was shown to interact with histones in intact nucleosomes, but showed no affinity for DNA, demonstrating that this effect must be mediated by the histones.

HAMLET was subsequently shown to disrupt nucleosome assembly, suggesting that the high affinity for HAMLET prevented the histones from binding to DNA. This was in contrast to the known nucleosome assembly protein-1 (Nap-1) which enhanced nucleosome assembly by delivery of the histone proteins (Ishimi *et al.*, 1987). We conclude that HAMLET differs from other histone binding proteins which act as chaperones during chromatin assembly and remodeling. These proteins depend on the reversibility of histone binding, as the protein must be delivered from the site of synthesis in the cytoplasm to the nucleus, and the chaperons are not to be part of the mature nucleosome complex. Instead HAMLET appears to freeze the chromatin due to the affinity for histones, and thus prevents the cell from transcription, replication and recombination (Düringer *et al.*, manuscript).

The strong affinity of HAMLET for histones offers a molecular explanation for the accumulation of HAMLET in nuclei of tumor cells. By preventing chromatin

assembly and by interfering with intact chromatin, HAMLET may cause irreversible

damage and cell death. As this process should be independent of the classical apoptotic

machinery of the cells, it may explain why HAMLET can trigger apoptosis in so many

different tumor cell types. By disrupting the fundamental cellular machinery, needed for

protein synthesis and chromatin assembly, HAMLET may ultimately upset the

replication of the genome.

HAMLET activates mitochondria and the caspase cascade. Early co-localization studies

with mitochondria specific markers showed that HAMLET interacts with mitochondria

in the cytoplasm of tumor cells, but not healthy cells (Fig. 8). The affinity was confirmed

using isolated mitochondria, where HAMLET triggered the depolarization of the

membrane potential, and release of cytochrome C (Kohler *et al.*, 1999; Kohler *et al.*, 2001).

HAMLET was shown to activate pro-apoptotic caspases including caspase 3 and

caspase 6, as shown by cleavage of specific substrates (Fig. 8), but HAMLET induced cell

death did not rely entirely on caspases, as the caspase inhibitor ZVAD did not prevent

apoptosis, and cell lines lacking caspase 3 did not show an increased resistance to

HAMLET-induced apoptosis (Table 1). Consistent with these results, ZVAD did not

stop HAMLET from moving through the cytoplasm to the nuclei (Gustafsson *et al*.

manuscript).

We conclude that caspases are activated in cells that die following exposure

to HAMLET, but that caspase inhibitors do not rescue cells from death. The role of

caspases as executors of HAMLET induced apoptosis remains undefined.

HAMLET binds ribosomal proteins. In a search for cell surface receptors, A549 cells which undergo apoptosis in response to HAMLET were fractionated. The membrane-containing fraction was subjected to SDS-PAGE and Coomassie stained. A parallel gel was blotted to a PVDF membrane, overlaid with radiolabelled HAMLET, and binding was quantified by PhosphoImager. At least eight distinct bands were detected with molecular weights ranging from 17 to 42 kDa.

The proteins interacting with HAMLET were identified by MALDI-TOF. To our surprise, all showed homology with ribosomal proteins, and were tentatively identified as ribosomal proteins. To verify this specificity, ribosomes were isolated (modified from Spedding, 1990), disrupted and the protein fraction was overlaid with radiolabelled HAMLET. HAMLET bound to proteins L4, L6, L8, L15, L13a, L30, L35a, S12 and L21, as shown by N-terminal sequencing and MALDI-TOF.

The human ribosome consists of two subunits, bound to the mRNA strand that is being translated. To examine if HAMLET could interact with intact ribosomes, mixtures of ribosomes were incubated with HAMLET, subjected to gel filtration on an S-300 column, and eluted with PBS. Selected fractions were analyzed for RNA and protein content. While the isolated ribosomes or HAMLET controls eluted as sharp peaks at different times, the mixture contained a new complex which eluted between the ribosome and the HAMLET peaks (Fig. 9).

We conclude that HAMLET binds to several ribosomal proteins, and that HAMLET can target intact ribosomes. HAMLET may thus upset the ribosome

organization, and potentially block translation. In addition, we speculate that ribosomal proteins may be involved in the nuclear targeting of HAMLET. The ribosomal proteins travel from the site of synthesis in the cytoplasm to the nuclei, and ribosomal subunits are assembled in the nucleolus and then exported back to the cytoplasm. Experiments addressing this hypothesis are ongoing.

Summary, cellular interactions. HAMLET differs from many other apoptosis-inducing agonists in that it shows broad selectivity for tumor cells, with no effect on healthy differentiated cells. HAMLET bypasses many known apoptosis-related signaling pathways, and induces apoptosis regardless of the *p53* or *bcl-2* status of the cells. HAMLET enters tumor cells, translocates to the perinuclear region and targets cell nuclei, where it interacts directly with the chromatin due to its specificity for histones. In addition, HAMLET targets the ribosomes that may disrupt translation. This unusual molecule thus disrupts critical organelles involved in the transcription of the genome and in the translation of RNA to protein. The result is a rapid halt to cellular activity, and activation of apoptosis.

IV. HAMLET – STRUCTURAL ASPECTS

Properties of α-lactalbumin. α-Lactalbumin is a member of the lysozyme protein family, but the proteins perform vastly different functions. Lysozyme hydrolyses bonds between specific muramic acid residues in the bacterial cell wall and kills the bacterium by lysis (Fleming, 1922), but α-lactalbumin functions as a substrate specifier for

galactosyl transferase aiding in the production of lactose (Brodbeck and Ebner, 1966; Ebner *et al.*, 1966). The two proteins are believed to have arisen by gene duplication from an ancestral gene and to have undergone divergent evolution. One continued to perform the original lysozyme function while the other underwent an independent series of mutations generating α-lactalbumin. The gene duplication is estimated to have occurred about 400 million years ago, and the divergence of α-lactalbumin from lysozyme preceded the divergence of fishes from tetrapods (Dautigny *et al.*, 1991), long before its function was utilized (Shaw *et al.*, 1993). α-Lactalbumin was discovered in 1939 (Sorensen and Sorensen, 1939), and in 1966 the lactose synthase complex was characterized (Brodbeck and Ebner, 1966).

The *ala* gene is 2.3 kbp, with four exons and three introns and was regionally assigned to chromosome 12q13 (Davies *et al.*, 1987). About 140bp upstream *ala* is the so called "milk box", for hormone-regulated expression of milk proteins, and α-lactalbumin is expressed exclusively in the secretory cells of the lactating mammary gland (Pike *et al.*, 1996).

α-Lactalbumin is a globular 14.2 kDa protein (Fig. 10) with four α-helices and a triple-stranded anti-parallel β-sheet (Permyakov and Berliner, 2000; Paci *et al.*, 2001). The molecule is stabilized by four disulphide bonds (Acharya *et al.*, 1991), and by the high affinity calcium binding site. The sequence is highly preserved among different species, and especially the calcium-binding residues suggesting the importance of this site for the "integrity" of the molecule. Calcium is required to form the native conformation (Rao and Brew, 1989; Ewbank and Creighton, 1993), and the bound

calcium ion is co-ordinated by the side chain carboxylates of Asp82, Asp87 and Asp88, the carbonyl oxygens of Lys79 and Asp84, a forming a distorted pentagonal bipyramidal structure with two water molecules (Acharya *et al.*, 1991). The K_{Dapp} for calcium binding is on the order of $10^{-7}M$ (Kronman *et al.*, 1981; Permyakov *et al.*, 1981).

Partially unfolded states. α-Lactalbumin has been studied as a model of protein folding, as it forms relatively stable folding intermediates. Molten globules constitute a particular species of stable intermediates (conformations in kinetic traps) (Dolgikh *et al.*, 1981; Ohgushi and Wada, 1983) and the structural characteristics include native like secondary structure but fluctuating tertiary structure. The acid denatured state (A-state) caused by low pH defines the partially unfolded "molten globule" (Finkelstein and Ptitsyn, 1977). The calcium-free form, often referred to as the apo form, represents another partially unfolded state and heat also causes a molten globule like conformation. These states share a native like secondary structure but lack specific tertiary packing of the side chains, cooperative thermal unfolding transition and compactness, and the radius of gyration is 5-10% greater than that of the native state. The apo form of the protein is very sensitive to the ionic strength. It is partially denatured at low ionic strength and resembles the low pH molten globule. However, at physiological salt concentrations, the apo state has a more well-defined structure, although clearly different from the native state and of lower stability (Kuwajima, 1989; Dolgikh et al., 1981; Dolgikh et al., 1985; Ikeguchi et al., 1986; Ewbank et al., 1995;

Alexandrescu et al., 1993; Peng et al., 1995; Wilson et al., 1996; Wu et al., 1995; Schulman et al., 1997; Schulman et al., 1997; Kuwajima, 1996).

The crystal structure of native, human α-lactalbumin has been known since 1991 (Acharya *et al.*, 1991), but less is known about the tertiary structure of the molten globules. Studies have suggested that the α-domain has a significant amount of residual structure and forms the core of the α-lactalbumin molten globule while the β-domain remains largely unstructured (Wu *et al.*, 1995; Paci *et al.*, 2001). This was confirmed recently, as the crystal structure of bovine apo α-lactalbumin was solved (Chrysina *et al.*, 2000b). In addition, the NMR spectra of the native and apo-conformers (Wijesinha-Bettoni *et al.*, 2001), revealed a significant structural change at the interlobe interface. The slight expansion of the calcium binding loop tilts the 3_{10} helix towards the C helix, resulting in the disruption of the aromatic cluster Trp 26, 60, 104, Phe 53 and Tyr 103. Most striking is the perturbation of Tyr 103 resulting in an opening of the cleft and the loss of the channel water molecule associated with the calcium-binding site.

HAMLET, a folding variant of α-lactalbumin causing tumor cell apoptosis. The discovery of HAMLET demonstrated that α-lactalbumin acquires novel biological activities after conformational switching. This discovery was made guided by the search for the biological activity, and showed that the apoptosis-inducing complex contained α-lactalbumin in a novel molecular form (Svensson *et al.*, 2000).

The activity was detected in casein, which is a fraction of human milk obtained at low pH (Hakansson *et al.*, 1995). The active complex was retained on the ion

exchange column, and eluted only after high salt. By N-terminal sequence, the fraction was shown to contain α-lactalbumin, but the native protein was inactive in the apoptosis assay.

To analyze structural basis for the novel biological activity, the active complex was compared to native α-lactalbumin. Chromatographic separation on a size-exclusion column revealed that the active complex had a tendency to form dimers, trimers and higher order oligomers. The multimers were stable enough to resist dissociation on SDS-Page gels. The variant purified from casein was therefore named MAL for multimerized α-lactalbumin (Hakansson *et al.*, 1995). The multimers are probably not important for the activity, however, as HAMLET is in a mostly monomeric state.

By mass spectrometry, no post-translations modifications were detected, suggesting that the new activity might be caused by a change in 3-D structure (Svensson *et al.*, 1999). Circular dichroism (CD) spectroscopy revealed that the variant had essentially retained secondary structure (strong signals in the far-UV, 250-185 nm range) but that the aromatic side-chains are more free to rotate (reduced signal in the near-UV, 320-250 nm range), compared to native α-lactalbumin. Fluorescence spectroscopy showed that the tryptophan side-chains are more accessible to solvent water in the variant (longer wavelength of the Trp emission maximum), which also has more accessible hydrophobic surfaces (seen as enhanced and blue-shifted ANS fluorescence) (Svensson *et al.*, 1999). We concluded that the apoptosis-inducing variant retained the

secondary structure of α-lactalbumin but had a more loosely organized tertiary structure than the native protein.

The similarity of the CD and fluorescence spectra of the variant to the molten globule state of α-lactalbumin was striking and raised the question if the molten globule state has novel biological properties. The novel form differed from the molten globules in that it was stable at neutral pH, at 25-37°C, in an oxidizing environment, and in the presence of calcium. This is in striking contrast to the low pH molten-globule state, which reverts to the native state if the pH is brought back to physiological values. Temperature denaturation of α-lactalbumin is also reversible and the native form is regained at ambient temperature. The reduced form can be oxidized back to the native state, and the apo state rapidly reverts to the native state if calcium is added (Kuwajima, 1996). We therefore assumed that the active fraction must contain a cofactor which stabilizes the altered conformation.

α-lactalbumin can be converted to an apoptosis-inducing complex only in the presence of a lipid cofactor. The cofactor was identified as a fatty acid. This was achieved by extraction of column matrices that had been used to purify the active complex from human milk casein. Lipids retained on the column were eluted with solvents, and eluted lipids were identified by GC-MS. Individual lipids were then used to condition clean column matrices to which α-lactalbumin was added. Conversion to the apoptosis inducing form was only achieved when the protein in its calcium-free state was applied to a column, that had been conditioned with a fatty acid cofactor identified as oleic acid

(C18: 1, cis). The folding change relative to native a-lactalbumin and the resulting loss of defined tertiary structure was confirmed by near UV CD spectroscopy and by increased ANS binding. The integration of the lipid and the protein into a novel molecular complex was proven by functional studies combined with NMR spectroscopy (Fig. 10). HAMLET was defined as the product of apo-α-lactalbumin and oleic acid.

The conversion experiments are important as they prove that the active complex is formed from pure components (α-lactalbumin and oleic acid), each of which is inactive in the apoptosis assays. They prove that the folding change is necessary for the protein to attain this new function and that lipid cofactors enable proteins to adopt stable novel conformations, and thus act as partners in protein folding.

The molten globule state of α-lactalbumin has been proposed to represent a folding intermediate on the pathway from denatured to native protein and that this is critical for the correct folding of the protein. We argue that the molten-globule state instead reflects the ability of α-lactalbumin to switch conformation and function.

Specificity of the lipid cofactor. The specificity of the lipid cofactor was investigated, using fatty acids differing in carbon chain length, saturation or cis/trans conformation. C18:1 fatty acids with a double bond in the cis conformation at position 9 or 11 were identified the optimal cofactors. Saturated C18 fatty acid or unsaturated fatty acids in the trans conformation were completely inactive. So were fatty acids with shorter carbon chains. We concluded that highly specific inter-molecular interactions are required for lipids to act as folding partners in this system.

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Tentative fatty acid binding sites were identified based on the three-dimensional structures of native and apo α-lactalbumin (Fig. 10). The native α-lactalbumin molecule is a hydrophilic, acidic protein, with two hydrophobic regions. One is located in the interface between the α-helical and the β-sheet domains, and the second is formed by residues internal to the α- domain (Fig. 10) (Saito, 1999; Wu and Kim, 1998). The crystal and NMR structures of bovine apo α-lactalbumin have revealed a significant structural change in the cleft between the two domains (Bettoni-Wijesinha *et al.*, 2001; Chrysina *et al.*, 2000a) when the protein adopts the apo-conformation (Chrysina *et al.*, 2000a). The α-domain, in contrast, remains structured in both the native and the apo-conformations, with near native side chain packing. We therefore hypothesize that the C18:1 fatty acid binds in the interface between the α and β domains, and thus stabilizes a molten-globule like conformation.

Tentative fatty acid binding site in HAMLET. Based on the information on other fatty acid binding proteins (Curry *et al.*, 1998; Cistola, 1998) and the structure of oleic acid and α-lactalbumin, we have located a tentative oleic acid binding site in the cavity between the α- and β-domain (Fig. 11). Hydrophobic amino acids in the pit of the cavity may bind the fatty acid tail, and arginine at position 70 (Arg 70), lysine at position 94 (Lys 94) and lysine at position 99 (Lys 99) may be the basic amino acids coordinating the head group of the fatty acid.

These residues were selected for mutagenesis as they are the basic amino acids tentatively coordinating the head group of the fatty acid. By substituting these

basic for acidic amino acids, we expect to loose or reduce fatty acid binding. The *ala* sequences were successfully mutated using the overlapping extension PCR strategy, and confirmed by DNA sequencing. Three mutated variants were produced where the basic amino acids, Arg 70 and Lys 94 and Lys 99, were substituted for acidic, aspartate at position 70 and glutamate at position 94 and 99. Substituting the same amino acids at position 70 and 99 or only at position 99 produced a double and single mutated variant.

The mutant proteins have been expressed in *E. coli* BL21 according to (Wu and Kim, 1998). We have previously shown that recombinant a-lactalbumin expressed in this manner is fully functional, and can be converted to HAMLET, with similar activity as protein derived from human milk whey. While we do not predict that the point mutation in the tentative fatty acid binding pocket will alter the 3-D structure of the native protein, this remains to be examined. When appropriately folded mutant proteins have been obtained, we will examine their structure and function in fatty acid binding and apoptosis induction.

The apo-conformation of a-lactalbumin does not induce apoptosis in the absence of the lipid cofactor. To clarify the role of the lipid cofactor, we investigated if α-lactalbumin alone can induce apoptosis following a change to the apo-conformation. Mutations in the Ca^{2+} binding site of bovine a-lactalbumin were used to obtain proteins that maintain the apo-conformation also at physiologic conditions. A point mutation at position D87A inactivated the Ca^{2+} binding site and caused a change in tertiary structure, locking the protein in the apo-conformation. This mutant was tested for activity in the apoptosis

assay. The mutant proteins did not induce apoptosis, but were efficiently converted to HAMLET, demonstrating that a conformational change in α-lactalbumin is not sufficient to trigger apoptosis. The mutant bovine proteins could be converted to a HAMLET like complex in the presence of oleic acid, however, demonstrating that the biological properties of HAMLET are defined both by the protein and the lipid cofactor. Interestingly, the activity of the converted mutant protein suggested that a functional calcium binding site is not required for the apoptotic function of this protein.

V. GENERAL DISCUSSION

The human genome sequence revealed fewer genes than expected, and fewer gene products than are needed for the functional diversity of the living organism. As a consequence, the prevailing dogma "one gene, one protein, one function" is rapidly changing. It is becoming obvious that single polypeptide chains must be able to vary their structure and function in order to provide the basis for biologic diversity. Changes in tertiary conformation are being recognized as a mechanism to achieve functional variation. In the case of a-lactalbumin, we have shown that α-lactalbumin in a molten globule like conformation, binds a fatty acid and changes into HAMLET that induces apoptosis in tumor cells. The native, folded protein acts as a substrate specifier in lactose synthesis, and lactose is needed for the nutrition of the baby and to maintain the fluidity of milk. Thus, the protein acquires very different functions depending on the environment, the three dimensional structure and the availability of cofactors.

HAMLET has unique biological properties, as it selectively purges malignant and immature cells by apoptosis, and apparently without harming normal tissues (Hakansson *et al.*, 1995).This may appear paradoxical, as protein folding variants have been discussed in the context of "misfolding" and have been recognized as causes of disease. The native prion protein alters its fold to a β-sheet rich conformation, forming the "prp-scrapie" disease isoform. Similarly, the families of proteins that form amyloid fibrils (lysozyme, amyloid protein b1, β2-microglobulin etc.) undergo a conformational change from a mixed α-helical to a β sheet rich conformation. In both cases, the conformational change causes the accumulation of β-sheet rich fibrils in the tissues, with tissue destruction through mechanisms that are only partly understood (Bucciantini *et al.*, 2002). The resulting diseases include Alzheimer's disease, Parkinson's disease, variant Creuzfeldt-Jacob disease, etc.

The propensity to form β-sheet rich fibrils has been proposed to be a generic property of all polypeptide chains (Dobson, 2001), but these are equilibrium states, that undergo reversions to the native fold. The present study introduces lipids as stabilizing cofactors in protein folding processes. The prions and amyloid fibrils are examples of unsuccessful protein processing, causing protein accumulation in peripheral tissues where damage is done (Dobson, 2001; McLaurin *et al.*, 2000; Pepys, 2001). It has been postulated that a cofactor, "factor x" or "protein x" is required for the transmission of human prions to transgenic mice, to form the nascent scrapie isoforms during prion propagation (Billeter *et al.*, 1997; Telling *et al.*, 1994; Telling *et al.*, 1995). By mutational analysis, the interaction with "protein x" was shown to depend on a discontinuous

epitope formed by the C terminal a helix, with residues 167 and 171 in an adjacent loop (Kaneko *et al.*, 1997), but the molecular nature of "factor x " has remained elusive. There is evidence that amyloid fibers contain a mixture of lipid species, but their role in the formation of fibrils remains to be defined (Kim *et al.*, 1967; McLaurin *et al.*, 2000). The present study suggests that lipids should be explored as cofactors that lock the prions and amyloid proteins in their β-sheet rich conformations.

The identification of the fatty acid cofactor raises the possibility of a "two component model," postulating that two requirements must be met, in order for a folding variant to attain a new, stable molecular state. First, the protein must meet an environment where the altered fold is preferred, and where the molecule unfolds. Second, the altered fold must be stabilized by a molecular species that prevents it from reverting to the native fold. Both requirements must be met in order for the folding variant to exert its effect in the local tissue. According to this hypothesis, the folding variants *per se,* are not biologically active, and thus not dangerous or beneficial as long as they can revert to the native conformation. The two components may serve to regulate the tissue specificity of the folding variants, and to protect the host from unwanted activity in tissues where the folding variant may arise, but where the cofactor may be missing. This is especially important, since once formed, the amyloid fibrils act as nuclei for the continued formation of the folding intermediates.

In this way, the availability of cofactors would influence if altered folds will be stable in different environments. In case of α-lactalbumin, the low pH of the stomach provides an environment fulfilling both requirements, allowing the molecule to be

formed in the nursing child. The low pH in the stomach, is known to cause partial unfolding of α-lactalbumin, and lipids are hydrolyzed by acid lipases to release oleic acid (Bernback *et al.*, 1990; Sarles *et al.*, 1992). It is interesting to speculate that the beneficial function of HAMLET has been a factor in the evolution of milk, underlying the special abundance of both α-lactalbumin (2mg/ml) (Heine *et al.*, 1991) and oleic acid (>50% of the fatty acid chains of triglycerides) (Jensen, 1996).

So what is the physiologic role of HAMLET in the breast fed child, and the long-term effect on disease in children and adults? Human milk is a unique source of infant nutrition and contains a rich variety of host defense molecules operating against different infectious agents. Our findings have added tumor cell apoptosis to this spectrum of protective activities exerted by molecules in milk. Breast-fed children show a lower incidence of childhood cancers, especially lymphomas (crude odds ratio of 8.19) and other tumors are about 2-fold less prevalent, than in bottle fed children, (Davis *et al.*, 1988; Mathur *et al.*, 1993) suggesting that ingestion of milk has a prophylactic effect. HAMLET offers a molecular mechanism that may contribute to this protective effect and reduce the rate of childhood cancers.

HAMLET is not present in newly secreted human milk, but the conditions required to form HAMLET are present in the stomach of the breast fed child. The low pH of gastric juice promotes the unfolding of the protein (Smith *et al.*, 1999), and triggers pH sensitive lipases to release the C18:1 fatty acid from milk phospho-lipids (Blackberg *et al.*, 1995). Even though we do not have formal proof that HAMLET is formed in the intestine, it is very likely to occur. Mixing experiments *in vitro,* have shown that

HAMLET may be formed in solution just from apo-a-lactalbumin and oleic acid, if the pH is adequate, demonstrating that these two molecules are capable of forming the active complex. We propose that HAMLET is formed *in vivo,* and that it acts as a natural tumor cell scavenger in infancy, with the mission of purging atypical or highly immature cells.

So, how could HAMLET influence tumor development many years after the weaning of the baby? Cell proliferation is extremely rapid during the first months of life, and high division rates increase the risk of mutation and malignant transformation in this rapidly growing cell population. Cells with a premalignant genotype may then act as founders for future tumor development. By purging those cells as they arise in the nursing child, HAMLET may exert a long-term protective effect. The milk targets the gastrointestinal tract which contains some of the most rapidly growing cell types, including the emterocytes and lymphocytes. The gut associated lymphoid tissue expands after birth in response to antigen and microflora, and the Peyer's patches are established. It is tempting to speculate that HAMLET reaches sites of proliferation, and assists in the purging of premalignant precursors which might explain the reduced childhood leukemia frequency that accompanies breast-feeding. High concentrations of HAMLET in the intestinal lumen may drive the lymphocyte population towards maturity, and away from malignancy. This effect remains to be proven *in vivo.*

This study provides evidence that a protein-folding variant may be used to prevent or treat malignant disease. Two extremely different models were used to test the therapeutic potential of HAMLET, and the results in both models were quite

promising. The glioblastoma model used a xenotransplant approach, to study of invasively growing, highly aggressive human brain tumors. A 24 hour infusion of HAMLET was sufficient to alter the tumor growth over an 8 week period, to reduce the tumor volume and to prolong the survival of the rats. In this model, we could obtain biopsies, and show that *in vivo* apoptosis development was tumor specific. This is remarkable, as HAMLET appears to selectively purge malignant cells, while leaving healthy cells, unharmed. This is an ideal outcome, as novel anti-tumor treatments should aim to selectively kill the tumor cells, and to avoid tissue toxicity by inducing apoptosis. We thus consider the results of the glioblastoma model promising enough to explore the potential of HAMLET treatment in patients with malignant gliomas.

The results of the skin papilloma studies in human are equally encouraging. A reduction in papilloma volume was obtained in all of the patients receiving active substance, but in only three of the patients receiving placebo. While skin papillomas usually are benign conditions, other papillomas involve a much greater risk for cancer development. The ease of local HAMLET application and the rapid effect suggest that a quite simple approach might be taken in such patient groups.

HAMLET offers a unique opportunity to learn from biology. By putting the ear to the ground and listening carefully to separate the signals from the noise, it may be possible to learn about the unique nature of cancer cells and about natural surveillance mechanisms evolved to protect the growing individual. It is a great challenge to listen to the HAMLET language of molecular adaptation, to explore why this exquisite mechanism has evolved and to identify molecular targets which explain the sensitivity

of tumor cells to HAMLET induced apoptosis. HAMLET may be just one of several molecules with a similar preventive function.

Acknowledgements

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Table 1. Sensitivity of tumor cells to HAMLET induced apoptosis.

Cell lines	MAL, LD$_{50}$[a]	HAMLET, LD$_{50}$[b]	*p53* status
Lymphoid cell lines	**0.45 (0.3-0.5)**	**0.013 (0.01-0.02)**	
Jurkat, human lymphocytic leukaemia (T)	0.4	0.01	mutant
L1210, mouse prolymphocytic leukaemia	0.3	0.01	mutant
B9, mouse lymphocytic leukaemia	0.4		c
HL-60, human promyeolcytic leukaemia	0.5		non-expressing
K562, human myeolgeneous leukaemia	0.5	0.02	non-expressing
U-937, human promyelocytic leukaemia	0.5		non-expressing
BT4C, mouse glioma	0.5		c
Thymocytes, rat	0.5		Wild-type
FL5.12, mouse lymphocytic leukaemia		0.01	
Carcinomas	**1.0 (0.75-1.75)**	**0.037 (0.02-0.05)**	
A549, human lung carcinoma (type II)	1.25	0.02	Wild-type
NCI, human bronchial carcinoma	1		Mutant
A-498, human kidney	1		Wid-type
GMK, monkey kidney	1		c
Vero, monkey kidney	1.75		c
MDCK, dog kidney	0.75		c
J 82, human bladder t carcinoma	0.75		Null
Caco-2, human intestine carcinoma	1		mutant
HT-29, human intestine carcinoma	1.25		mutant
MCF-7, human breast adenocarcinoma*	1.25		Wild-type,
SK-BR-3, human breast adenocarcinoma	1.25		mutant
MBD-231, human breast adenocarcinoma	1.25		c
T47d, human breast ductal carcinoma,	1.25		mutant
MBD-175VII, human breast carcinoma	1.5		c
PC-3, human prostate carcinoma	1		null
DU-145, human prostate carcinoma	1.25		non-expressing
WEHI, mouse fibrosarcoma	0.75		c
U37, human glioma	2		c
HCT116, human colon carcinoma		0.04	Wild-type
H1299, human lung carcinoma		0.05	Null
Gliomas		**0.037 (0.03-0.05)**	
U251		0.03	mutant
CRL 2356		0.03	c
D54		0.05	Wild-type
Embryonal cells			
Hel, human endothelial lung	>2.5		wt
HFF, human foreskin fibroblast	no apoptosis		wt
Healthy differentiated cells	no apoptosis		

Urinary tract epithelium, human	no apoptosis	wt
Nasopharyngeal epithelium, human	no apoptosis	wt
HRTEC, renal tubular epithelium, human	no apoptosis	wt
Granulocytes, human peripheral	no apoptosis	wt
Lymphocytes, human peripheral	no apoptosis	wt
Kidney, mouse	no apoptosis	wt
Bladder, mouse	no apoptosis	wt

[a] The concentration (mg/ml) required to kill 50% of the cells. Numbers in bold denote median values (range).

[b] The concentration (mM) required to kill 50% of the cells. . Numbers in bold denote median values (range).

[c] No data on *p53* status

* MCF-7 is caspase 3 depleted

FIGURE LEGENDS

Figure 1. HAMLET consists of a-lactalbumin and the C18:1, 9 cis fatty acid.

HAMLET is human α-lactalbumin made lethal to tumor cells. HAMLET is formed from native α-lactalbumin, purified from human milk (Lindahl and Vogel, 1984). The native protein can be converted to the apo-conformation by treatment with EDTA which removes Ca^{2+}, and by the addition of the fatty acid C18:1, 9 cis (Svensson *et al.*, 2000). The figure is based on the α-lactalbumin crystal structure (Acharya *et al.*, 1991).

Figure 2. Tumor cell apoptosis induced by a human milk complex.

A, B and C. A human milk fraction was shown to block the adherence of *S. pneumoniae* to the A549 lung cancer cell line (A), and to induce apoptosis (B and C). The light micrograph is taken at 40x magnification. Morphological changes include the loss of cytoplasm, nuclear condensation and apoptotic body formation. The confocal micrograph was obtained in a Bio-Rad MRC-1024 attached to a Nikon Eclipse E800 microscope. DNA is stained red by propidium iodide.

D. Apoptotic DNA fragments separated by agarose electrophoresis

Figure 3. HAMLET treatment reduces glioblastoma progression and prolongs the survival of xeno-transplanted rats.

A. Human glioblastoma spheroids were shown to undergo apoptosis in response to HAMLET. *In vitro* incubation of human glioblastoma biopsy spheroids with HAMLET

resulted in apoptosis, as shown by abundant TUNEL staining (green). α-Lactalbumin had no effect on cell viability (p<0.01).

B. Xenotransplant model used to study human glioblastomas. The biopsy spheroids were injected into the brains of immuno-deficient nu/nu rats, and allowed to establish for 7 days. HAMLET or α-lactalbumin (0.7 mM) was then infused by convection enhanced delivery (CED). Tumor volume was determined by MRI after 2 months.

C and D. The tumor volume was markedly reduced in HAMLET treated compared to α-lactalbumin treated rats as shown by MRI scans two months after infusion (p<0.01). HAMLET treated rats developed pressure symptoms later than the α-lactalbumin treated rats (p<0.01).

Figure 4. HAMLET treatment of human skin papillomas.

A. HAMLET was applied topically on human skin papillomas once a day for three weeks and the lesions were measured and photographed once a week during the treatment period and at follow up visits one and two months after completed treatment (→ indicates these time points). A double blind, placebo-controlled protocol was used.

B. HAMLET treatment removed or reduced skin papillomas.

C. HAMLET treatment reduced papilloma volume by ≥75% in 20/20 patients receiving HAMLET and 3/20 patients receiving placebo (p<0.001).

Figure 5. HAMLET induces apoptosis independent of bcl-2 and p53 genotype.

A. The human colon carcinoma HCT116 carries wild-type *p53* (+/+). The clone HCT116 (-/-) carries a *p53* deletion (Bykov *et al.*, 2002). HCT116 (+/+) and HCT116 (-/-) were exposed to HAMLET for 24 hours and viability was determined by Trypan blue exclusion (1 mg/ml corresponds to $7x10^{-5}$ M). Both cells died by apoptosis, at similar HAMLET concentrations.

B. The role of Bcl-2 on HAMLET-induced apoptosis was investigated using K562 cell clones over expressing Bcl-2. The myelogenous leukemia cell line K562 was stably transfected with the *bcl-2* sequence in the pc-DNA vector. The resulting clones S2 and S8 had increased BCL-2 expression compared to wild-type or vector control cells. The clones were exposed to different concentrations of HAMLET for 6 hours and the viability was assessed by trypan blue exclusion (1 mg/ml corresponds to $7x10^{-5}$ M). There was no difference in the kinetics of cell death or in other parameters of apoptosis, suggesting that Bcl-2, had no effect on HAMLET-induced apoptosis.

Figure 6. Subcellular localization of HAMLET in living A549 cells.

The morphological changes in tumor cells are shown by light transmission microscopy (left panels, a-d). The cellular outline changed from a smooth to an irregular, cytoplasmic vesicles and membrane blebs developed (b, c, and d) and the cell volume decreased (d). The cellular trafficking of Alexa-HAMLET was examined by real-time confocal microscopy (middle panels, red dye, e-h). Alexa-HAMLET was shown to bind to the cell surface (e), to enter the cytoplasm (f), to translocate to the perinuclear region (g) and to accumulate in nuclei (h). A schematic of this sequence is shown in the right

panel. Cells were incubated with 0.007 mM Alexa-HAMLET and analyzed by confocal microscopy.

Figure 7. HAMLET binds histones and upsets chromatin structure.

A. HAMLET was shown to recognize histones in nuclear extracts. A nuclear extract from A549 carcinoma cell was separated by SDS-PAGE, western blotted to a membrane and overlaid with ^{125}I-HAMLET. HAMLET recognized two protein bands in the nuclear extract and they were identified as histones by N-terminal sequencing and MALDI-TOF.

B. HAMLET binds native histones. Binding of HAMLET to purified bovine histones was analyzed by the overlay assay. HAMLET bound with high affinity to H3, with intermediate affinity to H4 and with lower affinity to H2A and H2B. HAMLET did not bind histone H1.

C. The crystal structure of the nucleosome. Generated using MolMol (Koradi *et al.*, 1996) and the coordinates from file 1AOI.pdb (Luger *et al.*, 1997). H3 in green, H4 in red, H2A in blue, H2B in yellow and DNA in grey and black.

Figure 8. Interaction of HAMLET with mitochondria.

A. HAMLET co-localizes with mitochondria. Jurkat cells were exposed to biotinylated HAMLET, fixed and incubated with FITC-streptavidin after saponin permeabilization (left panel). The cells were co-stained with Mitotracker red, specific for mitochondria (right panel). The center panel shows the merged images, demonstrating colocalization of HAMLET with mitochondria.

B. HAMLET induces cytochrome C release. Protein extracts from HAMLET treated Jurkat cells were subjected to western blot with cytochrome c-specific antibodies.

C. HAMLET activates caspase-3-like enzymes in Jurkat cells, as shown by cleavage of the fluorogenic substrate Ac-DEVD-amc.

Figure 9. HAMLET interacts with ribosomes.

A. Ribosomes or HAMLET, respectively, were incubated in PBS at 37°C for 60 minutes and subjected to gel filtration. The isolated ribosomes or HAMLET eluted as sharp peaks at different times. HAMLET peak time adjusted for differences between runs.

B. Ribosomes and HAMLET were mixed in PBS, incubated at 37°C for 60 minutes, and subjected to gel filtration. The mixture contained a new complex which eluted between the ribosome and the HAMLET peaks.

Figure 10. C18: 1 is the fatty acid needed to convert apo α-lactalbumin to the apoptosis-inducing form.

A. Whey derived or recombinant α-lactalbumin were subjected to ion exchange chromatography using a matrix preconditioned with C18: 1 fatty acid. Whey derived α-lactalbumin was added to the column in its native (solid blue line) or in the apo state (solid red line). The apo α-lactalbumin bound to the C18: 1 conditioned matrix and eluted as a sharp peak after 1 M NaCl. Native α-lactalbumin bound poorly to the matrix, with >50% in the void. Recombinant apo α-lactalbumin bound to the C18: 1 conditioned

matrix (solid green line) and eluted after 1 M NaCl. The NaCl gradient is shown by the dotted black line.

B. Near UV CD spectra of proteins eluting from the C18: 1 conditioned matrix. The spectrum of HAMLET (solid red line) and recombinant HAMLET (solid green line) strongly resembled the apo α-lactalbumin control (dashed black line). Native α-lactalbumin before (solid black line) and after (solid blue line) passage over the column had native properties.

C. ANS fluorescence spectra of material eluted from the C18: 1 conditioned column. HAMLET (solid red line) and recombinant HAMLET (solid green line) resembled the apo α-lactalbumin control (dashed black line). The native α-lactalbumin eluate off the C18: 1 conditioned column (solid blue line) and the native α-lactalbumin control (solid black line) showed low ANS binding.

D. DNA fragmentation and loss of cell viability in L1210 cells. Lane A = cell culture medium, lane B = whey derived native α-lactalbumin (0.07 mM), lane C = recombinant, native α-lactalbumin (0.07 mM), lane D = HAMLET (0.014 mM), lane E = recombinant HAMLET (0.014 mM), lane F = native α-lactalbumin eluate off a C18: 1 conditioned column (0.35 mM), lane G = lipids extracted from casein conditioned matrix (0.0035 mM) and lane H = 18:1 fatty acid (0.0018 mM). Material in lanes D and E induced apoptosis.

E. Subcellular distribution of HAMLET in L1210 cells. Cell surface binding of HAMLET and recombinant HAMLET was detected after 30 min, followed by translocation into the cytoplasm and accumulation in the cell nuclei (upper panel). Native α-lactalbumin

bound weakly to the cell surface and did not enter the cells (upper panel). The cellular outline is shown in blue reflection mode (lower panels).

Figure 11. Tentative fatty acid binding site in α-lactalbumin.

The α-domain of α-lactalbumin is dominated by α-helical secondary structure while the β-domain mainly consists of parallel β-sheets. The strongly bound Ca^{2+} ion is shown in red. The alpha-domain of α-lactalbumin contains four major a-helices (A:5-11, B:23-24, C:86-98 and D: 105-109) and three short 3_{10} helices (12-16, 101-104 and 115-119). The smaller beta-domain consists of a series of loops, a triple-stranded anti-parallel b-sheet (41-44, 47-50 and 55-56) and a 3_{10} helix (77-80) (Kuwajima, 1996), (Permyakov and Berliner, 2000), (Paci *et al.*, 2001). Four disulphide bonds, shown in black, connect the two domains (73-91 and 61-77) and stabilize the α-helical region (6-120 and 28-111) (Acharya *et al.*, 1991). The cleft between the α- and β-domain is a possible area for a fatty acid binding site. Basic amino acids and hydrophobic amino acids, suggested to coordinate the fatty acid, are marked blue and orange, respectively. The basic residues Lys 94, 99 and Arg 70 cap the cavity and might serve to coordinate the changed head group of the fatty acid.

Figure 12. Is HAMLET formed *in vivo*?

α-Lactalbumin and the C18: 1, cis fatty acid are major constituents of human milk (Heine *et al.*, 1996). In the mammary gland, α-lactalbumin acts as a coenzyme in lactose synthesis (Brew *et al.*, 1968), (Musci and Berliner, 1985). When milk reaches the stomach

of the nursing child the low pH precipitates casein and favors the conversion of native α-lactalbumin to the apo-conformation. In addition triglycerides are hydrolyzed by gastric lipase at low pH to release diglycerides and fatty acids (Hamosh *et al.*, 1978). Thus the conditions are optimal to form HAMLET.

Epidemiological studies suggest that breast-feeding protects against cancer. The overall incidence of childhood cancer is lower in breast-fed than in bottle-fed children, with an especially strong epidemiological association for lymphomas (crude odds ratio 8.19) (Davis *et al.*, 1988).
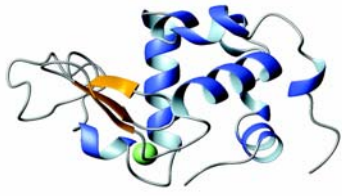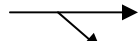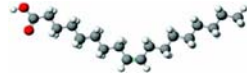
α-lactalbumin   Ca²⁺   +   C18:1,9 cis fatty acid   →   HAMLET

	light microscopy	electron microscopy	confocal microscopy	
Control				
Milk fraction				
	(A)	(B)	(C)	(D)

A. Biopsy speroids

HAMLET   α-lactalbumin

B. Xenotransplantation model

Human glioblastoma biopsy spheroids

Infusion :
HAMLET
α-lactalbumin

7 days
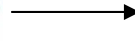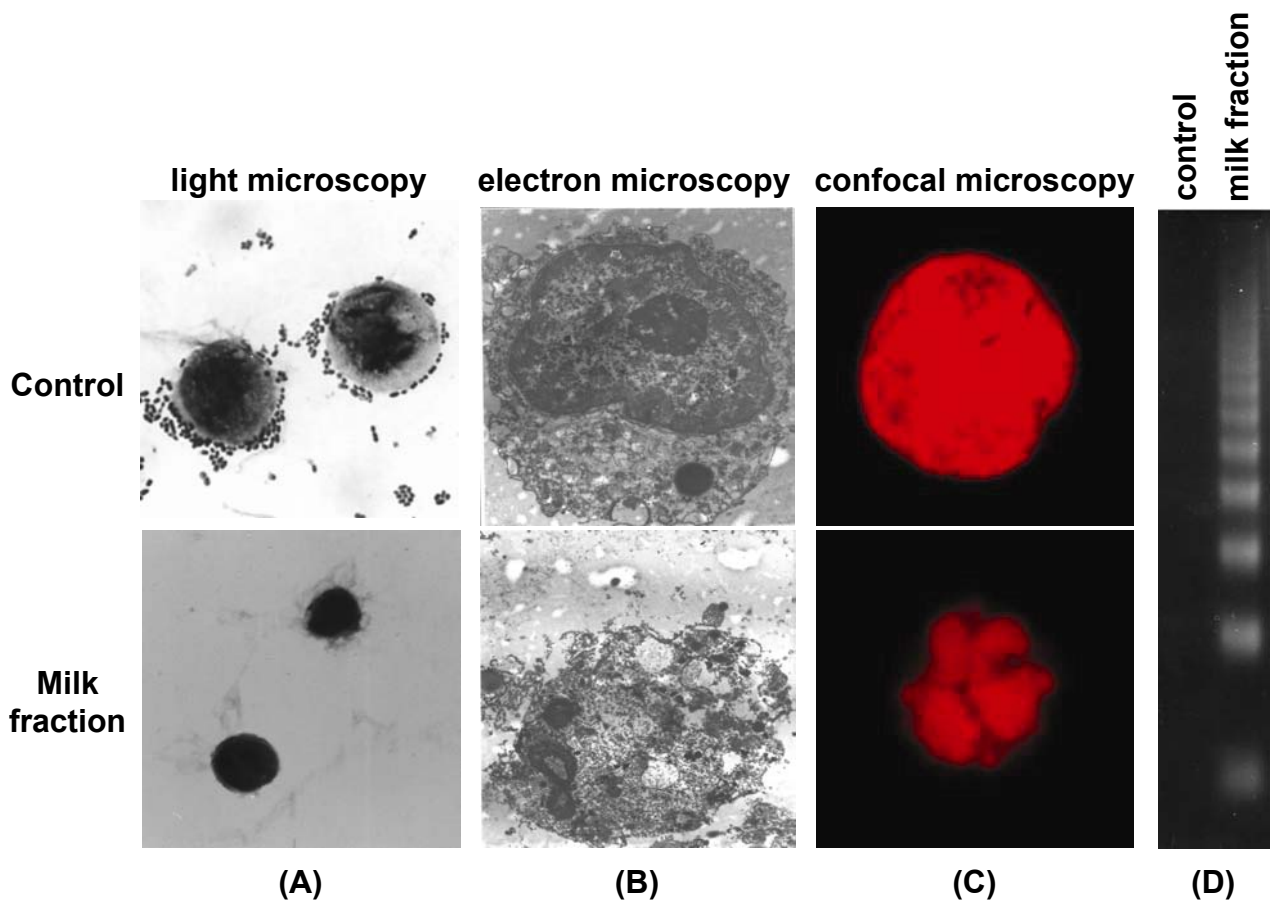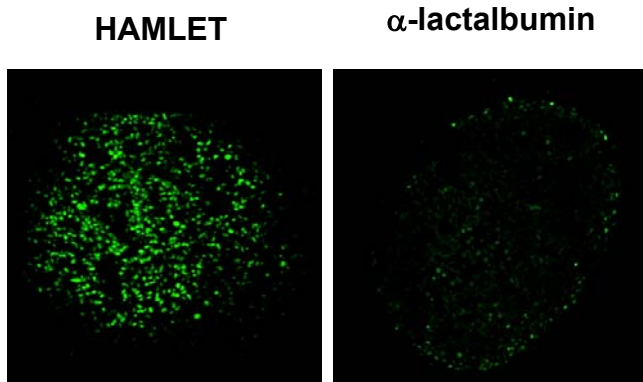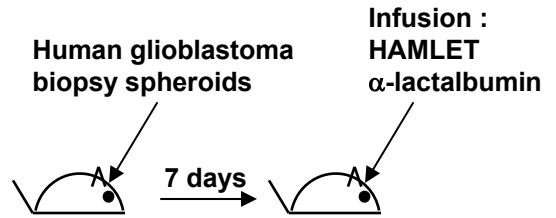
C. Tumour progression

α-lactalbumin   HAMLET
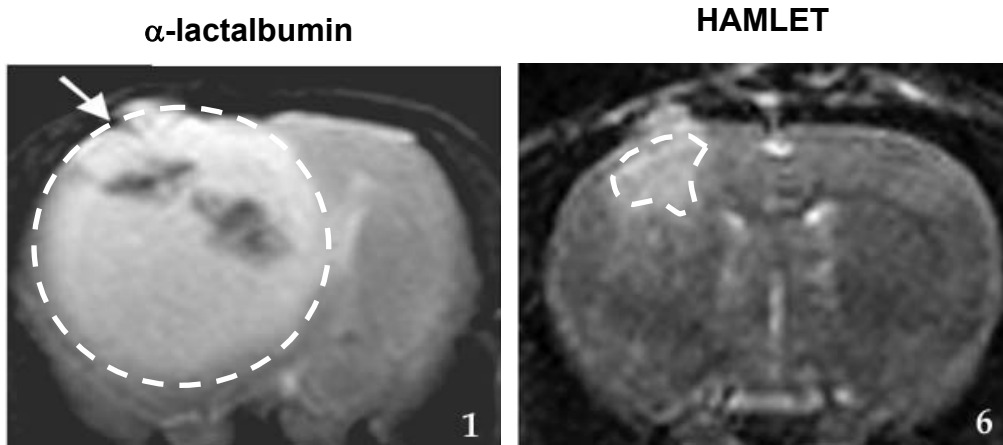
D. Tumour volume and survival

	Tumour volume (mean)	Pressure symptoms (days)
α-lactalbumin	455 mm^3	59
HAMLET	63 mm^3	76
	p< 0.01	p< 0.01

A. Study design

3 weeks   2 months

HAMLET or placebo
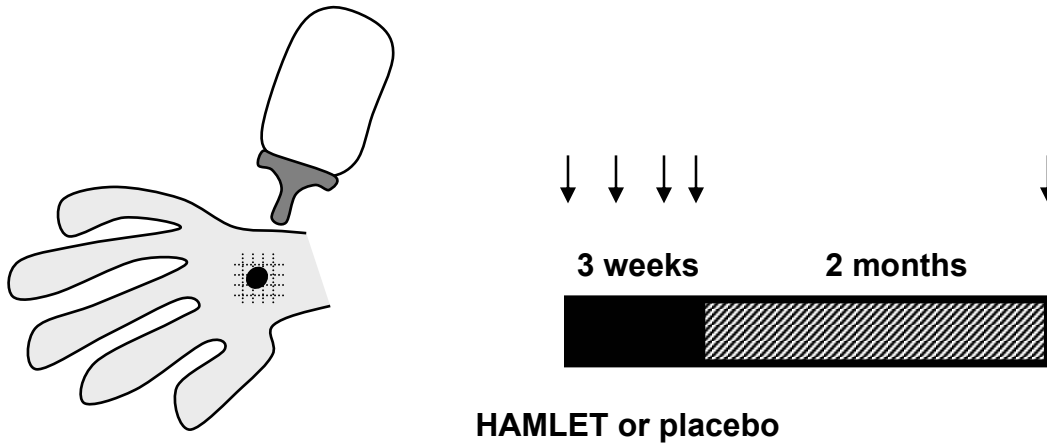
B. Morphology of papilloma before and after HAMLET treatment

Pre HAMLET   Post HAMLET
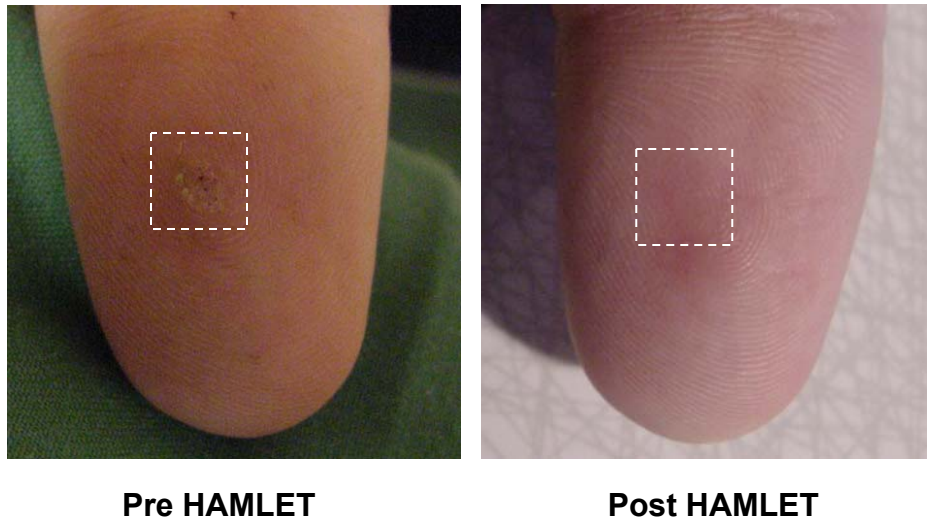
C. Treatment effect of HAMLET contra placebo

	Effect	No effect	Total
HAMLET	20	0	20
Placebo	3	17	20
Total	23	17	40

Effect = a mean volume decrease of ≥75%, p< 0.001

A. P53

deletion by homologous recombination

HCT116 +/+

wt p53
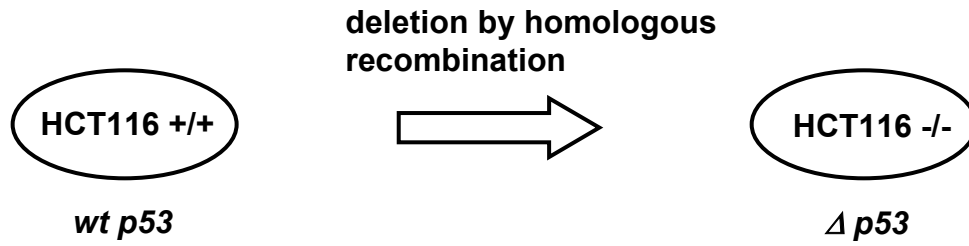
HCT116 -/-

Δ p53

Cell type	genotype
HCT116 +/+	*wild-type p53*
HCT116 -/-	*Δ p53*

B. Bcl-2

bcl-2

pcDNA

K562 *bcl-2*

S2, S8
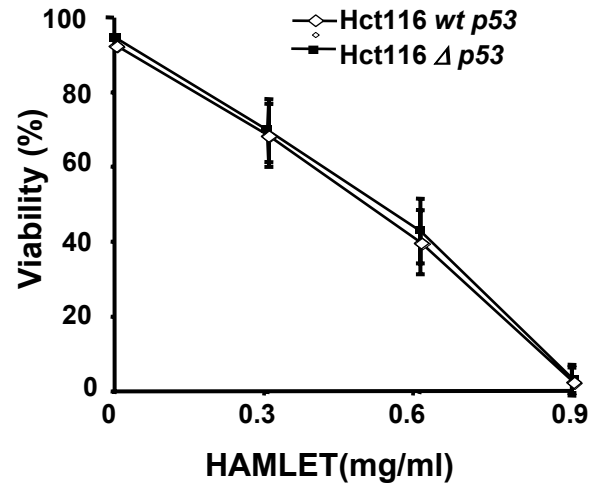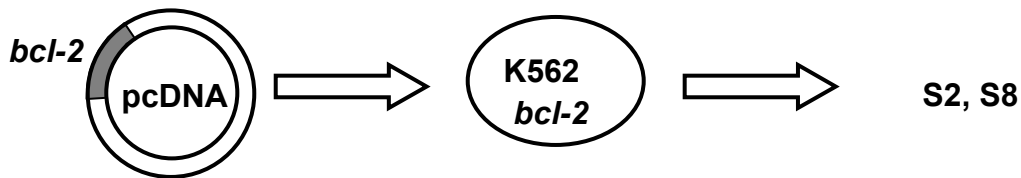
Cell type	Bcl-2 expression
K562, wild type	+
K562 C, vector control	+
Clone S2 , *bcl-2* transfectant	++
Clone S8, *bcl-2* transfectant	+++

| | Transmission | Confocal microscopy | Schematic |

a

e

b

f

c

g

d

h
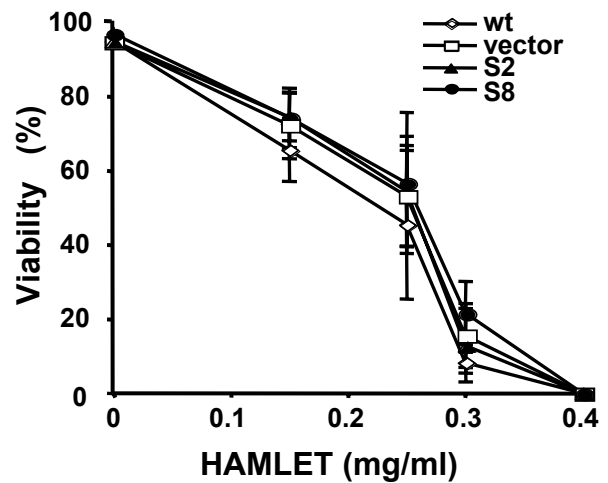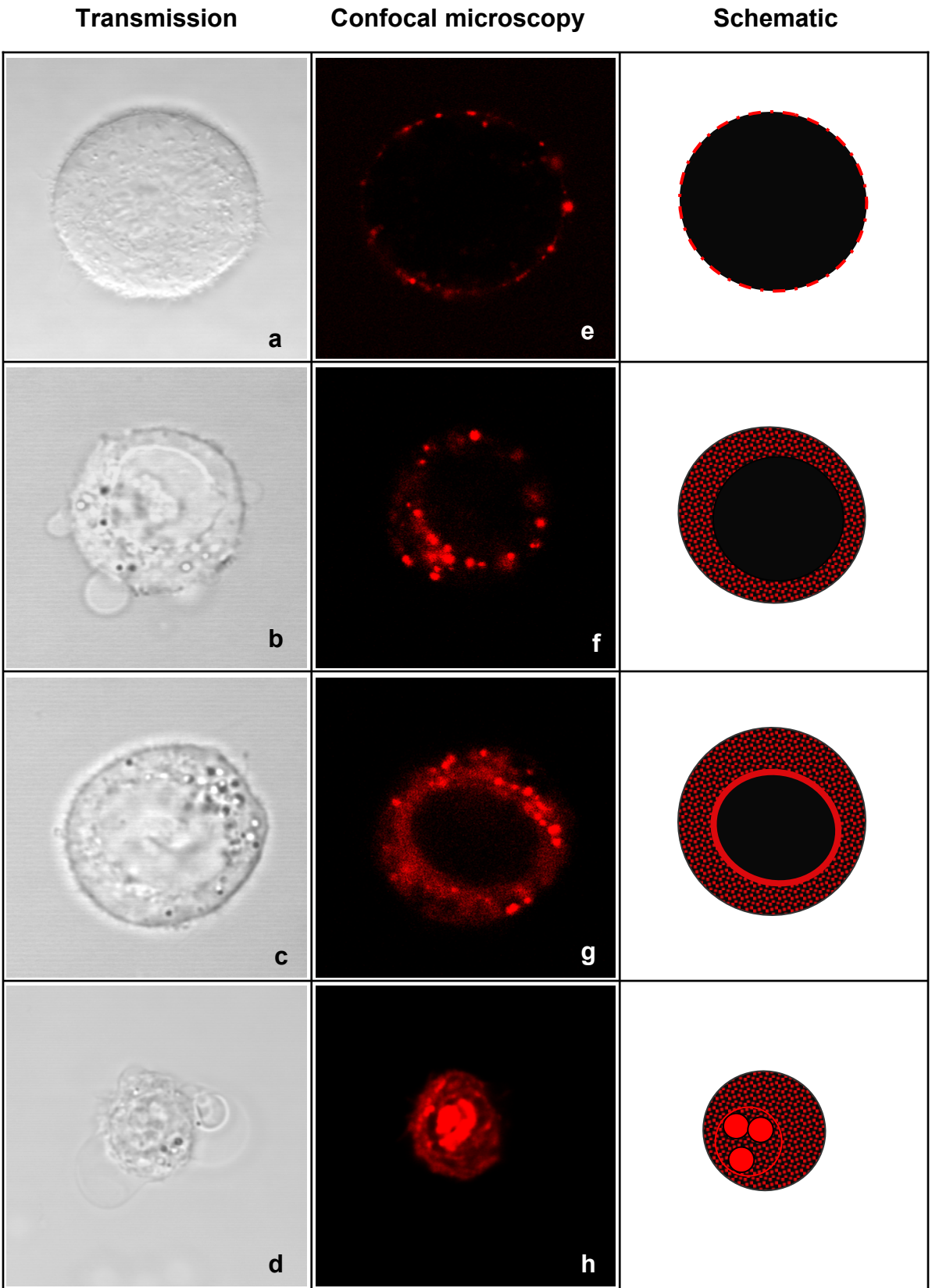
A. Nuclear extract
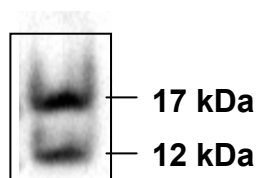
Band	Identity
17 kDa	Histone H3
12 kDa	Histone H4

C

B. Purified histones

**Coomassie stained
SDS-PAGE**
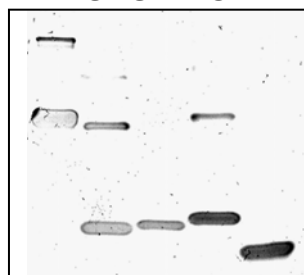
H1 H2A H2B H3 H4

$\downarrow$ **blot**

**Membrane incubated
with ^{125}I-HAMLET**
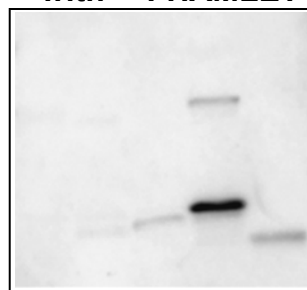
H1 H2A H2B H3 H4
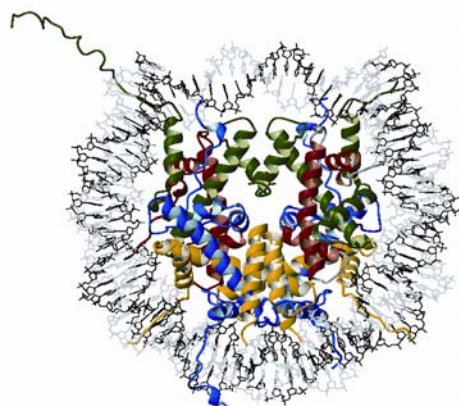
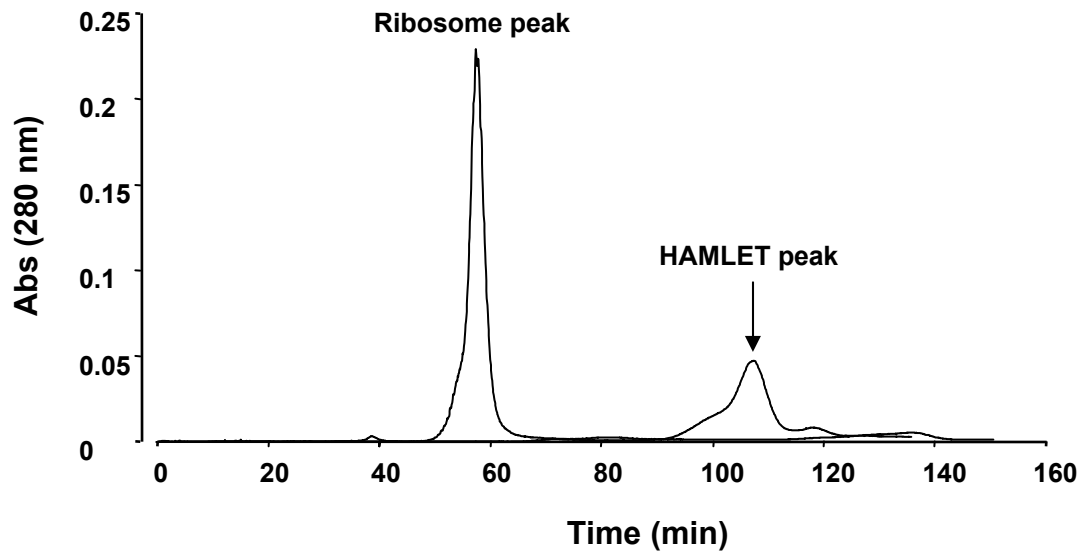## A. HAMLET co-localises with mitochondria
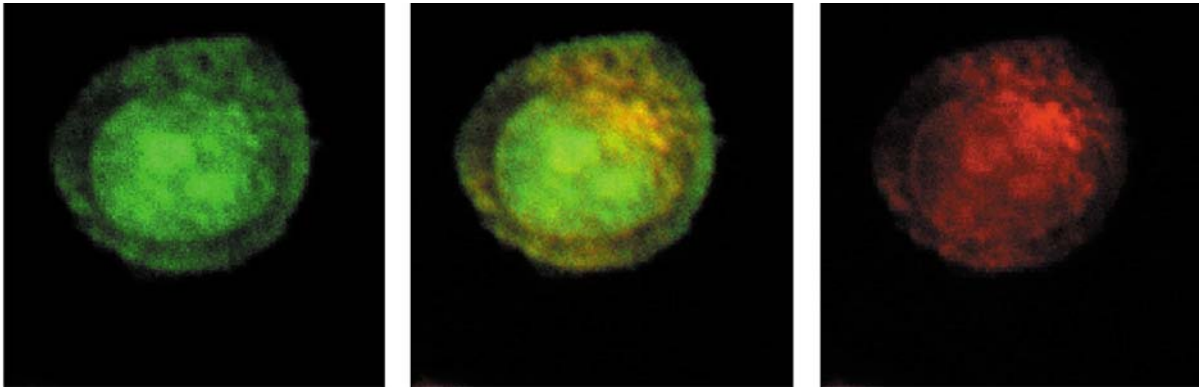
B. Cytochrome C release

Time (h)

| 0 | 1 | 2 | 4 | 6 |
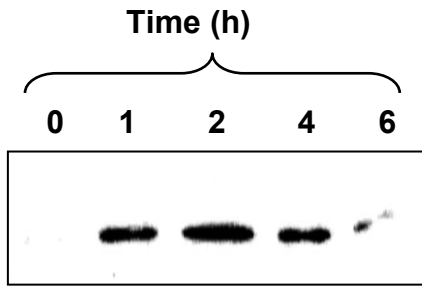
C. Caspase response
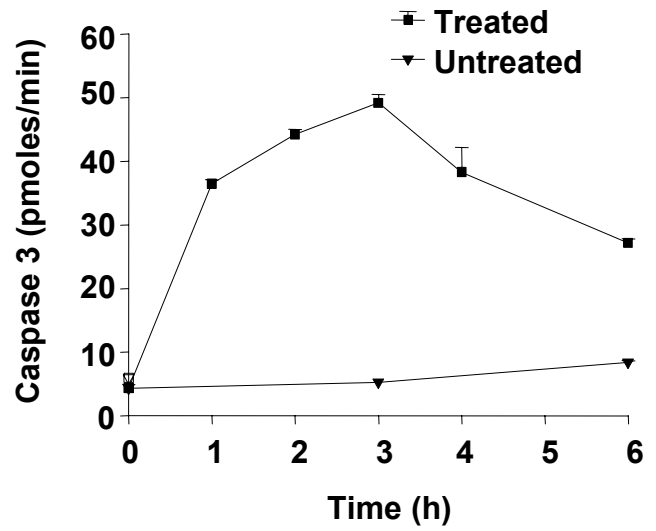
A

Individual proteins

B

Mixture of ribosomes and HAMLET

A

UV (absorbance 280 nm) / Conductivity (mS/cm) vs Time (min)

B

[θ], deg cm² dmol⁻¹ vs wavelength (240–320)

C

Fluorescence, arbitrary units vs Wavelenght (nm)

D

MW (kbp)

A B C D E F G H

1033

394

Cell viability (%) (98) (98) (96) (19) (14) (97) (98) (96)
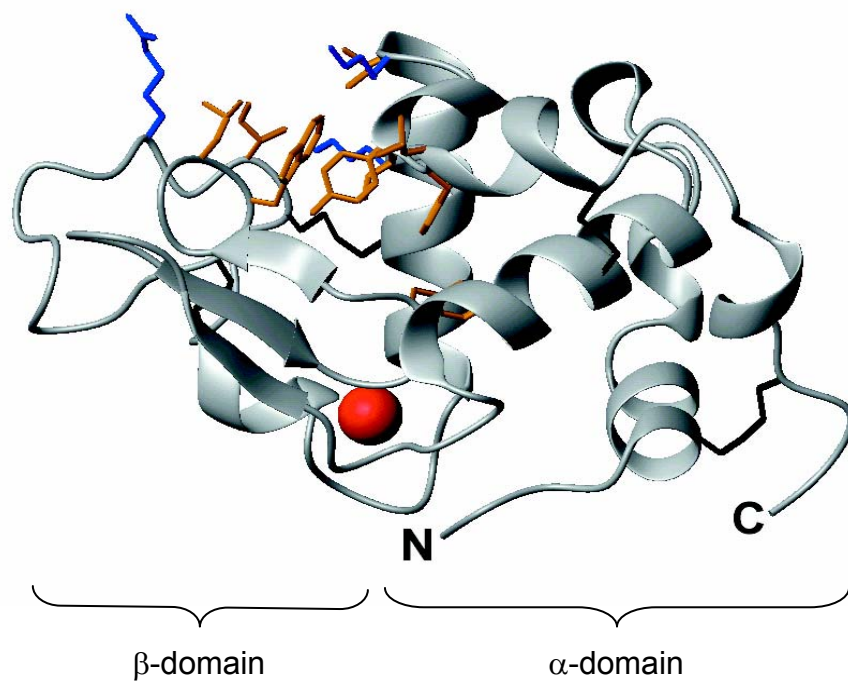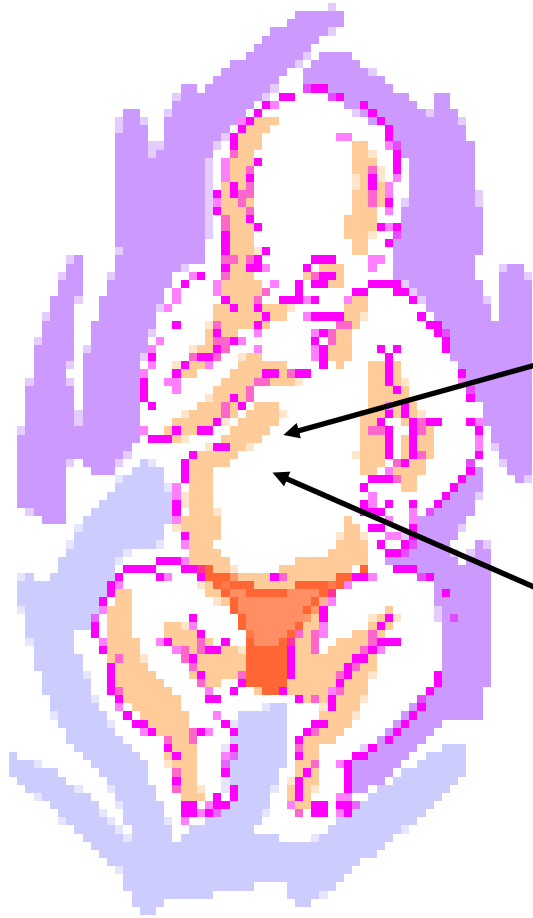
E

HAMLET   α-Lactalbumin

β-domain   α-domain
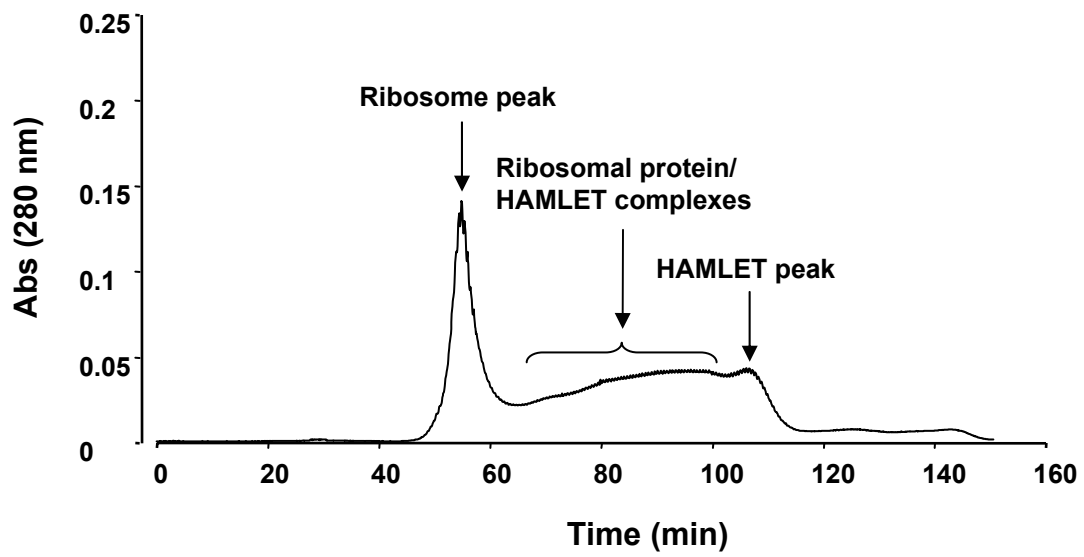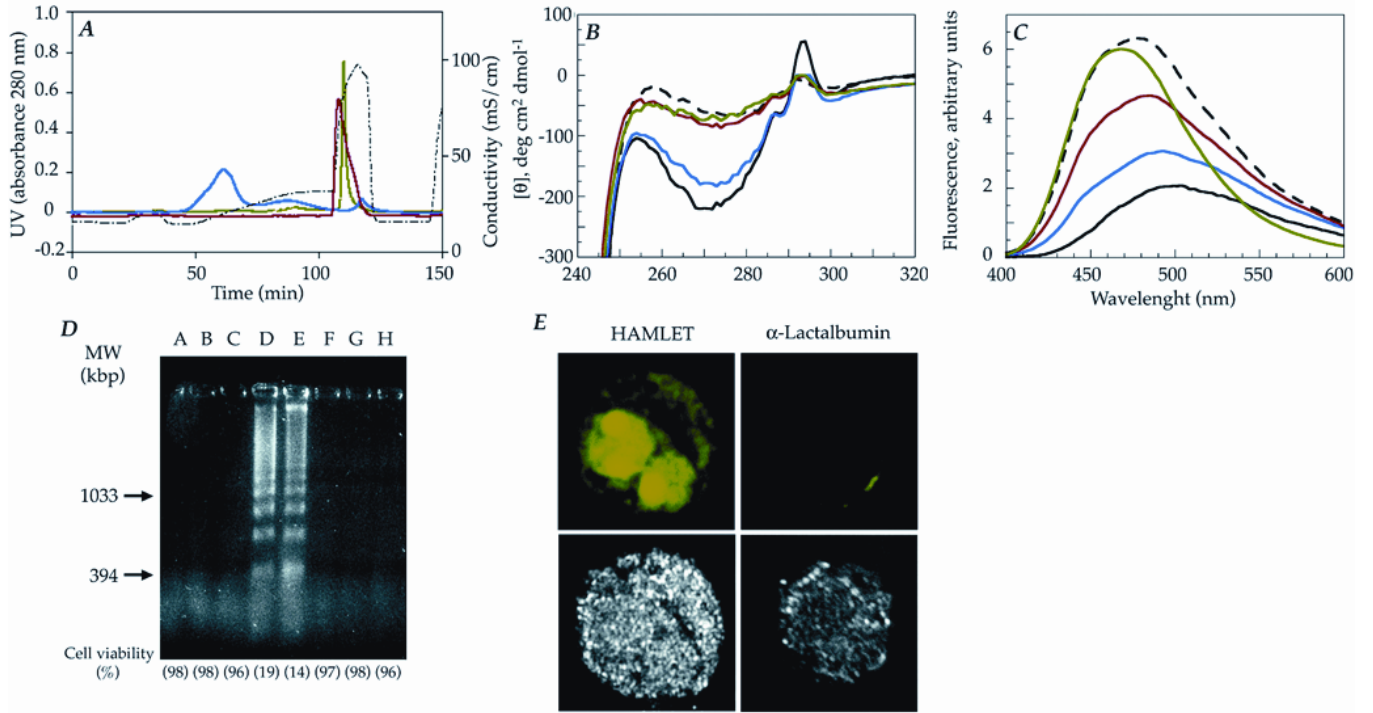
α-lactalbumin is the most abundant protein in human milk (2 mg/ml). Oleic acid is the domineering fatty acid in milk triglycerides.

Stomach:

1. At pH4 α-lactalbumin releases Ca^{2+} and adopts the apo-conformation.

2. Triglycerides are hydrolysed to release C18: 1.

1+2 may form HAMLET.

Intestine: Anti-tumor surveillance?