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HAMLET (Human α-lactalbumin Made LEthal to Tumor cells) kills human glioblastoma cells in brain xeno-grafts by apoptosis and prolongs survival.

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Abbreviation footnote: CED (convection enhanced delivery), GBM (Glioblastoma Multiforme), HAMLET (Human α–lactalbumin Made LEthal to Tumor cells), TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling

Abstract

New tumor treatments should aim to reach and selectively destroy tumor cells, without damaging normal tissues. HAMLET is a protein complex with remarkable properties in that it activates an apoptosis-like death program in malignant cells, but leaves fully differentiated cells unaffected. Here we show that HAMLET kills highly malignant human gliomas *in vitro*, and that intra-cerebral delivery of HAMLET to human glioblastoma xenografts, reduces the tumor volume and prolongs survival, compared to α-lactalbumin in the native, folded conformation. Apoptotic cells were only observed in the tumor, and the surrounding normal brain tissue remained intact. We propose HAMLET treatment as a novel approach to controlling tumor progression, with the dual advantage of selectivity for the tumor cells and death by apoptosis. As HAMLET is derived from human milk, this protective function may have evolved to purge malignant cells in the growing child, and to strengthen nature's own defense against cancer.

Introduction

Tumor cells are notoriously resistant to many of the agonists that limit the longevity of healthy differentiated cells. Thus, altered expression of specific genes in the apoptosis machinery of rapidly proliferating cells may be sufficient to cause tumor formation (1). For example, *p53* mutations impair the sensing of DNA damage, and altered expression of the *bcl-2* family of genes inhibits the mitochondrial response to classical apoptosis agonists (2), (3), (4), (5). Still, other molecular executors of apoptosis remain intact in many tumor cells, suggesting that it should be possible to circumvent the discrete ''road-blocks'' that prevent them from being activated (6). One productive approach to killing tumor cells may be to find agonists that synchronously activate multiple pathways of cell death in those cells.

HAMLET (Human α-lactalbumin Made LEthal to Tumor cells) is a protein-lipid complex that kills a wide range of tumor cells *in vitro* (7). Paradoxically, the tumor cells die through an apoptosis-like mechanism but healthy, differentiated cells are spared. The activity of HAMLET was discovered by serendipity after precipitation of human milk at low pH (7). The active fraction contained partially unfolded α-lactalbumin with less well-defined tertiary structure than the native state and in addition, the active complex contained a fatty acid co-factor that stabilizes this partially unfolded state (8). The altered fold was essential, as native α-lactalbumin was inactive in the cellular assays. HAMLET was defined as α-lactalbumin in a molten globule

like conformation bound to oleic acid (Fig. 1a) (9). The importance of the folding change for the novel biologic activity was proven by deliberate conversion of native α-lactalbumin, to the active HAMLET complex (9).

In this study, we have tested the therapeutic potential of HAMLET in an invasive brain tumor model. Glioblastomas (GBMs) have a most unfavorable prognosis, due to their invasive nature and diffuse infiltrative growth (10), (11), (12). The tumors are inaccessible to complete surgical removal and current modes of treatment are palliative, resulting in a mean survival time of <1 year (13), (14). Remarkably, HAMLET was shown to limit tumor progression and to prolong survival, with no signs of tissue toxicity.

Results

HAMLET induces apoptosis-like death in malignant glioma cells.

HAMLET kills a wide range of tumor cells by apoptosis, but healthy differentiated cells are resistant to this effect (Fig. 1b). The lymphoid tumor cells are the most sensitive, but carcinomas of different origins succumb to HAMLET, at concentrations well within the physiologic range.

The sensitivity of glioma tumor cells was first studied in cell culture. Three different glioma cell lines (D-54, U-251, CRL 2365) were compared to fully differentiated murine brain cells. The glioma cell lines were shown to undergo apoptosis in response to HAMLET, with reduced cellular viability and DNA fragmentation (Fig. 1b). The healthy brain cells, in contrast, remained viable also at concentrations $\geq$ 5 mg/ml (Fig. 1b).

By real time confocal microscopy, HAMLET was shown to invade the tumor cells, forming large cytoplasmic aggregates that moved to the nuclei (Fig. 1c). The cells proceeded to fragment their DNA, the nuclei became condensed, and there was cytoplasmic blebbing and formation of cellular fragments resembling apoptotic bodies (Fig. 1c). This pattern was not observed in the healthy cells, and not for α-lactalbumin; the native conformation of the same protein. α-Lactalbumin was shown to bind weakly to the cell surface and

very small amounts entered the cytoplasm, but α-lactalbumin did not reach the nuclei and DNA fragmentation did not occur (Fig. 1c).

We conclude that the malignant gliomal cells are sensitive to HAMLET induced cell death and that uptake and transport of HAMLET to the nuclei characterizes the tumor cells.

HAMLET induces apoptosis-like death in glioblastoma biopsy spheroids.

To verify that HAMLET killed malignant cells in intact tumors, GBM tumor biopsies were obtained from patients undergoing surgery. Spheroids of these tumors were cultured *in vitro*, and exposed to HAMLET with α-lactalbumin as a control. HAMLET triggered cell death throughout the GBM biopsy spheroids but spheroids derived from a benign meningioma did not die following HAMLET-exposure. By morphometry, 93 ±7 % (mean ±SD) of the GBM nuclei were TUNEL-positive (Fig. 1d, $p<0.001$). By histopathology, pycnotic and condensed nuclei were observed in the HAMLET-exposed GBMs (Fig. 1d).

Control GBM spheroids treated with α-lactalbumin were seen to shed a few apoptotic cells from the surface, but no TUNEL positive cells were seen in the interior of the spheroids, and there was no difference in the frequency of apoptotic cells between the GBM spheroids and the meningiomas exposed to α-lactalbumin (Fig. 1d)

We conclude that primary GBM tumor biopsies are sensitive to HAMLET-induced cell death, and that the protein complex is able to penetrate throughout biopsy spheroids.

HAMLET treatment of human brain tumors xeno-transplanted to nude rats.

Xeno-transplantation of human GBM biopsies into the nude rat brain was used to study the therapeutic effect of HAMLET. Biopsy spheroids of human gliomas maintain their invasiveness after xeno-transplantation (15), (16) and the nude rats develop pressure symptoms after 60 days with little variation. Infusion of therapeutic agents may be achieved by convection enhanced delivery (CED), allowing the extra-cellular compartment to acts as a substrate for convection of solutes to different parts of the brain, thus providing a standardized treatment model of the human disease (17), (18), (19). Other experimental approaches invariably produce intra-cerebral tumors, but they are not invasive *in vivo*, and thus less suitable as a model of the human disease (20), (21).

^{125}I -labeled HAMLET (2-10x10^6 PPM) was administered by CED into the striatum of healthy rats, and the distribution of HAMLET throughout the rat brain was verified by auto-radiography on serial brain sections in the entire infused hemisphere from the forebrain to the mesencephalon (Fig. 2a). We conclude that CED allowed HAMLET to reach the entire infused hemisphere.

HAMLET was then administered by CED to xeno-transplanted rats, one week after the injection of the tumor spheroids. Infusion was at the site of tumor injection for 24 hours (0.7 mM, n=10), and α-lactalbumin served as control (0.7 mM, n=10). Twelve hours later, four animals in each group were sacrificed for histology, TUNEL assay, and morphometric analyses. Two animals in each group died during anesthesia.

Tumor volumes were assessed by MRI in the remaining animals after seven weeks when the α-lactalbumin control animals developed symptoms (Fig. 2b). Large GBM-transplants with high T2-weighted signals could be observed in all the α-lactalbumin treated animals, with a mean tumor volume of 455 (range 292-485) mm^3. The HAMLET-infused rats showed significantly smaller tumor volumes (mean 63, range 10-131 mm^3, $p<0.01$). HAMLET treatment also delayed the onset of pressure symptoms. Rats receiving α-lactalbumin developed symptoms on day 59, and by day 65, all animals had been sacrificed. At this time, all animals in the HAMLET-treated group remained a-symptomatic (Fig. 2c, $p<0.01$). These rats eventually developed pressure symptoms (from day 76), however, and histology showed typical GBM tumors, with polymorphic cell types and pseudo-pallisading (Fig. 3).

Selective tumor cell apoptosis in the brain.

HAMLET treatment caused tumor cell death *in vivo* (33 ±7% of the HAMLET-treated GBM cells were TUNEL-positive compared to 2 ±2% in the α-lactalbumin group, Fig. 3, $p<0.001$). The host brain surrounding the tumor showed no evidence of cell death after CED of either HAMLET or α-lactalbumin, emphasizing the selectivity for the tumor cells. The apoptosis-like morphology of the dying cells was confirmed by routine histopathology, showing pycnotic and condensed nuclei in the HAMLET-treated tumors (Fig. 3).

The possible toxicity was examined by CED of α-lactalbumin or HAMLET into the brains of healthy animals. We detected no changes in behavior or motor performances of the animals and no signs of toxicity by histology or apoptosis by TUNEL.

Treatment of spheroids prior to transplantation verifies the killing effect of HAMLET.

In a parallel experiment, tumor spheroids were pulsed *in vitro* with HAMLET or α-lactalbumin for 3 hours prior to xeno-transplantation. The tumor size was estimated by magnetic resonance imaging (MRI) scans, 2 months after intra-cerebral transplantation of the pre-treated tumor spheroids. Tumors developed

in all the rats (n=6) treated with α-lactalbumin, but only in two of the six animals receiving HAMLET. The HAMLET treated rats had small tumors (mean volume of 31, range 28-34 mm^3) while the α-lactalbumin treated group developed large tumors (mean 496, range 286-696 mm^3). The two HAMLET treated rats with smaller tumors developed pressure symptoms after 77 days, but the four remaining animals in the HAMLET group were still a-symptomatic even at 210 days following transplantation (P<0.001). The α-lactalbumin treated rats developed symptoms after day 56.

We conclude that the cell death process that is initiated by HAMLET *in vitro* is continuing after injection of the treated cells into the brain.

Discussion

Malignant GBMs remain refractory to conventional therapies. They are not amenable to selective surgical removal due to their infiltrating growth, and are resistant to irradiation and chemotherapy. Regional infusion of a transferrin diphtheria toxin complex has been shown to decrease tumor volume, 1-14 months after the first treatment (22). This complex triggers necrosis, causing brain edema, and may destroy brain capillary endothelial cells, which express significant levels of transferrin-receptors. Other experimental therapies like gene therapy, anti-sense, engineered and defective viruses may be efficient in brain tumor models, but those few that have made it to clinical trials have not been promising (23). The limited success of these approaches emphasizes need for novel therapeutic approaches that selectively eliminate the tumor cells without damaging the surrounding brain.

HAMLET is promising, since it kills a wide range of malignant cells *in vitro* but leaves fully differentiated cells unaffected. Here we show that HAMLET maintains these properties *in vivo*. A 24-hour infusion of HAMLET into established human GBM tumors was sufficient to dramatically delay tumor development and the onset of pressure symptoms in nude rats. The effects of HAMLET on the established malignant brain tumors are promising, as the infusion of this protein complex did not appear to harm the normal brain or cause neurological symptoms. We conclude that HAMLET has the potential

to act as a selective anti-tumor agent, and propose regional therapy of HAMLET as a possible novel approach to control the progression of this highly malignant and invasive CNS tumor.

HAMLET consists of α-lactalbumin, in a molten globule-like conformation, and a fatty acid co-factor that stabilize this fold. Our findings show that a protein folding-variant may be beneficial, and even useful in the treatment of malignant disease, and appear especially surprising as protein folding variants mainly have been recognized as a cause of disease. The disease-causing iso-form of the prions accumulates in brain tissue (24), (25), and amyloid is formed when the partially unfolded complexes of proteins like β-amyloid (26), apo-lipoprotein or lysozyme (27), accumulate and disrupt cellular homeostatic functions**.** HAMLET, in contrast, appears not to be harmful to normal tissue but to selectively purge malignant and immature cells by apoptosis (7). It is possible that HAMLET acts as a natural tumor scavenger in infancy, with the mission of purging atypical or highly immature cells during normal development. Epidemiological studies have indeed shown that breast-fed children have a reduced incidence of childhood leukemia (28) and other cancers. The ability to regulate folding and to create functionally specialized conformers from a single amino acid sequence offers new possibilities to regulate the evolution of an organism and may be exploited to combat disease.

Materials and Methods

The HAMLET complex was produced from apo α-lactalbumin by ion exchange chromatography, on a DEAE-Trisacryl M (BioSepra, France) column preconditioned with oleic acid (C18: 1, 9 cis fatty acid) (9). C18: 1, 9 cis fatty acid was from Larodan, Sweden. For real-time confocal microscopy, HAMLET was conjugated to Alexa Fluor 568 (Molecular Probes Inc., Eugene, Oregon, U.S.A.) and ^{125}I-labelling was by the peroxidase method.

Cellular interactions. After exposure to HAMLET or α-lactalbumin, cell viability was determined by Trypan blue exclusion. DNA fragmentation was determined as described (7). Gliomal cell lines D54, U-251 and CRL 2365 were from ATCC. A single cell suspension of differentiated murine brain cells was prepared by placing tissue in DMEM (Gibco/BRL, Life Technologies Ltd. Paisley, Scotland, U.K.) with 1% Trypsin and 0.25% DNase in 1% FCS for 30 min at RT. After repeated washing the cells were suspended in DMEM at $4x10^6$/ml. The viability was >99%.

For sub-cellular localization by real-time confocal microscopy, cells incubated with Alexa Fluor labeled HAMLET (0.007 mM) were examined in an MRC-1024 confocal system attached to a Nikon Eclipse 800 upright microscope (Nikon, Kanagawa, Japan) and analyzed in Laser Sharp software, Version 3.2 (Bio-Rad Laboratories, Hemel-Hempstead, U.K.).

Human tumor biopsies were collected with the approval of the Medical Ethics Committee at the Haukeland University Hospital from a GBM of the right frontal lobe and a parasagittal meningioma. Spheroids (diameter 300 μm, 4-5/group) were moved to serum free medium, incubated for three hours with HAMLET or α-lactalbumin, transferred back to DMEM for another 21 hours and examined after serial sectioning by the TUNEL assay (F. Hoffmann-La Roche Ltd, Basel, Switzerland), with morphometry.

***In vivo* model of human GBM disease.** Xeno-transplantation of human GBMs to nude rats was approved by The National Animal Research Authority and conducted according to the European Convention for the Protection of Vertebrates Used for Scientific purposes. Nude rats (Han:rnu/rnu Rowett) bred at the Haukeland hospital, were anaesthetized, placed in a stereo-tactic frame (David Kopf, model 900, Tujunga, CA, U.S.A.) for trepanation, and about 5-10 μl of PBS containing 5 biopsy spheroids was injected into the striatum. Rats were monitored daily and sacrificed when they developed symptoms such as passivity, clumsiness and paresis. The tumor mass was quantified by MRI scans (1.5 Tesla Siemens Magnetom Vision, Erlangen, Germany), with a finger-coil for cerebral analysis.

The region of the tumor was infused with HAMLET or α-lactalbumin (0.7 mM in 0.9% NaCl), by CED through a 26 Gauge cannula connected to an

osmotic mini pump (AD01, Alzet Inc., Mountainview, CA, U.S.A., 8 μl/ hour over 24 hours).

Brains were embedded in Tissue-Tec (Sakura Finetek Inc., Torrance, CA, U.S.A.), frozen in liquid nitrogen, and sectioned on a Reichert Jung Cryostat (Reichert, Vienna, Austria). Apoptotic cells were detected by TUNEL, with counter-stained nuclei (propidium iodide, 10 μg/ml, 30 sec). Parallel sections were stained with Hematoxylin-Eosin (Merck, Darmstadt, Germany). Sections without freezing artifacts and with an acceptable signal/noise ratio for FITC (TUNEL) and TRITC (propidium iodide) were identified in a Leica scanner, and one representative section from the center of each tumor or spheroid was subjected to morphometric analysis. FITC and TRITC positive nuclear profiles were recorded. Results are expressed as TUNEL+/propidium iodide positive nuclei.

Groups were compared with one-way-ANOVA (post hoc LSD), and survival by Kaplan-Meier-analysis.

Figure Legends

Figure 1. *In vitro* studies of HAMLET.

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

HAMLET is formed from native α-lactalbumin by removal of Ca^{2+} and by the addition of the C18:1, 9 cis fatty acid (9). The figure is based on the α-lactalbumin crystal structure (33).

b. Broad anti-tumor spectrum of HAMLET. LD_{50} = concentration required to kill 50% of the cells in 6/24 hours.

c. Cellular trafficking of HAMLET in malignant gliomal cells. HAMLET (red, upper panels) binds to tumor cell surfaces, invades their cytoplasm, translocates to the perinuclear region and accumulates in the nuclei. Morphologic changes include membrane blebbing, nuclear condensation, vesicle formation, cell shrinkage, and formation of apoptotic bodies (light transmission, lower panels, magnification x180). α-Lactalbumin binds to the surface and some protein enters the cytoplasm, but no further translocation occurs. Trypan blue marks dead cells.

d. Apoptosis induction in human GBM biopsy spheroids. Dying cells were identified by the TUNEL-assay (green), with propidium iodide (red) counter-staining to visualize the total cell population. Glioblastoma cells died in spheroids exposed to HAMLET, but not in benign meningiomas (magnification x 360). Hyper-chromatic and pycnotic apoptotic cells (arrow) were detected in the

HAMLET-treated GBM spheroids (htx-eosin, magnification x 450). α-Lactalbumin did not induce cell death.

Figure 2. *In vivo* studies of HAMLET.

a. HAMLET reaches relevant areas of the brain following CED. Radio-labeled HAMLET was infused at a rate of 8 μl/ h, with the needle inserted in the striatum (arrow). Serial brain sections show radioactivity in the entire infused hemisphere, from the forebrain to the mesencephalon.

b. Therapeutic effect of HAMLET. The tumor sizes were assessed by MRI scans, seven weeks after CED of HAMLET or α-lactalbumin. The dotted line shows the approximate tumor outline.

c. Reduced tumor volume and prolonged survival in HAMLET-treated rats. The tumors were significantly smaller in the HAMLET-infused animals (green) than in the α-lactalbumin treated group (red, $p<0.001$). Symptoms of elevated intra-cranial pressure occurred after about 60 days in the α-lactalbumin controls (red). At this time, all rats receiving HAMLET were a-symptomatic (green) ($p<0.001$). These rats later developed GBM symptoms.

Figure 3. Selective tumor cell apoptosis in GBM.

Infusion of HAMLET caused abundant TUNEL staining (green, upper panels) within the tumor area. Co-localization with cellular DNA (red) showed yellow

double positive cells within the tumor (magnification x80). Approximate tumor boarder (dotted line). Pycnotic apoptotic tumor cell nuclei were also seen by histology (magnification x 600). The infusion artifact is shown as *.

Acknowledgements

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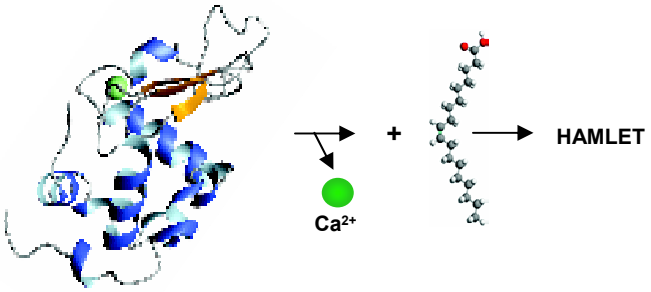
a. The HAMLET complex.

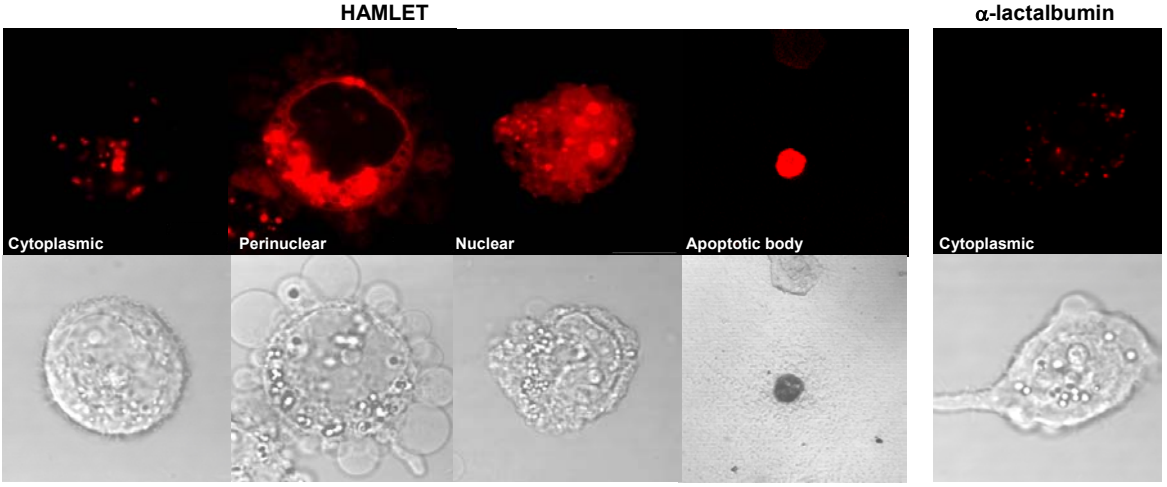


b. Broad anti-tumour spectrum of HAMLET.

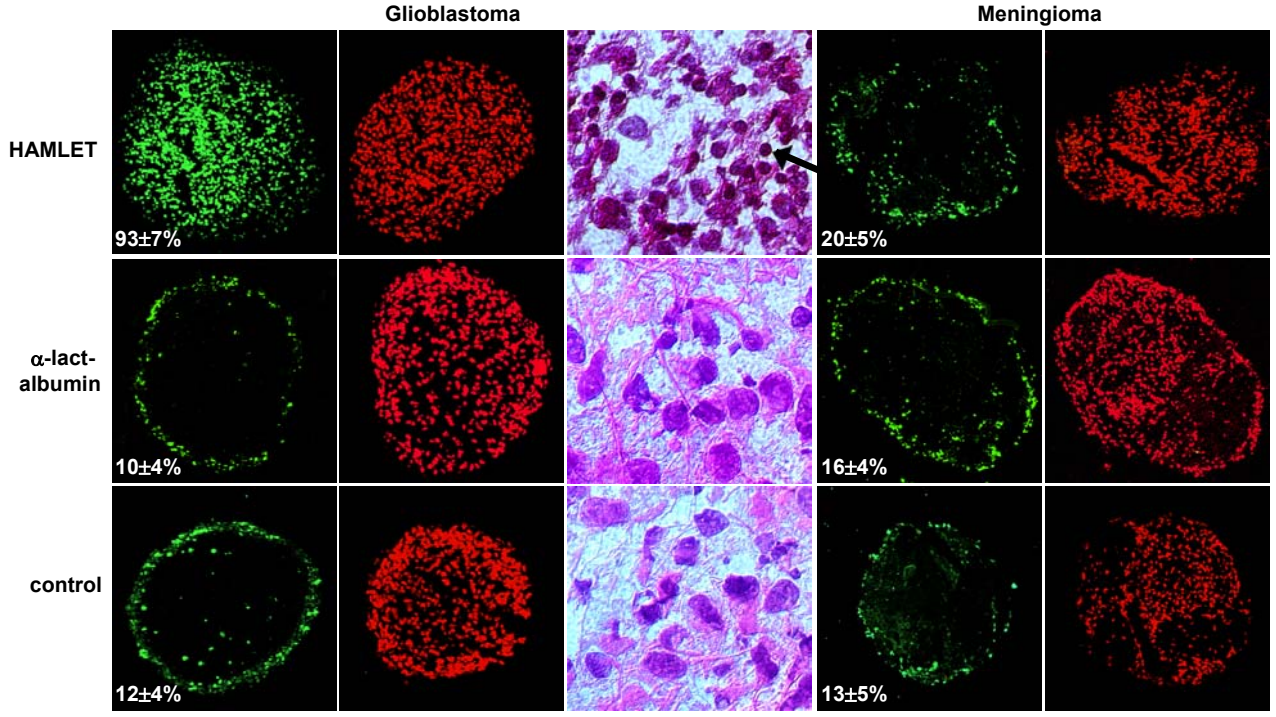
	LD_{50} (mM)
*Carcinomas	0.02
†Leukemias	0.01
*Glioblastomas	
U-251	0.03
CRL2365	0.03
D54	0.05
*Healthy brain cells	>0.35
Other healthy cells	>0.35

* incubation time 24h, † incubation time 6h
Concentration α-lactalbumin in milk 2 mg/ml = 0.14 mM

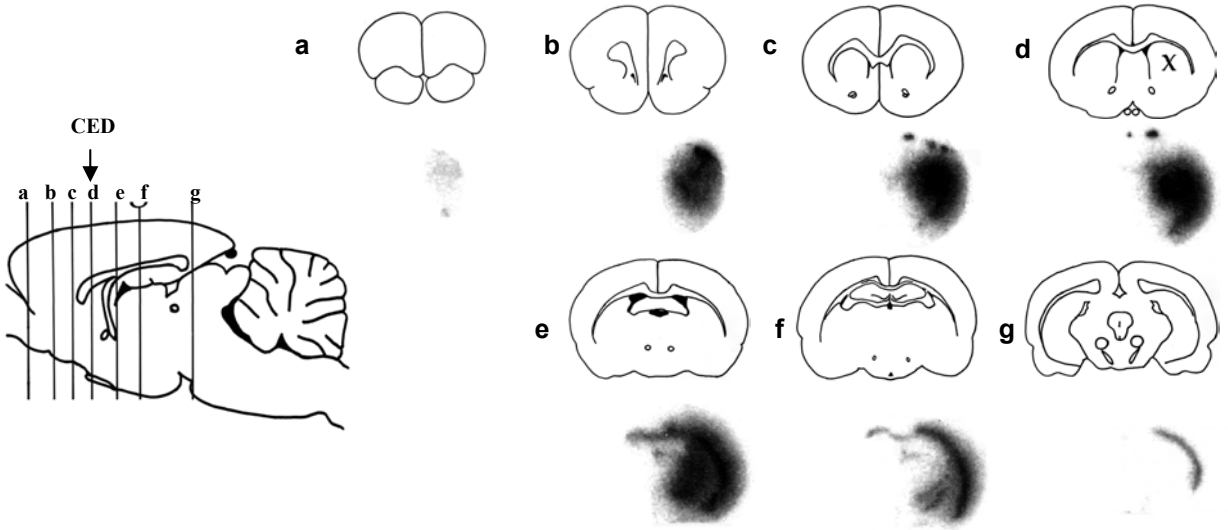
c. Cellular trafficking.



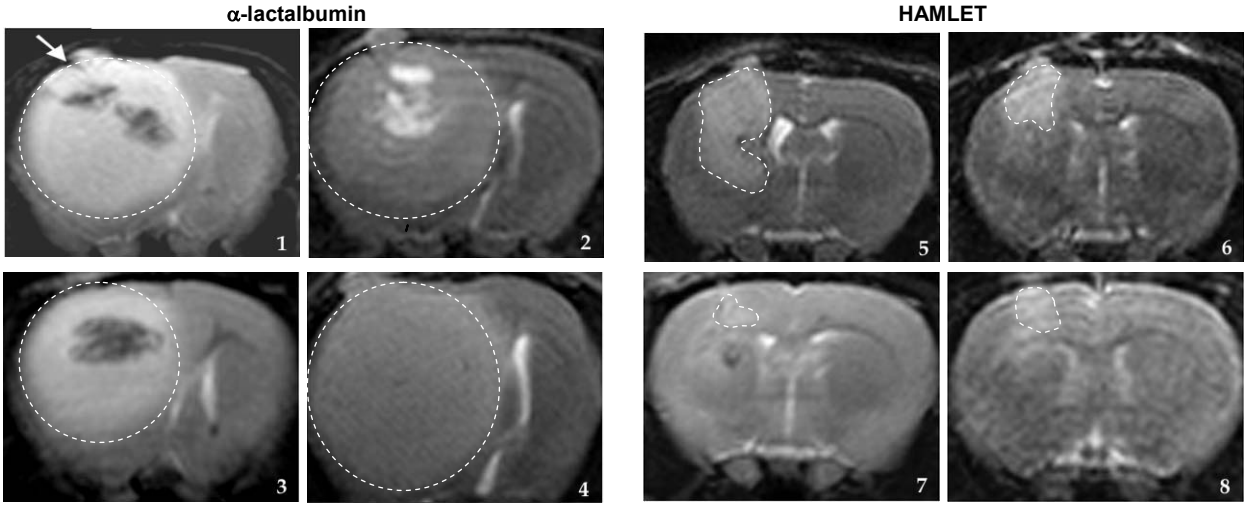
d. Apoptosis induction in human GBM biopsy spheroids.



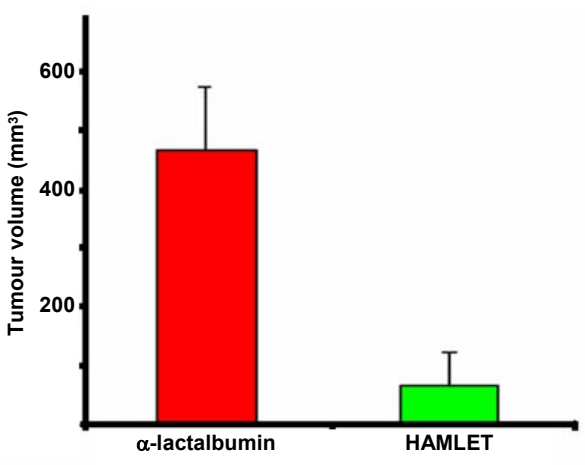
a. HAMLET reaches relevant areas of the brain following CED.



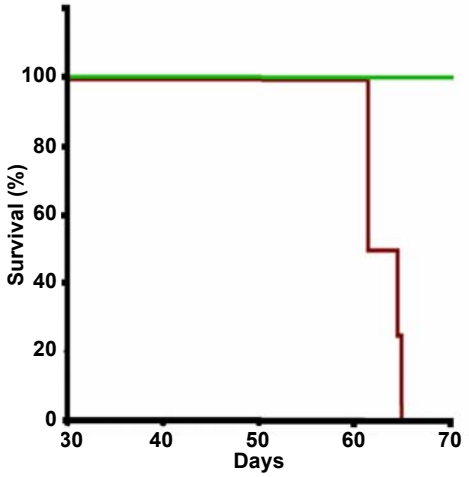
b. Treatment effect of HAMLET.



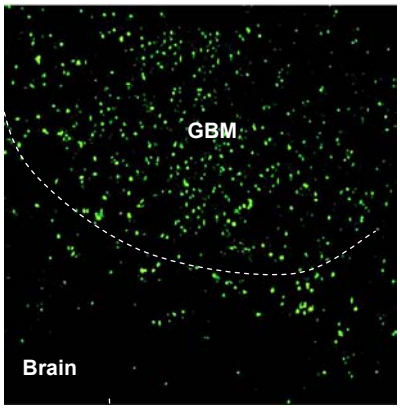
c. Reduced tumour volume in HAMLET- treated rats.

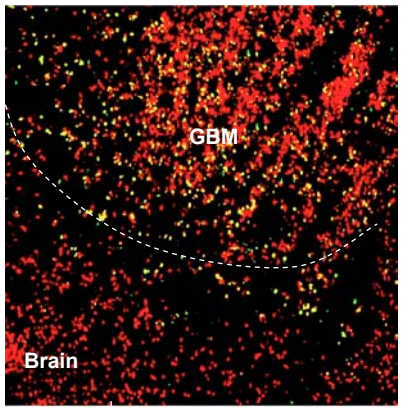


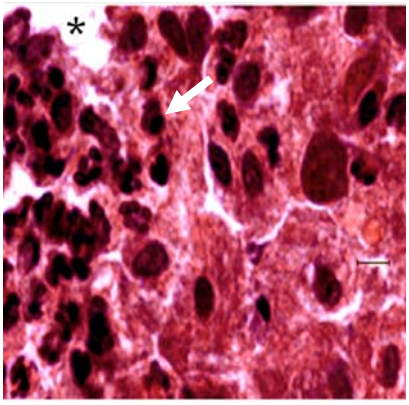
Prolonged survival in HAMLET-treated rats.



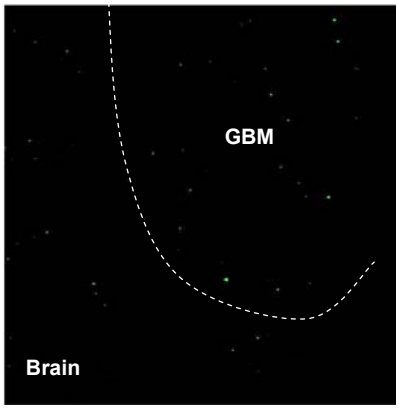
Selective tumor cell apoptosis in GBM.

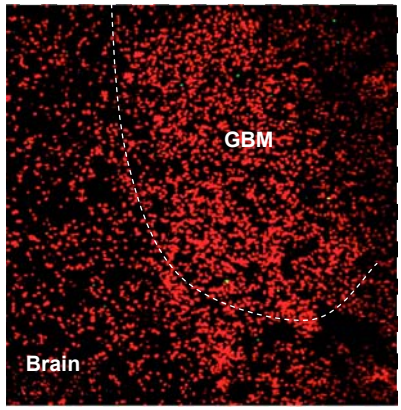


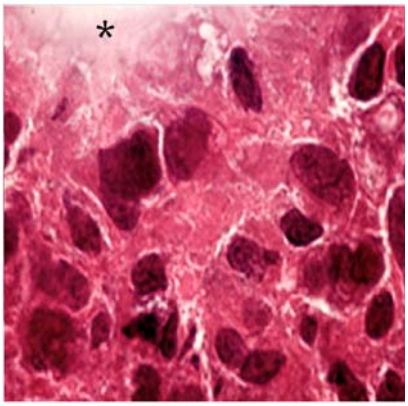




HAMLET







α-lact-albumin