The mechanism of HAMLET-induced cell death - cellular signalling, oncogenes and clinical perspectives

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The mechanism of HAMLET-induced cell death
- cellular signalling, oncogenes and clinical perspectives

Akademisk avhandling som med vederbörligt tillstånd från Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligt försvaras fredagen den 15e juni 2012 kl 9.00 i GK-salen.

Handledare: Professor Catharina Svanborg
Fakultetsoppponent: Professor Maria Masucci, Karolinska Institutet
Till mina tjejer
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# INTRODUCTION

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DISCUSSION

HAMLET acts as a "magic shotgun"

HAMLET induces a transcriptional response characterized by p38 MAPK signalling, cell death genes and ER stress

c-Myc and tumour cell metabolism determine HAMLET sensitivity

The HAMLET-activated ion channel remains elusive

HAMLET triggers ER stress in carcinoma cells

Glycolysis inhibition as a target for tumour therapy

MAP Kinases are critically involved in the tumouricidal response to HAMLET

HAMLET triggers an immunogenic cell death which should be optimal for in vivo anti-cancer activity

HAMLET shows therapeutic efficacy in a model of colon cancer

ACKNOWLEDGEMENT

REFERENCES
LIST OF PAPERS

I
Conserved features of cancer cells define their sensitivity to HAMLET-induced death; c-Myc and glycolysis


Oncogene (2011) 30, 4765–4779, *Equal contribution

II
Selective ion channel activation by HAMLET explains tumor cell death

Petter Storm, Thomas Kjaer Klausen, Maria Trulsson, James Ho Chin Shing, Marion Dosnon, Tomas Westergren, Yinxia Chao, Anna Rydström, Priscilla Lay Keng Lim, Henry Yang, Stine Falsig Pedersen and Catharina Svanborg

Submitted

III
Lipids as tumoricidal components of HAMLET; Unique and shared effects on signalling and death

James Ho CS*, Petter Storm*, Anna Rydström, Ben Bowen, Fredrik Alsin, Trent Northen and Catharina Svanborg

Manuscript, *Equal contribution

IV
Prevention of colon cancer development by peroral administration of HAMLET (human α-lactalbumin made lethal to tumor cells) in APCmin mice

Manoj K Puthia, Petter Storm, Aftab Nadeem, Godo Urbano, Catharina Svanborg

Manuscript
SUMMARY

Despite recent advances in cancer treatment, truly innovative approaches are required to move beyond the modest benefits achieved to date. HAMLET is a human protein-lipid complex originally discovered in breast milk with properties making it a suitable candidate for cancer therapy. Importantly, HAMLET has been shown to kill a wide range of tumour cells while leaving healthy, differentiated cells unaffected. HAMLET consists of α-lactalbumin, the most common protein in human breast milk, which has adopted a molten globular structure that enables it to bind oleic acid. The molecular mechanism of HAMLET induced cell death has been extensively studied but a unifying mechanism for the initiation and execution of cell death in response to HAMLET has not been identified. The aim of this thesis was to identify components dictating the HAMLET sensitivity of tumor cells and to define the events that lead to cell death in response to HAMLET. Additionally, the relative contribution of the fatty acid and the protein is discussed. Finally, the therapeutic effects of HAMLET are extended to colon cancer, in a mouse model with human relevance.

In Paper I we investigated the molecular basis of HAMLET sensitivity in tumour cells. By systematic knockdown of ~1600 cancer-implicated genes in an shRNA screen, c-Myc and glycolytic targets were identified as important determinants of HAMLET sensitivity. Knockdown of c-Myc with individual shRNAs in tumour cells rescued tumour cells from HAMLET induced cell death. In contrast, glucose starvation of tumour cells or the simultaneous application of glycolysis inhibitors significantly enhances tumour cell death. Potential binding partners to HAMLET were identified using a protein microarray approach. Hexokinase 1, the enzyme responsible for trapping glucose in the cytosol, was identified as a binding partner and HAMLET was shown to induce rapid metabolic paralysis with a reduced abundance of metabolites in the glycolysis pathways as well as accumulation of lipid metabolites. In conclusion, these results show that the activity of genes responsible for hallmarks of cancer, including oncogenes and the Warburg effect are important determinants of HAMLET sensitivity.

In paper II the initiating events for tumour cell death are elucidated. By rapid activation of a single ion channel the fluxes of cations were shown to be disturbed in tumour cells. These fluxes could be amended by pre-treatment with two broad ion channel inhibitors. These inhibitors also abrogated the majority of HAMLET induced cellular changes including uptake and cell shrinkage, transcriptional responses and ultimately, cell death. The transcriptional response to HAMLET was further elucidated and found to involve activation of the p38 MAPK signalling pathway. Activation of p38 MAPK was found to be crucially involved in tumour cell death as pre-treatment with p38-inhibitors or knockdown of p38...
reduced tumour cell death and morphological responses. In contrast to tumour cells, healthy differentiated cells did not activate p38 but an innate immune response. This provides a potential unifying mechanism for HAMLET’s tumoricidal effect.

Paper III analyses the relative contribution of the protein and lipid constituents to the tumoricidal effect of HAMLET. Oleate is the deprotonated form of oleic acid and was identified as the functional lipid in HAMLET formation and cell death, including ion channel activation, gene expression effects, morphological changes and cell death. Oleate alone did not account for the properties of the complex, as only minor effects on ion fluxes and morphology were observed. By metabolomic analysis, HAMLET-induced metabolic paralysis was not reproduced when cells were treated with oleate or oleic acid alone. These results show that the lipid acts on certain cellular targets in tumor cells, but that the protein and lipid are both necessary for the tumoricidal effect of HAMLET.

In Paper IV HAMLET was identified as a new colon cancer therapeutic, which, if given early in life, also acts prophylactically. The risk to develop colon cancer is increased in families carrying specific mutations and a colon cancer model has been established in Apc\(^{min/+}\) mice carrying the same mutation. Heterozygous APC mutations cause the Wnt-signalling pathway to be overactive and lead to adenoma formation in the mouse intestine. Peroral HAMLET treatment of established tumours caused a significant reduction in tumour numbers and mortality after 40 weeks, accompanied by a reduction in nuclear β-catenin levels by an ion channel dependent mechanism. Surviving tumours showed a reduction in a number of onco-proteins and increased expression of glycolic enzymes, indicating that active glycolysis might be essential to survive HAMLET challenge. Finally, prophylactic use of HAMLET in the drinking water was shown to prevent tumor formation in APC min mice.

In summary, this thesis defines the HAMLET-sensitive phenotype on basis of an oncogenic program re-written by c-Myc and other oncogenes. A unifying mechanism for HAMLET-induced cell death is also presented and shown to involve rapid ion channel activation propagated through p38 MAPK signalling. The charged form of oleic acid is shown to be the important agonist in HAMLET and metabolic effects are discussed. Finally, HAMLET is shown to be therapeutically and prophylactically active in a mouse model of human colon cancer.


Forskningen skapar ständigt nya idéer och möjligheter för cancerbehandling, och det gäller att utveckla dessa till nytt för patienten. I den här avhandlingen presenterar jag vad som skulle kunna kallas ”ett magiskt hagelgevär” för cancerceller. HAMLET är ett humant proteinderivat bestående av två i bröstmjölk rikligt förekommande


Är HAMLET därmed det sista skottet i kriget mot cancercellen som Nixon startade för 40 år sen? Det återstår att se men i denna avhandling har vi lagt viktiga bitar i HAMLET-pusslet genom att visa på mekanismer för tumörcellers död, egenskaper som gör dem känsliga för HAMLET samt visar att HAMLET är effektivt i en tjocktarmscancermodell.
INTRODUCTION

The Cancer Cell

The cancer cell is characterized by a gain of genetic alterations that enables the cancer cell to outgrow its non-transformed neighbours, evade cell death, sustain proliferation in a hostile environment and invade and metastasise into adjacent tissue. Hanahan and Weinberg proposed that these biological capabilities constitute the “hallmarks of cancer” [1], acquired by tumour cells during their multistep evolution from a single transformed cell into a metastasising tumour. Importantly, tumours are more than a mass of rapidly proliferating lump of cells but a complex mixture of cell types participating in distinct biological processes. In addition, tumours do not entail a single cell clone with identical genomic alterations but rather a complex genomic mix up where different cells within the tumour showing distinct advantages for proliferation, resistance to apoptosis or chemotherapy. In addition to the heterogeneity within tumours considerable heterogeneity exists between tumours [2]. Whereas two tumours originating from the same tissue or cell type, through a process analogous to Darwinian evolution of species they might end up with vastly different genotypes and phenotypes. Collectively these traits make eradication of a tumour with a single targeted agent a daunting task.

Oncogenes drive cancer progression

Arguably the most prominent fundamental characteristic of cancer cells is their ability to sustain chronic proliferation. The life and death of non-transformed cells is critically controlled by the production and release of growth-promoting and anti-growth signals thereby maintaining tissue homeostasis [3]. Cancer cells frequently abuse these signals to promote indefinite proliferation thereby becoming masters of their own destiny [4]. Whereas a normal cell is critically dependent on growth signals such as EGF and NGF for proliferation, a tumour cell avoids this limitation by deregulating pathways important for growth factor signalling.

Growth factor signalling starts by the engagement of a receptor on the cell surface. Numerous examples of overexpression of these receptors occur in tumour cells, for example HER2 in breast cancer [5] and EGFR in brain cancers. By upregulation of these receptors, ambient levels of growth factors might trigger cell proliferation or the upregulation itself might induce ligand-independent signalling. More complex mechanism of self-sustaining of growth signals might occur when downstream, cytoplasmic signalling nexus proteins are mutated. In particular, mutations in the RAS/RAF/MEK/ERK-pathway are found among a very large fraction of tumours [6]. For example, a large proportion of
melanomas carry a mutation in BRAF V599E, acting as phosphomimetic turning the BRAF constantly on thereby constantly bombarding the cell with mitogenic signals forcing the cell into a constant program of cell cycling and cell division. It might be argued that activation of growth signalling pathways might be present in all tumours although this is hard to prove formally. In colon cancer, as an example, KRAS is found mutated in around 50% of clinically assessed samples. The remaining tumours could acquire growth signals by for example receptor over expression, mutations in other downstream effectors or copy number alterations of critical transcription factors.

One fundamental oncogene commonly altered to sustain proliferation is c-Myc, a basic helix-loop-helix zipper (bHLHZ) motif–transcription factor. Cellular levels of c-Myc are tightly regulated at multiple steps including transcription, translation and protein. The activity is also tightly regulated by its direct binding to another bHLHZ protein MAX [7]. Cancers frequently shows overactive c-Myc due to gene rearrangements and amplifications. Over activity can also stem from mutations in the Ras/Raf/MEK/ERK pathway, causing increased c-Myc mRNA transcription as well as stabilization of the c-Myc protein. c-Myc activation can lead to transcriptional activation or repression of specific genes. c-Myc plays a role in multiple signalling pathways including those involved in cell growth, cell proliferation, metabolism, microRNA regulation, cell death, and cell survival.

**Tumour cells alter their metabolism**

One fundamental consequence of oncogene activation seems to be a re-wiring of tumour cell metabolism. Originally discovered in the 1920s and extended in the 1950s [8], Otto Warburg made the striking observation that, even in the presence of ample of oxygen, tumour cells favoured to metabolize glucose by glycolysis rather than oxidative phosphorylation. In the presence of oxygen, non-transformed cells metabolize glucose into pyruvate and then completely oxidize pyruvate into CO$_2$ during the process of oxidative phosphorylation [9]. This is a highly efficient process generating a net of up to 36 ATP molecules per molecule of glucose. In a non-transformed cell low on oxygen, pyruvate is redirected from the mitochondrial oxidative phosphorylation and instead converted into lactate, a highly inefficient process generating a net output of only 2 molecules of ATP. What Warburg observed was that tumour cells, regardless of oxygen availability, favoured glucose to lactate conversion. This phenomenon, termed “Warburg effect”, was later shown to be almost omnipresent in tumours and the concomitant increase in glucose uptake has been exploited clinically for the detection of tumours by flourodeoxyglucose positron emission tomography.
Warburg was initially unable to explain the altered metabolism and early research focused on defective mitochondria as the culprit for the Warburg effect [10]. Recent research has however shown that most cancer cells have fully functional mitochondria and that this is not the primary explanation [11]. One alternative hypothesis would be that the hypoxic environment within the tumour favours an oxygen-independent metabolism. However, tumours cells with ample of oxygen, such as those in blood and the lungs, also favours aerobic glycolysis [9]. Modern research indicates that aerobic glycolysis is not an adaptation of tumour cells to a harsh environment or an inborn error but that the altered metabolism is beneficial for the tumour cell in that it allows the diversion of glycolytic intermediates into various biosynthetic pathways [12]. In essence, the Warburg metabolism is a metabolism fine-tuned for the assembly of new cells, arguably the fundamental hallmark of cancer.

Importantly, signalling pathways activated by oncogenes have been shown to directly control metabolic pathways. For example, activation of the PI3K/Akt-pathway, a well characterised downstream effector of growth factor receptors, cause upregulation of glucose transporters, trapping of glucose intracellularly by hexokinase and commitment of glucose to glycolysis by activation of phosphofructokinases [13,14]. Additionally, Ras, which is frequently mutated in a number of tumours, increases glucose influx by upregulation of GLUT1 [15]. Also c-Myc has prominent effects not only on the expression of glycolytic genes but has also effect on mitochondrial biogenesis and glutamine utilization [16]. A molecular explanation of the Warburg effect was recently provided by Cantley and colleagues [17], where they provided evidence that an alternative splice form of pyruvate kinase acts as gatekeeper for aerobic glycolysis. This splice-form, termed PKM2, directs pyruvate towards lactate and was found solely in tumour cells.

The identification of an altered cancer cell metabolism opens the avenue for new, targeted therapies. Given that all cells rely on glycolysis for survival it could be expected that a metabolism-targeting agent would have precluding side effects and that the therapeutic window would be narrow [18]. However, tumour cells have an unprecedented appetite for glucose and molecules targeting either this weakness or drugs that forces tumours into a normal-cell metabolism are plausible as effective anti-cancer agents.

**Ion channels as targets for anti-tumour therapy**

Plasma membrane ion channels are involved in all basic cellular processes important for tissue homeostasis, proliferation, differentiation and cell death [19]. The major mechanisms by which ion channels contribute to these effects include influx of essential signalling ions, cellular volume regulation and maintenance of membrane potential. Ion
channels can roughly be classified into three groups, according to the mode of activation (gating). Voltage-gated channels are a group of ion channels whose activity changes with the transmembrane voltage. The binding of substances to the ion channel regulates the opening of ligand-gated channels. Mechanosensitive channels comprise a group of channels gated by mechanical force that is generally generated by membrane stretch. Despite the variation in the stimulus type the outcome is the same: channel opening and closing.

Ample of evidence for the involvement of ion channels in cancer development are beginning to emerge [20]. In particular, K⁺ channels and their regulation of membrane potential, which in turn regulates transmembrane Ca²⁺ fluxes, have gained considerable interest [21]. For example, members of the Transient Receptor Potential (TRP) channels have been shown to be upregulated and involved in androgen insensitivity and apoptosis resistance in prostate cancer cells [22]. K⁺ also seems to be critically involved in regulation of metastasis and cell movement, by regulation of downstream signalling pathways involving tyrosine kinases and GTPases [23]. Ion channels also regulate intracellular pH [24], which is fundamental to promote a proliferative phenotype, but also to counteract negative feedback from the cancer metabolism depicted above.

Ion fluxes are also involved in the regulation of cell death [25,26]. In particular the cell shrinkage, an early event in apoptotic cell death, is attributable to the efflux of K⁺ ions [27]. Concomitantly, activation of caspases, mitochondrial depolarization and endonuclease activation is dependent on K⁺ efflux. High concentrations of extracellular K⁺ have been shown to abrogate both the extrinsic and intrinsic cell death programs by inhibition of cytochrome c release [28]. In addition to K⁺, Ca²⁺ seems to play an important role in apoptosis induction [29]. Cytosolic Ca²⁺ are usually kept at a low levels (~100 nM) through shuttling of intracellular calcium into the ER and Ca²⁺ extrusion into extracellular space by the plasma membrane Ca²⁺-ATPase. Ca²⁺ overload or perturbation of intracellular calcium stores by for example ER stress or activation of plasma membrane Ca²⁺ channels is able to initiate a number of apoptosis-related events, including endonuclease activation and Ca²⁺ activated cysteine proteases, such as calpains.

Ion channels are widely used as therapeutic targets for a wide range of diseases, for example calcium blockers for myopathies and lidocain (a sodium blocker) for local anaesthetics. However, the application of ion channels for tumour therapy is still in its infancy [30]. In principle, an ion channel drug could work by a multitude of mechanisms, for example, by binding to the agonist site or blocking the pore. It could also act indirectly by binding to allosteric sites or affect the binding of the ion channel to downstream signalling partners. The K⁺ channel hERG1 has been under
particular scrutiny as it is frequently overexpressed and known to control a number of behaviours related to cancer cells [31]. hERG1 blockers have been tested and shown to decrease proliferation in vitro and also decrease the growth of tumour engraftments in mice [32]. However, the hERG1 blockers also induced life threatening cardiac repolarization side effects, highlighting the pitfalls of targeting widely expressed channels for tumour therapy. The identification of tumour specific ion channel aberrations, for example upregulation, isoform assembly or mutations, would be a great aid for the development of tumour therapy. Concomitantly, agents acting specifically for the activation or inhibition could have immense clinical benefit.

The involvement of stress and MAPK signalling cancer

The extensive rewiring of normal pathways for the benefit of the cancer cell is stressful for the cellular machinery. Such stresses include DNA damage/replication stress, proteotoxic stress, metabolic stress and oxidative stress, collectively called the “stress phenotype of cancers” [33]. The DNA damage response originates from the highly unorganized state of the tumour cell genome. Tumour cells frequently show alterations of telomeres causing the formation of abnormal chromosomes and numerous amplification and deletion events. In addition to this, DNA damage repair pathways are usually inactivated or non-functional in tumour cells. The highly glycolytic state of tumour cells will also put the metabolic machinery under heavy stress, leading to a build up of unwanted metabolites as well as reactive species. Finally, the constantly dividing cancer cell shows a high protein production rate, exhausting the protein synthesis machinery and leading to ER stress.

Mitogen activated proteins kinases (MAPKs) are crucial for the cellular response to stress [34]. Three MAPK signalling units have been characterized in detail: the extracellular signal-regulated kinases (ERKs), the c-Jun amino-terminal kinases (JNK) or stress-activated protein kinases (SAPK), and the p38 MAPKs (p38). Mitogens, inflammatory cytokines, and growth factors are known to activate various MAPK signalling pathways, whereas cellular stresses such as UV light, heat, or osmotic shock selectively induce the JNK/SAPK and p38 MAPK pathways. A shared feature among MAPKs is their activation by phosphorylation of both threonine and tyrosine residues by a dual-specificity serine-threonine MAPK. In turn, MAPKs frequently phosphorylate their substrates at serine or threonine residues adjacent to prolines.

Fundamental outcomes of cellular stress include cell cycle arrest, commitment to apoptosis, the activation of DNA-repair pathways, regulation of protein translation, and the initiation of immune responses [35]. p38 orchestrates these responses by direct phosphorylation of a
number of substrates including the transcription factors ATF2 (a bZIP family transcription factor with diverse roles in development, cell growth and survival), MEF2 (important for cell differentiation and organogenesis), DDIT3 (produced in response to DNA damage), and the tumour suppressor p53.

**Exploiting the hallmarks of cancer for therapy**

The explosion of knowledge in cancer pathogenesis during the past decades has evolved the paradigm of cancer therapy from relatively nonspecific cytotoxic agents to selective, mechanism-based therapies [36]. Whereas traditional cancer chemotherapies were initially identified by their ability to kill rapidly dividing cells, their efficacy is severely limited by a narrow therapeutic index, significant toxicity due to their unspecific mode of action and cancer cells ability to acquire resistance [37]. Targeted therapies in contrast act by blocking essential pathways or mutant proteins found exclusively in tumour cells. The first targeted agent, was directed to a specific translocation (t(9;22)(q34;q11)) occurring in around 95% of patients with chronic myelogenous leukaemia and giving rise to the fusion gene BCR-ABL1 [38]. Inhibiting the oncogenic kinase BCR–ABL1 is a paradigm for clinically successful targeted therapy. A more recent success is the treatment of melanoma, a cancer form with a dismal prognosis. Overactivity of the RAS/RAF/MEK/ERK pathway feeds cells with a constant bombardment of proliferative signals [39] and melanomas frequently turn on this pathway by a specific mutation in BRAF. Vemurafenib was developed to specifically inhibit BRAF bearing a mutation at V599E present in around half of all melanomas [40]. The observation that inhibition of a single mutant protein might eradicate an entire tumour might seem counterintuitive, however. To explain this phenomena the “oncogene addiction” hypothesis has been proposed. This dogma entails that tumour cells for their well-being are highly addicted to the activation of a single oncogene and that disturbance of this oncogene alone is sufficient to kill a tumour cell.

Even though some of the targeted therapies have shown impressive initial clinical response they usually fail to give long-term clinical benefits. This probably reflects the high heterogeneity existing both between different tumours originating from the same organ but also intra-tumoural heterogeneity. For effective cancer therapy targeting a single oncogenic aberration is unlikely to be enough, a more comprehensive attack on the tumour is likely to be necessary.
HAMLET

The discovery of HAMLET and molecular structure

HAMLET was discovered by serendipity while studying anti-adhesive molecules in human milk [41]. Tumour cells were shown to undergo morphological changes consistent with apoptosis when treated with the casein-containing fraction. The protein component responsible for the tumouricidal activity was identified as α-lactalbumin [42], the most abundant protein in human breast milk with a well-known function as a substrate specifier in the lactose synthase complex. However, a tumouricidal function for α-lactalbumin had not been previously described. Early studies of the α-lactalbumin complex suggested that a multimeric form of α-lactalbumin was responsible for the activity and the complex was hence named multimeric α-lactalbumin (MAL) [43]. However, MAL could not completely explain the effects seen and it was soon realized that a cofactor was involved in the conversion of α-lactalbumin into a cytotoxic entity. Mass spectrometry ruled out a covalent modification whereas CD spectroscopy identified a stable, partially unfolded form of α-lactalbumin. The factor responsible for the stabilization was identified as oleic acid, the most common fatty acid in human breast milk.

The structure of the HAMLET complex

HAMLET consist of a single molecule of α-lactalbumin protein complexed with 4-7 oleic acid residues [44]. α-lactalbumin is the most abundant protein in human breast milk and is made up of 123 amino acids [45]. In its native conformation α-lactalbumin is a tightly packed globular protein that is stabilized by a calcium ion and four disulphide bridges [46]. Structurally, α-lactalbumin can be divided into two α-helical domains in the C- and N-terminal ends of the molecule and one bridging β-sheet domain. When the calcium is removed from the protein, using for example low pH or EDTA, the protein will adopt a molten globule structure with retained secondary structure but loss of tertiary structure.
By unfolding of $\alpha$-lactalbumin, new epitopes with the ability to bind oleic acid is presented [47,48]. Structural analysis of the HAMLET complex by ANS and CD spectroscopy, and more recently using SAXS (Ho et al., unpublished), has shown that the HAMLET complex retains a partially unfolded state but with most of the secondary structure intact. SAXS analysis identified a two-domain structure with the c-terminal end of the polypeptide forming an extended leg, which might create a conformation that is responsible for the cellular effects.

**Molecular mechanism of HAMLETs effects**

Unlike targeted therapies, by some designated as magical bullets, HAMLET affects multiple cellular targets. HAMLET has been shown to interact with artificial membranes suggesting that specific receptor interactions are not required for these interactions to occur [49]. When rounded, defined vesicles were challenged they changed morphology to amorphous shapes reflecting the formation of long membrane distensions and increased fluidity. A similar response to HAMLET was observed in plasma membrane vesicles from carcinoma cells, suggesting that direct membrane effects might contribute to the tumouricidal effect of HAMLET. HAMLET has also been shown to rapidly enter tumour cells but not non-transformed cells, through a mechanism that remains to be explained [50]. Recent results also suggest that HAMLET interacts with cellular proteins important for cell attachment [51]. Trulsson and co-workers showed that HAMLET-binding to $\alpha$-actinin disrupts focal adhesions, causes detachment and effects signalling downstream of focal adhesions.

Once intracellular, HAMLET reaches multiple targets. Early studies identified histones as critical targets in tumour cells [52]. HAMLET was found to strongly bind histone H3 and to lesser extent histones H4 and H2B using immuno-precipitation and surface plasmon resonance technology. *In vitro*, HAMLET formed insoluble precipitates with histones probably defining the final act of the cell death program. In congruence with these effects, pretreatment of tumour cells with histone de-acetylase inhibitors (HDIs) was shown to enhance the lethal effect of HAMLET [53] and the histone hyperacetylation response to HDIs increased even further after HAMLET treatment. Internalized HAMLET is also targeted to 20S proteasomes and inhibits proteasome activity and perturbs proteasome structure [54]. In the same study, HAMLET was also shown to be relatively resistant to degradation by proteasomes when compared to $\alpha$-lactalbumin.
Mechanism of tumour cell death

Although HAMLET is able to activate an apoptotic response and caspases in tumour cells, this activation is not critical for cell death as caspase knock-out cells or cells pretreated with zVAD-fmk are still HAMLET sensitive [55]. The notion that classical apoptosis is not critically involved is further emphasized by the fact that neither p53 nor Bcl-2 affects HAMLETs activity. Autophagy seems to play some role in the response to HAMLET as HAMLET-treated tumour cells show definite signs of autophagy, such as granular LC3-II staining and reduced levels of active mTOR [56]. However, the contribution of autophagy to cell death is not fully understood as opposing effects has been reported [57].

Jäättelä and colleagues identified the lysosome as one critical component of the cell death pathway activated by HAMLET [57]. Using BAMLET, the bovine counterpart of HAMLET, they were able to show that stabilization of the lysosome by overexpression of HSP70 abrogated the cytotoxic effect of HAMLET. Interestingly, HAMLET was recently shown to kill bacteria with characteristics similar to that of apoptosis [58]. *Streptococcus pneumoniae* death was accompanied by apoptosis-like morphology such as cell shrinkage, DNA condensation and degradation. The effects on the bacteria were similar to those observed on eukaryotic mitochondria, and both these effects were linked to calcium transport.

Therapeutic aspects of HAMLET

In addition to its efficiency as a cancer cell killer *in vitro*, HAMLET has shown great promise as a therapeutic agent *in vivo*. In a placebo-controlled clinical study, therapeutic efficacy against skin papillomas was observed [59]. The lesion volume was reduced by 75% or more in all 20 patients treated with HAMLET compared to only 3 of 20 patients receiving placebo. Rapid topical effects on human bladder cancers were also seen in human patients receiving intra-uteral instillations of HAMLET [60] with the long-term effects established in murine bladder cancer model [61]. Whole body fluorescence imaging showed that Alexa-labelled HAMLET was retained in the bladder of tumour bearing mice for more than two days whereas tumour-free mice rapidly discarded the HAMLET-solution. Local infusion of HAMLET into rat brains with invasively growing human glioblastoma xenografts delayed tumour development and prolonged survival [62]. Apoptosis was mainly confined to the tumour area, although HAMLET diffused throughout the infused hemisphere. In conclusion, clinical studies reported so far have established HAMLET as a therapeutic agent inducing tumour specific cell death and uptake and with no toxic effects on healthy tissues.
PRESENT INVESTIGATIONS

Aim

The overall aim of this investigation was to identify the mechanism underlying HAMLET sensitivity as well as to identify critical components explaining HAMLETs tumouricidal activity. The second objective was to understand the mechanism of cell death, from the initial interaction of HAMLET with the tumour cell to the final execution of death. Finally, the translation of HAMLET into a therapy and the molecular mechanism explaining HAMLETs in vivo effects were studied.

Paper I – Conserved features of cancer cells define their sensitivity to HAMLET-induced death; c-Myc and glycolysis

Aim and Background

The genomes of all cancer cells carry somatic mutations. However, tremendous heterogeneity exists both within tumours and between tumours, an observation that has been unequivocally shown by recent large scale sequencing efforts of wide ranges of tumours [2]. Interestingly, HAMLET seems to identify characteristics shared by all cancer cells. In this paper we investigated the basis for tumour cell sensitivity.

Results

Using a reverse-genetics approach we identified c-Myc as well as several proteins related to the glycolytic flux as important modifiers of HAMLET sensitivity. Using retroviral infection, an shRNA library was introduced into A549 cells targeting 1600 cancer related genes. By treating these cells with HAMLET and assessing the relative abundance of each shRNA before and after HAMLET exposure we identified a candidate list of genes important for HAMLET sensitivity. Among genes giving rise to resistance to HAMLET, c-Myc was one of the most prominent hits. c-Myc expression reflected the difference in HAMLET sensitivity, as lung and kidney carcinoma cells show higher expression of c-Myc than healthy cells. In addition, knockdown of c-Myc using individual shRNAs conferred significant resistance to HAMLET. c-Myc is known to drive cell cycle progression but this effect was not crucial for determining HAMLET sensitivity. Instead, c-Mycs potential effect on the metabolic state was investigated. Depletion of glucose or addition of the glycolysis inhibitor 2-deoxyglucose significantly sensitized tumour cells to HAMLET. Potential binding partners of HAMLET in the glycolytic pathway were identified using a high-content functional protein array containing 8000 human recombinant proteins. Hexokinase 1, with a
pivotal role in trapping glucose within the cytosol, was identified as a potential binding partner. The interaction between HAMLET and Hexokinase 1 was confirmed using dot blots and confocal microscopy. Finally, the overall impact of HAMLET on tumour cell metabolism was investigated using a metabolomics approach. HAMLET-treated cells were shown to contain high amount of oleic acid but also other fatty acid metabolites, indicating a saturation of the lipolytic machinery. In addition, a general shutdown of metabolism was evident 1 hour after HAMLET treatment.

**Conclusion**

The sensitivity of tumour cells to HAMLET reflects the Hallmarks of cancer, including oncogene expression and the Warburg effect. Susceptibility was modified by c-Myc expression and by Hexokinase, both affecting the metabolic state of tumour cells. HAMLET caused a general shutdown of metabolism, as supported by the metabolomics screen.

**Paper II - Selective ion channel activation by HAMLET explains tumor cell death**

**Background**

HAMLET has been shown to enact a rapid, tumour cell-specific cell death response. However, the identification of a "HAMLET-receptor" has remained elusive. In this study we aimed to reconcile recent observation of a tumour membrane effect with early observation of an increase in intracellular calcium [41]. We present a unifying mechanism for HAMLET-induced cell death.

**Results**

Exposure of tumour cells to HAMLET was shown to induce a rapid efflux of potassium and an influx of calcium and sodium. Broad inhibitors of ion channels, including amiloride and barium chloride, were shown to abrogate the fluxes, indicating the activation of an ion channel. Patch clamping experiments confirmed these findings as evidence of activation of a single ion channel with permeability for all three cations were observed. Importantly, the ion channel activation could not be reproduced using the free fatty acid or unfolded protein alone. Inhibitors that prevented the current also prevented all aspects accompanying
tumour cell death, including ATP drop, shrunken morphology and signalling. Using transcriptomics we identified a transcriptional program activated by HAMLET, characterised by activation of the p38 MAPK pathway and upregulation of cell death and ER stress genes. The activation of p38 was confirmed by phospho-specific antibodies and was shown to be time and dose dependent. Activation of p38 is an integral part of the cell death program as inhibition of p38, either by small molecule inhibitors or siRNA, drastically reduced cell death. Healthy cells responded to HAMLET challenge with innate immunity and survival rather than a p38-dependent death response.

**Conclusion**

For the first time we provide a potentially unifying mechanism for HAMLET induced cell death. A single ion channel is shown to be activated by HAMLET and blocking ion fluxes abrogates most downstream effects. These results also indicate that HAMLET may be used to identify ion channels as attractive targets for cancer therapy.

**Paper III - Lipids as tumoricidal components of HAMLET; contributions to signalling and death**

**Background**

The importance of the fatty acid for HAMLETs activity in relation to the full complex remains controversial. Cis-monounsaturated fatty acid has been identified as the optimal co-factor for HAMLET formation but the relative contributions of the fatty acid, the unfolded protein and the complex remains to be fully elucidated.

**Results**

HAMLET was formed with oleate or oleic acid and the resulting complexes were compared in terms of their structure and ability to kill tumour cells. Oleic acid or oleate formed HAMLET complexes with similar efficiency and with structures that closely resembled each other. Oleic acid was largely inert to tumour cells whereas oleate was able to reproduce some of the effects of the HAMLET complex. Transcriptional responses to HAMLET and high concentrations of oleate were largely overlapping even though HAMLET caused upregulation of a distinct set of DNA damage genes. By mass spectrometry analysis of the cellular metabolome, both oleate and HAMLET triggered an increase in intracellular lipid levels. However, only HAMLET triggered a general
metabolic paralysis. In addition, HAMLET caused a depletion of cellular carnitine levels and concomitant increase in oleoyl-carnitine levels.

**Conclusion**

Both oleic acid and oleate are efficient cofactors for formation of the HAMLET complex. Cell appeared largely to be inert to oleic acid, but oleate reproduced some of the complex responses to HAMLET. The full complex consisting of both the fatty acid and the unfolded protein was necessary for the full cell death response to occur.

**Paper IV- HAMLET treatment of colon cancer in APCmin mice; peroral application and disruption of the Wnt/β-catenin-signalling pathway**

**Background**

Previous studies have established HAMLET as an anti-cancer agent with clinical potential. Colorectal tumours are frequently initiated by inactivation of the APC tumour suppressor gene, which renders the Wnt-signalling pathway overactive. Based on HAMLETs properties as a milk protein and previously documented resistance to gastric enzymes, we used APC^Min/+^ mice, which carry a germline mutation in APC, to study the therapeutic potential of HAMLET in colon cancer.

**Results**

HAMLET was administered per-orally for ten days to tumour-bearing mice and tumour development was assessed five weeks after the final HAMLET administration. A significant reduction in tumour numbers, tumour size, as well as in a number of key markers for proliferation and oncogenesis were observed after HAMLET administration. HAMLET was also shown to accumulate in tumour tissue but no gross side effects were observed in adjacent healthy tissue. Transcriptomic analysis of remaining tumour tissue identified a highly glycolytic phenotype in surviving tumour cells, indicating that HAMLET might target glycolysis for its effect. Untreated tumours showed higher expression of T-cell marker genes, indicating that HAMLET might purge tumours of T-cells. In vitro, HAMLET was shown to cause caspase, as well as ion channel, dependent degradation of β-catenin and cause nuclear exit.
**Conclusion**

These results indicate that HAMLET has potential as an anti-cancer agent in colon cancer. It also reveals the Wnt pathway as one possible HAMLET target. It also establishes the Warburg phenotype as important for HAMLET sensitivity as well as confirms the importance of ion channels for *in vivo* effects of HAMLET. The work confirms and extends previous studies have established HAMLET as an anti-cancer agent with clinical potential.

**DISCUSSION**

**HAMLET acts as a “magic shotgun”**

Contemporary targeted therapies have been described as magic bullets, indicating that they targets tumour specific aberrations and thereby specifically kills tumour cells. They are all designed to hit one single target. HAMLET, in contrast appears to have multiple targets and this feature is inherent to HAMLET’s tumoricidal effect. Since the discovery of HAMLET, mitochondria, calcium signalling, histones, proteasomes, α-actinins and the plasma membrane have been identified as HAMLET targets. The cell death modality has been discussed as a function of caspase activation, DNA damage, autophagy and lysosomal cell death programs.

These mechanisms do not explain HAMLET-induced cell death, however. Since the discovery of HAMLET we and others have searched for THE events that initiate cellular attack by HAMLET, hoping for a unifying mechanism of action. Knowing that the entire process of HAMLET induced cell death is multifaceted and complex, is it still possible that there are key molecular interactions that trigger the downstream events, that converge on cell death? The ion fluxes induced by HAMLET suggest that there are distinct early events, which, if inhibited, prevent subsequent cellular responses. The molecular details of such interactions need further study, however.

Acting on multiple targets and through multiple pathways is traditionally not viewed as something positive. Concerns are associated with unclear modes of action for drug development and litigation as well as complex side effects. On the other hand, it is becoming increasingly clear that targeting a single oncogenic aberration will rarely be curative, as resistance due to acquired mutations develops rapidly.
By the engagement of multiple pathways and cellular compartments, HAMLET achieves several beneficial goals. Resistance does not develop readily, as shown in vitro in cellular propagation studies. Loss of a single target is unlikely to create resistance as many other interactions potentially can take over and kill the cells. This spectrum of interactions may also explain the broad effects of HAMLET against many different tumours and the increased resistance of healthy, differentiated cells.

**HAMLET induces a transcriptional response characterised by p38 MAPK signalling, cell death genes and ER stress**

In the outset of this project we defined the transcriptional response to HAMLET using microarray technology. By the simultaneous quantification of all mRNA transcripts in one single experiment microarray technology enables an unbiased approach to the study of cell biology. By treatment of a carcinoma cell line with HAMLET, purification of totalRNA and subsequent labelling and hybridisation to a whole-genome human microarray we identified transcriptional regulation falling in predominantly three categories; p38 MAPK signalling, cell death genes and the ER stress pathway. HAMLET increased expression of genes in the p38 MAPK signalling pathway. Cell survival and p38 signaling are transcriptionally and post-transcriptionally regulated, through death receptors, survival pathways or pro- and anti-apoptotic Bcl-2 proteins, which may activate p38 by secondary routes, e.g., by the production of reactive oxygen species (ROS) [63]. The second category of genes activated by HAMLET was involved in cell death. HAMLET has been shown to increase the expression of for example KLF6, DDIT3, GADD45 and ATF3, all key signaling components in cell death pathways. Further analysis of the exact transcriptional regulation of cell death genes will enable us to pinpoint more specifically the mechanism of cell death execution by tumour cells after HAMLET treatment. Genes in the ER stress pathway were also regulated by HAMLET, as discussed in detail below. It can be debated if transcriptional regulation is crucial for HAMLET induced cell death. However, when the p38 MAPK pathway was blocked either using siRNA or pharmacological inhibitors, cells failed to undergo cell death showing that microarray technology is indeed useful to elucidate both cell death pathways but also bystander phenomena.

Importantly, using transcriptomic technology, healthy cells were shown to be much less responsive to HAMLET challenge. HAMLET was identified as an immune activator, mostly targeting signaling pathways involved in innate immunity. As a consequence, pro-inflammatory cytokines were produced in healthy cells but this immune response was lower or absent in tumor cells. The functional importance of these pathways in healthy cells remains to be explored.
c-Myc and tumour cell metabolism determine HAMLET sensitivity

Deregulated expression of c-Myc has been shown to occur in a wide range of human cancers and is often associated with poor prognosis, indicating a key role for this oncogene in tumour progression [64]. Strikingly, it has recently been shown that the enhanced expression of c-Myc contributes to almost all every aspect of cancer cell biology. The ability of c-Myc to drive cellular proliferation and to inhibit cellular differentiation has been known for a long time, but c-Myc’s importance for vasculogenesis, cell adhesion, cellular metabolism, metastasis and genomic instability has recently gained considerable interest [65]. Consistent with this, disruption of c-Myc in c-Myc-induced transgenic tumours triggers proliferative arrest and re-differentiation of tumour cells usually resulting in rapid tumour regression [66]. Despite this, targeting c-Myc for pharmacological therapy has proven challenging for a number of reasons [67]; c-Myc exerts its effect through protein-protein and protein-DNA interaction, which are usually not tractable with small molecule inhibitors. Secondly, c-Myc is usually not mutated itself but its effects are a consequence of induction by upstream oncogenic signals. Finally, c-Myc is essential for proliferation of stem cells, indicating that systemic blocking of c-Myc’s function might trigger irreversible side effects.

In paper I the activity of HAMLET was shown to be critically linked to the expression of c-Myc. The dependence on c-Myc overexpression as a determinant of HAMLET sensitivity was related to the metabolic program employed by tumour cells. It should be noted that c-Myc has widespread effects and is also known to induce expression of genes driving cell proliferation. This effect is unlikely to explain the c-Myc-dependence as cell cycle arrest of lung carcinoma cells (by serum starvation or double-thymidine block) did not significantly alter HAMLET sensitivity.

c-Myc does not only increase the expression of glycolysis related genes but is also heavily involved in regulation of glutamine metabolism. Glutamine is together with glucose the main substrate for catabolic processes and tumour cells seems to have an extreme appetite for glutamine, even so that some cancer patients shows depressed plasma glutamine levels [68]. The knockdown of c-Myc is likely to force tumour cells into a less “glutaminophilic” phenotype. Whether this effect of c-Myc has any relation to c-Mycs effect on HAMLET sensitivity remains to be elucidated.

Finally, c-Myc is also a critically regulated by β-catenin [65]. In paper IV, intestinal tumours overexpressing β-catenin are shown to be sensitive to
HAMELT, giving confidence to the in vivo significance of c-Myc expression for HAMLETs activity.

The HAMLET-activated ion channel remains elusive

In paper II we identify perturbations to ion fluxes as early events in the interaction between HAMLET and tumour cells. Furthermore, these fluxes are directly involved in the cell death response, as pretreatment with broad ion channel inhibitors (amiloride, BaCl₂) abrogated many changes accompanying tumour cell death. Patch clamping experiments identified one single ion channel with permeability for Ca²⁺, Na⁺ and K⁺. Importantly, our whole cell patch clamp data showed a marked time- and voltage-dependent inactivation, and a permeability profile not consistent with simple diffusion, strongly arguing against that HAMLET merely forms pores in the membrane. The biophysical and pharmacological characteristics of the ion channel activated by HAMLET differ from those of classical TRP-, ENaC- or CNG-channels. Detailed experiments ruled out the CNG-channels, as prototypical activators of this channel had a signature not resembling the one activated by HAMLET.

The results suggest that HAMLET triggers ion fluxes through a novel channel but the exact identity of the activated ion channel remains unknown. Aside from the patch clamping, a number of experiments have been carried out to identify the ion channel. First, siRNA knockdown of a number of channels inhibited by amiloride and BaCl₂ has been performed, but no knockdown has significantly affected HAMLET induced cell death. Secondly, the use of a small library consisting of 80 highly specific inhibitors was unable to resolve the responsible ion channel, aside from amiloride.

Currently we are employing two approaches to identify the responsible channel. Using a forward genetics approach we try to isolate a clone of A549 lung cancer cells that are significantly less sensitive to HAMLET by repeated exposure. Having a resistant clone of A549 cells would allow us to ask a number of questions to address the action of HAMLET. By RNA-seq we would identify pathways being transcriptionally up/downregulated as well as mutations that alter HAMLET sensitivity. The second approach entails a focused siRNA library targeting all known ion channels, provided that the novel channel is represented in this library.

One interesting possibility is that HAMLET is the ion channel. It has recently been shown that amyloid fibrils are able to form cation-permeable ion channels. Aβs forms pore-like complexes in cell membranes, detected by electrophysiological recordings in artificial and biological membranes [69]. Additionally, electron microscopy of post-
mortem brains have revealed porelike Aβ structures in cell membranes of postmortem brains from patients with Alzheimer’s disease [70]. HAMLET has been shown to form annual oligomers under in vitro conditions [71], but their presence in a cellular or in vivo setting remains to be established. In addition, if HAMLET-pores exist they need to be shown to transport ions. Crucial experiments To define if HAMLET actually acts as an ion-channel, patch clamping of artificial lipid vesicle is needed as well as detailed studies, using electron microscopy, of cell membranes of HAMLET-treated membranes.

**HAMLET triggers ER stress in carcinoma cells**

HAMLET internalization exposes carcinoma cells to partially unfolded α-lactalbumin, which is partially resistant to proteasome degradation and remains intracellular [54]. Endoplasmic reticulum stress (ER stress) is elicited if the cell is burdened with an overwhelming amount of unfolded proteins and activates a set of signaling pathways collectively known as the Unfolded Protein Response (UPR). UPR is mediated by three transmembrane sensors spanning the ER membrane: pancreatic eIF2-α kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1). These three sensors each initiate signaling cascades aimed at reducing protein synthesis and the unfolded protein load.

The ER stress response identified in the transcriptomic analysis in Paper II has been characterized further but was not included as a part of that paper. A lethal dose of HAMLET in lung carcinoma cells caused a marked increase in the transcription of several ER stress-related genes, including ATF4, BiP, IRE1, PERK and XBP1 (top...
To confirm the activation of the ER stress in carcinoma cells we studied activation of the ER stress sensors. We detected a rapid increase in phosphorylated eIF2α in lung carcinoma cells after HAMLET exposure, which was sustained for at least three hours. A decrease in ATF6 and an increase in cleaved ATF6 were detected after three hours (middle panels). Activation of IRE1 results in splicing of the XBP1 mRNA to its active form, XBP1s, which restores normal ER function through diverse targets [72]. XBP1 splicing was detected by PCR in HAMLET-treated carcinoma cells, which contained an RNA band of 442 bp corresponding to XBP1 and a smaller band, lacking 26 base pairs, corresponding to XBP1s (lower left panel). Taken together, our results show that HAMLET is indeed triggering a rapid ER stress response. To determine whether the ER stress response is important for cell death, we quantified mRNA levels of CHOP/DDIT3, a pro-apoptotic transcription factor strongly implicated in ER stress-induced cell death [73]. CHOP/DDIT3 mRNA was increased 30-fold, three hours after HAMLET treatment.

The specific mechanisms by which HAMLET activate ER stress are yet to be completely understood, but several interactions may contribute. We have recently shown that HAMLET interacts directly with proteasomes [54], which play a crucial role in ER stress and the unfolded protein response [74]. Additionally, in preliminary experiments, HAMLET was found to interact directly with ER stress sensors. HAMLET may also indirectly perturb the protein folding capability of the cell by decreasing ATP levels [75] and by causing mitochondrial damage and permeabilization [76].

**Glycolysis inhibition as a target for tumour therapy**

The ATP-dependent phosphorylation of glucose to form glucose-6-phosphate (G-6-P) is the first and rate-limiting reaction in glycolysis [77] and is catalyzed by hexokinase. Paper I indicated that HAMLET might interact directly with hexokinase, thus interfering with its function. Inhibition of hexokinase would be predicted to reduce glucose phosphorylation, thereby creating a state similar to glucose deprivation in cancer cells. Several studies have indicated that hexokinase plays a critical role in initiation and maintaining the high catabolic rate of tumour cells [78]. Hexokinase is found within the cytosol or bound to the mitochondrial outer membrane. At the mitochondrial membrane, hexokinase interacts with voltage dependent anion channels (VDACs), facilitating the transport of ATP over the mitochondrial membrane [79]. VDACs has been shown to be important in the initiation of mitochondrial permeability transition and release of apoptotic factors such as cytochrome c [80]. As HAMLET has been shown to interact with and cause mitochondrial permeability transition [76], it is plausible that
HAMLET-hexokinase interactions at the mitochondrial membrane help to initiate cell death. If this is true, overexpression of either hexokinase or VDACs should protect tumour cells from HAMLET-induced cell death. Consistent with this, siRNA mediated inhibition of hexokinase 1 was shown to reduce the sensitivity to HAMLET. However, other mechanisms such as ER stress and DNA damage might also cooperate to cause mitochondrial damage.

**MAP Kinases are critically involved in the tumouricidal response to HAMLET**

In paper II we show that HAMLET activates a rapid dose and time-dependent activation of p38 MAPK. P38 MAPK activation has previously been shown to be activated by numerous cellular stress responses, including cytokines, osmotic stress and irradiation [81]. Interestingly, a close relationship exists between the p38 MAPK activation and inactivation of the ERK signaling pathway. For example, overexpression of ERK in NIH 3T3-cells abrogates p38 dependent induced cell death following UV irradiation. Thus it seems plausible that the ultimate outcome in terms of cell survival is closely knitted to the balance between p38 and ERK activation.

We have gathered unpublished data pointing to a direct inactivation of the RAS/RAF/MEK/ERK-pathway by direct binding of HAMLET to RAF proteins. In a proteomic screen, HAMLET bound to cRAF and BRAF, which was confirmed in co-immunoprecipitation experiments. The binding was further studied in vitro using surface plasmon resonance techniques, showing a binding constant between HAMLET and BRAF in the low micromolar range (left panel below). Importantly, binding of BRAF to HAMLET was shown to inhibit its kinase activity (right panel below). The direct effect of HAMLET on BRAF suggests a parallel mechanism of ERK inactivation but the cause-effect relationship between p38 activation and ERK inactivation remains to be fully elucidated.

![Graph showing BRAF activity inhibition by HAMLET](image)

In addition to the cation imbalance and HAMLETs effect on RAF signaling, the unfolded protein response could be an activator of p38 MAPK. ER stress activates ASK1 [82], a well-known MAP kinase kinase...
in the p38-signaling pathway [83] providing a plausible intermediate between p38 and ER stress. A link between p38 cell death and ER stress-induced cell death has previously been observed in carcinoma cells after infection with Japanese encephalitis virus [84]. Furthermore, in a screen for ER stress inhibitors, p38 activation was identified as an essential event of ER stress-induced cell death [85]. The Unfolded Protein Response is primarily cytoprotective, but when the burden of misfolded/unfolded protein reaches beyond the critical level for ER homeostasis, it triggers cell death [86]. Cell death in response to ER stress has apoptosis-like characteristics, including DNA fragmentation, chromatin condensation and cell shrinkage, as seen in response to HAMLET.

**HAMLET triggers an immunogenic cell death which should be optimal for in vivo anti-cancer activity**

Intuitively, simply killing the tumour cells, by for example induction of apoptosis, should be enough to remove the tumours. This view has however been challenged. Guido Kroemer and colleagues have identified and characterized immunogenic cell death [87], whereby a tumour cell exposes calreticulin (CRT), mediated by an ER stress dependent pathway and critically regulated by phosphorylation of eIF2α. When tumourigenic cell death is initiated, by for example oxaliplatin the CRT-exposure pathway is initiated by pre-apoptotic ER stress and phosphorylation of eIF2α by the ER resident kinase PERK. This is followed by caspase 8-mediated cleavage of BAP31, activation of Bax and Bak, transport of CRT from the ER to the Golgi and finally presentation of CRT on the cell membrane. Importantly, perturbations to ER calcium levels have been shown to be critically involved as it controls the conformation of CRT [88]. The importance of an immunogenic cell death becomes apparent when you consider the daunting task that the immune system has to challenge when tumour therapy is initiated. If all dying cells were immunogenic, the problem with autoimmunity would be extremely high, making the proposition of a well-defined immunogenic cell death plausible.

As HAMLET has been shown to induce a multifaceted cell death response with characteristics similar to that of an immunogenic cell death it is feasible to believe that HAMLET is indeed killing cells by an immunogenic cell death. HAMLET has been shown to induce a strong and rapid eIF2α activation, the key player for immunogenic cell death. Additionally, the activation of an immune response in healthy, differentiated cells could be one important factor for the regulation of the immune response to HAMLET-killed tumour cells. Key experiments to further address the possibility of an immunogenic cell death activated by HAMLET include identification of cleaved BAP31 as well as CRT on the cell membrane of HAMLET-exposed tumour cells.
HAMLET shows therapeutic efficacy in a model of colon cancer

Virtually all cases of colon cancer harbour mutations in the Wnt signalling pathway [89]. In paper IV we show that HAMLET is therapeutically active in a mouse colon cancer animal model that closely mimics the aberration seen in patients. In a non-transformed cell, β-catenin associates with a multi-protein complex that contains the adenomatous polyposis coli (APC) protein. APC functions as a scaffold to coordinate β-catenin phosphorylation and degradation by the proteasome. When cells are exposed to Wnt, β-catenin escapes proteasomal degradation, and is chaperoned to the nucleus by APC. Inherited APC mutations give rise to familial adenomatous polyposis, a disease where afflicted individuals are burdened by thousands of intestinal polyps early in adulthood.

The effects on β-catenin were shown to be linked to ion channel activation as well as caspases in in vitro experiments. The translation of the pathways activated in vitro to the in vivo effects of HAMLET remains to be fully established. For example, the high-affinity binding of HAMLET to histones previously reported to dictate the end stage of tumour cell death remains to be observed in vivo. However, using transcriptomic analysis of tumours surviving HAMLET challenge, an increase in expression of genes in the glycolytic machinery was observed. This goes well with the findings reported in paper I were HAMLET was shown to attack hexokinase, the rate limiting step in glycolysis. These observations also indicate that combinatorial treatment of tumours with glycolytic inhibitors, such as 2-deoxyglucose or metformin, might potentiate HAMLETs effects in vivo. The importance of ion channel activation in vivo would also give important knowledge of how to optimally apply HAMLET in a clinical setting. However, observing ion fluxes in vivo is technically challenging.
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