The human milk protein-lipid complex HAMLET disrupts glycolysis and induces death in
*Streptococcus pneumoniae*

Hazeline Roche-Hakansson1#, Goutham Vansarla2, Laura R. Marks3, and
Anders P. Hakansson2*

From 1Department of Microbiology and Immunology, University at Buffalo, State University of New
York, Buffalo, NY, 2Division of Experimental Infection Medicine, Department of Translational Medicine,
Lund University, Malmö, Sweden and 3Department of Medicine, Barnes-Jewish Hospital, Washington
University, St. Louis, MO, USA

Running title: Pneumococcal glycolysis inhibition by HAMLET

# Present address: Allergan Inc, New Jersey, NJ, USA

*To whom correspondence should be addressed: Anders P. Hakansson, Division of Experimental
Infection Medicine, Department of Translational Medicine, Lund University, 53 Inga Marie Nilsson
Street, SE-21428, Malmö, Sweden, Phone. +46-725-190255; Fax. +46-40-337044; E-mail:
anders_p.hakansson@med.lu.se.

Keywords: *Streptococcus pneumoniae*, glycolysis, cell death, human milk, alpha-lactalbumin, metabolism,
HAMLET (human α-lactalbumin made lethal to tumor cells), oleic acid, antibiotic

**ABSTRACT**

HAMLET is a complex of human α-lactalbumin (ALA) and oleic acid and kills several Gram-
positive bacteria by a mechanism that bears resemblance to apoptosis in eukaryotic cells. To
identify HAMLET’s bacterial targets, here we used *Streptococcus pneumoniae* as a model organism
and employed a proteomic approach that identified several potential candidates. Two of these targets
were the glycolytic enzymes fructose bis-phosphate aldolase (FBPA) and glyceraldehyde 3-phosphate
dehydrogenase (GAPDH). Treatment of pneumococci with HAMLET immediately inhibited their ATP and lactate production, suggesting that HAMLET inhibits glycolysis. This observation was supported by experiments with recombinant bacterial enzymes, along with biochemical and bacterial viability assays, indicating that HAMLET’s activity is partially inhibited by high glucose-mediated stimulation of glycolysis but enhanced in the presence of the glycolysis inhibitor 2-deoxyglucose. Both
HAMLET and ALA bound directly to each glycolytic enzyme in solution and solid phase
assays and effectively inhibited their enzymatic activities. In contrast, oleic acid alone had little to
no inhibitory activity. However, ALA alone also exhibited no bactericidal activity and did not block
glycolysis in whole cells, suggesting a role for the lipid moiety in the internalization of HAMLET into
the bacterial cells to reach its target(s). This was verified by inhibition of enzyme activity in whole
cells after HAMLET but not ALA exposure. The results of this study suggest that part of HAMLET’s
antibacterial activity relates to its ability to target and inhibit glycolytic enzymes, providing an
example of a natural antimicrobial agent that specifically targets glycolysis.

HAMLET (Human alpha-lactalbumin made lethal
Pneumococcal glycolysis inhibition by HAMLET

to tumor cells) consists of human alpha-lactalbumin (ALA) in a partially unfolded state, stabilized by binding the human milk-specific fatty acid oleic acid (OA; C18:1 cis-9) (1). HAMLET was first purified from human milk casein and shown to kill tumor cells by induction of apoptosis, while sparing healthy cells (2, 3). Part of HAMLET’s activity is related to its ability to enter cancer cells and interact with mitochondria to induce depolarization and permeability transition that leads to apoptosis execution (4, 5). Using an shRNA-based screen in tumor cells, Svanborg and collaborators demonstrated that glycolytic enzymes were required for full HAMLET activity (6). It was therefore suggested that HAMLET interferes with glycolysis to mediate a change in cancer cell metabolism as part of its death induction.

HAMLET also kills several species of bacteria (the evolutionary predecessor to mitochondria), with its highest activity against Streptococcus pneumoniae (7, 8), an aerotolerant, anaerobic organism that lacks the Krebs’ cycle and oxidative phosphorylation and relies primarily on glycolysis and fermentation for energy production (9). Interestingly, akin to features seen in eukaryotic cells, HAMLET-induced death in bacteria is accompanied by depolarization of the bacterial membrane that requires sodium-dependent calcium transport, cell shrinkage, DNA condensation and fragmentation, as well as other biochemical hallmarks of mitochondria-induced apoptosis (8, 10). These phenotypes are also present when these bacteria die from other physiological stimuli, such as starvation, and represent a novel and general bacterial death mechanism that we are currently exploring. This suggests that physiological cell death pathways in prokaryotic and eukaryotic cells may be more similar than previously considered.

Besides its direct antibacterial activity, HAMLET can also potentiate the effect of antibiotics to render bacterial species sensitive to antibiotics they are resistant to (11, 12). This effect is not limited to species sensitive to HAMLET but can also be seen for antibiotic-resistant organisms of species that are not directly killed by HAMLET (12). HAMLET’s potentiating effect was shown to also require ion transport and membrane depolarization and HAMLET-treatment resulted in an increased binding/association of the antibiotics used to the bacterial population treated (12).

In this study, we show that HAMLET disrupts glycolysis in whole pneumococci and that this disruption is partially responsible for, and clearly potentiates, HAMLET’s bactericidal activity. Unlike previous studies associating the bactericidal activity of HAMLET with the OA component, this study showed that the protein component (ALA) was responsible for HAMLET’s inhibitory effect on glycolysis in whole cells. We show that HAMLET binds to and inactivates two key glycolytic enzymes, fructose-bis phosphate aldolase (FBPA) and glyceraldehyde-3 phosphate dehydrogenase (GAPDH), both in vitro and in vivo. This inhibition results in an impaired production of ATP that augments activation of HAMLET’s cell death mechanism. A detailed understanding of HAMLET’s inhibition of glycolysis could provide future leads to therapeutic intervention against a broad range of bacterial species.

RESULTS

Bactericidal activity of HAMLET, ALA and oleic acid

HAMLET was used to treat S. pneumoniae D39 in a time-kill assay over 1 hour. This treatment caused a concentration-dependent death of pneumococci with a 3 log₁₀ reduction in bacterial viability at approximately 100 µg/ml (6 µM) and eradication of the inoculum reached at 250 µg/ml (15 µM). (Fig. 1, solid line). As HAMLET is a complex of ALA and oleic acid, we also tested each component separately. Native ALA had no bactericidal activity (Fig. 1, dashed line) and we know from previous experience that no death occurs even after exposure to 10,000 µg/ml (7, 10). Oleic acid (OA), however, has been shown to have
bactericidal activity by itself (13). Measurements of HAMLET:OA stoichiometry consistently show a molar ratio of approximately 1:5 (14, 15) with OA constituting approximately 9-11% of the total complex weight. To understand the role of OA in the bactericidal activity of HAMLET we treated the bacteria with OA at the concentration present in the complex (based on a 10% w/w OA content). Although OA had bactericidal activity by itself, the concentration associated with various concentrations of HAMLET-complex consistently resulted in significantly lower bactericidal activity than the HAMLET-complex itself (Fig. 1, dotted line). These results suggest that although lipid is required for HAMLET’s bactericidal activity, both ALA and OA are necessary and contribute to bacterial killing.

HAMLET inhibits glycolysis and ATP production in pneumococci

To investigate the role of HAMLET-treatment on glycolysis activity and ATP production, D39 pneumococci were washed and resuspended in PBS without any carbon source. At T = 0 min the bacteria were energized by adding 10 mM glucose and at T = 5 min or T = 10 min, 125 µg/ml of HAMLET (a concentration that resulted in a 4 log₁₀ reduction in viability over 1 hour) was added to subsets of energized bacteria, and lactate secretion from the cells and ATP content in the cells were measured over time (Fig. 2A and B). HAMLET addition resulted in an immediate and drastic inhibition of both ATP and lactate production over time, resulting in significantly lower ATP accumulation and lactate production in the cells at the 30 minute time point (Fig. 2).

To make sure that this was not just an effect of the bacteria dying rapidly in the presence of high concentrations of HAMLET, the cells were treated with sub-lethal concentrations of HAMLET (25, 37.5, and 50 µg/ml). HAMLET was added 5 minutes after energizing the cells with glucose and ATP content of the bacteria was measured (Fig. 2C). No effect was seen for the lowest HAMLET concentration but both 37.5 and 50 µg/ml HAMLET significantly reduced the ATP production in the cells after 30 minutes of incubation (P < 0.05 and P < 0.01 for 37.5 µg/ml and 50 µg/ml, respectively).

Treatment with native ALA had no effect on either ATP or lactate production (Fig. 2A and B). Similarly, addition of 12.5 µg/ml OA (equivalent to the amount of lipid present in 125 µg/ml of HAMLET that resulted in an >2 log₁₀ reduction in bacterial viability) did not significantly affect ATP production in the pneumococci (Fig. 2D), suggesting that bacterial death induced by OA was not directly associated with inhibition of glycolysis or energy production.

Modulation of glycolysis function affects pneumococcal sensitivity to HAMLET

We next addressed the role of HAMLET on glycolysis by testing HAMLET’s activity in the presence of a high concentration of glucose that would accelerate glycolysis or in the presence of 2-deoxyglucose, a glucose analog that competitively inhibits glycolysis as it cannot be hydrolyzed for energy. Bacterial cells in buffer alone (PBS) served as a control. None of the treatments had any impact on bacterial survival by themselves over the 1-hour time course of the assay. HAMLET was added at increasing concentrations and incubated for 1 hour at 37°C and viability was monitored by viable plate counts (Fig. 3A). Addition of glucose in the buffer significantly reduced HAMLET-induced death at all concentrations tested, with 1.3 log₁₀ less death occurring at the highest concentration compared with cells treated in the absence of carbohydrate (P < 0.01), and 2.7 log₁₀ less death compared with cells treated with the glycolysis inhibitor 2-deoxyglucose (P < 0.01), indicating that modulation of glycolysis activity affects HAMLET’s bactericidal activity.

Treatment with bactericidal concentrations of OA was not affected by the presence of glucose or 2-deoxyglucose in the media (Fig. 3B), again suggesting that OA-induced death do not act by
directly regulating glycolytic activity and energy production in the pneumococcal cells.

**HAMLET binds to FBPA and GAPDH**

To identify potential bacterial targets responsible for HAMLET’s glycolytic inhibition, we first separated bacterial lysate from strain D39 by one-dimensional SDS-PAGE, transferred it to nitrocellulose membrane and overlaid the blot with biotinylated HAMLET (biotinylation did not affect HAMLET’s bactericidal activity as shown in Fig. S1). Several bands were identified as potential interaction partners for HAMLET (Fig. 4A) and the identities of the two strongest bands were determined by N-terminal sequencing using Edman degradation. Only FBPA (31.4 kDa) and GAPDH (35.9 kDa) were possible candidate proteins based on the N-terminal sequence data and on having appropriate molecular mass in the blot (Fig. 4A).

In a second approach, biotinylated HAMLET conjugated with NHS-LC-Diazirine, a UV-excitable cross-linker, was added to pneumococcal cell lysate. After incubation, the reactions were cross-linked by UV light, and HAMLET and its bound targets were captured on streptavidin-conjugated magnetic beads and captured proteins were identified by LC-MS/MS. Two independent assays were performed, and the results of the assay confirmed 3 peptides and 1 peptide, respectively, with perfect match for FBPA and 8 peptides and 5 peptides, respectively, with perfect match for GAPDH in experiment 1 and 2. As the identification of captured proteins were performed by independent peptide sequencing the identity will have >99.9% certainty if two or more peptides are identified (ProtTech). In conclusion, both assays identified two central enzymes in glycolysis (FBPA and GAPDH) as potential HAMLET targets.

To ensure a direct interaction between HAMLET and the two glycolytic proteins, pneumococcal, recombinant FBPA and GAPDH were produced and separated by SDS-PAGE, transferred to nitrocellulose, and overlaid with biotinylated HAMLET or ALA. Interestingly, both HAMLET and ALA (that had no effect on cell viability, ATP or lactate production) bound strongly to FBPA and GAPDH (Fig. 4B). Binding of GAPDH and FBPA to HAMLET was also observed in solution after capturing the His-tagged enzymes by magnetic beads (Fig. S2). To confirm that HAMLET’s interaction with FBPA and GAPDH was specific, binding assays employing the glycolytic enzyme glucokinase (GK; also known as hexokinase) and dihydrolipoamide dehydrogenase (DLDH; the E3 component of 2-oxo acid dehydrogenases) were performed in solution. No binding was observed to glucokinase; however, some binding was observed to DLDH (Fig. S2).

**HAMLET specifically inhibits FBPA and GAPDH enzymatic activity**

As HAMLET bound strongly to FBPA and GAPDH, two central glycolysis enzymes, we were interested in investigating whether this binding resulted in inhibition of their activity as a mechanism of blocking ATP and lactate production. Recombinant enzymes were assayed for enzymatic activity in the presence of increasing concentrations of HAMLET, ALA, and OA. Immunoglobulin G (IgG) was used as an unrelated protein control for comparison (Fig. 5).

HAMLET and ALA both showed an effective inhibition of FBPA activity with the IC50 being approximately 2 µg/ml for both proteins and with over 75% of inhibition seen at a concentration of 8 µg/ml. The inhibitory activity of OA was negligent with around 5% inhibition seen at the highest concentration tested that represents 80 µg/ml HAMLET (8 µg/ml OA; Fig. 5A). IgG did not inhibit FBPA activity at all (Fig. 5A).

For GAPDH the general inhibitory activity was somewhat lower with around 50% inhibition seen at 6 µg/ml of HAMLET and around 40% inhibition seen for ALA at the same concentration (Fig. 5B). OA inhibited GAPDH to a significantly lower degree (22%; P < 0.01 compared with HAMLET’s inhibitory activity) at the
concentration present in the HAMLET complex (Fig. 5B). Again, IgG lacked any inhibitory activity. This indicates that HAMLET and ALA show strong inhibitory activity of both enzymes whereas OA shows only a slight inhibitory activity against GAPDH.

HAMLET did not inhibit the activity of glucokinase even at high concentrations (100 µg/ml), which was consistent with a lack of binding between the two proteins (Fig. S3A). Similarly, even though HAMLET bound to DLDH, this binding was not associated with any inhibitory activity even at concentrations of HAMLET of up to 100 µg/ml (Fig. S3B), suggesting that interaction in vitro does not automatically correlate with inhibitory activity.

**HAMLET but not ALA can access its target enzymes in vivo**

Although ALA effectively inhibited both FPBA and GAPDH activity in vitro (Fig. 5), it had no activity on the production of ATP and lactate when added to whole cells (Fig. 2). This suggested indirectly that HAMLET, but not ALA, may gain entry to the bacterial cells, reach the respective targets to inhibit glycolysis, similar to its ability to traverse the membrane and enter tumor cells (4).

To provide further evidence that HAMLET directly targets the glycolytic pathway intracellularly in vivo, we measured FBPA activity in whole cell extracts from D39 pneumococci pre-treated for 10 min with various concentrations of HAMLET and compared the activity to cells treated with ALA or OA (Fig. 6). Cells were treated with HAMLET, ALA, OA or buffer alone for 10 minutes at 37°C and were immediately washed in 4°C PBS to remove unassociated protein or lipid and bacterial lysates were produced. Lysates from cells treated with ALA showed no significant change in FBPA activity compared with untreated D39 pneumococci (FBPA activity of 421 mU/mg and 408 mU/mg for lysates from ALA-treated and untreated bacteria, respectively). However, pre-treatment of D39 pneumococci with HAMLET for 10 minutes, resulted in a significant reduction in FBPA activity in treated lysates (94 and 2.1 mU/mg in extracts from bacteria treated with 50 and 100 µg/ml of HAMLET, respectively). Similar to its inability to inhibit FBPA activity in vitro (Fig. 5A), OA had no inhibitory activity on FBPA activity in this assay at the concentrations present in the HAMLET complex (396 mU/mg at 12.5 µg/ml OA). The results suggest that HAMLET can traverse the membrane and access the glycolytic machinery associated with the inner leaflet of the membrane (16), which ALA cannot access.

**DISCUSSION**

In this study we show for the first time that the antimicrobial molecule, HAMLET, targets glycolysis as part of its antibacterial activity. Although this study was limited to the bacterial pathogen *S. pneumoniae*, the results are likely more generally applicable to Gram-positive bacteria. HAMLET is known to have direct bactericidal activity against several Gram-positive organisms as well as having the ability to sensitize a broader array of both Gram-positive and Gram-negative organisms to antibiotics (7, 11, 12). In all these organisms, HAMLET-treatment induces identical initial changes, such as membrane depolarization and ion transport, suggesting a conserved activation pathway. Based on this pathway conservation and the fact that the pneumococcal targets identified in this study are highly conserved between bacterial species, it is likely that the mechanisms described in this study are also present in other HAMLET-sensitive bacteria. This is interesting from several perspectives.

As HAMLET also targets glycolysis in tumor cells (but not in healthy cells; 6, 17), this suggests a conserved mechanism that induces death in such diverse cells as bacteria and eukaryotes. Although the specific glycolytic enzymes targeted by HAMLET in this study (FBPA and GAPDH) and the study by Storm et al. (Hypoxia-inducible factor 1α, hexokinase and 6-phospho-2-
Pneumococcal glycolysis inhibition by HAMLET

Fructokinase / fructose-2,6-bis-phosphatase) were not identical, this merely emphasizes the complexity and potential divergence through evolution of glycolytic regulation. In eukaryotes, glucose is imported primarily through GLUT transporters, which renders the hexokinase especially prone to regulation by glucose levels in the blood in conjunction with glucose-6 phosphate levels in the cell. Also, the irreversible enzyme phosphofructokinase is regulated both by its substrate and cAMP levels in the cell.

In Gram-positive bacteria, regulation is somewhat different. The majority of glucose enters cells through carbohydrate phosphoenolpyruvate: phospho-transferase (PTS) systems where glucose upon import is phosphorylated directly into glucose-6-phosphate, thus circumventing hexokinase (18, 19). Regulation of hexokinase therefore is of less importance. Instead, components of the PTS system, such as HPr, as well as metabolites, such as fructose-2, 6-bis phosphate and glucose-6-phosphate, regulate catabolic activity through transcriptional regulation of genes using the major transcription factor CcpA (20). Both HAMLET-targets in this study, FBPA and GAPDH, are central and essential enzymes in glycolysis in many bacterial species, including pneumococci and at least GAPDH’s function is regulated indirectly through CcpA, and, at least in *Staphylococcus aureus*, through the CcpA-dependent factor GapR (21, 22). Although the regulatory framework is different in eukaryotes and bacteria, HAMLET still targets glycolysis in both systems, and it is possible that findings from the less complex bacterial system can provide leads to potential mechanisms involved also in tumor cell death, and vice versa.

Furthermore, glycolysis as a target for tumor cell death has become a topic of rising interest during the last couple of years with the renaissance of the Warburg effect and its role in cancer metabolism (23, 24). Otto Warburg described already in the 1920s that tumor cells are more glycolytically active than healthy cells and showed that tumor cells exhibited increased fermentation rather than aerobic respiration even when oxygen was present in abundance (aerobic glycolysis) (25, 26). Interference with glycolysis would provide a selective advantage in targeting tumor cells and is therefore a topic of great interest (27, 28). Inhibition of glycolysis by compounds such as 2-deoxyglucose can inhibit growth of tumor cells and its therapeutic potential has been investigated in the treatment of certain solid tumors (29, 30). Targeting glycolysis and its central enzymes could also be effective for a multitude of bacterial species, such as *S. pneumoniae*, that produce energy primarily from glycolysis and fermentation (9, 31). Based on the structural and functional differences of glycolytic enzymes between eukaryotes and bacteria, especially with regard to FBPA (32, 33), screening for potential molecules that target the activity of bacterial FBPA from several different species have been conducted in vitro (34–37) but no such efficacy tests have so far been done using whole bacteria. The known difference in structure between eukaryote and prokaryote FBPA and an evolutionary divergence in GAPDH genes between most eukaryotes and bacteria (38) suggest an explanation for the specific targeting of these enzymes by HAMLET only in bacteria. The structural motifs involved in HAMLET binding and its ensuing inhibition of enzymatic activity are not known but are worth future studies as they could provide clues for improved future therapeutic strategies, especially taking into account the high level of conservation of glycolytic enzymes in bacteria affected by HAMLET-treatment.

Pneumococci lack the Krebs’ cycle and oxidative phosphorylation and thus, the absolute majority of ATP is produced from glycolysis and fermentation of carbohydrates obtained from the harsh environment of the host (31). Engagement primarily of glycolysis is also true for many other facultative anaerobes, many of which are sensitive to HAMLET’s direct bactericidal effects or its ability to potentiate the activity of antibiotics (7).
These carbohydrates enter the bacterial cells through their many and effective transport systems and eventually feed into glycolysis. One of the vital roles of ATP is to maintain a polarized membrane, through ATP-driven transporters that extrude or import ions, such as sodium, potassium, hydrogen and calcium, to retain a gradient over the membrane, resulting in the production of an optimal membrane potential and proton motive force. These gradients are then used to promote transport of substrates, volume regulation, as well as other aspects of cell function and integrity.

The mechanism of HAMLET-induced death of tumor cells and bacteria is not fully characterized. However, we know that HAMLET causes depolarization of the bacterial and mitochondrial membranes and that HAMLET-induced death requires an influx of calcium in both systems (5, 8, 10). By inhibiting ATP-production from glycolysis, it is likely that HAMLET sensitizes the bacteria by disrupting their ability to maintain membrane polarity and specific ion gradients, that when dissipated will activate a downstream cascade of signaling, including kinase and protease activity, that results in apoptosis-like morphological features in pneumococcal cells (10).

Additionally, a reduced ATP-production also has the potential to both increase the membrane permeability to antibiotics as a result of dissipation of membrane polarity and may also decrease activity of drug efflux pumps. These processes, combined, could explain the increased activity of antibiotics in pneumococci as well as other bacteria treated with sub-lethal doses of HAMLET, leading to accumulation or increased association of antibiotics with bacterial cells (11, 12).

This study emphasized a crucial role of the protein component of the HAMLET complex. In this study we found that the protein component per se is crucial for the glycolytic inhibition. Both HAMLET and native ALA effectively inhibited the activity of both FBPA and GAPDH in vitro, whereas OA had no activity. However, in most mechanistic studies performed to date, we and others have shown that the oleic acid component alone has the ability to induce bactericidal activity, just like HAMLET, albeit at a higher concentration than found within the HAMLET complex (See Fig. 1 for death induction and 39, 40). A better understanding of the role of the lipid and protein components of the HAMLET complex during bacterial death or for increased activity during combination treatment with antibiotics is therefore of great interest. The prior studies indicate that HAMLET provides a way to effectively solubilize and/or present the lipid to targets in the bacterial cell, most likely the bacterial membrane. This effect is not confined to oleic acid, as it has been shown that active HAMLET complex can be made with other long-chained, unsaturated fatty acids in the cis-configuration, such as vaccenic (C18:1 cis-11) linoleic (C18:2 cis-9, 12), and linolenic acid (C18:3 cis-9, 12, 15) (41). These fatty acids, as well as other shorter chained fatty acids, have long been known to have general bacteriostatic or bactericidal activity in and of themselves against several bacterial species (42), albeit at higher concentration than found in the HAMLET complex.

At the same time, HAMLET’s effect on cell membrane polarity and ion transport are reminiscent of other antimicrobial peptides, such as defensins, lantibiotics and some antibiotics of the lipopeptide and glycopeptide classes. These agents associate directly with the bacterial membrane to induce lysis and some peptides have been suggested to translocate into the cytoplasm and have intracellular targets (43, 44). However, they also target cell wall and peptidoglycan biosynthesis through direct inhibition of biosynthetic enzymes or binding to lipid II or bactoprenol-coupled cell wall precursors (45–48). The identified cell wall targets of these antimicrobial peptides, such as MraY, MurM, PBP2, LytR, and others, are conserved in pneumococci (49, 50) but their role in HAMLET-induced death is not known. Preliminary studies in our laboratory has shown an initial interaction of HAMLET with the pneumococcal cell wall that appear to be required for subsequent downstream
effects. The role of these interactions both for HAMLET’s bactericidal activity and in HAMLET translocation into the bacterial cell will be of great interest for future studies.

As ALA had no activity when added exogenously, this suggested that only when ALA is bound to OA in the correct conformation does it have activity, which is most likely associated with its ability to associate with membranes (51) that promotes internalization into the bacteria, similar to some other antimicrobial peptides (43). This is very similar to the internalization of HAMLET into tumor cells (4), where HAMLET is found to colocalize primarily with mitochondria. The efficiency of internalization could not be determined in this study as intracellular visualization of HAMLET could not be observed directly due to membrane association. Still, considering that the IC₅₀ in vitro of both FBPA and GAPDH was approximately 1-2 µg/ml (60-120 nM) which was 50-100 times lower than the concentration added exogenously, this provides a physiological possibility that inhibitory amounts of HAMLET will reach its targets. Additionally, the inhibitory effect of HAMLET was stronger when HAMLET was added exogenously to pneumococci than when added in combination with each enzyme alone in vitro. It has been shown by mass spectrometry analysis after in-solution digestion that the glycolytic enzymes in pneumococci bind to each other and are clustered together in a complex associated with the pneumococcal membrane (52, 53). Thus, the binding of HAMLET simultaneously to both FBPA and GAPDH present in a glycolytic complex inside the bacterial cell may lead to more effective inhibition of glycolysis than the interaction with either enzyme alone in vitro.

As both FBPA and GAPDH are essential proteins in pneumococci (54, 55) as well as other organisms (56–59), we were not able to produce mutants lacking one or the other protein to better understand the mechanistic aspects of HAMLET’s interaction with either enzyme inside the cells and the impact of that interaction on glycolysis inhibition. As we have also been unable over the years to produce HAMLET-resistant bacterial mutants in the laboratory, this approach to gain a better understanding of the mechanisms involved in glycolysis inhibition was also not possible.

In conclusion, this study shows that HAMLET’s bactericidal activity is partly dependent on inhibition of glycolysis and ATP production, similar to what has been described in tumor cells. HAMLET inhibits ATP production from glycolysis by entering the bacterial cell and interacting with and inhibiting the activity of the glycolytic enzymes FBPA and GAPDH. HAMLET’s inhibitory effect on glycolysis in whole cells is associated with the ALA component of the complex as OA had no effect on either ATP production in whole cells or glycolysis enzyme inhibition either in vitro or in whole cells. This is different from most other studies that have identified oleic acid as the cytotoxic component of the complex. Still, the lipid component of the complex was required for its inhibitory activity in whole bacterial cells, emphasizing the role of both ALA and oleic acid in the bactericidal mechanism.

Agents that inhibit glycolysis and other energy producing systems in bacterial cells represent a novel class of antimicrobial agents in this era of rapidly increasing antibiotic resistance development. Importantly, they offer novel and promising therapeutic potential against targets where there are no existing resistant mechanisms.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Protein markers were from Thermo Fisher Scientific (Waltham, MA, USA) and enhanced chemiluminescence substrate were from GE Healthcare Bio-Sciences Corp (Piscataway, NJ, USA). Plasmid purification reagents, expression vectors, Ni-NTA agarose, and anti-RGS-His antibody were from Qiagen (Valencia, CA, USA). Anti-ALA antibody was from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-conjugated...
anti-IgG antibody and magnetic Dynabeads conjugated with streptavidin or histidine were from Invitrogen (Carlsbad, CA, USA). Bacto-Todd Hewitt media and Bacto-yeast extract, were from BD (Franklin Lakes, NJ, USA). Remaining reagents were from Sigma Chemicals Co (St. Louis, MO, USA).

HAMLET was produced as described (1) by complexing EDTA-treated ALA, purified from human milk, with oleic acid on an anion-exchange matrix (see Fig. S4 for the staining pattern of HAMLET and ALA after separation by SDS-PAGE gel electrophoresis). Lipid content of the HAMLET batch used was tested with the free fatty acid quantitation kit (Sigma), resulting in a stoichiometry of 6.1 molecules of oleic acid per molecule ALA, representing 10.8% of the complex (w/w).

**Bacterial strains**

The bacterial strains, plasmids, and primers used for the study are listed in Table 1. *S. pneumoniae* strain D39 (60) was used throughout the study. Pneumococci were stored at -80°C in the presence of 15% glycerol and grown at 37°C in Todd-Hewitt broth supplemented with 0.5% Yeast Extract (THY) or on tryptic soy agar (TSA) supplemented with 5% sheep blood, as appropriate.

*Escherichia coli* XL1-Blue (Stratagene / Agilent Technologies, Santa Clara, CA, USA) was used for cloning of recombinant protein constructs and *E. coli* M15 (Qiagen, Valencia, CA, USA) was used to express recombinant proteins. Ampicillin (100 µg/ml) and Kanamycin (50 µg/ml) were added to Luria-Bertani (LB) growth medium and LB agar plates to provide required selection, when appropriate.

**Expression of recombinant proteins**

Recombinant DLDH was produced from M15 *E. coli* harboring the pQE30 plasmid containing the full-length *dldh* sequence (SPD_1026 in the D39 genome, (61)), as described (62). The *fba* (SPD_0526), *gapA* (SPD_1823) and *gki* (SPD_0580) genes were amplified by PCR using primer pairs Fba-pQE-F/ Fba-pQE-R, Gap-pQE-F/Gap-pQE-R, or Gki-pQE-F/ Gki-pQE-R, respectively (Table 1) and chromosomal DNA from *S. pneumoniae* D39 (27) as template. The amplified fragments were cloned into the pQE30 expression vector and transformed into XL-1 Blue cells. Clones carrying the insert of interest were selected on LB agar containing ampicillin (100 µg/ml) and verified by restriction digestion and sequencing of purified plasmid. Verified plasmids were then transformed into *E. coli* M15 cells for protein expression.

Overnight cultures of M15 cells carrying each of the four enzymes were used to seed fresh cultures and the cultures were grown shaking at 37°C to an OD₆₀₀ of 0.5. Expression of plasmid-borne protein sequences was induced with 1 mM IPTG for 4 hours and the overexpressed proteins were purified from bacterial lysates by affinity chromatography using Ni-NTA agarose (Qiagen) according to the manufacturer's instructions. The proteins were analyzed by SDS-PAGE (see Figure S4) and Western blot and were quantified using the
BioTek Synergy 2 plate reader with the Take3 microdrop addition (Biotek, Winooski, VT, USA).

**Binding assays and Western blot**

For binding assays in solution, recombinant His-tagged enzymes were produced as described above and 3.25 µg of rFBPA and 6.5 µg of the remaining three enzymes was incubated with 5 µg HAMLET in a total volume of 0.5 ml. After 30 min incubation at room-temperature, 20 µl His-coupled magnetic beads (Dynabeads, Invitrogen, Carlbad, CA, USA) were added, the beads were collected by a magnet, washed five times in PBS, and beads were boiled and supernatant was run by gel electrophoresis and blotted as below.

Protein sources (1-4 µg purified enzymes or 10 µg bacterial lysate, as determined by the Bradford assay) were run on 4-12% Bis-Tris gels (Invitrogen) under denaturing conditions and the gels were electroblotted to a nitrocellulose membrane (GE Healthcare, Piscataway, NJ, USA) using transfer buffer (Invitrogen) for 1 h. Blotted membranes were blocked in PBS containing 0.05% Tween-20 (PBS-T) with 2.5% skim milk for 1 h at room-temperature and washed three times with PBS-T (PBS containing 0.2% Tween-20). To detect HAMLET binding in solution, membranes were both incubated with anti-ALA antibody (1:500 dilution) to detect the capture of HAMLET by the recombinant proteins and with anti-His antibody (1:1,000 dilution) to assure the capture of the recombinant protein by the beads. The blots were further incubated with rabbit-anti-mouse IgG (His-blot) or goat-anti-rabbit IgG (ALA-blot) antibody conjugated with HRP for detection. Detection of bands on each blot was done using a GelDoc EZ system (Bio-Rad Laboratories) with the Image Lab 6 software, set to automatic mode for optimal capture of signal in each blot image.

To determine binding on solid phase, membranes were incubated with biotinylated HAMLET or ALA (5 µg/ml), washed in PBS-T and further incubated with streptavidin conjugated with HRP (1:5,000 dilution in PBS-T) for 1 hour at room temperature. After washing, the membrane was developed as described above.

**Capture compound mass spectrometry**

HAMLET (1 mg in 200 µl volume) was sequentially labeled with NHS-LC-Diazirine, a UV-excitable cross-linker, and amine-reactive biotin from the EZ-link Sulfo-NHS-LC biotinylation kit (both from Pierce/Thermo Scientific, Rockland, IL, USA) according to the manufacturer’s instructions. After quenching with 10 mM Tris-HCl, pH 8, to terminate the reaction, HAMLET carrying biotin and cross-linker was purified over a Zeba desalting column and the final protein concentration was determined using a BioTek Synergy 2 plate reader with the Take3 microdrop addition (Biotek).

D39 pneumococci (10 ml) were grown in THY to an optical density (OD₆₀₀nm) of 0.6, the bacteria were pelleted by centrifugation at 1,500 x g for 10 min, washed once in PBS, and resuspended in 900 µl of PBS. Bacteria were lysed by addition of 100 µl 4% sodium deoxycholate and incubation for 5 min at room temperature, and the resulting lysate was purified through a Zeba desalting column (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration of the final eluate was determined as above.

Bacterial lysate (1 mg in 100 µl) was incubated with tagged HAMLET carrying cross-linker and biotin (12.5 µg in 50 µl) for 30 min on ice. Proteins in the vicinity of HAMLET were cross-linked by exposing the sample to UV light (365 nm) for 10 min and the reaction was incubated with 50 µl Streptavidin-coupled magnetic beads (Dynabeads, Invitrogen, Carlbad, CA, USA), collected by a magnet, washed five times in PBS, and captured proteins were identified by nano-liquid chromatography-mass spectrometry (Prot Tech, Norrisville, PA, USA).

**Enzyme assays**

For ATP and lactate production, bacteria were grown in THY to an OD₆₀₀nm of 0.6, pelleted...
and washed twice in PBS by centrifugation at 8,000 x g for 2 min, and resuspended in PBS to the original volume. After treatment, bacterial ATP production was determined using the ATP determination kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer’s instructions with slight modification. To determine intracellular ATP concentrations, 10 µl of bacteria were added to 90 µl of reaction buffer containing 0.5% Triton-X100 required to lyse the bacteria, and the luciferin conversion was measured by chemiluminescence as a measure of ATP in the sample using a Synergy 2 plate reader (Biotek). Triton-X100 was used to lyse bacteria as both SDS and deoxycholate inhibited the luciferase in the assay as has been previously described (63).

Lactate produced by the bacteria was determined essentially as described (64) by measuring the production of NADH in Equation 1.

\[ \text{NAD}^+ + \text{lactate} \leftrightarrow \text{NADH} + \text{H}^+ + \text{pyruvate} \] (1)

Bacteria grown as described above were treated and 250 µl bacteria were pelleted per sample and the supernatant containing lactate was immediately saved at -20°C until further analysis. To determine lactate, 100 µl of sample was mixed with 200 µl reaction buffer (100 mM potassium phosphate (pH 9), 320 mM phenyl hydrazine, 2.4 mM NAD+, and 2 U/ml L-lactate dehydrogenase) and the absorbance of formed NADH was measured at 340 nm in a Synergy 2 plate reader (Biotek). The concentration of lactate was determined from a standard curve of known concentrations of sodium lactate determined on the same plate.

Fructose bis-phosphate aldolase (FBPA, EC 4.1.2.13) activity was determined as described (65) by measuring the breakdown of NADH in the linked reaction presented in Equations 2-4, where FBPA catalyzes reaction 2, and reaction 3 and 4 are catalyzed by triose-phosphate isomerase (EC 5.3.1.1) and alpha-glycerophosphate dehydrogenase (EC 1.1.1.8), respectively.

D-fructose-1,6-phosphate ↔ Glycerone phosphate + D-glyceraldehyde-3 phosphate (2)

D-glyceraldehyde-3 phosphate ↔ Glycerone phosphate (3)

Glycerone phosphate + NADH + H+ ↔ sn-glycerol-3-phosphate + NAD+ (4)

Recombinant FBPA (rFBPA) was mixed with reaction buffer (50 mM Tris-HCl (pH 8), 100 mM KAc, 0.4 mM NADH, 5 mM fructose-1,6-phosphate, 0.6 U each of triose-phosphate isomerase and alpha-glycerophosphate dehydrogenase. The activity was measured as a decrease in absorbance at 340 nm in a Synergy 2 plate reader (Biotek) and quantified in units (conversion of substrate in µmol min⁻¹) per mg enzyme using an extinction coefficient of 6.22 mM⁻¹cm⁻¹ of NADH at 340 nm. For inhibition studies, rFBPA was pre-incubated with various concentrations of HAMLET or other potential inhibitor compounds for 15 minutes prior to initiating the reaction.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) activity was measured according to Cori et al (66) by measuring the production of NADH in reaction 5.

D-glyceraldehyde-3-phosphate + NAD+ + P_i ↔ 3-phospho-D-glyceroyl phosphate + NADH + H+ (5)

Recombinant GAPDH (rGAPDH) was mixed with reaction buffer (15 mM potassium pyrophosphate and 30 mM sodium arsenate set to pH 8.5 with 10% phosphoric acid, 1.25 mM NAD+, 3.3 mM dithiothreitol, and 1 mM D-glyceraldehyde-3 phosphate). Free D-glyceraldehyde-3 phosphate was prepared from a diethyl acetate barium salt where the diethyl acetate barium was cleaved off on a DOWEX resin as described by the manufacturer (Sigma, product G5376). The activity was
measured as an increase in absorbance at 340 nm in a Synergy 2 plate reader (Biotek) and activity was quantified in U/mg enzyme using the extinction coefficient of 6.22 mM$^{-1}$cm$^{-1}$ of NADH at 340 nm. For inhibition studies, rGAPDH was pre-incubated with various concentrations of HAMLET or other potential inhibitor compounds for 15 minutes prior to starting the reaction.

Glucokinase activity (GKI, EC 2.7.1.1) was determined by measuring the production of NADH in the linked reaction presented in equations 6 and 7 where glucokinase catalyzes reaction 6 and reaction 7 is catalyzed by glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (67)

\[
\text{D-Glucose + ATP \rightleftharpoons D-Glucose-6-Phosphate + ADP (6)}
\]

\[
\text{D-Glucose-6-phosphate + NAD\ensuremath{^+} \rightleftharpoons 6-phospho-D-glucono-1,5-lactone + NADH + H\ensuremath{^+} (7)}
\]

Recombinant GK (rGK) was mixed with reaction buffer (50 mM Tris-HCl (pH 7.4), 10 mM MgSO₄, 20 mM NAD\ensuremath{^+}, 1 mM D-glucose, 1 mM ATP, and 10 U/ml glucose-6-phosphate dehydrogenase). The activity was measured as an increase in absorbance at 340 nm in a Synergy 2 plate reader (Biotek) and activity was determined in U/mg enzyme using the extinction coefficient of 6.22 mM$^{-1}$cm$^{-1}$ of NADH at 340 nm. For inhibition studies, rGK was preincubated with various concentrations of HAMLET or other inhibitors for 15 minutes prior to starting the reaction.

Recombinant dihydrolipoamide dehydrogenase (rDLDH) activity was measured as the oxidation of NADH in the presence of 6,8-thioctic acid amide (lipoamide), visualized as decreased absorbance at 365 nm according to equation 8 as described (62).

\[
\text{NADH + H\ensuremath{^+} + Lipoamide \rightleftharpoons NAD\ensuremath{^+} + Dihydrolipoamide (8)}
\]

Statistical analysis — Group comparisons were examined with the unpaired Student’s $t$-test using the Prism 8 Software (GraphPad Software, Inc, La Jolla, CA).
ACKNOWLEDGEMENTS: The authors would like to thank Dr. Anki Mossberg for assistance with production of HAMLET for some of the experiments.

CONFLICT OF INTEREST: The authors declare that they have no conflicts of interest with the contents of this article

AUTHOR CONTRIBUTIONS: HRH, GV, and LRM designed, performed, analyzed and interpreted experiments in the manuscript. APH was responsible for designing the study, performing some enzyme experiments, analyzing and interpreting data and writing the manuscript. All authors read and approved the manuscript.

REFERENCES

1. Svensson, M., Håkansson, A., Mossberg, A. K., Linse, S. and Svanborg, C. (2000) Conversion of alpha-lactalbumin to a protein inducing apoptosis. *Proc Natl Acad Sci U S A* 97, 4221-4226

2. Hakansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H. and Svanborg, C. (1995) Apoptosis induced by a human milk protein. *Proc Natl Acad Sci U S A* 92, 8064-8068

3. Svensson, M., Düringer, C., Hallgren, O., Mossberg, A. K., Håkansson, A., Linse, S. and Svanborg, C. (2002) Hamlet--a complex from human milk that induces apoptosis in tumor cells but spares healthy cells. *Adv Exp Med Biol* 503, 125-132

4. Hakansson, A., Andréasson, J., Zhivotovsky, B., Karpman, D., Orrenius, S. and Svanborg, C. (1999) Multimeric alpha-lactalbumin from human milk induces apoptosis through a direct effect on cell nuclei. *Exp Cell Res* 246, 451-460

5. Köhler, C., Gogvadze, V., Håkansson, A., Svanborg, C., Orrenius, S. and Zhivotovsky, B. (2001) A folding variant of human alpha-lactalbumin induces mitochondrial permeability transition in isolated mitochondria. *Eur J Biochem* 268, 186-191

6. Storm, P., Aits, S., Puthia, M. K., Urbano, A., Northen, T., Powers, S., Bowen, B., Chao, Y., Reindl, W., Lee, D. Y., Sullivan, N. L., Zhang, J., Trulsson, M., Yang, H., Watson, J. D. and Svanborg, C. (2011) Conserved features of cancer cells define their sensitivity to HAMLET-induced death; c-Myc and glycolysis. *Oncogene* 30, 4765-4779

7. Hakansson, A., Svensson, M., Mossberg, A. K., Sabharwal, H., Linse, S., Lazou, I., Lönnerdal, B. and Svanborg, C. (2000) A folding variant of alpha-lactalbumin with bactericidal activity against *Streptococcus pneumoniae*. *Mol Microbiol* 35, 589-600

8. Clementi, E. A., Marks, L. R., Duffey, M. E. and Hakansson, A. P. (2012) A novel initiation mechanism of death in *Streptococcus pneumoniae* induced by the human milk protein-lipid complex HAMLET and activated during physiological death. *J Biol Chem* 287, 27168-27182

9. Tettelin, H., Nelson, K. E., Paulsen, I. T., Eisen, J. A., Read, T. D., Peterson, S., Heidelberg, J., DeBoy, R. T., Haft, D. H., Dodson, R. J., Durkin, A. S., Gwinn, M., Kolonay, J. F., Nelson, W. C., Peterson, J. D., Umayam, L. A., White, O., Salzberg, S. L., Lewis, M. R., Radune, D., Holtzapple, E., Khouri, H., Wolf, A. M., Utterback, T. R., Hansen, C. L., McDonald, L. A., Feldblyum, T. V., Angiuli, S., Dickinson, T., Hickey, E. K., Holt, I. E., Loftus, B. J., Yang, F., Smith, H. O., Venter, J. C., Dougherty, B. A., Morrison, D. A., Hollingshead, S. K. and Fraser, C. M. (2001) Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. *Science* 293, 498-506

10. Hakansson, A. P., Roche-Hakansson, H., Mossberg, A. K. and Svanborg, C. (2011) Apoptosis-like death in bacteria induced by HAMLET, a human milk lipid-protein complex. *PLoS One* 6, e17717
11. Marks, L. R., Clementi, E. A. and Hakansson, A. P. (2012) The Human Milk Protein-Lipid Complex HAMLET sensitizes bacterial pathogens to traditional antimicrobial agents. *PLoS ONE* 7, e43514

12. Marks, L. R., Clementi, E. A. and Hakansson, A. P. (2013) Sensitization of *Staphylococcus aureus* to methicillin and other antibiotics in vitro and in vivo in the presence of HAMLET. *PLoS ONE* 8, e63158

13. Speert, D. P., Wannamaker, L. W., Gray, E. D. and Clawson, C. C. (1979) Bactericidal effect of oleic acid on group A streptococci: mechanism of action. *Infect Immun* 26, 1202-1210

14. Pettersson-Kastberg, J., Mossberg, A. K., Trulsson, M., Yong, Y. J., Min, S., Lim, Y., O’Brien, J. E., Svanborg, C. and Mok, K. H. (2009) alpha-Lactalbumin, engineered to be nonnative and inactive, kills tumor cells when in complex with oleic acid: a new biological function resulting from partial unfolding. *J Mol Biol* 394, 994-1010

15. Meikle, V., Mossberg, A. K., Mitra, A., Hakansson, A. P. and Niederweis, M. (2019) A protein complex from human milk enhances the activity of antibiotics and drugs against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 63, e01846-18

16. Gutowicz, J. and Terlecki, G. (2003) The association of glycolytic enzymes with cellular and model membranes. *Cell Mol Biol Lett* 8, 667-680

17. Fang, B., Zhang, M., Fan, X. and Ren, F. Z. (2016) The targeted proteins in tumor cells treated with the α-lactalbumin-oleic acid complex examined by descriptive and quantitative liquid chromatography-tandem mass spectrometry. *J Dairy Sci* 99, 5991-6004

18. Postma, P. W., Lengeler, J. W. and Jacobson, G. R. (1993) Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev* 57, 543-594

19. Vadeboncoeur, C. and Pelletier, M. (1997) The phosphoenolpyruvate:sugar phosphotransferase system of oral streptococci and its role in the control of sugar metabolism. *FEMS Microbiol Rev* 19, 187-207

20. Gorke, B. and Stulke, J. (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol* 6, 613-624

21. Purves, J., Cockayne, A., Moody, P. C. and Morrissey, J. A. (2010) Comparison of the regulation, metabolic functions, and roles in virulence of the glyceraldehyde-3-phosphate dehydrogenase homologues gapA and gapB in *Staphylococcus aureus*. *Infect Immun* 78, 5223-5232

22. Fillinger, S., Boschi-Muller, S., Azza, S., Dervyn, E., Branlant, G. and Aymerich, S. (2000) Two glyceraldehyde-3-phosphate dehydrogenases with opposite physiological roles in a nonphotosynthetic bacterium. *J Biol Chem* 275, 14031-14037

23. Gogvadze, V., Zhivotovsky, B. and Orrenius, S. (2010) The Warburg effect and mitochondrial stability in cancer cells. *Mol Aspects Med* 31, 60-74

24. Diaz-Ruiz, R., Rigoulet, M. and Devin, A. (2011) The Warburg and Crabtree effects: On the origin of cancer cell energy metabolism and of yeast glucose repression. *Biochim Biophys Acta* 1807, 568-576

25. Warburg, O. (1956) On the origin of cancer cells. *Science* 123, 309-314

26. Koppenol, W. H., Bounds, P. L. and Dilda, P. J. (2011) Otto Warburg’s contributions to current concepts of cancer metabolism. *Nat Rev Cancer* 11, 325-337

27. Ferrin, G., Linares, C. I. and Muntane, J. (2011) Mitochondrial drug targets in cell death and cancer. *Curr Pharm Des* 17, 2002-2016

28. Ramsay, E. E., Hogg, P. J. and Dilda, P. J. (2011) Mitochondrial metabolism inhibitors for cancer therapy. *Pharm Res* 28, 2731-2744

29. Pelicano, H., Martin, D. S., Xu, R. H. and Huang, P. (2006) Glycolysis inhibition for anticancer treatment. *Oncogene* 25, 4633-4646

30. Seymour, C. B. and Mothersill, C. (1988) The effect of glycolysis inhibitors on the radiation response of CHO-K1 cells. *Radiat Environ Biophys* 27, 49-57

31. Hewitt, L. F. (1932) Bacterial metabolism: Glucose breakdown by pneumococcus variants and the effect of phosphate thereon. *Biochem J* 26, 464-471
Pneumococcal glycolysis inhibition by HAMLET

32. Marsh, J. J. and Lebherz, H. G. (1992) Fructose-bisphosphate aldolases: an evolutionary history. *Trends Biochem Sci* **17**, 110-113

33. Katebi, A. R. and Jernigan, R. L. (2015) Aldolases utilize different oligomeric states to preserve their functional dynamics. *Biochemistry* **54**, 3543-3554

34. Labbé, G., Krismanich, A. P., de Groot, S., Rasmusson, T., Shang, M., Brown, M. D., Dmitrienko, G. I. and Guillemette, J. G. (2012) Development of metal-chelating inhibitors for the Class II fructose 1,6-bisphosphate (FBP) aldolase. *J Inorg Biochem* **112**, 49-58

35. Capodagli, G. C., Sedhom, W. G., Jackson, M., Ahrendt, K. A. and Pegan, S. D. (2014) A noncompetitive inhibitor for *Mycobacterium tuberculosis*’s class IIa fructose 1,6-bisphosphate aldolase. *Biochemistry* **53**, 202-213

36. Yadav, P. K., Singh, G., Gautam, B., Singh, S., Yadav, M., Srivastav, U. and Singh, B. (2013) Molecular modeling, dynamics studies and virtual screening of Fructose 1, 6 bisphosphate aldolase-II in community acquired- methicillin resistant *Staphylococcus aureus* (CA-MRSA). *Bioinformation* **9**, 158-164

37. Tiwari, K., Jamal, S., Grover, S., Goyal, S., Singh, A. and Grover, A. (2016) Cheminformatics based machine learning approaches for assessing glycolytic pathway antagonists of *Mycobacterium tuberculosis*. *Comb Chem High Throughput Screen* **19**, 667-675

38. Martin, W. F. and Cerff, R. (2017) Physiology, phylogeny, early evolution, and GAPDH. *Protoplasma* **254**, 1823-1834

39. Permyakov, S. E., Knyazeva, E. L., Khasanova, L. M., Fadeev, R. S., Zhadan, A. P., Roche-Hakansson, H., Hakansson, A. P., Akatov, V. S. and Permyakov, E. A. (2012) Oleic Acid is a Key Cytotoxic Component of HAMLET-like Complexes. *Biol Chem* **393**, 85-92

40. Frislev, H. S., Boye, T. L., Nylundsted, J. and Otzen, D. (2017) Liprotides kill cancer cells by disrupting the plasma membrane. *Sci Rep* **7**, 15129

41. Svensson, M., Mossberg, A. K., Pettersson, J., Linse, S. and Svanborg, C. (2003) Lipids as cofactors in protein folding: stereo-specific lipid-protein interactions are required to form HAMLET (human alpha-lactalbumin made lethal to tumor cells). *Protein Sci* **12**, 2805-2814

42. Kabara, J. J., Swieczkowski, D. M., Conley, A. J. and Truant, J. P. (1972) Fatty acids and derivatives as antimicrobial agents. *Antimicrob Agents Chemother* **2**, 23-28

43. Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* **3**, 238-250

44. Shai, Y., Makovitzky, A. and Avrahami, D. (2006) Host defense peptides and lipopeptides: modes of action and potential candidates for the treatment of bacterial and fungal infections. *Curr Protein Pept Sci* **7**, 479-486

45. Schneider, T. and Sahl, H. G. (2010) Lipid II and other bactoprenol-bound cell wall precursors as drug targets. *Curr Opin Investig Drugs* **11**, 157-164

46. Sass, V., Schneider, T., Wilmes, M., Körner, C., Tossi, A., Novikova, N., Shamova, O. and Sahl, H. G. (2010) Human beta-defensin 3 inhibits cell wall biosynthesis in Staphylococci. *Infect Immun* **78**, 2793-2800

47. Münch, D., Müller, A., Schneider, T., Kohl, B., Wenzel, M., Bandow, J. E., Maffioli, S., Sosio, M., Donadio, S., Wimmer, R. and Sahl, H. G. (2014) The lantibiotic NAI-107 binds to bactoprenol-bound cell wall precursors and impairs membrane functions. *J Biol Chem* **289**, 12063-12076

48. Singh, M., Chang, J., Coffman, L. and Kim, S. J. (2017) Hidden mode of action of glycopeptide antibiotics: Inhibition of wall teichoic acid biosynthesis. *J Phys Chem B* **121**, 3925-3932

49. Massidda, O., Anderluzzi, D., Friedli, L. and Feger, G. (1998) Unconventional organization of the division and cell wall gene cluster of *Streptococcus pneumoniae*. *Microbiology* **144**, 3069-3078

50. Johnsborg, O. and Havarstein, L. S. (2009) Pneumococcal LytR, a protein from the LytR-CpsA-Psr family, is essential for normal septum formation in *Streptococcus pneumoniae*. *J Bacteriol* **191**, 5859-5864
51. Mossberg, A. K., Puchades, M., Halskau, Ø., Baumann, A., Lanekoff, I., Chao, Y., Martinez, A., Svanborg, C. and Karlsson, R. (2010) HAMLET interacts with lipid membranes and perturbs their structure and integrity. PLoS One 5, e9384

52. Sun, X., Jia, H. L., Xiao, C. L., Yin, X. F., Yang, X. Y., Lu, J., He, X., Li, N., Li, H. and He, Q. Y. (2011) Bacterial proteome of Streptococcus pneumoniae through multidimensional separations coupled with LC-MS/MS. OMICS 15, 477-482

53. Sun, X., Yang, X. Y., Yin, X. F., Yu, G., Xiao, C. L., He, X. and He, Q. Y. (2011) Proteomic Analysis of Membrane Proteins from Streptococcus pneumoniae with Multiple Separation Methods Plus High Accuracy Mass Spectrometry. OMICS 15, 683-694

54. Song, J. H., Ko, K. S., Lee, J. Y., Baek, J. Y., Oh, W. S., Yoon, H. S., Jeong, J. Y. and Chun, J. (2005) Identification of essential genes in Streptococcus pneumoniae by allelic replacement mutagenesis. Mol Cells 19, 365-374

55. Jensen, P. A., Zhu, Z. and van Opijnen, T. (2017) Antibiotics disrupt coordination between transcriptional and phenotypic stress responses in pathogenic bacteria. Cell Rep 20, 1705-1716

56. de la Paz Santangelo, M., Gest, P. M., Guerin, M. E., Coinçon, M., Pham, H., Ryan, G., Puckett, S. E., Spencer, J. S., Gonzalez-Juarrero, M., Daha, R., Lenzaerts, A. J., Schnappinger, D., Therisod, M., Ehrt, S., Sygusch, J. and Jackson, M. (2011) Glycolytic and non-glycolytic functions of Mycobacterium tuberculosis fructose-1,6-bisphosphate aldolase, an essential enzyme produced by replicating and non-replicating bacilli. J Biol Chem 286, 40219-40231

57. Gerdes, S. Y., Scholle, M. D., Campbell, J. W., Balázsi, G., Ravasz, E., Daugherty, M. D., Somera, A. L., Kyrides, N. C., Anderson, I., Gelfand, M. S., Bhattacharya, A., Kapral, V., D’Souza, M., Baev, M. V., Grechkin, Y., Mseeh, F., Fonstein, M. Y., Overbeek, R., Barabási, A. L., Olsvai, Z. N. and Osterman, A. L. (2003) Experimental determination and system level analysis of essential genes in Escherichia coli MG1655. J Bacteriol 185, 5673-5684

58. Jacobs, M. A., Alwood, A., Thaipsuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C., Levy, R., Chun-Rong, L., Guenther, D., Bovee, D., Olson, M. V. and Manoil, C. (2003) Comprehensive transposon mutant library of Pseudomonas aeruginosa. Proc Natl Acad Sci USA 100, 14339-14344

59. Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., Boland, F., Brignell, S. C., Cron, S., Bunai, K., Chapuis, J., Christiansen, L. C., Danchin, A., Dérivieux, M., Deuerling, E., Devine, K., Devine, S. K., Dreessen, O., Errington, J., Fillinger, S., Foster, S. J., Fujita, Y., Galizia, A., Gardan, R., Eschevins, C., Fukushima, T., Haga, K., Harwood, C. R., Hecker, M., Hosoya, D., Julio, M. F., Nakamuta, K., Karamata, Y., Kawamura, F., Koga, K., Koski, P., Kuwana, R., Imamura, D., Ishihara, M., Ishizawa, S., Ishio, I., Le Coq, D., Masson, A., Maule, C., Meima, R., Melloid, R. P., Moir, A., Moriya, S., Nagakawa, E., Nanamiya, H., Nakai, S., Nyaagaard, P., Ong, M., Ohanian, T., O’Reilly, M., O’Rourke, M., Pragai, Z., Pooley, H. M., Rapoport, G., Rawlinson, J. P., Rivas, L. A., Rivolta, C., Sadaie, A., Sadaie, Y., Sarvas, M., Sato, T., Saxild, H. H., Scanlan, E., Schumann, W., Seegers, J. F., Sekiguchi, J., Sekowska, A., Séror, S. J., Simon, M., Stragier, P., Studer, R., Takamatsu, H., Tanaka, T., Takeuchi, M., Thomaides, H. B., Vagner, V., van Dijl, J. M., Watabe, K., Wipat, A., Yamamoto, H., Yamamoto, Y., Yamane, K., Yata, K., Yoshida, K., Yoshikawa, H., Zuber, U. and Ogasawara, N. (2003) Essential Bacillus subtilis genes. Proc Natl Acad Sci USA 100, 4678-4683

60. Avery, O. T., Macleod, C. M. and McCarty, M. (1944) Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a deoxyribonucleic acid fraction isolated from pneumococcus type III. J Exp Med 79, 137-158

61. Lanie, J. A., Ng, W. L., Kazmierczak, K. M., Andrzejewski, T. M., Davidsen, T. M., Wayne, K. J., Tettelin, H., Glass, J. I. and Winkler, M. E. (2007) Genome sequence of Avery’s virulent serotype 2 strain D39 of Streptococcus pneumoniae and comparison with that of encapsulated laboratory strain R6. J Bacteriol 189, 38-51
62. Hakansson, A. P. and Smith, A. W. (2007) Enzymatic characterization of dihydrolipoamide dehydrogenase from *Streptococcus pneumoniae* harboring its own substrate. *J Biol Chem* **282**, 29521-29530

63. Simpson, W. J. and Hammond, J. R. (1991) The effect of detergents on firefly luciferase reactions. *J Biol Chem* **282**, 97-106

64. Lundholm, L., Mohme-Lundholm, E. and Vamos, N. (1963) Lactic acid assay with L(plus)lactic acid dehydrogenase from rabbit muscle. *Acta Physiol Scand* **58**, 243-249

65. Tunio, S. A., Oldfield, N. J., Berry, A., Ala’Aldeen, D. A., Wooldridge, K. G. and Turner, D. P. (2010) The moonlighting protein fructose-1, 6-bisphosphate aldolase of *Neisseria meningitidis*: surface localization and role in host cell adhesion. *Mol Microbiol* **76**, 605-615

66. Cori, G. T., Slein, M. W. and Cori, C. F. (1948) Crystalline d-glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle. *J Biol Chem* **173**, 605-618

67. Pakoskey, A. M., Lesher, E. C. and Scott, D. B. (1965) Hexokinase of *Escherichia coli*. Assay of enzymatic activity and adaptation to growth in various media. *J Gen Microbiol* **38**, 73-80

**FOOTNOTES**

Funding for this work was provided by the Bill and Melinda Gates Foundation (Grant 53085), The American Lung Association (Grant RG-123721-N), The Swedish Medical Research Council (VR; Grants no. K2015-99X-22878-01-6 and 2018-05947, and the Alfred Österlund Foundation, Malmö, Sweden (APH), and The Royal Physiographic Society, Lund, Sweden (GV).

Abbreviations used in this manuscript: ALA, alpha-lactalbumin; DLDH, dihydrolipoamide dehydrogenase; FBPA, fructose-bis phosphate aldolase; GAPDH, glyceraldehyde tri-phosphate dehydrogenase; GKI, glucokinase (hexokinase); HAMLET, Human Alpha-lactalbumin Made LEthal to Tumor cells; HRP, horseradish peroxidase; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry; OA, oleic acid.
Table 1. Strains and primers used in this study.

| Strain or plasmid | Description | Reference |
|-------------------|-------------|-----------|
| Strains           |             |           |
| D39               | Pneumococcal serotype 2 wild-type strain | (60) |
| XL-1 Blue         | *E. coli* strain used for cloning | Stratagen |
| M15               | *E. coli* strains used for recombinant protein expression | Qiagen |
| Plasmids          |             |           |
| pQE30             | *E. coli* expression vector | Qiagen Inc. |
| pSH-rDLDH         | pQE30 vector containing fulllength *dldh* sequence | (62) |
| pHH-rFba          | pQE30:: XbaI-HindIII Fba-pQE-F/R PCR product | This study |
| pHH-rGapA         | pQE30::BamHI-HindIII Gap-pQE-F/R PCR product | This study |
| pHH-rGki          | pQE30::BamHI-PstII Gki-pQE-F/R PCR product | This study |
| Primers           |             |           |
| Fba-pQE-F         | TGCTCTAGAGGGTGGCTGCTAAATACA | This study |
| Fba-pQE-R         | CCCAAGCTTTGGTCTACGCTAACTCTCTGG | This study |
| Gap-pQE-F         | CGCGGATCCGTAGTTAAAGTTGGTTAATTCG | This study |
| Gap-pQE-R         | CCCAAGCTTTGTATTAGCAATTTTTGC | This study |
| Gki-pQE-F         | CGCGGATCCATGAGTCAAAAAGATTATCGG | This study |
| Gki-pQE-R         | CCCCTGCAGTTATTGCAAACAGTGATG | This study |
Figure 1. Bactericidal activity of HAMLET, ALA and oleic acid. 
*S. pneumoniae* D39 was treated with native alpha-lactalbumin (ALA) and HAMLET at the concentrations indicated, or with oleic acid (OA) using a concentration equivalent to the concentration present in HAMLET (11% w/w) and incubated for 1 hour at 37°C. Viable organisms were assessed after plating dilutions of each sample onto blood agar plates and enumerating colony forming units after overnight growth. The results represent 6 individual experiments and are presented as the mean with error bars representing the standard deviations. Statistical analysis was performed using the Student’s unpaired *t*-test with * = *P* < 0.05, *** = *P* < 0.001 and ns = non-significant.
Figure 2. Inhibition of glycolysis and energy production in HAMLET-treated *S. pneumoniae*. *S. pneumoniae* D39 were energized with 10 mM glucose and treated with 125 µg/ml of ALA after 5 min or 125 µg/ml of HAMLET after either 5 or 10 min (see arrows) and (A) intracellular ATP production and (B) lactate secretion were recorded over time. (C) D39 pneumococci were energized with 10 mM glucose and treated with sublethal concentrations of HAMLET (25, 37 and 50 µg/ml) that were added 2 min post addition of glucose. (D) D39 pneumococci were energized with 10 mM glucose and treated with the oleic acid (OA) concentration present in 125 µg/ml of HAMLET (12.5 µg/ml) that was added after either 5 or 10 min. All results represent 3 individual experiments with duplicate samples and are presented as the mean with error bars representing the standard deviations. Statistical analysis was performed using the Student’s unpaired *t*-test with * = *P* < 0.05, ** = *P* < 0.01, *** = *P* < 0.001 and ns = non-significant.
Figure 3. Modulation of glycolysis affects HAMLET-sensitivity.

D39 pneumococci were washed in PBS and resuspended in PBS alone or PBS with the addition of either 25 mM glucose or 25 mM 2-deoxy glucose. After 15 min, (A) increasing concentrations of HAMLET or (B) oleic acid were added to the bacterial cells and viable organisms were determined by viable counts after 1 hour. All results represent 3 individual experiments with duplicate samples and are presented as the mean with error bars representing the standard deviations. Statistical analysis was performed using the Student’s unpaired t-test with * = P < 0.05, ** = P < 0.01, *** = P < 0.001 and ns = non-significant.
Figure 4. Binding of HAMLET to FBPA and GAPDH. (A) D39 whole bacterial lysates were separated by SDS-PAGE and blotted to nitrocellulose membrane. The blot was blocked and incubated in the presence of buffer alone (S) or biotinylated HAMLET (HL) and after addition of HRP-conjugated streptavidin, binding was detected in the presence of ECL-reagents. The two major bands detected were cut out and identified by Edman degradation. The (*) indicates the presence of biotin carboxyl carrier protein (17.0 kDa), a protein that is naturally biotinylated and thus reacts directly with the HRP-conjugated streptavidin and does not represent HAMLET binding. The blots were photographed and are displayed without any image correction. (B) Pneumococcal recombinant FBPA or GAPDH were separated by SDS-PAGE, blotted to nitrocellulose membrane, and overlaid with HAMLET, alpha-lactalbumin (ALA) or buffer alone (Streptavidin). After incubation with HRP-conjugated streptavidin, binding was detected in the presence of ECL-reagents. The blots were captured by a GelDoc system set to automatic mode for optimal capture of signal in each blot image and blots are presented without image correction.
Figure 5. HAMLET and ALA inhibits FBPA and GAPDH activity.

Recombinant FBPA (A) or GAPDH (B) were preincubated with buffer alone, or decreasing concentrations of HAMLET, ALA, OA or mouse IgG for 15 min. At this time the mixtures were added to reaction buffer and the reaction was started by adding NADH (for FBPA activity) and NAD (for GAPDH activity) and activity was monitored by a decrease and increase, respectively in the absorbance at 340 nm. The results are presented as the percentage activity compared with the buffer alone control. All results represent 3 individual experiments with duplicate samples and are presented as the mean with error bars representing the standard deviations. Statistical analysis was performed using the Student’s unpaired t-test with \( * = P < 0.05, ** = P < 0.01, *** = P < 0.001 \) and ns = non-significant.
Figure 6. HAMLET inhibits FBPA activity in whole cells.
D39 pneumococci were exposed to buffer alone (D39), ALA (100 µg/ml), HAMLET (50 or 100 µg/ml), or OA (12.5 µg/ml) for 10 min, bacterial cells were washed in pre-cooled buffer, pneumococcal lysates were produced at 4°C and tested for FBPA activity presented as the breakdown of NADH at 340 nm over time. The results represent 3 individual experiments with duplicate samples and are presented as the mean (center line) with standard deviations depicted as the hatched lines surrounding the center line. Statistical analysis was performed by comparing the enzymatic activity (in mU/mg lysate) over the first 10 minutes with Student’s unpaired $t$-test. ** = $P < 0.01$ when comparing D39 lysate with Lysate in the presence of 50 µg/ml HAMLET, and *** = $P < 0.001$ when comparing D39 lysate with lysate in the presence of 100 µg/ml HAMLET.
The human milk protein-lipid complex HAMLET disrupts glycolysis and induces death in *Streptococcus pneumoniae*

Hazeline Roche-Hakansson, Goutham Vansarla, Laura R Marks and Anders P Hakansson

*J. Biol. Chem.* published online November 6, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.009930

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts