



# A Protein Complex from Human Milk Enhances the Activity of Antibiotics and Drugs against *Mycobacterium tuberculosis*

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**ABSTRACT** *Mycobacterium tuberculosis*, the causative agent of human tuberculosis (TB), has surpassed HIV/AIDS as the leading cause of death from a single infectious agent. The increasing occurrence of drug-resistant strains has become a major challenge for health care systems and, in some cases, has rendered TB untreatable. However, the development of new TB drugs has been plagued with high failure rates and costs. Alternative strategies to increase the efficacy of current TB treatment regimens include host-directed therapies or agents that make *M. tuberculosis* more susceptible to existing TB drugs. In this study, we show that HAMLET, an  $\alpha$ -lactalbumin–oleic acid complex derived from human milk, has bactericidal activity against *M. tuberculosis*. HAMLET consists of a micellar oleic acid core surrounded by a shell of partially denatured  $\alpha$ -lactalbumin molecules and unloads oleic acid into cells upon contact with lipid membranes. At sublethal concentrations, HAMLET potentiated a remarkably broad array of TB drugs and antibiotics against *M. tuberculosis*. For example, the minimal inhibitory concentrations of rifampin, bedaquiline, delamanid, and clarithromycin were decreased by 8- to 16-fold. HAMLET also killed *M. tuberculosis* and enhanced the efficacy of TB drugs inside macrophages, a natural habitat of *M. tuberculosis*. Previous studies showed that HAMLET is stable after oral delivery in mice and nontoxic in humans and that it is possible to package hydrophobic compounds in the oleic acid core of HAMLET to increase their solubility and metabolic stability. The potential of HAMLET and other lipotides as drug delivery and sensitization agents in TB chemotherapy is discussed here.

**KEYWORDS** HAMLET,  $\alpha$ -lactalbumin, lipotides, macrophages, multidrug resistance, oleic acid, potentiation, sensitization

*Mycobacterium tuberculosis*, the causative agent of human tuberculosis (TB), has surpassed HIV/AIDS as the leading cause of death worldwide from a single infectious agent. In 2016, 10.4 million people were infected with *M. tuberculosis*, resulting in 1.7 million deaths (1, 2). A concerning development is that multidrug-resistant TB (MDR-TB) cases tripled between 2009 and 2016 (3). Treatment of MDR-TB and extensively drug-resistant TB (XDR-TB) requires the use of less efficient and more expensive drugs, which often have side effects (4–6). This increases treatment times and the risk of developing new drug-resistant *M. tuberculosis* strains. A striking example is bedaquiline, which was the first new TB drug approved by the FDA since 1974. Bedaquiline resistance was reported only 3 years after its approval in 2012 (7). Furthermore, drug-resistant TB poses a large economic burden on health care systems. In the United States, the average patient treatment costs are approximately \$80,000 and \$250,000 for MDR- and XDR-TB, respectively (8). In developing countries, the treatment of MDR- and XDR-TB alone can consume up to one-third of the TB program resources (6). Thus, new TB treatment strategies including new drugs are urgently needed (9). However, TB is notoriously difficult to treat due to the slow growth of *M. tuberculosis*,

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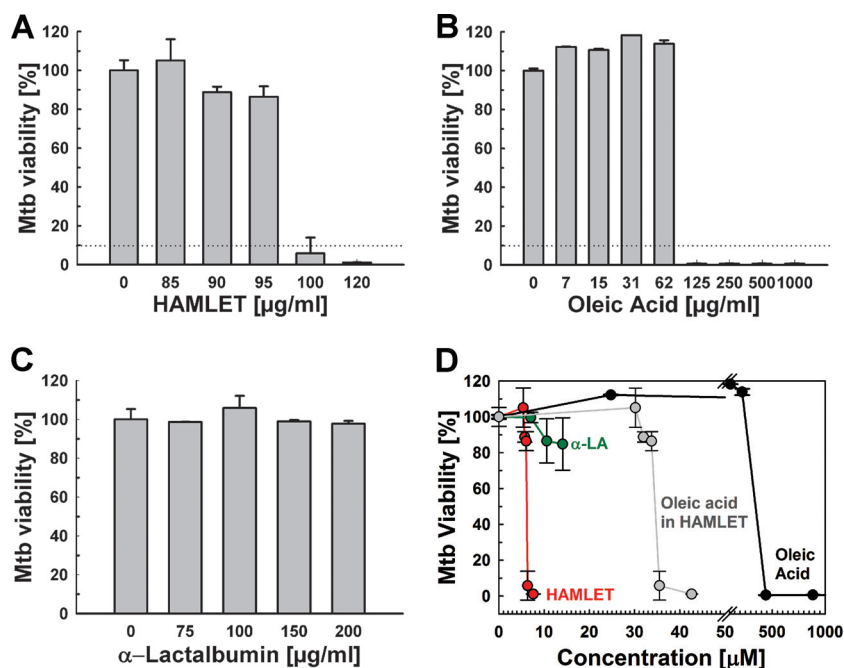
a formidable permeation barrier established by its outer membrane in combination with other resistance mechanisms (10, 11), and its ability to survive in a persistent drug-resistant state after infection (12, 13). A consequence of the unusually high intrinsic resistance of *M. tuberculosis* to toxic compounds is that many current antibiotics are not efficient in treating TB (14, 15). Furthermore, high-affinity inhibitors of essential targets developed by *in vitro* drug screening approaches were not active against *M. tuberculosis* because of permeability problems (9). In contrast, compounds identified in phenotypic screens using whole bacterial cells are active against *M. tuberculosis* but may work via nonspecific mechanisms (9). These challenges increase the failure rate and costs of TB drug development.

A promising alternative strategy is to make *M. tuberculosis* more susceptible to existing TB drugs by increasing their efficacy. The most widely studied approach is the use of drug efflux pump inhibitors (16, 17). For example, the efflux pump inhibitor verapamil reduced the MIC of bedaquiline against *M. tuberculosis in vitro* (18, 19) and augmented its bactericidal activity in mice (20, 21). Thioridazine is an FDA-approved drug to treat psychotic disorders, enhances the activity of a standard regimen to treat susceptible *M. tuberculosis* in mice (22), and was used to cure 10 of 12 XDR-TB patients in Buenos Aires, Argentina (23). Thioridazine appears to have multiple beneficial effects in TB chemotherapy, as follows (24): it has moderate activity against *M. tuberculosis in vitro* (25, 26), enhances the cell permeability of *M. tuberculosis* (27), and inhibits  $\text{Ca}^{2+}$  channels in the phagosomal membrane, leading to an acidification of the phagosome and increased killing of *M. tuberculosis* (28).

In this study, we examined the activity of HAMLET (human  $\alpha$ -lactalbumin made lethal against tumor cells) against *M. tuberculosis*. HAMLET is a protein-lipid complex with anticancer activity, derived from human milk, and composed of  $\alpha$ -lactalbumin and milk fatty acids (29–31). Surprisingly, HAMLET is bactericidal against some bacteria (32) and has also been shown to reverse the antibiotic resistance of both HAMLET-sensitive and -resistant species (33, 34). Here, we show that HAMLET has bactericidal activity against *M. tuberculosis in vitro* and at sublethal concentrations potentiates the effect of several first- and second-line TB drugs both in *in vitro* and inside macrophages.

## RESULTS

**Activity of HAMLET against *M. tuberculosis*.** Previous studies showed that HAMLET has bactericidal activity against a select set of Gram-positive and Gram-negative bacteria, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, respectively (32, 35). However, HAMLET was not active against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, or *Enterococcus faecalis* (32). The first aim of this study was to examine whether HAMLET exhibits direct antibacterial activity against *Mycobacterium tuberculosis*. To this end, HAMLET was prepared from  $\alpha$ -lactalbumin obtained from human milk and complexed with oleic acid, as described previously (36). We used microplate alamarBlue assays, growth curve measurements, and agar plate-based drop assays to examine whether HAMLET has any activity against *M. tuberculosis*. The virulent strain *M. tuberculosis* H37Rv was first grown in Hartmans-de Bont (HdB) minimal medium containing increasing concentrations of HAMLET, and the bacterial viability was determined using the alamarBlue assay. Surprisingly, considering the extraordinary low permeability of the mycobacterial outer membrane, HAMLET inhibited the growth and/or viability of *M. tuberculosis*, with an  $\text{MIC}_{90}$  of 100  $\mu\text{g}/\text{ml}$  (Fig. 1A). Since HAMLET is prepared through complexing of  $\alpha$ -lactalbumin with oleic acid (36) and oleic acid is known to be toxic for *M. tuberculosis* (37–40), we determined the antibacterial activities of oleic acid and  $\alpha$ -lactalbumin separately against *M. tuberculosis*. While the  $\text{MIC}_{90}$  of oleic acid was lower than 125  $\mu\text{g}/\text{ml}$  for *M. tuberculosis* H37Rv (Fig. 1B), no significant activity of human  $\alpha$ -lactalbumin was observed up to concentrations of 200  $\mu\text{g}/\text{ml}$  (Fig. 1C). Fatty acid quantification revealed that, on average, a single  $\alpha$ -lactalbumin protein bound 5.6 oleic acid molecules in the HAMLET preparation used in these experiments. The growth-inhibitory concentration of HAMLET was 6.3  $\mu\text{M}$ , which corresponds to an average

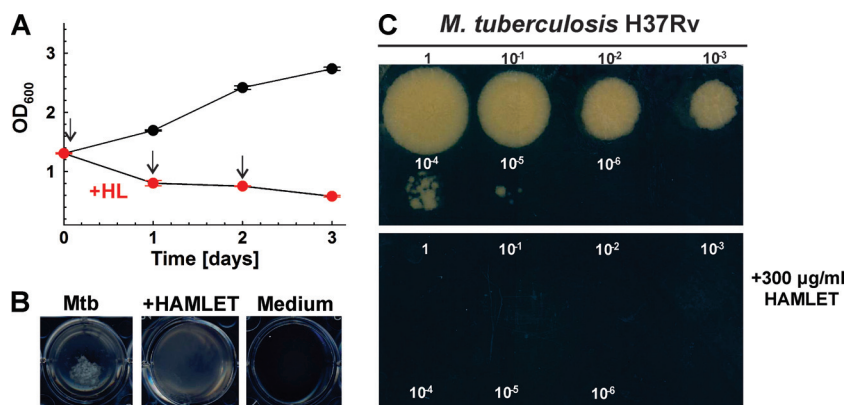


**FIG 1** HAMLET is active against *M. tuberculosis*. The viability of *M. tuberculosis* H37Rv in Hartmans-de Bont (HdB) minimal medium in the presence of increasing concentrations of HAMLET (A), oleic acid (B), and human  $\alpha$ -lactalbumin (C) was determined by the microplate alamarBlue assay. Error bars represent standard errors of the mean values of biological triplicates. The MIC<sub>90</sub> is represented by dotted lines in panels A and B. (D) Analysis of the viability of *M. tuberculosis* H37Rv with increasing molar concentrations of HAMLET (A), oleic acid (B) and human  $\alpha$ -lactalbumin ( $\alpha$ -LA) (C), with data obtained from the experiments shown in panels A to C.

concentration of 35  $\mu$ M oleic acid (Fig. 1D). This is  $\sim$ 13-fold less than the MIC<sub>90</sub> of 443  $\mu$ M of oleic acid alone against *M. tuberculosis* (Fig. 1B). This result suggested that  $\alpha$ -lactalbumin most likely acts by more efficiently delivering oleic acid to the bacteria and thereby increases its activity against *M. tuberculosis*. The increased activity of HAMLET against *M. tuberculosis* compared to that of oleic acid was described previously for other bacteria (32, 41).

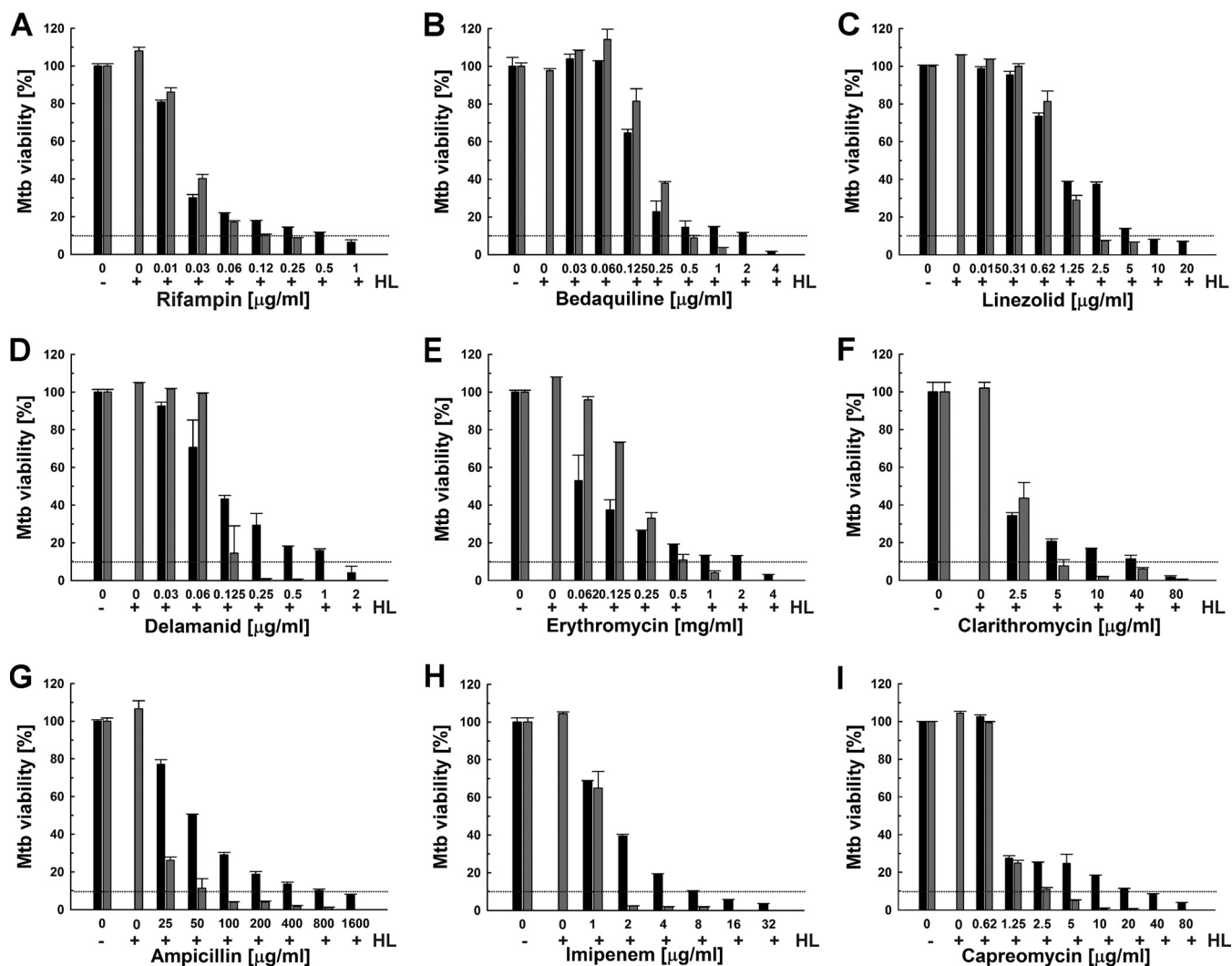
**HAMLET kills *M. tuberculosis* in vitro.** To examine whether HAMLET is bacteriostatic or has bactericidal activity, *M. tuberculosis* was first grown in HdB minimal medium to an optical density at 600 nm (OD<sub>600</sub>) of 1.3. This culture was then split into two separate cultures, one of which was treated three times with 300  $\mu$ g/ml HAMLET at 24-h intervals. On day 3, the OD<sub>600</sub> of the treated culture declined to 0.58, while the untreated culture continued to grow to an OD<sub>600</sub> of 2.75 (Fig. 2A). A visual inspection of the wells showed the complete absence of the typical cell clumps in the cultures treated with HAMLET, indicating a drastic change or death of *M. tuberculosis* cells (Fig. 2B). Each day, aliquots were removed from the HAMLET-treated and untreated cultures, and the growth of *M. tuberculosis* was examined by adding drops of serially diluted samples on agar plates. While growth of untreated *M. tuberculosis* was detected using a 10,000-fold diluted sample (Fig. 2C), no growth was observed for any HAMLET-treated culture even when an undiluted sample was plated after only one treatment (day 1). These results indicated that a single treatment with 300  $\mu$ g/ml HAMLET killed *M. tuberculosis*. Bactericidal activity of HAMLET was previously also observed for *S. pneumoniae* (32).

**HAMLET enhances the activity of antibiotics and TB drugs against *M. tuberculosis*.** Previous studies showed that HAMLET enhances the activity of some antibiotics against *S. pneumoniae* and *S. aureus* (33, 34). After we established that HAMLET is active against *M. tuberculosis*, we examined whether HAMLET would also potentiate the efficacy of antibiotics and TB drugs against *M. tuberculosis*. First, we determined that the



**FIG 2** Bactericidal activity of HAMLET (HL) against *M. tuberculosis*. (A) *M. tuberculosis* H37Rv was grown in HdB minimal medium to an OD<sub>600</sub> of 1.3, and then 5 ml of culture was seeded into 12-well microplates. *M. tuberculosis* was treated with 300 µg/ml HAMLET on days 0, 1, and 2 as indicated by the arrows. The bactericidal activity of HAMLET was determined by measuring the optical density at 600 nm. Error bars represent standard errors of mean values of biological triplicates. (B) Images of the wells were taken on the second day of HAMLET treatment. (C) Tenfold serial dilutions of cultures of untreated and HAMLET-treated *M. tuberculosis* strains were plated on Middlebrook 7H10 agar after the first treatment with HAMLET (day 0). Agar plates were imaged after incubation at 37°C for 15 days.

MIC<sub>90</sub> of the new HAMLET preparation against the susceptible *M. tuberculosis* H37Rv strain was 125 µg/ml, using the microplate alamarBlue assay. Thus, this HAMLET preparation was slightly less active than that used in the previous experiments (Fig. 1). To examine whether HAMLET synergizes with antibiotics and TB drugs, we kept the concentration of HAMLET below its MIC to observe synergistic effects without the confounding factor of the direct bactericidal activity of HAMLET against *M. tuberculosis*. To this end, we tested several sublethal HAMLET concentrations for synergistic effects with rifampin and a few other TB drugs. The highest HAMLET concentration which did not affect the viability of *M. tuberculosis* was 90 µg/ml (~75% of the MIC<sub>90</sub>), while no synergizing effect was observed for 60 µg/ml HAMLET (~50% of the MIC<sub>90</sub>). Then, the viability of *M. tuberculosis* H37Rv treated with antibiotics and TB drugs alone or in combination with 90 µg/ml HAMLET was measured using the microplate alamarBlue assay. This sublethal concentration of HAMLET sensitized *M. tuberculosis* to a large number of structurally diverse antibiotics and TB drugs by lowering the MIC<sub>90</sub> between 2- and 16-fold (Fig. 3 and Table 1). Importantly, the activity of rifampin, one of the first-line TB drugs, and of its analog rifabutin were enhanced 4- to 8-fold in the presence of HAMLET (Fig. 3A and Table 1; see also Fig. S2 in the supplemental material). Surprisingly, large sensitization effects between 8- and 16-fold-reduced MIC<sub>90</sub> values were also observed for the new TB drugs bedaquiline and delamanid, the important second-line TB drug capreomycin (42), and ampicillin (Table 1 and Fig. 3B, D, G, and I). Bedaquiline, delamanid, and capreomycin are used in the treatment of multidrug-resistant and extensively drug-resistant tuberculosis (43–45), while ampicillin has been proposed as an alternative treatment option in combination with β-lactamase inhibitors. HAMLET potentiated the efficacy of the macrolide antibiotics erythromycin and clarithromycin by 8- and 16-fold (Table 1 and Fig. 3E and F), respectively, whereas the efficacies of second-line β-lactam imipenem (Fig. 3H) and the oxazolidone linezolid (Fig. 3C) were both potentiated by 4-fold (Table 1). The efficacies of fluoroquinolones (levofloxacin and ofloxacin) and aminoglycosides (streptomycin and amikacin) were only slightly enhanced or did not change. The first-line TB drugs isoniazid, ethambutol, and pyrazinamide were not compatible with the alamarBlue assay and were therefore not included in this analysis. Taken together, these results show that HAMLET enhances the efficacy of several broad-spectrum antibiotics and TB drugs. It should be noted that the susceptibility of *M. tuberculosis* to oleic acid (46), the active component in HAMLET, and to drugs (47) depends on the growth medium.



**FIG 3** HAMLET potentiates antibiotics and TB drugs against *M. tuberculosis*. *M. tuberculosis* H37Rv was incubated with increasing concentrations of rifampin (A), bedaquiline (B), linezolid (C), delamanid (D), erythromycin (E), clarithromycin (F), ampicillin (G), imipenem (H), and capreomycin (I) in the absence (black bars) or presence (gray bars) of 90 µg/ml HAMLET (75% of the MIC<sub>90</sub>). The viability of *M. tuberculosis* H37Rv was determined by the microplate alamarBlue assay. In each panel, the MIC<sub>90</sub> is represented by dotted lines. Error bars represent standard errors of the mean values of biological triplicates.

**Production and activity of recombinant HAMLET.** The HAMLET preparation used in the experiments described above was produced from  $\alpha$ -lactalbumin obtained from human milk, a heterogenous fluid in limited supply that may not be suitable for future drug development. In order to avoid using human samples and to have easy access to large quantities of HAMLET, we developed a standardized method of producing HAMLET from recombinant  $\alpha$ -lactalbumin. First, we synthesized a gene encoding modified human  $\alpha$ -lactalbumin. Human  $\alpha$ -lactalbumin is a Ca<sup>2+</sup>-binding metalloprotein whose native state is stabilized by four disulfide bonds (48). It has been shown that all cysteine residues in  $\alpha$ -lactalbumin can be mutated to alanines and that this mutated  $\alpha$ -lactalbumin forms a complex with oleic acid and retains the tumoricidal activity of HAMLET produced from  $\alpha$ -lactalbumin obtained from human milk (49). Since serine is structurally similar to cysteine, we opted to substitute all cysteines of  $\alpha$ -lactalbumin with serines (C6S, C28S, C61S, C73S, C77S, C91S, C111S, and C120S) to maintain the chain size and hydrophobic properties, while eliminating the formation of the disulfide bridges. Moreover, a mutation of aspartate 87 eliminates Ca(II) binding and results in partial unfolding, with a loss of tertiary structure (50). A bovine  $\alpha$ -lactalbumin with aspartate 87 substituted for alanine was also shown to bind oleic acid and to form a

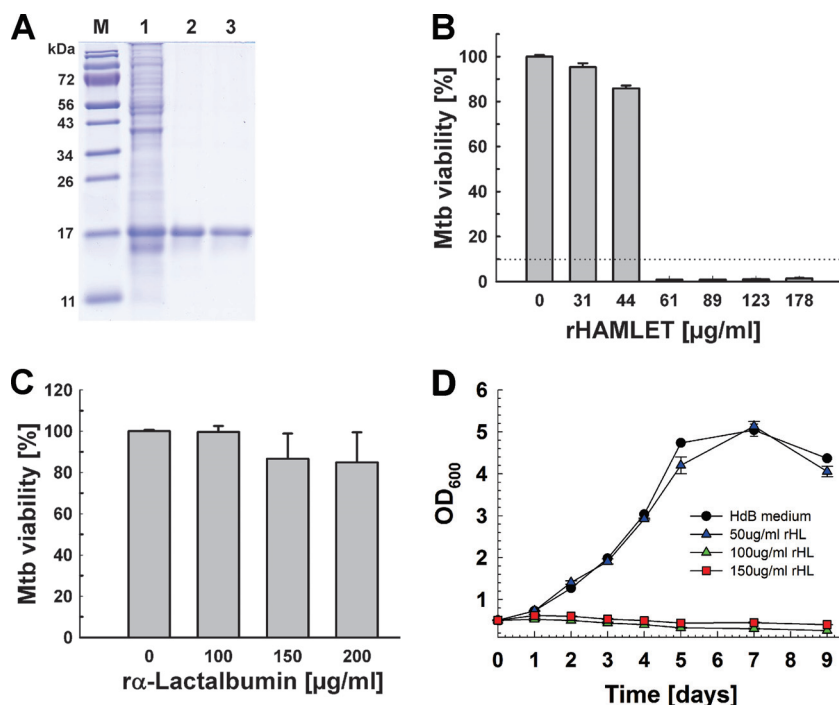
**TABLE 1** Potentiating effect of HAMLET on drugs and antibiotics against *M. tuberculosis*<sup>a</sup>

Antibiotic	MIC <sub>90</sub> (μg/ml) for:		Potentiation factor
	Drug	Drug + HAMLET	
Ampicillin	1,600	100	16
Bedaquiline	4	0.5	8
Capreomycin	40	2.5	16
Clarithromycin	80	5	16
D-Cycloserine	12.5	6.25	2
Delamanid	2	0.25	8
Erythromycin	4,000	500	8
Imipenem	8	2	4
Linezolid	10	2.5	4
Rifampin	1	0.125–0.25	4–8
Rifabutin	1.2	0.15	8
Streptomycin	1	0.5	2
Tetracycline	8	4	2

<sup>a</sup>The MIC<sub>90</sub>s of the antibiotics and TB drugs against *M. tuberculosis* H37Rv were determined using the microplate alamarBlue assay from experiments shown in Fig. 3. HAMLET was added at a concentration of 90 μg/ml (75% of the MIC<sub>90</sub>). This concentration did not affect the viability of *M. tuberculosis* (Fig. 3).

complex with oleic acid and have tumoricidal activity (50). Thus, the D87A mutation was included in our recombinant protein because α-lactalbumin unfolding is required for the conversion to HAMLET (50). The human α-lactalbumin gene encodes a signal peptide which is not part of the mature protein in human milk (51) and was therefore omitted in our construct. Finally, we added an N-terminal histidine tag for affinity purification. This synthetic gene encodes a recombinant α-lactalbumin with a theoretical molecular mass of 15 kDa and was cloned into the T7 polymerase-driven expression vector pET21a+ to yield pML3438 (Fig. S1). Recombinant α-lactalbumin was produced in *E. coli* BL21(DE3) and was purified from urea-solubilized inclusion bodies by nickel affinity chromatography under denaturing conditions to apparent homogeneity (Fig. 4A). We obtained ~43 mg recombinant α-lactalbumin from a 250-ml culture of *E. coli*. To produce recombinant HAMLET (rHAMLET), oleic acid (OA) was added to purified recombinant α-lactalbumin at a 5-fold molar excess in the presence of 2 M urea. This was followed by dialysis against phosphate-buffered saline (PBS) buffer with 2 M urea to remove free oleic acid (Fig. 4A, lane 3). After the second dialysis against only PBS, oleic acid was not detected anymore in the dialysis buffer. Furthermore, increasing the dialysis by 24 h did not change the oleic acid bound to α-lactalbumin. Both results indicated that the oleic acid in the rHAMLET preparation was strongly bound to lactalbumin and not freely available. The oleic acid bound to rHAMLET was determined using a coupled enzyme assay based on reactions catalyzed by acyl coenzyme A synthetase and acyl coenzyme A oxidase and a fluorescent probe which yielded a 6.5-fold molar excess of oleic acid over α-lactalbumin. Recombinant HAMLET (HLML1; final yield, 6.4 mg) was then lyophilized and stored as a powder at -80°C. We also prepared HAMLET from recombinant α-lactalbumin using 3- and 10-fold molar excesses of oleic acid. However, these preparations were less and overly active, respectively, compared to HLML1 and were not used in subsequent experiments.

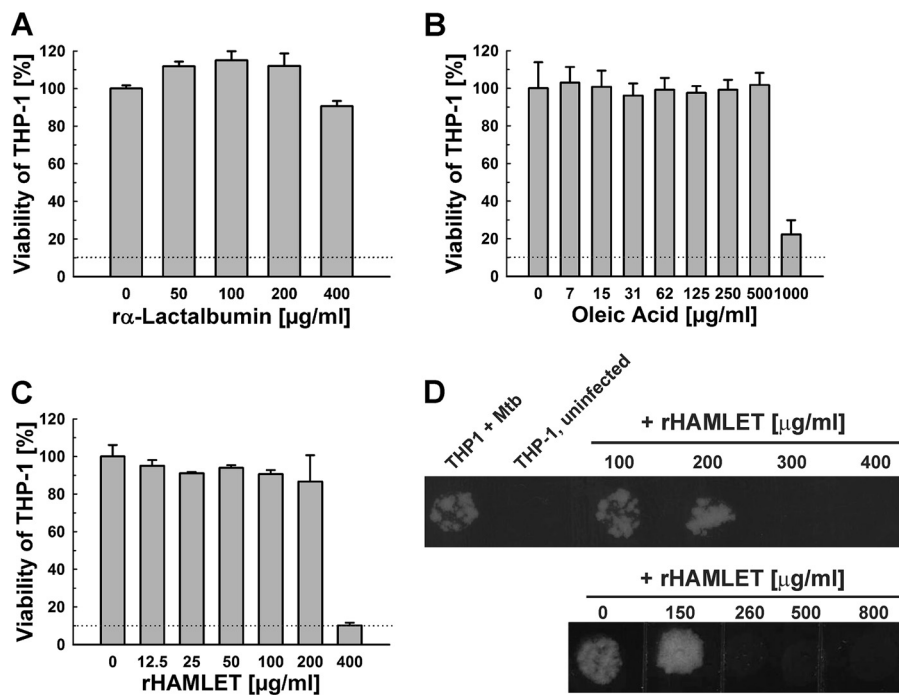
The activity of recombinant HAMLET against *M. tuberculosis* was then determined by an alamarBlue assay, as described above. Recombinant HAMLET strongly inhibited the growth of *M. tuberculosis*, with an MIC<sub>90</sub> of <60 μg/ml (Fig. 4B), whereas we did not observe a significant effect on the viability of *M. tuberculosis* using recombinant α-lactalbumin up to concentrations of 200 μg/ml (Fig. 4C). Recombinant HAMLET also inhibited the growth of *M. tuberculosis* in liquid cultures at a concentration of 100 μg/ml (Fig. 4D). These results demonstrated that the mutations of recombinant α-lactalbumin compared to the human protein and that the affinity tag did not interfere with the activity of recombinant HAMLET against *M. tuberculosis*. The higher activity of recombinant HAMLET compared to HAMLET derived from human α-lactalbumin might be due to its larger oleic acid load, based on the observation that the bactericidal activity of HAMLET is directly correlated with the type



**FIG 4** Production of recombinant HAMLET (rHL) from *E. coli*. (A) Analysis of purified recombinant HAMLET by gel electrophoresis. Lane 1, cell lysate of *E. coli* BL21(DE3) with the plasmid pML3438 encoding recombinant human  $\alpha$ -lactalbumin ( $\alpha$ -lactalbumin); lane 2, pooled  $\alpha$ -lactalbumin fractions after nickel affinity purification; lane 3, purified  $\alpha$ -lactalbumin after complexation with a 5-fold molar excess of oleic acid to generate HAMLET; lane M, molecular mass in kilodaltons. (B and C) The viability of *M. tuberculosis* H37Rv grown in Hartmans-de Bont (HdB) minimal medium in the presence of increasing concentrations of recombinant HAMLET (B) and  $\alpha$ -lactalbumin (C) was determined using the microplate alamarBlue assay. Error bars represent standard errors of mean values of biological triplicates. The MIC<sub>90</sub> is represented by a dotted line. Tukey's HSD following an F-test ( $P < 0.02$ ) revealed that the differences between 100, 150, and 200  $\mu$ g/ml  $\alpha$ -lactalbumin are not significant ( $P = 0.16$ ). (D) *M. tuberculosis* H37Rv was grown in HdB medium to an optical density at 600 nm of 0.5. Cells were harvested, split, and then treated with fresh HdB medium alone or with 50, 100, and 150  $\mu$ g/ml rHL at day 0.

and the amount of complexed fatty acid (52, 53). This assumption is supported by our finding that a larger molar excess of oleic acid during the formation of recombinant HAMLET increased its activity. However, we cannot exclude that some mutations might also increase the activity of recombinant HAMLET. Our approach to use recombinant  $\alpha$ -lactalbumin modified to associate with oleic acid without additional treatment is fast and yields large quantities of HAMLET, thus providing an efficient alternative to produce HAMLET which does not require human materials.

**HAMLET is active against *M. tuberculosis* in macrophages.** To examine the activity of recombinant HAMLET in macrophages, we first determined its cytotoxicity. Human THP-1 cells were treated with increasing concentrations of  $\alpha$ -lactalbumin, oleic acid, and HAMLET, and the viability of the THP-1 cells was determined using an alamarBlue assay, as described above. Recombinant  $\alpha$ -lactalbumin was not cytotoxic for THP-1 cells up to concentrations of 400  $\mu$ g/ml (Fig. 5A), whereas oleic acid exhibited cytotoxic effects at concentrations greater than 500  $\mu$ g/ml (Fig. 5B). The toxicity of recombinant HAMLET for THP-1 cells was observed at 400  $\mu$ g/ml (Fig. 5C). To examine whether recombinant HAMLET is active against *M. tuberculosis* within macrophages, THP-1 cells were infected with *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of 10. The infected THP-1 cells were then treated with 2-fold increasing concentrations of HAMLET for 3 days. After lysis of the THP-1 cells, the viability of *M. tuberculosis* was determined by plating on agar plates. In a first experiment, we observed that HAMLET concentrations up to 245  $\mu$ g/ml did not affect the growth of *M. tuberculosis* (not shown). In a subsequent experiment, we found that 260  $\mu$ g/ml HAMLET completely



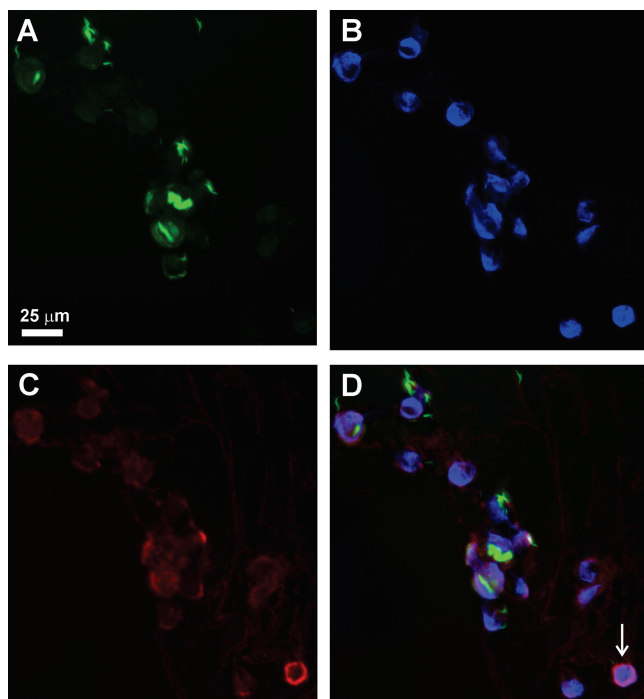
**FIG 5** Activity of recombinant HAMLET against *M. tuberculosis* in macrophages. The viability of differentiated THP-1 macrophages in the presence of increasing concentrations of recombinant  $\alpha$ -lactalbumin (A), oleic acid (B), and recombinant HAMLET (C) was determined using the microplate alamarBlue assay. Error bars represent standard errors of mean values of biological triplicates. The MIC<sub>90</sub> is represented by dotted lines. (D) Differentiated THP-1 macrophages were infected with *M. tuberculosis* H37Rv at an MOI of 10 for 4 h. Then, recombinant HAMLET was added at the indicated amounts, and the viability of *M. tuberculosis* was determined for two days using a drop assay on Middlebrook 7H10 agar plates. The uninfected macrophages are shown as a negative control.

inhibited the growth of *M. tuberculosis* (Fig. 5D). It cannot be excluded that the viability of some macrophages was affected by HAMLET at this concentration (Fig. 5C).

**HAMLET enters the cytoplasm of macrophages.** It has been previously observed that HAMLET produced from human milk binds to eukaryotic and prokaryotic cell membranes, is rapidly internalized, and accumulates in the nucleus (54–56). In order to examine whether this is also the case for recombinant HAMLET, we first confirmed that an antiserum against human  $\alpha$ -lactalbumin recognized the mutated recombinant  $\alpha$ -lactalbumin in an immunoblot (Fig. S1B). Then, human THP-1 cells were infected with *gfp* expressing *M. tuberculosis* and treated with 150  $\mu$ g/ml recombinant HAMLET for 16 h. The THP-1 cells were then stained with the anti- $\alpha$ -lactalbumin antibody (red) and 4',6-diamidino-2-phenylindole (DAPI; blue) to visualize the nucleus. The immunofluorescence images clearly showed that *M. tuberculosis* (green) is found in the cytoplasm of the THP-1 cells, presumably in phagosomes, and that recombinant HAMLET accumulates around the nucleus (Fig. 6). The accumulation of  $\alpha$ -lactalbumin around the nucleus was not caused by the infection of the macrophages with *M. tuberculosis*, since the same observation was made with uninfected THP-1 cells (not shown). These experiments demonstrate that recombinant HAMLET is capable of entering macrophages similar to HAMLET produced from human milk.

**HAMLET enhances the activities of bedaquiline, clarithromycin, and delamanid against *M. tuberculosis* in macrophages.** Since we established that recombinant HAMLET is active against *M. tuberculosis* in macrophages (Fig. 5), we examined whether it also potentiates the efficacies of antimycobacterial compounds within macrophages. To this end, we determined the effect of noninhibitory concentrations of recombinant HAMLET (200  $\mu$ g/ml) in the presence of TB drugs against *M. tuberculosis* within infected macrophages. *M. tuberculosis*-infected THP-1 cells were treated with 200  $\mu$ g/ml recombinant HAMLET in combination with various concentrations of each antibiotic in



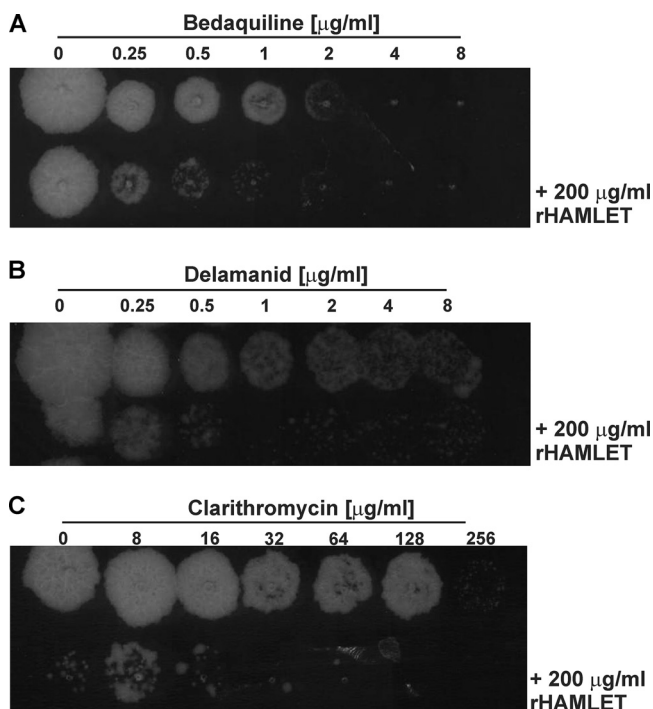


**FIG 6** HAMLET enters the cytoplasm of macrophages. THP-1 macrophages were infected with green fluorescent protein (GFP)-expressing *M. tuberculosis* mc<sup>2</sup>6206 at an MOI of 10:1 and treated with HAMLET (150  $\mu$ g/ml) for 16 h. The fluorescence is shown for the individual channels: (A) *M. tuberculosis* (green, GFP); (B) nuclei (blue, DAPI staining); (C) monoclonal human  $\alpha$ -lactalbumin antibody coupled with Alexa Fluor 594 (red). The overlay is shown in panel D.

microtiter plates. After 3 days of treatment, the viability of *M. tuberculosis* was determined by drop assays on Middlebrook 7H10 agar plates. Recombinant HAMLET increased the activities of the novel TB drugs bedaquiline and delamanid by 4-fold and 8-fold, respectively (Fig. 7), similar to the potentiation effect of HAMLET *in vitro* (Fig. 3B and D). The MIC<sub>90</sub> values of clarithromycin were previously found to be between 64 and 128  $\mu$ g/ml against different strains of *M. tuberculosis* (57), consistent with the MIC<sub>90</sub> of 80  $\mu$ g/ml determined in our study (Table 1). Since the efficacy of clarithromycin against *M. tuberculosis* *in vitro* was strongly enhanced by HAMLET, we included clarithromycin in our experiments. The MIC<sub>90</sub> of clarithromycin for *M. tuberculosis* in THP-1 cells was reduced from 256  $\mu$ g/ml to 16 to 32  $\mu$ g/ml in the presence of 200  $\mu$ g/ml recombinant HAMLET (Fig. 7C). Thus, HAMLET enhances the efficacy of clarithromycin against *M. tuberculosis* by 8- to 16-fold both *in vitro* and in macrophages. In contrast, and similar to the *in vitro* experiments performed with HAMLET derived from human milk, we did not observe any effect of recombinant HAMLET on the efficacies of levofloxacin, D-cycloserine, and isoniazid against *M. tuberculosis* in THP-1 macrophages (data not shown).

## DISCUSSION

**HAMLET has bactericidal activity against *M. tuberculosis*.** In this study, we showed that HAMLET, a complex of  $\alpha$ -lactalbumin and oleic acid derived from human milk, has bactericidal activity against *M. tuberculosis* (MIC<sub>90</sub>, 100 to 125  $\mu$ g/ml, or 6 to 7.5  $\mu$ M), as previously found for *S. pneumoniae* and other bacteria (32, 33, 56). This effect is specific for HAMLET, as the individual components of the complex had no activity under the same conditions. At first glance, this is a surprising result considering that mycobacteria have the lowest cell permeability known among bacteria (10, 58), while Gram-positive bacteria, such as *S. pneumoniae*, have a rather high cell permeability (59). However, it is known that *M. tuberculosis* is susceptible to low concentrations of oleic acid (60). HAMLET and similar protein-oleic acid complexes, called lipotides,



**FIG 7** Recombinant HAMLET increases the efficacy of bedaquiline, delamanid, and clarithromycin against *M. tuberculosis* in infected macrophages. Differentiated THP-1 cells were infected with *M. tuberculosis* H37Rv at an MOI of 10, washed, and treated with serial dilutions of bedaquiline (A), delamanid (B), and clarithromycin (C) in the presence and absence of 200  $\mu\text{g/ml}$  recombinant HAMLET for two days. Ten-microliter drops of lysed macrophages were then plated on Middlebrook 7H10 agar plates which were incubated for 13 days at 37°C.

form structures consisting of a micellar oleic acid core surrounded by a shell of partially denatured protein (61, 62). When lipotides contact lipid membranes, they transfer their entire fatty acid content within 20 s (63) and release the protein monomers (62). Numerous studies indicate that the fatty acids are the toxic components in HAMLET (52, 53, 64) and in other lipotides (41, 65). This is consistent with our finding that HAMLET was more active against *M. tuberculosis* with increasing oleic acid content. The function of the protein component, e.g.,  $\alpha$ -lactalbumin in HAMLET, is believed to increase the solubility of the fatty acid by shielding it from water (61, 62, 66). Our observation that HAMLET is approximately 10-fold more efficient in killing *M. tuberculosis* than the same molar amount of oleic acid, while  $\alpha$ -lactalbumin had no significant effect on the viability of *M. tuberculosis in vitro* (Fig. 1D) is consistent with the lipotide model, in which  $\alpha$ -lactalbumin increases the solubility and stability of the oleic acid micelle core and transports it to lipid membranes. The bactericidal activity of oleic acid against *M. tuberculosis* (67) might also explain the toxicity of HAMLET for *M. tuberculosis*.

It is conceivable that the lack of activity of HAMLET against many other bacteria (32) is related to their resistance to oleic acid. For example, *Staphylococcus aureus* and *Streptococcus pyogenes* (68) are resistant to oleic acid concentrations up to 6 mM. *Escherichia coli* (69), *Pseudomonas aeruginosa* (70), and *Enterococcus faecalis* (71) are used in large-scale conversions of oleic acid to hydroxy fatty acids, also indicating a significant resistance to oleic acid. A prominent exception appears to be *S. pneumoniae*, which is susceptible to HAMLET (32) but resistant to 6 mM oleic acid (68).

**HAMLET synergizes with TB drugs.** In our study, we showed that HAMLET at concentrations which did not affect the growth of *M. tuberculosis* (Fig. 3) potentiates the efficacies of a remarkably broad array of TB drugs and antibiotics by 2- to 16-fold against *M. tuberculosis* H37Rv (Table 1). The capability of HAMLET to sensitize bacteria to antibiotics was observed previously (33, 34). For example, sublethal concentrations of HAMLET increased the efficacy of penicillin, erythromycin, and gentamicin against

sensitive *S. pneumoniae* strains between 3- and 5-fold (33). HAMLET increased the efficacies of methicillin, erythromycin, and vancomycin against *S. aureus* strains sensitive to these antibiotics by 2-fold, although HAMLET showed no detectable activity by itself (34). Remarkably, HAMLET showed larger synergy effects between 8- and 300-fold against drug-resistant *S. pneumoniae* strains and 4- and 32-fold against drug-resistant strains of *S. aureus*. The rather large synergy of HAMLET with TB drugs and antibiotics against the drug-susceptible *M. tuberculosis* H37Rv strain suggests that HAMLET has the potential to make drug-resistant strains of *M. tuberculosis* susceptible to standard TB chemotherapy regimens. The molecular mechanism by which HAMLET augments the efficacy of antibiotics and drugs against *M. tuberculosis* is unknown. It is conceivable that HAMLET might increase the membrane permeability of *M. tuberculosis*, as has been described for other cells (55, 72, 73). HAMLET was also shown to decrease the bacterial proton motive force that may reduce the activity of efflux pumps and cause an accumulation of antibiotics within bacterial cells (32, 33). However, intracellular effects of HAMLET might also play a role, e.g., HAMLET-induced sensitization of *S. pneumoniae* was found to require calcium influx and kinase activation that may synergize with antibiotics and other drugs to increase their bactericidal activity (33).

Sensitization of bacterial pathogens to existing antibiotics has been mainly explored by using drug efflux pump inhibitors (74, 75). This approach also appears to be promising for *M. tuberculosis*, which encodes the largest number of drug efflux pumps compared to its genome size among known bacteria (76). Indeed, numerous studies have shown the role of efflux pumps in drug resistance of *M. tuberculosis* (16, 77). In particular, thioridazine was shown to synergize with TB drugs by enhancing the efficacy of isoniazid and rifampin by 64- and 128-fold (78), has sterilizing activity in combination with a first-line regimen against acute murine tuberculosis (79), and cured 17 out of 18 XDR-TB patients in Argentina (80). However, thioridazine and other phenothiazines inhibit  $\text{Ca}^{2+}$  ion channels and are used as antipsychotic drugs (24); hence, they have side effects and are cytotoxic at low concentrations. For example, the 50% inhibitory concentration ( $\text{IC}_{50}$ ) of thioridazine for human macrophages is 5 to 8  $\mu\text{g}/\text{ml}$  (24, 78, 81). An additional cytotoxic mechanism of many drug efflux pump inhibitors is that they impair host transporter proteins (82).

**HAMLET is active against *M. tuberculosis* in macrophages.** The activity of HAMLET against *M. tuberculosis* in THP-1 cells (Fig. 5D) is remarkable because it indicates that oleic acid, the toxic component of HAMLET (52, 64), is released in sufficient quantities into the macrophage cell to impair growth or kill *M. tuberculosis*. Interestingly, we also observed that  $\alpha$ -lactalbumin accumulated around the nucleus of both uninfected and *M. tuberculosis*-infected THP-1 cells after treatment with HAMLET (Fig. 6). Since THP-1 cells were derived from the peripheral blood of a patient with acute monocytic leukemia (83), cell entry of  $\alpha$ -lactalbumin and accumulation in the nucleus of THP-1 cells after treatment with HAMLET is consistent with previous observations for tumor cells (30, 84). Treatment of tumor cells with HAMLET leads to a loss of the mitochondrial membrane potential, induces mitochondrial swelling and the release of cytochrome *c* (31), disrupts chromatin by strongly interacting with histones (54), perturbs the structure and integrity of lipid membranes (55, 72), and triggers signaling cascades resulting in apoptotic cell death (85, 86). However, it is unknown whether and how these mechanisms contribute to the growth restriction of *M. tuberculosis* in HAMLET-treated THP-1 cells. Further experiments are needed to establish whether HAMLET is also active against *M. tuberculosis* in primary human macrophages.

**Can HAMLET be used in TB treatment regimens?** While the bactericidal activity of HAMLET against *M. tuberculosis* is surprising, the potentiating effect for a broad range of antibiotics and TB drugs is certainly more useful from a therapeutic point of view. It should be noted that it is unlikely that HAMLET alone will play a role in TB chemotherapy due to the rather high concentration needed to kill *M. tuberculosis* in macrophages ( $\sim 260 \mu\text{g}/\text{ml}$ ). In addition, these concentrations might be cytotoxic for human cells, as we found that HAMLET kills THP-1 cells at concentrations between 200 and

400  $\mu\text{g/ml}$ . For medical applications, the finding that HAMLET augmented the activities of bedaquiline, delamanid, and clarithromycin inside macrophages by factors similar to those *in vitro* is more relevant because lung macrophages constitute the initial habitat of *M. tuberculosis* after infection in the host lung (87). Importantly, clinical studies showed that topical applications of HAMLET were safe and that HAMLET had tumor-specific activity, resulting in an effective treatment of bladder and skin cancer in humans (88, 89). *In vitro* studies showed that HAMLET is assembled at low pH and is protease resistant (90), suggesting that it can survive the harsh conditions in the stomach. Indeed, oral applications of HAMLET not only reduced tumor progression and mortality in mice with colon cancer, but it also reduced tumor development when given prophylactically in drinking water (91, 92). Taken together, these results suggest that HAMLET might be a useful reagent in current TB regimens or in new combinations including drugs which are more efficient in the presence of HAMLET (Table 1). Probably the most exciting potential application is based on the recent findings that protein complexes, such as HAMLET, can package hydrophobic molecules, such as cholesterol, tryptophan, and the hydrophobic vitamins D and E in their fatty acid micelle core (93–95). Packaging into lipotides not only drastically increased the solubility and stability of these molecules in water but also delivered them to lipid membranes (63, 93, 95). Thus, incorporation of the very hydrophobic TB drugs, such as rifampin, rifabutin, bedaquiline, and/or delamanid, into HAMLET would not only make these drugs more water-soluble and likely increase their chemical and metabolic stability, but it would also provide a transport vehicle to cells and a mechanism to exploit the considerable synergy of HAMLET with these drugs (Table 1). Another beneficial property of HAMLET is its potential to reduce the emergence of drug-resistant strains by interfering with the slow evolution of drug resistance in *M. tuberculosis* (96) in a manner similar to that shown for the drug efflux pump inhibitors verapamil (97) and thioridazine (98). Indeed, HAMLET inhibited resistance development when *S. aureus* was repeatedly exposed to methicillin in the presence of HAMLET (34). However, access of drugs to *M. tuberculosis* in granulomas, the primary sites of *M. tuberculosis* in the lungs of TB patients, and the phenotypic resistance of *M. tuberculosis* in the cavity of granulomas are key determinants of the efficacy of TB drugs (13, 99) and represent major hurdles in TB drug development. The unique property of HAMLET and other lipotides to interact with lipid membranes and rapidly unload their content into target cells (63) might improve granuloma penetration of drug cargo and might also reduce potential side effects.

**Conclusions.** HAMLET is a promising new agent that directly kills *M. tuberculosis* and augments the activity of several TB drugs and antibiotics against *M. tuberculosis*. The synergistic effect of HAMLET with hydrophobic drugs could be exploited in coencapsulation formulations (93) and might enable the use of drugs which have become ineffective against drug-resistant strains. The observations that HAMLET is stable after oral delivery in mice (91) and is nontoxic in human studies (88) highlight the potential of HAMLET as a TB drug delivery and sensitization agent. These findings justify experiments to examine the activity of HAMLET coadministered with current TB drugs and of HAMLET-drug complexes in animal models of TB.

## MATERIALS AND METHODS

**Chemicals and enzymes.** All chemicals were purchased from AdooQ Bioscience, ABM, GE Healthcare, or Sigma at the highest purity available. Enzymes for DNA restriction and modification were purchased from New England BioLabs. Isolation and modification of DNA were performed as described previously (100). The  $\alpha$ -lactalbumin genes were obtained from GenScript USA, Inc. Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Table S1).

**Bacterial strains and growth conditions.** All bacterial strains used in this study are listed in Table S2. *Escherichia coli* DH5 $\alpha$  was used for all cloning experiments and was routinely grown in LB medium at 37°C. Initial cultures of *M. tuberculosis* were grown at 37°C in Middlebrook 7H9 liquid medium (Difco Laboratories) supplemented with 10% Middlebrook OADC enrichment (0.5 g/liter oleic acid, 50 g/liter albumin, 20 g/liter dextrose, 0.04 g/liter catalase, 8.5 g/liter sodium chloride; Difco Laboratories), 0.2% glycerol, and 0.025% tyloxapol. Then, *M. tuberculosis* was grown in Hartmans-de Bont (HdB) minimal medium (101) with 0.02% tyloxapol for *in vitro* susceptibility experiments or suspended in cell culture medium (RPMI 1640) for experiments with macrophages. For testing the viability of *M. tuberculosis*,

samples were plated on Middlebrook 7H10 agar (Difco Laboratories) supplemented with 0.2% glycerol, unless stated otherwise. Antibiotics were used as described in the text.

**Production and characterization of HAMLET from human milk.** Human  $\alpha$ -lactalbumin purified from human milk was converted into HAMLET by complexing EDTA-treated apo-protein with oleic acid (C<sub>18:1</sub>; Sigma) on an ion-exchange matrix, and the HAMLET complex was eluted with salt, as described previously (32). The complexed material was dialyzed with water to remove salt, and the protein-lipid complex was lyophilized and saved at  $-80^{\circ}\text{C}$  until use. The oleic acid content of the HAMLET batches was determined using a coupled enzymatic reaction (catalog no. MAK044; Sigma-Aldrich). The fluorescence at 585 nm was measured using an excitation at 535 nm. Before dilution of HAMLET for each experiment, the concentration of the stock solution was determined by measuring the absorption at 280 nm using the theoretical extinction coefficient for  $\alpha$ -lactalbumin in a NanoDrop spectrophotometer (Thermo Scientific).

**Susceptibility of *M. tuberculosis* to HAMLET, antibiotics, and TB drugs by the alamarBlue assay.**

To determine the MICs of HAMLET, oleic acid,  $\alpha$ -lactalbumin, antibiotics, and TB drugs, we used the microplate alamarBlue assay (MABA) as described previously (102, 103). Briefly, *M. tuberculosis* was grown at  $37^{\circ}\text{C}$  in supplemented Middlebrook 7H9 liquid medium. Then, a 500- $\mu\text{l}$  aliquot of the culture was added to 10 ml of Hartmans-de Bont (HdB) minimal medium (101) with 0.02% tyloxapol until the culture reached an OD<sub>600</sub> of 1. The culture was then diluted by 20-fold in HdB medium with 0.02% tyloxapol to an OD<sub>600</sub> of 0.05. Aliquots of 100  $\mu\text{l}$  were added to the wells of a 96-well microplate containing 100  $\mu\text{l}$  of HAMLET, oleic acid,  $\alpha$ -lactalbumin, antibiotics, antibiotics plus HAMLET, or HdB medium as a control. Dilutions of HAMLET, oleic acid,  $\alpha$ -lactalbumin, and antibiotics were prepared in HdB medium. Antibiotics and drugs were serially 2-fold diluted from the following starting concentrations: rifampin, 1  $\mu\text{g}/\text{ml}$ ; bedaquiline, 4  $\mu\text{g}/\text{ml}$ ; linezolid, 20  $\mu\text{g}/\text{ml}$ ; delamanid, 2  $\mu\text{g}/\text{ml}$ ; erythromycin, 4 mg/ml; clarithromycin, 80  $\mu\text{g}/\text{ml}$ ; ampicillin, 1,600  $\mu\text{g}/\text{ml}$ ; imipenem, 32  $\mu\text{g}/\text{ml}$ ; capreomycin, 80  $\mu\text{g}/\text{ml}$ ; 4-salicylic acid, 80  $\mu\text{g}/\text{ml}$ ; streptomycin, 32  $\mu\text{g}/\text{ml}$ ; tetracycline, 64  $\mu\text{g}/\text{ml}$ ; rifabutin, 1.2  $\mu\text{g}/\text{ml}$ ; D-cycloserine, 100  $\mu\text{g}/\text{ml}$ ; ofloxacin, 4  $\mu\text{g}/\text{ml}$ ; amikacin, 1.25  $\mu\text{g}/\text{ml}$ ; and levofloxacin, 128  $\mu\text{g}/\text{ml}$ . After incubation of the microplates for 3 days at  $37^{\circ}\text{C}$ , resazurin was added to each well at a final concentration of  $\sim 90 \mu\text{M}$ . After an additional incubation of 6 h, the fluorescence of the metabolically converted resazurin dye was measured at 590 nm after excitation at 530 nm using a Cytation3 imaging reader (BioTek). The MICs (Table 1) were defined as the lowest concentration of antibiotic which reduced the viability of *M. tuberculosis* by at least 90%.

**Activity of HAMLET against *M. tuberculosis* in liquid medium.** *M. tuberculosis* H37Rv was grown in a 60-ml bottle with Hartmans-de Bont (HdB) minimal medium to an OD<sub>600</sub> of 1.3. Then, 10 ml of the culture was centrifuged, and the pelleted cells were resuspended in the same volume of fresh HdB medium. The culture was split, and 2.5 ml was transferred to each of two wells of a 12-well microplate. One culture was treated with a final concentration of 300  $\mu\text{g}/\text{ml}$  HAMLET each day for 3 days. The microplates were incubated at  $37^{\circ}\text{C}$  for 14 days. HdB medium in a third well served as a background control for the optical density of medium alone. Each day, the OD<sub>600</sub> was measured, and 10  $\mu\text{l}$  of each sample of serial 10-fold dilutions was dropped on Middlebrook 7H10 agar plates. The agar plates were incubated for 15 days at  $37^{\circ}\text{C}$ .

The activity of recombinant HAMLET against *M. tuberculosis* was determined in 30-ml bottles, each containing 5 ml of HdB medium. After *M. tuberculosis* was grown to an OD<sub>600</sub> of 0.5, recombinant HAMLET was added to final concentrations of 50, 100, and 150  $\mu\text{g}/\text{ml}$ . The OD<sub>600</sub> was measured every day for 10 days.

**Cytotoxicity of oleic acid,  $\alpha$ -lactalbumin, and HAMLET for macrophages.** To determine the cytotoxicity of oleic acid,  $\alpha$ -lactalbumin, and HAMLET for macrophages, we used the microplate alamarBlue assay. THP-1 cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, and 0.5% nonessential amino acids in a 5% CO<sub>2</sub> environment at  $37^{\circ}\text{C}$ . Cells were seeded at 100,000 per well in 96-well plates, washed in supplemented RPMI 1640 medium without fetal bovine serum, and differentiated by the addition of 0.4% phorbol 12-myristate 13-acetate (PMA) for 17 h. To determine the toxicity of oleic acid in macrophages, a 100 mg/ml solution of oleic acid in 100% ethanol was diluted 100-fold in the supplemented RPMI 1640 medium without fetal bovine serum, as described above. The serial dilutions were prepared in tubes before addition to the macrophages. As a negative control, ethanol was diluted in the same medium and added to macrophages. Oleic acid micelles were visualized using a light microscope. Serial dilutions of recombinant  $\alpha$ -lactalbumin in RPMI 1640 medium supplemented as described above were added to the macrophages. To determine the toxicity of HAMLET or TB drugs, alone or in combination, compounds were diluted in same supplemented RPMI 1640 medium and then added to the macrophages. The microplates were incubated at  $37^{\circ}\text{C}$  in a 5% CO<sub>2</sub> incubator for up to 48 h. Resazurin dye was added to each well at a final concentration of  $\sim 90 \mu\text{M}$ . After an additional incubation of 6 h, the fluorescence of the metabolically converted resazurin dye was quantified using a Cytation3 imaging reader (BioTek).

**Intracellular activity of HAMLET against *M. tuberculosis*.** To determine the intracellular activity of HAMLET, *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 medium to an OD<sub>600</sub> of about 1.0, pelleted, and resuspended in the supplemented RPMI 1640 medium. THP-1 cells were grown in a flask and then differentiated with 0.4% PMA in 96-well microplates, as described above. Then, the differentiated THP-1 cells were infected with *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of 10:1 for 4 h. Uninfected THP-1 cells were used as a control. The microplate was washed two times with RPMI 1640 medium, and 200  $\mu\text{l}$  RPMI 1640 medium alone or dilutions of different HAMLET concentrations in RPMI 1640 medium were added to the wells and incubated at  $37^{\circ}\text{C}$  in a 5% CO<sub>2</sub> for 2 days. Then, the macrophages were lysed by adding 100  $\mu\text{l}$  of  $1\times$  PBS and 0.025% SDS per well. Serial 10-fold dilutions in  $1\times$  PBS were made in another 96-well microplate. Five microliters of each dilution was dropped on Middlebrook 7H10 agar plates, which were incubated for 15 days at  $37^{\circ}\text{C}$ . The agar plates were imaged using a scanner.

**Construction of an expression vector for production of recombinant human  $\alpha$ -lactalbumin from *E. coli*.** *E. coli* DH5 $\alpha$  (Table S2) was used for plasmid construction. The DNA encoding the mutated human  $\alpha$ -lactalbumin (C6S, C28S, C61S, C73S, C77S, D87A, C91S, C111S, and C120S) with an N-terminal histidine tag comprising eight histidines was synthesized by GenScript. This DNA fragment was cloned into the pET-21(b)+ plasmid using the restriction sites NdeI and HindIII, resulting in the expression plasmid pML3438 (Table S3). The DNA sequence of the mutated  $\alpha$ -lactalbumin gene was confirmed by sequencing (Heflin Center for Genomic Science, UAB). The sequence of the final recombinant  $\alpha$ -lactalbumin protein is HHHHHHHHKQFTK**SELSQLLKIDIDYGGI**ALPEL**ISTMFHTSGYDTQA**IVENNESTEYGLFQISNKLW**SKSSQVPQSRNISKD**IS**SKD**FLDD**ITADIM**SAKKILDIKGIDYWLAKHKA**LST**EKLEQWL**SEKL**. The positions of the mutated residues are in bold and underlined. The calculated isotopically averaged molecular mass of the recombinant  $\alpha$ -lactalbumin protein is 15,003 kDa.

**Purification of recombinant lactalbumin from *E. coli* and production of HAMLET.** *E. coli* BL21(DE3) carrying the  $\alpha$ -lactalbumin expression plasmid pML3438 was cultured for 24 h in ZYP-5052 autoinduction medium (104) supplemented with 100  $\mu$ g/ml ampicillin at 37°C. The cells were harvested by centrifugation, washed, and lysed by sonication in PBS (pH 7.5) containing 10 mg/ml lysozyme, 200 mM phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor cocktail (cComplete Mini; Sigma). The insoluble fraction was collected by centrifugation and dissolved in 6 M urea in PBS (pH 7.5). The protein solution was loaded onto a Ni(II) affinity column (HisTrap HP; GE Healthcare) in the same buffer, and the protein was eluted using 250 mM imidazole. The fractions containing  $\alpha$ -lactalbumin (15 kDa) were pooled, and oleic acid dissolved in 100% ethanol was added at a 5-fold molar excess over  $\alpha$ -lactalbumin. This mixture was dialyzed against PBS (pH 7.5) containing 2 M urea for 24 h. This step was followed by a second dialysis against only PBS (pH 7.5) for 24 h to remove free oleic acids and urea. Recombinant HAMLET was then lyophilized and kept as a powder at  $-80^{\circ}\text{C}$  until used. Before performing the experiments, the biological activity of HAMLET was tested as described above. HAMLET was dissolved in the appropriate medium or buffer and then filtered using a polyvinylidene difluoride (PVDF) filter with a pore size of 0.22  $\mu\text{m}$  (Millipore). Additionally, the concentration of HAMLET was determined photometrically before each experiment by measuring the absorption at 280 nm using the theoretical extinction coefficient of 22,460  $\text{M}^{-1}\text{cm}^{-1}$  for recombinant  $\alpha$ -lactalbumin and a NanoDrop spectrophotometer (Thermo Scientific).

**Fluorescence microscopy of macrophages.** Uninfected THP-1 cells and THP-1 cells infected with *gfp*-expressing *M. tuberculosis* (105) were treated with 150  $\mu$ g/ml human  $\alpha$ -lactalbumin or HAMLET. The cells were incubated in an 8-well glass slide (Lab-Tek II Chamber Slide) for 16 h at 37°C. The coverslips were washed with warm fresh RPMI 1640 medium and fixed for 10 min at room temperature with 4% paraformaldehyde. The THP-1 cells were washed three times with PBS and then incubated with 0.2% Triton X-100 for 5 min to permeabilize the cells. The cells were washed again with PBS, blocked with 3% normal goat serum (NGS) in 3% bovine serum albumin (BSA) for 45 min at room temperature, and then incubated with a 1:300 dilution of an anti- $\alpha$ -lactalbumin monoclonal mouse antibody overnight at 4°C. The cells were washed three times with PBS and incubated for 1 h with a 1:500 dilution of a goat anti-mouse antibody coupled with Alexa Fluor Plus 594 (gift from Elizabeth Sztul) at room temperature. Then, the cells were washed three times with PBS, incubated with 300 nM 4',6-diamidino-2-phenylindole (DAPI) for 5 min, washed with PBS, and kept at 4°C in PBS. For fluorescence microscopy, the THP-1 cells were covered with FluorSave medium (Calbiochem) and a coverslip. Images were taken using Zeiss AxioXam MRm Rev3 and/or MRC cameras attached to a Zeiss AxioImager Z1 epifluorescence microscope (Carl Zeiss, Thornwood, NY).

**Statistical analysis.** SigmaPlot (Systat Software) was used for graph development and statistical analysis. Where applicable, statistical significance was determined by Tukey's honestly significant difference (HSD) test following an F-test. *P* values less than 0.05 are considered significant. All data presented are mean values, with error bars representing the standard error of the mean values of biological triplicates.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01846-18>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

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