J. vet. Pharmacol. Therap. **30**, 151–156, 2007.

Intracellular killing of mastitis pathogens by penethamate hydriodide following internalization into mammary epithelial cells

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Almeida, R. A., Patel, D., Friton, G. M., Oliver, S. P. Intracellular killing of mastitis pathogens by penethamate hydriodide following internalization into mammary epithelial cells. *J. vet. Pharmacol. Therap.* **30**, 151–156.

Penethamate hydriodide was highly effective in killing *Streptococcus uberis*, *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *Staphylococcus aureus* that internalized mammary epithelial cells. At higher concentrations (32 μg/mL to 32 mg/mL), killing rates ranged from 85% to 100%. At lower concentrations (0.032 μg/mL to 3.2 μg/mL), killing rates ranged from 0 to 80%. Results of this proof-of-concept study demonstrated that: (1) penethamate hydriodide is capable of entering mammary epithelial cells and killing intracellular mastitis pathogens without affecting mammary epithelial cell viability, (2) the *in vitro* model used is capable of quantifying the fate of mastitis pathogens internalized into mammary epithelial cells, and (3) this *in vitro* model can be used to determine the effectiveness of antibiotics at killing bacteria within the cytoplasm of mammary epithelial cells.

(Paper received 25 September 2006; accepted for publication 8 December 2006)

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INTRODUCTION

Research from our laboratory has shown that several bovine mastitis pathogens adhere to and internalize into bovine mammary epithelial cells (Matthews *et al.*, 1994; Almeida & Oliver, 1995; Almeida *et al.*, 1996a,b). We hypothesize that adherence and internalization are important virulence attributes of bovine mastitis pathogens. Adherence is important for bovine mastitis pathogens once they gain entry into the udder. Otherwise bacteria would be removed quite easily by fluid flow during milking. Internalization of bovine mastitis pathogens into the cytoplasm of mammary epithelial cells allows pathogens an opportunity to reach the intracellular microenvironment. There is indirect evidence of internalization into bovine tissue. Oliver *et al.* (1998) demonstrated that the same clonal type of *Streptococcus uberis* was isolated from the same mammary quarter for more than one lactation period. Once internalized, mastitis pathogens could infect deeper regions of the affected bovine mammary gland resulting in the development of chronic intramammary infections. Following internalization, components of the immune system such as polymorphonuclear neutrophils, macrophages and antibodies; nonspecific antibacterial factors present in bovine milk; and antibiotics that do not cross mammary epithelial cell membranes are essentially ineffective. Therefore, it would appear that central to the control of several bovine mastitis pathogens is the ability of antibiotic preparations used in the treatment of mastitis to penetrate into host cells and kill intracellular bacteria within the cytoplasm of mammary epithelial cells.

We have developed cell culture techniques that allow the study of bovine mammary epithelial cell–bovine mastitis pathogen interactions that occur during the early stages of pathogenesis as well as the fate of mastitis pathogens once they are internalized into mammary epithelial cells (Matthews *et al.*, 1994; Almeida & Oliver, 1995; Almeida *et al.*, 1996a,b). It is likely that internalization into epithelial cells provides bacterial pathogens a better chance to become persistent, and therefore cause chronic mastitis.

The objective of the present study was to use these techniques to assess the intracellular killing activity of penethamate hydriodide, a diethylaminoethyl ester of benzylpenicillin which is licensed in various countries worldwide for the systemic treatment of mastitis in cattle. To our knowledge, this is the first study of its kind to assess the activity of an antibiotic preparation used in the treatment of mastitis to penetrate into host cells and kill intracellular bovine mastitis pathogens within the cytoplasm of bovine mammary epithelial cells.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Streptococcus uberis (UT888), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (UT19), and *Staphylococcus aureus* (UT23) isolated

from dairy cows with mastitis were used. All mastitis pathogens were characterized extensively by biochemical analysis and PCR-based DNA fingerprinting, and have been used extensively in our laboratory. A nonpathogenic strain of *Escherichia coli* (DH5α; Gibco, Grand Island, NY, USA) was used as a negative control. Gram-positive bacteria were cultivated in Todd–Hewitt broth (THB; Becton Dickinson and Co., Franklin Lakes, NJ, USA) and *E. coli* was cultivated in Luria broth (LB). All bacteria were subcultivated on blood agar. For internalization assays, bacterial lawns were harvested, washed and resuspended at approximately 10^7 colony-forming units per milliliter (cfu/mL) in Dulbecco's Modified Eagle's medium (DMEM, Gibco). The concentration of bacteria and strain purity were determined by standard plate count techniques.

Fluorescent labeling of bacteria

Procedures for immunofluorescence staining and confocal laser microscopy (CLM) followed previously described protocols (Barker *et al.*, 1997) with modifications. Briefly, mastitis pathogens and *E. coli* DH5α kept at −70 °C were thawed at room temperature, plated onto blood agar, and incubated overnight at 37 °C in 5% CO_2:95% air (v/v). After incubation, the bacterial lawn was harvested with 0.5 mL of THB or LB, seeded into 9.5 mL of the same media (THB or LB), and incubated for 1 h at 37 °C with orbital shaking at 150 r.p.m. After incubation, bacterial suspensions were washed three times by centrifugation (20 800 ***g*** for 3 min at 4 °C) with phosphate-buffered saline (PBS) (pH 7.4) and fluorescently labeled (LIVE/DEAD *Bac*Light Bacterial Viability Kits L-7007; Molecular Probes, Eugene, OR, USA) following manufacturer instructions.

Mammary epithelial cell culture

A bovine mammary epithelial cell line (MAC-T, kindly provided by J.D. Turner, McGill University, Canada) was used (Huynh *et al.*, 1991). MAC-T cells were grown in 24-well cell culture plates (Corning Inc., Corning, NY, USA) or 8-well slides (Lab-Tek II; Nalge Nunc International Corp., Rochester, NY, USA) at 37 °C in 5% CO_2:95% air (v/v) using cell growth media described previously (Almeida *et al.*, 1996b). Mammary epithelial cell viability was monitored by trypan blue dye exclusion.

Internalization and intracellular bactericidal assay

Fluorescent-labeled or untreated mastitis pathogens and *E. coli* DH5α were co-cultured with MAC-T cells in DMEM. Following incubation [2 h at 37 °C in 5% CO_2:95% air (v/v)], monolayers were washed three times with PBS (pH 7.4) and then incubated (60 min at 37 °C CO_2:95% air (v/v)] with and without addition of several 10-fold dilutions of penethamate hydriodide (32 mg/mL, 3.2 mg/mL, 0.32 mg/mL, 32 μg/mL, 3.2 μg/mL, 0.32 μg/mL, and 0.032 μg/mL; Mamyzin®; Boehringer Ingelheim Animal Health GmbH, Ingelheim/Rhein, Germany). Each vial contained 10 g of penethamate that when dissolved with the solvent provided a concentration of approximately 32 mg/mL. Tests were started using 32 mg/mL and 10-fold dilutions were used until having reached the lowest concentration in which the lowest killing activity was detected (0.032 μg/mL). The lower concentrations used (0.32–3.2 μg/mL) included concentrations that penethamate reaches in milk under natural conditions (Friton *et al.*, 2003).

As a control, co-cultures were incubated with DMEM containing gentamicin (100 μg/mL; Sigma Chemical Co., St Louis, MO, USA) and penicillin (100 IU/mL, Sigma) or no antibiotic. Penicillin and gentamicin do not penetrate into mammary epithelial cells and are used in the standard internalization protocol to eliminate bacteria that are outside of mammary epithelial cells and allow discrimination between intracellular and extracellular micro-organisms (Isberg & Falkow, 1985; Rubens *et al.*, 1992). After removing media containing antibiotics, MAC-T cell monolayers were washed and lysed. MAC-T cell lysates were 10-fold serially diluted, plated in triplicate on blood agar, and incubated overnight. Intracellular survival was evaluated by determining the number of cfu/mL in MAC-T cell lysates. For fluorescent assays, after incubation with penethamate hydriodide, slides were washed with PBS (pH 7.4) and mounted. Cover slips were then sealed onto slides with nail polish and kept at 4 °C until visualization by CLM (Leica TCS SP2; Leica Microsystems, Heidelberg, Germany). Internalization and intracellular survival assays were performed in triplicate three times.

Image analysis

Red/green images were collected and overlaid using Leica Lite software (Leica Microsystems, Heidelberg, Germany). Further image analysis was conducted using IMAGE J software (http://www.rsb.info.nih.gov/ij/).

Transmission electron microscopy

MAC-T cell monolayers grown on plastic cell culture chamber slides (Lab-Tek II, Nalge Nunc International Corp.) were co-cultured with mastitis pathogens and *E. coli* DH5α. After incubation (2 h at 37 °C in 5% CO_2:95% air (v/v)], monolayers were washed three times with PBS (pH 7.4) and treated with penethamate hydriodide (32 μg/mL, Ingel-Mamyzin®; Boehringer Ingelheim Animal Health GmbH). Monolayers were fixed in 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer for 2 h at room temperature. Monolayers were then washed three times with 0.1 M cacodylate buffer (pH 7.2) for 30 min. Postfixation was done in 1% osmium tetraoxide (Sigma) and samples were dehydrated through a graded alcohol series. Monolayers were then embedded in Epon 812 resin (Polysciences, Warrington, PA, USA), thin-sectioned along the sagittal axis, and stained with uranyl acetate and lead citrate. Ultrathin sections were examined by transmission electron microscopy (TEM).

Statistical analysis

Unless otherwise noted, assays were performed three times with each condition tested in triplicate. Mean values from each experiment were analyzed by two-way analysis of variance and those showing statistically significant differences ($P \leq 0.05$) were further analyzed by Student's *t*-test using PROSTAT (Poly Software International, Salt Lake City, UT, USA) statistical software.

RESULTS

Internalization of mastitis pathogens

Initial work conducted by Isberg and Falkow (1985) showed that the inability of penicillin and gentamicin to penetrate into eukaryotic cells could be used to discriminate between internalized and extracellular bacteria. This technique was initially used in gram-positive bacteria by Rubens *et al.* (1992) to show that group B streptococci was able to internalize into host cells. Previous work conducted in our lab showed that *S. uberis*, *S. dysgalactiae* subsp. *dysgalactiae* and *S. aureus* internalize into bovine mammary epithelial cells in a time-dependent manner reaching high numbers of internalized bacteria after 2 h of co-culture (Matthews *et al.*, 1994; Almeida & Oliver, 1995; Almeida *et al.*, 1996a). Results of the present study showed that the mean number of bacteria internalized into mammary epithelial cells was 1.2×10^6, 8.6×10^6, 2.5×10^5, and 0 for *S. uberis*, *S. dysgalactiae* subsp. *dysgalactiae*, *S. aureus*, and *E. coli* DH5α, respectively. Transmission electron and confocal microscopy used in this study verified that large numbers of intracellular bacteria were detected after 2 h of co-culture.

Intracellular antimicrobial activity

The antimicrobial activity of penethamate hydriodide was evaluated by determining its killing activity against intracellular bovine mammary pathogens using several 10-fold dilutions of the antimicrobial. The ability of different concentrations of penethamate hydriodide to kill intracellular *S. uberis*, *S. dysgalactiae* subsp. *dysgalactiae*, and *S. aureus* are shown in Fig. 1. A dose-dependent rate of intracellular bacterial killing was observed with each bovine mastitis pathogen evaluated. Viability of mammary epithelial cells was not affected by addition of any of the concentrations of penethamate hydriodide based on confocal microscopy and trypan blue dye exclusion.

Streptococcus uberis

The highest concentration of penethamate hydriodide (32 mg/mL) killed all intracellular *S. uberis*. Penethamate hydriodide at 3.2, 0.32 and 0.032 mg/mL killed 98.9 ± 0.8%, 97.8 ± 2.0% and 85.3 ± 3.4% of intracellular *S. uberis*, respectively. These results were statistically significant ($P < 0.01$) when compared with untreated controls. The lowest concentration of penethamate hydriodide where bacterial killing was observed was 0.32 μg/mL, which killed 52.7 ± 2.0% of intracellular *S. uberis* ($P \leq 0.01$).

Streptococcus dysgalactiae subsp. dysgalactiae

Similar to *S. uberis*, penethamate hydriodide at 32 mg/mL killed all intracellular *S. dysgalactiae* subsp. *dysgalactiae*. At 3.2, 0.32 and 0.032 mg/mL, the percentage of intracellular killing was 99.7 ± 0.05%, 95.8 ± 1.5%, 95.8 ± 1.5% respectively. These results were statically significant ($P < 0.01$) when compared with untreated controls. The lowest concentration of penethamate hydriodide where bacterial killing was observed was 0.32 μg/mL, which killed 56.2 ± 2.0% of intracellular *S. dysgalactiae* subsp. *dysgalactiae* ($P \leq 0.01$).

Staphylococcus aureus

Percentages of intracellular killing of *S. aureus* at the higher concentrations of penethamate hydriodide (32 and 3.2 mg/mL)

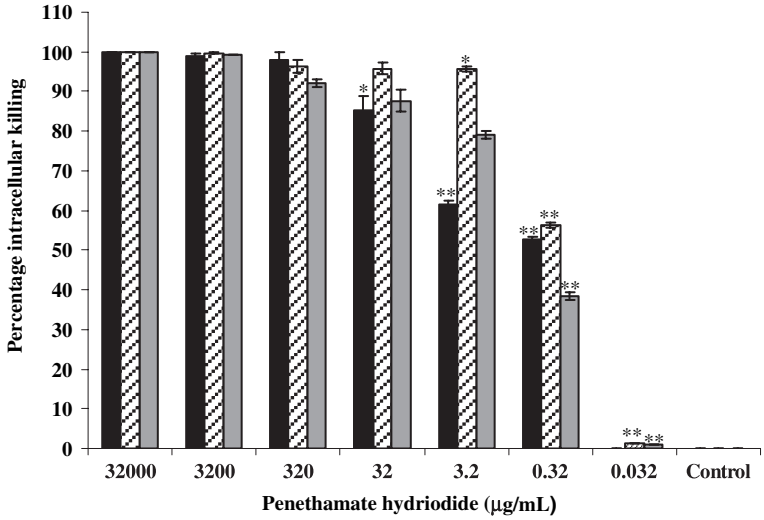

Fig. 1. Percentage intracellular killing of mastitis pathogens following addition of varying concentrations of penethamate hydriodide. MAC-T mammary epithelial cell monolayers were co-cultured with *Streptococcus uberis* (■), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (▨) or *Staphylococcus aureus* (▩) and treated with various concentrations of penethamate hydriodide (32 mg/mL to 0.032 μg/mL) and percentage of viable bacteria was calculated. Data are presented as percentage of penethamate untreated control. Errors bar represent the standard error of the mean (SEM) of three experiments run in triplicate. $^*P \leq 0.05$, $^{**}P \leq 0.01$ – statistically different from the highest intracellular killing rate of that particular pathogen.

evaluated were 100% and 99.3 ± 0.02% and similar to that observed for *S. uberis* and *S. dysgalactiae* subsp. *dysgalactiae*. At 0.32 mg/mL, the percentage of intracellular killing of *S. aureus* was 92.1 ± 1.0%, which was slightly lower than corresponding values for *S. uberis* and *S. dysgalactiae* subsp. *dysgalactiae* (97.8% and 96.3%, respectively). The percentage of intracellular killing at 32 μg/mL was 87.7 ± 2.7%, which was lower than that observed for *S. dysgalactiae* subsp. *dysgalactiae* (95.8%) but higher than that observed for *S. uberis* (85.3%). Despite these differences, killing effect of penethamate at concentrations of 32 mg/mL to 0.32 μg/mL was statistically significant ($P < 0.01$) when compared with untreated controls. The lowest concentration of penethamate hydriodide where bacterial killing was observed was 0.32 μg.

Confocal laser and transmission electron microscopy

In order to verify results obtained with the intracellular killing assay, studies using CLM and TEM were conducted. For CLM, MAC-T cells co-cultured with fluorescent-labeled bacteria were treated with 32 μg/mL of penethamate hydriodide and compared with untreated controls. Under these conditions, live bacteria were green, whereas dead bacteria were visualized as red corpuscles. Results presented in Fig. 2a,c,e depict MAC-T cells that contained green corpuscles (live bacteria) before penethamate hydriodide treatment. Fig. 2b,d,f shows MAC-T cells that contained red corpuscles (dead bacteria), which represent the killing effect of penethamate hydriodide on internalized bacteria (Fig. 2).

Similar to CLM, TEM results presented in Fig. 3 demonstrate the killing effect of penethamate hydriodide on internalized bacteria. Internalized mastitis pathogens observed before treatment (Fig. 3a,c,e) were not seen after MAC-T monolayers were treated with 32 μg/mL of penethamate hydriodide (Fig. 3b,d,f). Results of both microscopy procedures agree with data from intracellular killing assays for each of the mastitis pathogens evaluated.

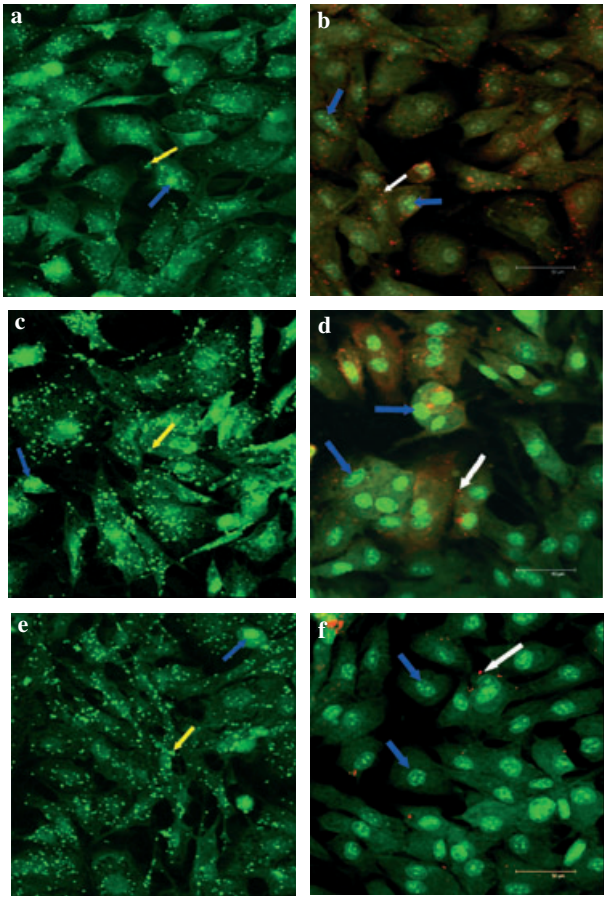

Fig. 2. Confocal laser micrographs of mastitis pathogens internalized into MAC-T mammary epithelial cells before and after addition of penethamate hydriodide. *Streptococcus uberis* (a, b), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (c, d) and *Staphylococcus aureus* (e, f) were fluorescent labeled, co-cultured with MAC-T cell monolayers and kept as untreated controls (a, c, and e) or treated with penethamate hydriodide (32 μg/mL, b, d and f). Live bacteria appear green (yellow arrows), whereas dead bacteria are visualized as red corpuscles (white arrows). Light blue arrows indicate fluorescent green-stained epithelial cells nuclear DNA. Bar = 50 μm.

DISCUSSION

Adherence to and internalization into bovine mammary epithelial cells are important virulence strategies of bovine mammary pathogens. Adherence allows these pathogens to resist removal by fluid flow during milking while internalization provides bacteria a safe environment where components of the immune system such as polymorphonuclear neutrophils, macrophages and antibodies are essentially ineffective. In addition, many host antimicrobial factors present in milk as well as antibiotics used to treat cows with mastitis are ineffective because of their inability to exert their antibacterial activity intracellularly thus allowing mastitis pathogens that have internalized mammary epithelial cells to persist and cause chronic infections. Therefore, to effectively control intracellular bacteria, antibiotics should be able to cross host cell membranes and kill intracellular pathogenic bacteria. In order to achieve this goal, efficacious systemic antibiotic therapy requires specific physical–chemical properties of the drug being used (Ziv, 1980a; Ziv & Storper, 1985). Penethamate hydriodide, unlike penicillin salts, is a weak base that exists in unionized form in plasma. After intramuscular injection, it is rapidly adsorbed from the injection site. Due to the pH gradient present between milk and plasma (pH 6.6–6.8 vs. pH 7.2) combined with its highly liposoluble properties, and its basic state (p*K*a = 8.4), undissociated penethamate easily crosses the blood–milk barrier (Ziv, 1980a). During diffusion of the drug through the udder tissues, it partially dissociates into penicillin G (p*K*a = 2.8) and diethylaminoethanol. Remaining undissociated penethamate effectively penetrates the gland parenchyma and into the mammary cells (Ziv, 1980b). Once inside, penethamate is rapidly ionized into penicillin G (p*K*a = 2.8) which limits its return into interstitial fluid thus getting trapped intracellularly and intramammary in increasing concentrations (Rasmussen, 1959). As these chemical properties seem to assure passage of

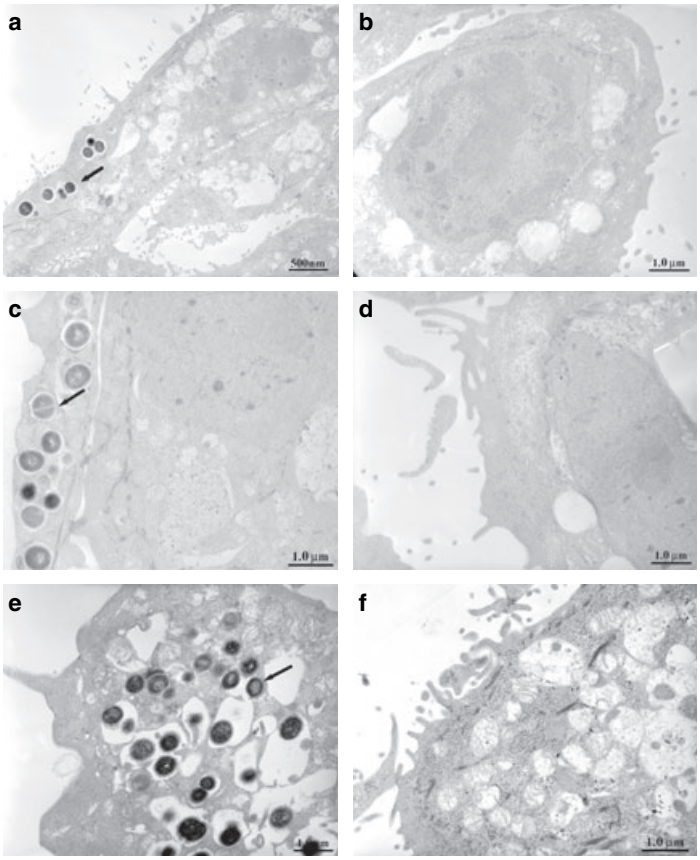

Fig. 3. Transmission electron micrographs of mastitis pathogens internalized into MAC-T mammary epithelial cells before and after addition of penethamate hydriodide. *Streptococcus uberis* (a, b), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (c, d) and *Staphylococcus aureus* (e, f) were co-cultured with MAC-T cell monolayers and kept as untreated controls (a, c, and e) or treated with penethamate hydriodide (32 μg/mL, b, d and f). Arrows indicate viable bacteria. Bar = 1.0 μm.

penethamate hydriodide into intracellular compartments, we decided to evaluate the efficacy in killing internalized mastitis pathogens. Results demonstrated that penethamate hydriodide at concentrations ≥32 μg/mL killed more than 87% of intracellular *S. uberis*, *S. dysgalactiae* subsp. *dysgalactiae* and *S. aureus*, which suggests that penethamate hydriodide was highly effective against intracellular bacteria. At 0.32 μg/mL, intracellular killing was still within a range of 40–60% depending on the pathogen. Confocal laser and TEM confirmed what was observed when intracellular killing assays were used, reaffirming the effective intracellular killing activity of penethamate hydriodide.

It should be considered that data generated under *in vitro* conditions cannot be directly applied to *in vivo* conditions. Results from a pharmacokinetic study in lactating cows showed that the maximum mean milk concentration of penethamate hydriodide was 0.81 μg/mL at 5.9 h after the first injection, and 0.437 μg/mL at 4.6 h after the third injection (Friton *et al.*, 2003). Field studies that investigated the efficacy of systemic penethamate hydriodide treatment in cows with subclinical and clinical mastitis demonstrated high bacteriological cure rates for staphylococci and streptococci (St. Rose *et al.*, 2003; Sérieys *et al.*, 2005). Based on milk concentrations of penethamate reported in the pharmacokinetic study (Friton *et al.*, 2003), penethamate hydriodide concentrations approaching levels observed in milk (3.2, 0.32, and 0.032 μg/mL) were tested for intracellular killing activity. At 3.2 μg/mL, the percentage of intracellular killing was 61.4%, 95.7% and 79.1% for *S. uberis*, *S. dysgalactiae* subsp. *dysgalactiae* and *S. aureus*, respectively. When MAC-T cell monolayers were treated with 0.32 μg/mL of penethamate hydriodide, the percentage of intracellular killing was 52.7%, 56.2% and 38.4% for *S. uberis*, *S. dysgalactiae* subsp. *dysgalactiae* and *S. aureus*, respectively. Data collected from several field studies on mastitis isolates indicated that penicillin-sensitive staphylococci and streptococci generally have MIC_{90} values (≤ 0.07 μg/mL). In addition, data collected in several European countries showed that sensitivity of mastitis-isolated staphylococci and streptococci isolated to penicillin was consistently below 0.1 μg/mL suggesting a strong sensitivity of these organisms to penicillin (Ganiere *et al.*, 1988; Lohuis *et al.*, 1995; Watts, 1996). Thus, when penethamate hydriodide was adjusted to concentrations simulating those obtained in milk following injection into healthy lactating cows, concentrations of penethamate hydriodide were above MIC_{90} values for staphylococci and streptococci. It is important to recognize that the killing effect of penethamate hydriodide was determined after 1 h of incubation and it is very likely that the killing percentage increased with extended incubation time. Future work should address if killing percentage increases with extended incubation time as well as the effect of other variables such as pH, bacterial load or media (i.e. milk, serum) used to conduct these assays. In addition, research should investigate the relationship and interpretation of data from *in vitro* studies and their impact on *in vivo* conditions and vice versa.

In conclusion, results of this proof-of-concept study demonstrate that: (1) penethamate hydriodide is capable of entering mammary epithelial cells in culture and effective at killing

intracellular mastitis pathogens at doses of 32 mg to 0.32 μg/mL without affecting mammary epithelial cell viability, (2) this *in vitro* model system is capable of objectively quantifying mammary epithelial cell–mastitis pathogen interactions that occur during the early stages of pathogenesis as well as the fate of mastitis pathogens once they are internalized into mammary epithelial cells, and (3) this model system can be used to determine whether antibiotics penetrate into host cells and kill intracellular bacteria within the cytoplasm of mammary epithelial cells.

ACKNOWLEDGMENTS

This study was supported, in part, by a grant from Boehringer Ingelheim Animal Health GmbH, and by The University of Tennessee Food Safety Center of Excellence; the Tennessee Agricultural Experiment Station; and The University of Tennessee, College of Veterinary Medicine, Center of Excellence Research Program in Livestock Diseases and Human Health.

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