Extended Nasal Residence Time of Lysostaphin and an Anti-Staphylococcal Monoclonal Antibody by Delivery in Semisolid or Polymeric Carriers

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Purpose. Eradication of *Staphylococcus aureus* nasal colonization reduces the risk of nosocomial and community acquired infections with this organism. This study describes the formulation and use of lysostaphin and BSYX-A110, an anti-lipoteichoic acid monoclonal antibody, for eradication of *S. aureus* nasal colonization.

Methods. Lysostaphin was formulated into a hydrophilic cream that forms an emulsion with the secretions of the nasal mucosa, and aqueous formulations of BSYX-A110 were made containing the mucoadhesive polymers polystyrene sulfonate and chitosan. Intranasal pharmacokinetics of the drugs was measured in mice and cotton rats.

Results. Lysostaphin formulated in the cream increased nasal retention of the drug by 10-fold at 3 h post-cream installation and 50-fold at 24 h as compared to lysostaphin in saline drops. Furthermore, the levels of lysostaphin in the nose 24 h post-cream instillation are still above the minimum bactericidal concentration for most bacterial strains. The liquid polymer formulations also resulted in prolonged retention of antibody in the nose, with 4-fold higher levels at 3 h post-instillation as compared to antibody in saline drops.

Conclusions. These results demonstrate that cream and polymer delivery systems significantly decrease the clearance rate of lysostaphin and BSYX-A110 from the nose, thereby enhancing their therapeutic potential for eradicating *S. aureus* nasal colonization.

KEY WORDS: cream; lysostaphin; mucoadhesive polymer; nasal; *Staphylococcus*.

INTRODUCTION

Staphylococcus aureus is one of the most important pathogens in nosocomial and community acquired infections (1,2), leading to life-threatening diseases such as osteomyelitis, organ abscesses, and endocarditis (3,4). Coagulase-negative staphylococci are the most common cause of nosocomial sepsis, followed by *S. aureus* with an incidence of 15% and mortality of 25% (5). Cross-sectional surveys in healthy adults show that nasal carriage of *S. aureus* occurs in about 20–55% of the population (6–9). Because nosocomial staphylococcal infections are predominantly caused by the same strain of bacteria that resides in the patients own nasal flora (10–12), elimination of nasal carriage could drastically reduce the incidence of nosocomial infections by this organism.

The current standard for eradication of *S. aureus* nasal colonization is Bactroban Nasal (mupirocin calcium ointment 2%, SmithKline Beecham). A recent study demonstrated that prophylactic use of Bactroban Nasal significantly decreased nosocomial infections caused by *S. aureus* in those patients who were identified as nasal carriers of this bacteria (13). However, resistance to mupirocin is a growing problem (14,15) and the time needed for *S. aureus* clearance is slow, requiring several treatments over 5 days. There is a clear need for new antimicrobial agents that are effective against resistant *S. aureus* strains and can rapidly eradicate nasal colonization.

Lysostaphin is a 27-kDa, glycylglycine endopeptidase capable of specifically cleaving the cross-linking pentaglycine bridges in the cell walls of staphylococci, leading to cell lysis and death (16). Lysostaphin is highly effective in lysing *S. aureus* because the cell wall bridges of this species contain a high proportion of pentaglycine cross-bridges. Furthermore, lysostaphin's activity is not dependent on the growth cycle of the cell and can therefore kill bacteria more rapidly than antibiotics that target protein synthesis or cell division. Nasal eradication of *S. aureus* by lysostaphin was successfully tested in the late 1960s and early 1970s (17–19), but the enzyme was difficult to produce, the recombinant form was not available, potency varied from lot to lot, and resistance to the cheaper, traditional antibiotics was not yet a major problem. Now, with recombinant lysostaphin readily available and the rising incidence of resistance to antibiotics, lysostaphin is once again being investigated for clinical use. We have also developed antibodies that bind to various cell wall components of *Staphylococcus* and show promise for preventing and treating infection. BSYX-A110 is a chimeric, monoclonal antibody (mAb) that binds to *S. aureus* lipoteichoic acid (LTA), which is an important molecule for the initial attachment of bacteria to epithelial cells (20–22). Antibodies to LTA also block adherence of staphylococci to fibrin platelet clots (23) and suggest that such antibodies may be useful in preventing, and possibly disrupting, bacterial colonization at other sites such as the surface of the nasal mucosa.

The primary ecological niche for *S. aureus* in humans is the anterior third of the nares (12,24–26). Clearance of nasal carriage will require effective delivery and retention of lysostaphin in this region of the nose. Traditionally, intranasal drug delivery has been investigated as an alternative to other systemic delivery routes such as oral, intravenous, and intramuscular. Many of these delivery systems use synthetic, semisynthetic, and natural polymers that are mucoadhesive or have absorption promoting affects. Examples include chitosan, poly-L-arginine, cyclodextdrins, cellulose derivatives, polylactic acid, polymethacrylate, and polyethylene glycol (27–31). The most common goal of these delivery systems is to attain rapid and high levels of drug absorption through the nasal mucosa (primarily the distal, ciliated epithelium) and into systemic circulation. The target indication for lysostaphin is exactly the opposite. Our goal is to attain high drug levels on the mucosa of the anterior nares with minimal systemic absorption. To this end, we have developed a cream and two saline-based mucoadhesive polymer formulations that significantly prolong the residence time of lysostaphin in the nose while also preserving the bactericidal potency of the drug.

MATERIALS AND METHODS

Materials

Lysostaphin (Ambicin L) was obtained from AMBI, Inc., Purchase, NY, BSYX-A110 was manufactured by Biosyn-

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exus, Inc., Gaithersburg, MD, Chitosan was purchased from Pronova Biomedical, Oslo, Norway, (PROTOSAN G 213; MW 460,000), and sodium polystyrene sulfonate from Scientific Polymer Products, Ontario, NY, (MW 500,000). MIGLYOL 812 N (caprylic/capric triglyceride) and SOFTISAN 649 (bis-diglyceryl polyacyladipate-2) were provided by Sasol (Witten, Germany).

Instillation of BSYX-A110 in Mouse Noses

Female Hsd:ICR (CD-1) mice were purchased from Harlan, Indianapolis, IN. The mice were sedated with an intraperitoneal injection of ketamine (1.6 mg) and xylazine (0.64 mg) to minimize expulsion of the test solutions from the nose. The mice were held in a supine position while the various test articles were administered to the nose in a dropwise fashion; 20 μl total divided between both nares in 5 μl increments. BSYX-A110 was administered at doses of 50 or 100 μg/ml in one of three formulations: normal saline, 0.5% (w/w) chitosan in saline, or 0.5% (w/w) sodium polystyrene sulfonate (SPSS) in saline. At various time points post-instillation (5, 30, 60, 120, and 180 min), the mice were sacrificed by CO_2 inhalation, and the noses were surgically removed. The nostrils were bisected with scissors, and then the excised nose was placed in 200 μl of phosphate-buffered saline (PBS) containing 0.5% (v/v) Tween-20 (Sigma, St. Louis, MO) to aid in release of antibody from the surface of the nasal mucosa. The amount of antibody persisting in the nose was then measured by an LTA-binding ELISA. Briefly, 96-well immunoassay plates were coated with *S. aureus* LTA followed by incubation with dilutions of the nasal wash solutions. Goat anti-human IgG labeled with horseradish peroxidase was then used to detect the BSYX-A110. After TMB (BioFX) colorimetric development, the plates were measured at an absorbance of 450 nm in a SpectraMAX Plus plate reader (Molecular Devices; Sunnyvale, CA, USA).

Cream Synthesis and Lysostaphin Instillation in Cotton Rat Noses

The cream designed for intranasal administration of protein antimicrobials was formulated by first melting together the following waxes and oils, with all excipients listed as percent w/w: 36% (w/w) MIGLYOL 812 N, 24.2% (w/w) SOFTISAN 649, 27.5% (w/w) white petrolatum (Ultra Chemical Inc., Red Bank, NJ, USA), 3.4% (w/w) paraffin (Sigma, St. Louis, MO), 3.4% (w/w) beeswax, bleached, white (Kosterkeunen, Watertown, CT, USA), and 0.5% (w/w) zinc stearate, (Sigma, St. Louis, MO). After partial cooling, the remaining 5% of the cream was formulated by adding lysostaphin dissolved in phosphate-buffered saline at 100 or 250 mg/ml, which yields final lysostaphin cream concentrations of 0.5% (5 mg/ml) or 1.25% (12.5 mg/ml). The available animal model in mice was not suitable for cream instillations, and therefore a cotton rat model was developed to investigate the pharmacokinetics and efficacy of the cream formulation. The nasal cavity of the rats is larger than in mice so intranasal instillation of the cream was technically easier than in mice, and rats were more tolerant to cream administration than mice. Cream installation had been previously attempted in mice but was found to physically obstruct airway passages and caused respiratory distress in the animals. Cream instillation into the nose of cotton rats was performed as previously described (32). Six-week-old female cotton rats (*Sigmadon hispidus*, bred at the Biosynexus Inc. breeding facility, U.S. Department of Agriculture Certificate 51-R-0075) were anesthetized with a combination of xylazine hydrochloride, acepromazine maleate, and ketamine (2.5 mg/kg, 2.5 mg/kg, and 25 mg/kg, respectively). Cream formulations were administered to anesthetized cotton rats using a 1-ml syringe fitted with a flexible 23-gauge Angiocath (Becton Dickinson). The catheter was inserted 2 to 3 mm into each nostril and then drawn back slowly as about 15 μl of the cream was injected into each nostril. The nose of the animal was massaged well to ensure even distribution of the cream throughout the nares and to prevent obstruction of the airway. The noses were harvested at 5 min and 3, 24, and 48 h post-instillation and processed as described above for the mice. An anti-lysostaphin capture ELISA was used to measure lysostaphin persistence in the nose (33). Briefly, 96-well microtiter plates were coated overnight with a polyclonal rabbit anti-lysostaphin antibody followed by incubation with dilutions of the nasal wash solutions. Lysostaphin binding was detected with biotin-labeled, polyclonal rabbit anti-lysostaphin followed by extravidin-HRP incubation and TMB colorimetric detection. The plates were measured at an absorbance of 450 nm in a SpectraMAX Plus plate reader.

Efficacy of Lysostaphin and BSYX-A110 in Cotton Rat *S. aureus* Nasal Colonization Model

Cotton rats were nasally colonized with *S. aureus* as previously described (32). Briefly, a clinical isolate of *S. aureus*, strain MBT 5040, was grown overnight on Columbia agar supplemented with 2% NaCl and then resuspended in PBS (percent transmittance = 10 at 650 nm; Spectonic 200+; Spectonic Instruments). Six-week-old female cotton rats (*Sigmodon hispidus*; bred at Biosynexus, Inc; U.S. Department of Agriculture certificate 51-R-0075) were anesthetized with a 2.5 mg/kg xylazine hydrochloride, 2.5 mg/kg acepromazine maleate, and 25 mg/kg ketamine. Ten microliters of the *S. aureus* suspension was instilled in the cotton rat nose in a dropwise fashion, divided equally between the two nostrils. Five days after nasal instillation of *S. aureus*, one of four intranasal treatments was applied to anesthetized cotton rats. Each nose was instilled with 20 μl, divided equally between both nostrils, of the following therapies: 5 mg/ml lysostaphin in PBS, 5 mg/ml lysostaphin formulated with SPSS, 5 mg/ml lysostaphin formulated with chitosan, and 5 mg/ml BSYX-A110 formulated with SPSS. Twenty-four hours after nasal instillation, cotton rats were sacrificed, the external area around the nose was cleansed with a 70% alcohol wipe, and the noses were surgically removed. The nostrils were bisected and then placed in 500 μl of PBS-Tween 20 containing 10 mg/ml of proteinase K as a lysostaphin neutralizer. The noses were vortexed vigorously, and then 100 μl of the supernatant was streaked onto tryptic soy agar plates supplemented with 7.5% NaCl and Streptomycin.

RESULTS

Intranasal Residence Time of BSYX-A110 in Chitosan and SPSS

The nasal pharmacokinetics of BSYX-A110 was initially investigated in normal saline with no carrier polymers.

BSYX-A110 was administered to the nose of mice in PBS at a total dose of 50 or 100 μg. The initial half-life of the antibody on the surface of the nasal mucosa was quite rapid, about 45 min (Fig. 1). The dose-response for this range of antibody concentrations was also linear, with about half as much antibody recovered from the nose dosed with 50 μg as from the nose dosed with 100 μg at every time-point evaluated. Intranasal clearance of a single dose of lysostaphin in PBS was also measured in cotton rats (Fig. 2). Later time-points were examined than for BSYX-A110 in mice and demonstrate that intranasal clearance is biphasic. The half-life in the distribution phase was about 40 min and increased to 200 min in the elimination phase.

Increasing the residence time of antibody on the surface of the nasal mucosa would reduce the dosing frequency and total amount of drug needed to clear or block nasal colonization with *S. aureus*. Several mucoadhesive polymers were investigated for their potential to increase BSYX-A110 persistence in the nose of mice: poly-L-arginine, hydroxylpropyl cellulose, microcrystalline cellulose, sodium polystyrene sulfonate (SPSS), and chitosan. Only SPSS and chitosan were found to significantly improve intranasal retention of the antibody and were subsequently compared head-to-head against the antibody in saline formulation (Fig. 3). The initial (t = 5 min) concentration of BSYX-A110 is equivalent for all dosage forms but the intranasal retention, especially in the first 60 min, is greatly increased for the SPSS and chitosan dosage forms compared to normal saline delivery of antibody. The total area under the curve (AUC) from 5 to 180 min for each different delivery vehicles is as follows: 143 μg · min for saline, 222 μg · min for chitosan, and 298 μg · min for SPSS. Therefore, the amount of antibody residing on the surface of the nasal mucosa for the first 3 h after administration is effectively doubled when applied in the SPSS carrier compared to the saline carrier.

Intranasal Residence Time of Lysostaphin Delivered in a Hydrophilic Cream

Because *S. aureus* predominantly resides in the anterior third of the nose, a semisolid dosage form was judged to be the most practical delivery vector for drugs targeting this population of bacteria. A hydrophilic cream was developed that offers good compatibility with protein drugs and forms an emulsion with the secretions of the nasal mucosa. The intranasal retention of lysostaphin delivered to cotton rat noses in cream or saline formulations showed similar clearance profiles to antibody in SPSS, chitosan, or saline administration in mice, respectively (Fig. 2). The cream significantly increased persistence of lysostaphin on the surface of the nasal mucosa compared to saline administration, particularly within the first few hours post-instillation. At the 0.5% dose, there was 10 times the amount of lysostaphin in the nose 3 h after cream instillation compared to saline drops and 50 times more at 24 h. Furthermore, the amount of lysostaphin recovered from the cream-treated noses 24 h post-instillation was sufficiently high to eradicate a high inoculum of *S. aureus,* whereas lysostaphin recovered from the saline-treated noses at 24 h is ineffective (Ref. 32 and unpublished data). The clinical dose of lysostaphin is anticipated to be between 0.5% and 1.25% (w/w) in the cream formulation, so the change in clearance kinetics was examined for 0.5% and 1.25% lysostaphin nasal creams (Fig. 2). The amount of intranasal lyso-

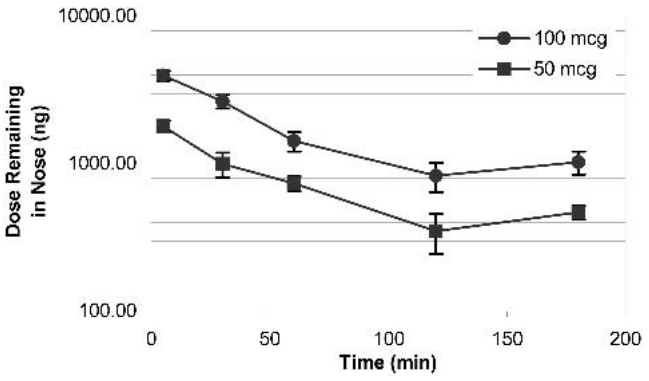

Fig. 1. Intranasal clearance for two different doses of the anti-LTA monoclonal antibody BSYX-A110 administered as saline drops to mouse noses (n = 4 per time point).

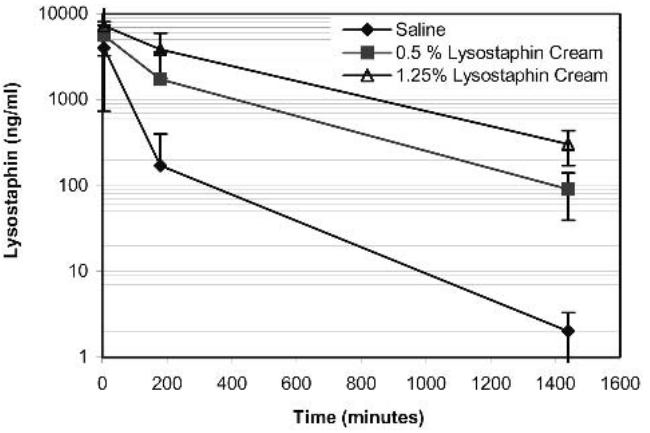

Fig. 2. Nasal residence time of 0.5% and 1.25% (w/w) lysostaphin formulated in a hydrophilic cream and instilled into the nose of cotton rats. Lysostaphin clearance in the cream is compared to an equivalent dose of the drug administered in saline.

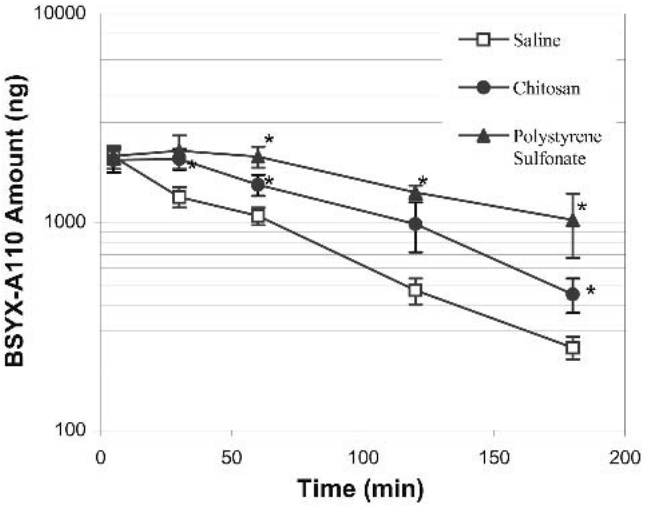

Fig. 3. Clearance of BSYX-A110 in mouse noses when formulated with SPSS or chitosan (n = 5 per time point). These mucoadhesive polymers increased the intranasal retention of antibody, especially within the first hour after instillation. *Difference in data point compared to saline is statistically significant ($p < 0.02$; ANOVA test).

staphin was proportionately higher at each time-point examined for the 1.25% cream. The AUC for the 1.25% cream was also 2.7 times greater than the 0.5% cream (2211 μg · min vs. 827 μg · min, respectively), which is virtually identical to the 2.5 times difference in total dose.

Efficacy of BSYX-A110 and Lysostaphin Formulations in *S. aureus* Nasal Colonization Model

We have previously shown that lysostaphin formulated in the nasal cream completely eradicates *S. aureus* nasal colonization in cotton rats (32). In contrast, the same dose of lysostaphin administered in the form of saline drops was only partially effective, reducing colonizing CFUs by about 1 log. Table I shows that lysostaphin and BSYX-A110 formulated in SPSS and chitosan is also more effective at clearing nasal colonization than in saline alone. The efficacy of lysostaphin in SPSS, chitosan, and saline closely mimics the kinetics of intranasal clearance observed with the carriers (Fig. 2). Lysostaphin in SPSS was the most effective, eradicating *S. aureus* nasal colonization in all but one animal. The chitosan formulation was only slightly less effective, clearing just one of five animals but reducing colonization to only a few CFU (a 2.8 log reduction); however, each formulation was superior to that of saline alone, which only reduced the mean nasal CFU by 0.8 logs. Though BSYX-A110 was not as effective as lysostaphin in the SPSS carrier, nasal *S. aureus* colonization was greatly reduced, and formulated antibody was still more effective than unformulated lysostaphin.

DISCUSSION

Pharmaceutical agents delivered through the intranasal route are typically targeted to the posterior, ciliated regions of the nose for systemic absorption or presentation to the nasal mucosal immune system. Eradication of *S. aureus* nasal colonization offers a unique challenge in that drug formulations must target the anterior third of the nasal passage, and long-term residence of the drug at this site of action, with little systemic absorption, is preferable. We are currently investigating two drugs that have high affinities and potent bactericidal activity against this organism. BSYX-A110 is a monoclonal, anti-LTA antibody that opsonizes staphylococcal species and stimulates immune-mediated bacterial killing, and lysostaphin is a cell wall endopeptidase that specifically targets *S. aureus* and lyses bacteria within minutes upon exposure. Liquid formulations that would be compatible with nasal spray devices and have wide-angle plume geometries were initially investigated to increase persistence on the surface of the nasal mucosa compared to simple saline formulations.

Table I. Efficacy of 0.5% Lysostaphin and BSYX-A110 Liquid Formulations in a Cotton Rat *S. aureus* Nasal Colonization Model (n = 9 for Controls; n = 5 for All Other Groups)

Treatment	Mean CFU recovered/nose	Standard deviation
Control	3536	467
Lysostaphin in SPSS	1	0.4
Lysostaphin in Chitosan	6	0.8
Lysostaphin in PBS	548	238
BSYX-A110 in SPSS	126	42

Several mucoadhesive polymers were considered for increasing nasal retention of BSYX-A110 and lysostaphin, but only two were found to significantly increase persistence in mouse noses: chitosan and sodium polystyrene sulfonate (SPSS). The major retention activity of these polymers appears to occur within the first hour after administration, virtually 100% retention for BSYX-A110 in SPSS and 82% in chitosan compared to 64% in saline, with an apparent equalization of clearance rates from 1 to 3 h for all three vehicles. However, this difference in retention during the first hour after treatment leads to large absolute differences in antibody amounts in the nose at 3 h; the AUC for antibody in SPSS over the whole 3-h period was twice that for antibody in saline. The nasal pharmacokinetics shown in mice also represents a "worst case" scenario for persistence because the formulations were applied drop-wise as opposed to a wide-angle spray that targets the anterior third of the nose. Drops are known to clear faster than sprays, and mucus flow in the posterior, ciliated region of the nose is much more rapid, 8–100 mm/h, than in the anterior, nonciliated region of the nose, 1–2 mm/h (34,35). Therefore, we would expect even slower clearance of drug than that shown in Fig. 3 when the formulations are applied as sprays directed to the anterior third of the nose.

There are many advantages to using a spray formulation for eradication of *S. aureus* nasal colonization (e.g., simple formulation and controlled dosing), but it is difficult to deliver sprays to the most extreme anterior areas of the nose, and some bacterial colonies may escape this type of treatment. On the other hand, a cream formulation can be applied manually to just the anterior portion of the nose to completely and evenly cover the colonized region. Therefore, we developed a cream formulation that would be compatible with protein drugs and also form an emulsion with the secretions of the nasal mucosa to increase bioavailability of the drug. The available animal model in mice was found to be lacking for cream instillations and therefore a cotton rat model was developed to investigate the pharmacokinetics and efficacy of the cream formulation. There were several reasons for this change: the nasal cavity of the rats is larger than in mice so intranasal instillation of the cream was technically easier than in mice; rats were more tolerant to cream administration than mice; and *S. aureus* nasal colonization is far more effective in the rats (32). Lysostaphin was mixed into the cream at concentrations of 0.5% to 1.25% (w/w), and intranasal pharmacokinetics was measured in the noses of cotton rats. The cream greatly increased the persistence of lysostaphin in the nose compared to saline formulations, with 50 times as much drug retained in the nose 24 h after administration. Furthermore, the amount of drug in the nose 24 h after cream instillation remains above its minimum therapeutic value, which is important for two reasons. Lysostaphin cream could be administered on a once-daily dosing schedule compared to multiple doses per day for simple saline formulation, thus improving patient compliance and lowering treatment costs. Second, resistance to antibiotics often arises when concentrations of the drug fall below the minimum therapeutic value for extended periods of time. If lysostaphin cream were to be applied daily for the course of treatment, then intranasal concentrations of the drug would never fall below this threshold, and the potential for emergence of lysostaphin resistant *S. aureus* would be minimized.

Increasing intranasal residence time of lysostaphin and BSYX-A110 with liquid mucoadhesive and cream formulations does not guarantee that they will be more effective than simple saline formulations. The improved efficacy of SPSS, chitosan, and cream formulations of lysostaphin and BSYX-A110 was therefore verified in a cotton rat *S. aureus* nasal colonization model (32). The results shown in Table I support the claim that increased retention of anti-staphylococcal drugs on the surface of the nasal mucosa increases their efficacy when attempting to eradicate *S. aureus* nasal colonization. A single application of lysostaphin in saline alone was effective at reducing the level of intranasal colonization by an average of 85%, but addition of mucoadhesive polymers to the formulation reduced the mean CFU recovered an additional 100-fold. Furthermore, the SPSS formulation of lysostaphin eradicated colonization in all but one animal, and only 5 colonies were recovered from the nose of that animal. BSYX-A110 formulated in SPSS was not as potent as lysostaphin in the same carrier, but was nevertheless very effective at reducing nasal colonization and was superior to the saline lysostaphin formulation. The efficacy of the chitosan-lysostaphin formulation was also suggestive from its nasal PK profile (data not shown) and was much more effective than saline, reflected by the 100-fold reduction in mean CFU recovered.

We have previously demonstrated treatment of *S. aureus* nasal colonization in cotton rats with the cream-lysostaphin formulation. The efficacy of the cream is similar to that of SPSS and chitosan formulations, eradicating colonization in >90% of noses with mean CFU in the remaining animals of less than 10 (32). These data when taken together suggest that the retention time of lysostaphin and BSYX-A110 on the surface of the nasal mucosa, whether by semisolid or mucoadhesive liquid formulations, greatly enhances the drugs' efficacy against *S. aureus* that reside in the nasal cavity. Although the cream and mucoadhesive formulations appear to be equally efficacious, the two formulations have different practical advantages that may give each importance for actual clinical therapy. The lysostaphin-cream formulation can be directly applied to the most anterior region of the nose, which is where the preponderance of nasal bacterial colonies resides. Once this initial clearance is rapidly achieved by the cream, a mucoadhesive spray formulation containing lysostaphin or BSYX-A110, which would be cheaper to produce and easier to use by the patient, could be used to maintain clearance for extended periods of time. Future studies will involve human volunteers in clinical trials to prove the safety and efficacy of lysostaphin and BSYX-A110 formulations for eradicating *S. aureus* nasal colonization in at-risk populations. The objective of these clinical trials is to eradicate nasal colonization and thereby prevent spread of antibiotic-resistant staphylococci both in and outside of hospital settings.

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