2015

Imidazolium salts as small-molecule urinary bladder exfoliants in a murine model

Patrick O. Wagers
University of Akron

Kristin M. Tiemann
Washington University School of Medicine in St. Louis

Kerri L. Shelton
University of Akron

William G. Kofron
University of Akron

Matthew J. Panzner
University of Akron

See next page for additional authors

AAC Journals.ASM.org

Imidazolium Salts as Small-Molecule Urinary Bladder Exfoliants in a Murine Model

Patrick O. Wagers,[a] Kristin M. Tiemann,[b] Kerri L. Shelton,[a] William G. Kofron,[a] Matthew J. Panzner,[a] Karen L. Wooley,[c] Wiley J. Youngs,[a] David A. Hunstad[b,d]

Department of Chemistry and Center for Silver Therapeutics Research, University of Akron, Akron, Ohio, USA[a]; Department of Pediatrics, Washington University, St. Louis, Missouri, USA[b]; Department of Chemistry, Department of Chemical Engineering, and Department of Materials Science and Engineering, Texas A&M University, College Station, Texas, USA[c]; Department of Molecular Microbiology, Washington University, St. Louis, Missouri, USA[d]

We present a novel family of small-molecule urinary bladder exfoliants that are expected to be of great value in preclinical studies of urologic conditions and have improved potential for translation compared with prior agents. There is broad urologic interest in the therapeutic potential of such exfoliating agents. The primary agent used in preclinical models, the cationic peptide protamine sulfate (PS), has limited translational potential due to concerns including systemic adverse reactions and bladder tissue injury. Intravesical application of a safe, systemically nontoxic exfoliant would have potential utility in the eradication of *Escherichia coli* and other uropathogens that reside in the bladder epithelium following cystitis, as well as in chronic bladder pain and bladder cancer. Here, we introduce a family of imidazolium salts with potent and focused exfoliating activity on the bladder epithelium. Synthesis and purification were straightforward and scalable, and the compounds exhibited prolonged stability in lyophilized form. Most members of the compound family were cytotoxic to cultured uroepithelial cells, with >10-fold differences in potency across the series. Upon topical (intravesical) administration of selected compounds to the murine bladder, complete epithelial exfoliation was achieved with physiologically relevant imidazolium concentrations and brief contact times. The exfoliative activity of these compounds was markedly improved in comparison to PS, as assessed by microscopy, immunofluorescence, and immunoblotting for uroplakins. Bladder uroepithelium regenerated within days to yield a histologically normal appearance, and no toxicity was observed. Finally, the chemical scaffold offers an opportunity for inclusion of antimicrobials or conjugation with chemotherapeutic or other moieties.

The urinary bladder in higher mammals is uniquely formed and structured to collect filtered waste products from the kidneys and to provide a means for retention and storage of this waste until micturition is convenient for the animal. The bladder lumen is lined by a multilayered, pseudostratified transitional epithelium, underlain by the basement membrane and lamina propria. Smooth muscle deep to this, under parasympathetic neural influence, directs contraction of the bladder, which in conjunction with sphincter relaxation allows for controlled micturition. Importantly, the tissues of the bladder are protected from potentially toxic effects of urine components by a highly ordered array of uroplakins, glycoproteins which coat the apical surfaces of superficial bladder epithelial cells (also termed facet or umbrella cells) and comprise an impermeable barrier to urine and its solutes (1).

These uroplakins are decorated with mannose moieties, which are co-opted as receptors for binding by uropathogenic *Escherichia coli* (UPEC), the chief cause of urinary tract infections (UTI) (2). Bacterial binding to mannosylated uroplakin is the initiating event of cystitis (3, 4) and leads to the internalization of UPEC by superficial bladder epithelial cells during acute bacterial cystitis in murine and human hosts (5–8). These cells serve as a protected niche for UPEC to replicate while sheltered from the phagocytic activity of neutrophils summoned in response to infection (9). Exfoliation of the superficial cell layer is observed later in the acute phase and represents a host defense mechanism capable of eliminating large numbers of replicating bacteria (7, 10). However, this process also exposes additional cells to infection, as facet cell exfoliation triggers underlying cells to initiate expression of uroplakins (11). When the acute phase of experimental cystitis has resolved, many hosts remain infected with a small number of organisms residing in quiescent intracellular reservoirs within superficial epithelial cells (10). These bacteria apparently resist immune clearance and are not eliminated by systemic antibiotic therapy (12–14). As these bacteria are thought to serve as a seed for recurrent cystitis caused by the same strain (15, 16), there is interest in developing novel strategies for their eradication.

One concept for such a strategy is intentional exfoliation of the superficial epithelial layer, which would eliminate many resident bacteria in the urine and force the emergence of remaining reservoir bacteria into the luminal compartment, where systemically administered antibiotics might be effective (15). Though there are few data to confirm the effectiveness of this concept, one exfoliative agent has predominated in use in a variety of preclinical models for conditions other than UTI (e.g., chronic bladder pain, interstitial cystitis). This agent, protamine sulfate (PS), is an

Received 24 April 2015 **Returned for modification** 29 May 2015
Accepted 19 June 2015

Accepted manuscript posted online 29 June 2015

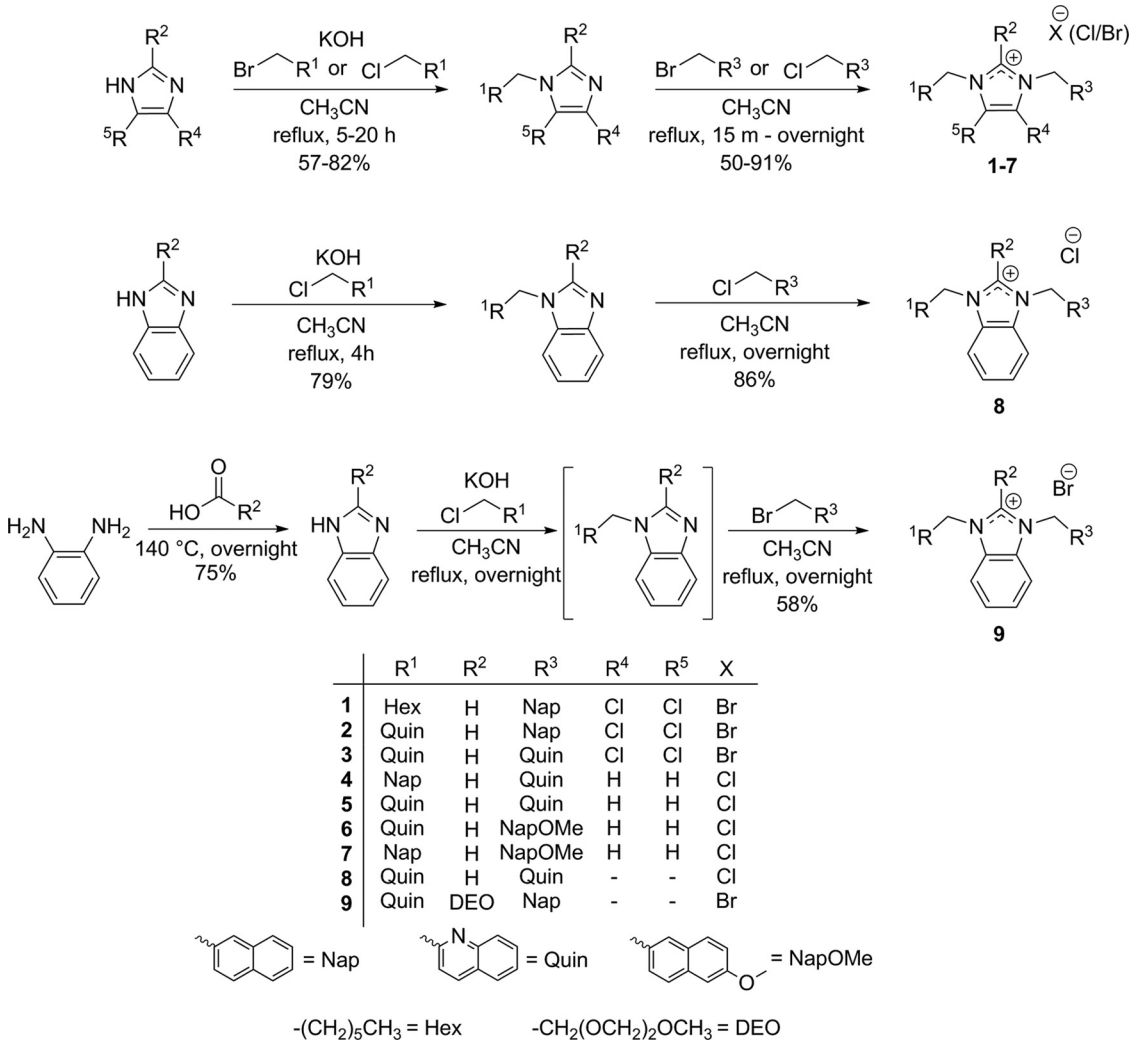
Citation Wagers PO, Tiemann KM, Shelton KL, Kofron WG, Panzner MJ, Wooley KL, Youngs WJ, Hunstad DA. 2015. Imidazolium salts as small-molecule urinary bladder exfoliants in a murine model. Antimicrob Agents Chemother 59:5494–5502. doi:10.1128/AAC.00881-15.

Address correspondence to David A. Hunstad, dhunstad@wustl.edu.

P.O.W. and K.M.T. contributed equally to this work.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.00881-15.

doi:10.1128/AAC.00881-15



	R^1	R^2	R^3	R^4	R^5	X
1	Hex	H	Nap	Cl	Cl	Br
2	Quin	H	Nap	Cl	Cl	Br
3	Quin	H	Quin	Cl	Cl	Br
4	Nap	H	Quin	H	H	Cl
5	Quin	H	Quin	H	H	Cl
6	Quin	H	NapOMe	H	H	Cl
7	Nap	H	NapOMe	H	H	Cl
8	Quin	H	Quin	-	-	Cl
9	Quin	DEO	Nap	-	-	Br

FIG 1 Synthetic scheme for imidazolium salts, compounds 1 to 7 (top row), compound 8 (middle row), and compound 9 (bottom row). The identities of substituents R^1 through R^5 in these compounds are indicated in the table, using abbreviations for substituent groups as indicated below the table. Abbreviations: Hex, hexyl; Quin, quinolyl; Nap, naphthyl; NapOMe, 6-methoxynaphthyl; DEO, di(ethyleneoxide).

~5-kDa representative of a family of arginine-rich cationic peptides isolated from fish sperm; in humans, recombinant PS is given intravenously for reversal of heparin-based anticoagulation. Importantly, systemic administration of PS in humans can provoke significant allergic (IgE-mediated) and anaphylactoid reactions in several patient populations, including diabetics and infertile men (17–20). Its application in moderate to high concentrations to the mouse or rat bladder elicits substantial loss of superficial epithelial cells (21–23), and it has been used in combination with other irritants (e.g., lipopolysaccharide) in models of chronic bladder inflammation (24–26). Further pursuit of combined strategies targeting chronically resident uropathogens, therefore, would benefit greatly from an optimized exfoliant.

We sought to identify and develop small-molecule topical intravesical agents exhibiting enhanced exfoliative effects and higher potency while requiring short contact time and inducing minimal local inflammation. Here, we present a series of imidazolium salt derivatives demonstrating a cytotoxic effect after limited incubation with cultured bladder transitional epithelial cells. Several representative compounds from this series, selected on the basis of their *in vitro* activity, were shown to induce near-complete exfoliation of the bladder superficial epithelial cell layer in murine hosts, without overt inflammatory changes or apparent injury to underlying cells. It is expected that these imidazolium salts will prove to be useful in preclinical studies of bladder biology while also holding promise as adjunctive therapeutics for chronic and recurrent UTI. The compounds exfoliate the mammalian bladder rapidly, safely, and effectively and can be adapted structurally to carry other biocides (e.g., silver moieties) in a combined agent. In addition, the chemical backbone provides a tractable platform for future diagnostic and therapeutic application to other urinary tract conditions, such as bladder epithelial cancers.

MATERIALS AND METHODS

Imidazolium compounds. All reagents and solvents used in the synthesis of the compounds were purchased from commercial sources and used without purification with the exception of 2-(chloromethyl)-6-methoxynaphthalene, which was synthesized according to a published

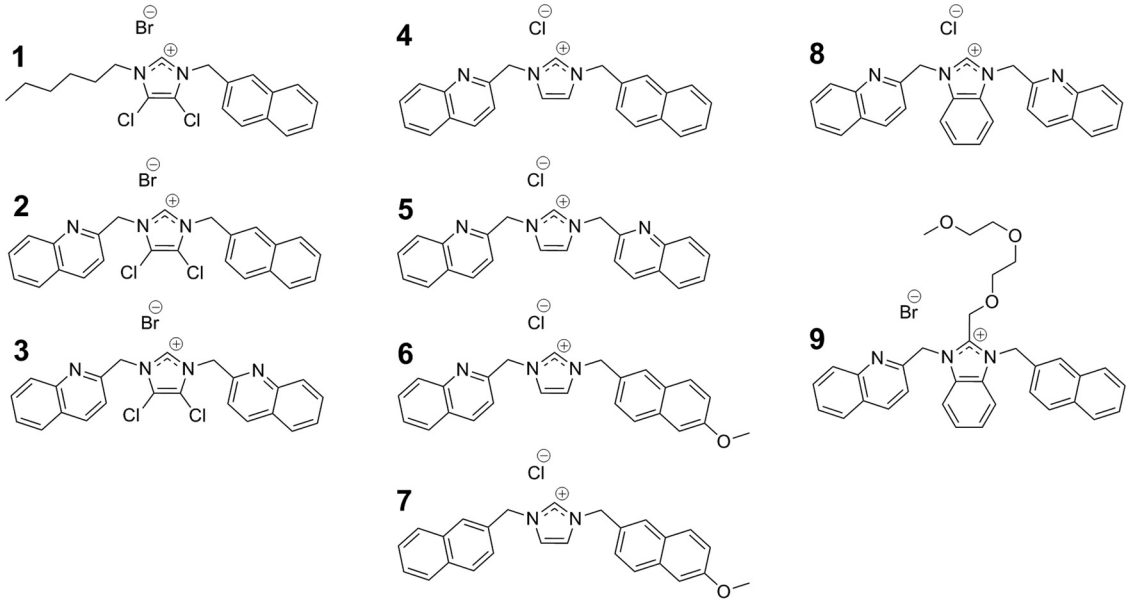


FIG 2 Chemical structures of compounds 1 to 9.

method (27). Column chromatography utilized silica gel (60 Å; ICN Medicals) embedded with fluorescence indicator green 254 nm (Fluka Analytical). Melting points were determined on a MelTemp apparatus. ^{1}H and ^{13}C{^{1}H} nuclear magnetic resonance (NMR) spectra were collected on a Varian instrument operated at 500 MHz for ^{1}H and 125 MHz for ^{13}C, with all spectra referenced to residual solvent protons or the ^{13}C signal in dimethyl sulfoxide-d_6 (DMSO-d_6) (Cambridge Isotope Laboratories) ($\delta = 2.50$ ppm or 39.51 ppm, respectively). Mass spectrometry was performed by the University of Akron mass spectrometry laboratory (Akron, OH). Elemental analysis was performed by Atlantic Microlabs, Inc. (Norcross, GA) or by the University of Akron Department of Geology (Akron, OH).

Single crystals of compounds 3 to 6 and 8 were coated in paratone oil, mounted on a CryoLoop, and placed on a goniometer under a stream of nitrogen. Crystal structure data sets were collected with a Kappa APEX II SuperDuo charge-coupled-device (CCD) system (Bruker AXS Inc., Madison, WI) equipped with a Mo ImuS source ($\lambda = 0.71073$ Å) and a Cu ImuS microfocus source equipped with QUAZAR optics ($\lambda = 1.54178$ Å). Unit cells were determined by using reflections from three different orientations. Data sets were integrated using SAINT (Bruker). An empirical absorption correction and other corrections were applied to the data sets using multiscan SADABS (Bruker). Structure solution, refinement, and modeling were accomplished by using the Bruker SHELXTL software package (28). The structures were determined by full-matrix least-squares refinement of F2 and the selection of the appropriate atoms from the generated difference map. Hydrogen atom positions were calculated and Uiso(H) values were fixed according to a riding model.

Lyophilized compounds were stored in ambient conditions on the benchtop until the day of use. Compounds were dissolved in dimethyl sulfoxide (Sigma) and then brought up to a working concentration of 1 mM in sterile molecular biology-grade water (Fisher) (final concentration, 1% DMSO). For mouse inoculations, compounds were diluted to 800 μM in water. For tissue culture experiments, compounds were diluted from the working 1 mM stock in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS) (Sigma). Protamine sulfate (PS) (Sigma) (50 mg/ml in water) was used as an established comparator method for bladder epithelial exfoliation (29), and 0.8% DMSO in water was used as a vehicle-only control.

Cell viability assays. Human bladder epithelial cells (5637 [ATCC HTB-9]; American Type Culture Collection) were maintained in RPMI 1640 medium with 10% FBS. The viability of cultured cells was analyzed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (TACS; Trevigen) according to the manufacturer's instructions. Briefly, cells were plated (5×10^4 per well) and grown to confluence at 37°C with 5% CO_2. On the day of the experiment, the medium was removed, and compounds and controls (in medium as described above) were added at the indicated doses. Following 15- or 60-min incubation, treatment medium was removed; cells were incubated with fresh medium containing the MTT reagent at 37°C for 3 h, until an intracellular purple precipitate was visible by microscopy. Detergent was then added to each well, and plates were kept at room temperature in the dark overnight. Absorbance at 570 nm was measured on a BioTek Synergy 2 microplate reader, and cell viability was determined by subtracting blanks and dividing the A_{570} value of treated cells by cells incubated with medium only.

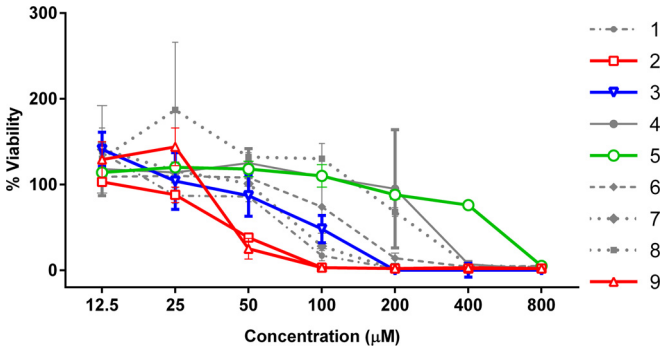


FIG 3 Dose-dependent effects of imidazolium salts on the viability of cultured uroepithelial cells. Bladder epithelial cells were treated for 60 min with the indicated concentrations of compounds 1 through 9, and viability was measured by a standard MTT assay. Relative cytotoxicity, normalized to that of untreated cells lysed subsequently with detergent, of each compound and dose is shown. Values are means ± standard deviations (SD) (error bars). Compounds 2 and 9 displayed the highest potency, while compound 5 induced cytotoxicity only at the highest tested concentration. Data are representative of two or three independent experiments per compound, with triplicate wells. Data from 15-min incubations are given in Fig. S2 in the supplemental material.

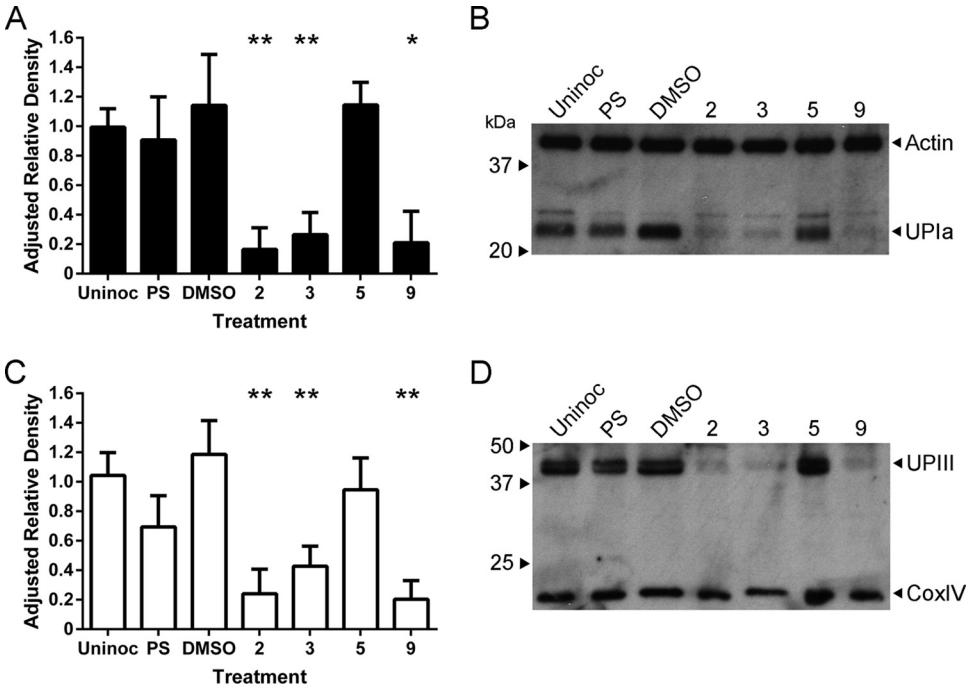


FIG 4 Exfoliation of murine bladder epithelium upon treatment with selected imidazolium salts. Murine bladders were treated twice (12.5 h apart) with protamine sulfate (PS) (50 mg/ml), vehicle only (DMSO), or compound 2, 3, 5, or 9 at 800 μM. Uninoculated (Uninoc) bladders were also similarly analyzed. (A to D) Exfoliation was assessed by adjusted relative density on Western blots of whole-bladder homogenates for uroplakin Ia (A and B) or uroplakin III (C and D). (A and B) Cumulative ImageJ quantitation (A) and a representative Western blot for UPIa with actin control (B). (C and D) Cumulative ImageJ quantitation (C) and a representative Western blot for UPIII with CoxIV control (D). Compounds 2, 3, and 9 elicited statistically significant exfoliation compared with the value for vehicle only (DMSO). Values that are significantly different from the value for vehicle only (DMSO) by paired *t* test are indicated by asterisks as follows: *, $P = 0.02$; **, $P < 0.001$. Data in panels A and C are derived from six or more mice per treatment on two separate days with independent compound preparation.

Data shown are representative of two or three independent experiments per compound, with triplicate wells.

Mice. All animal procedures were done with prior approval from the Animal Studies Committee at Washington University. Female C57BL/6 mice 7 to 8 weeks of age (Jackson Laboratories) were anesthetized with inhaled isoflurane, and 50 μl of compound or control was delivered to the bladder via transurethral catheterization (30). Anesthesia was maintained for 15 min following administration to decrease the chance of immediate voiding of bladder contents. A second dose was similarly administered 12.5 h after the initial inoculation. Mice were euthanized after 16 h of compound exposure (3.5 h after the second inoculation), a schedule based on recent studies with PS (29).

Immunoblotting. Exfoliation was quantified by blotting bladder homogenates for epithelial uroplakins using methods we developed previously (31). Briefly, bladders were harvested aseptically and homogenized in a bead beater (Bullet Blender Storm 2.4; Next Advance, Inc.) using sterile stainless steel beads. Homogenates were stored long-term at −80°C, and working aliquots were diluted in SDS loading buffer and maintained at −20°C. Prior to blotting, homogenates were thawed and loaded without boiling into 12% SDS-polyacrylamide gels. The samples were then semidry transferred to polyvinylidene difluoride (PVDF) membranes, blocked in 2% dry milk with 2% bovine serum albumin (BSA) in blocking buffer (0.5% Tween 20, 3% sodium chloride, 0.13% Tris base [pH 8.2]), and probed for uroplakin la (UPIa) (SC-15173; Santa Cruz Biotechnology) (diluted 1:250) and actin (SC-1616; Santa Cruz Biotechnology) (1:400) or for uroplakin III (UPIII) (SC-33570; Santa Cruz Biotechnology) (1:2,000) and CoxIV (4844S; Cell Signaling) (1:4,000). Different control proteins were used for the parallel Western blots so that, given the molecular weights, the bands could be clearly imaged and quantified. Alkaline phosphatase-conjugated anti-goat IgG (Sigma) (1:1,000) or anti-rabbit IgG (Sigma) (1:2,000) was used as a secondary antibody, respectively. Blots were developed with Tropix CDP-Star (Applied Biosystems) and exposed to film. Band density was quantified by ImageJ analysis, and uroplakin levels were normalized to the loading control bands (actin or CoxIV). Adjusted band densities were compared by paired *t* test; *P* values of <0.05 were considered significant. Data are from two experiments completed in triplicate on different days with freshly prepared compounds. Results were obtained from a total of six mice per compound.

Histology. The bladders were removed aseptically and fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid). The tissues were then set in 2% agar, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Images were captured using an Olympus DP25 camera and BX40 light microscope. For each compound included in this analysis (compounds 2, 3, 5, and 9), two or three separate bladders were examined and compared to multiple uninoculated, DMSO-treated, and PS-treated bladders.

Immunofluorescence. Bladder sections were deparaffinized with mixed xylenes and rehydrated with isopropanol prior to antigen retrieval via boiling in 10 mM sodium citrate. Once the slides were cooled, they were blocked with 1% BSA and 0.3% Triton X-100 in phosphate-buffered saline (PBS) and then incubated with rabbit anti-uroplakin III (diluted 1:100) for 1.5 h. Following primary antibody removal, Alexa Fluor 488-conjugated goat anti-rabbit antibody (catalog no. A11008; Life Technologies) (1:500) and Syto 61 (Molecular Probes) (1:1,000) were added together for 30 min at room temperature. Slides were mounted with ProLong Gold (Invitrogen) and examined by epifluorescence microscopy using a Zeiss Axioskop instrument. For each compound included in this analysis (compounds 2, 3, 5, and 9), two or three separate bladders were examined and compared to multiple uninoculated, DMSO-treated, and PS-treated bladders.

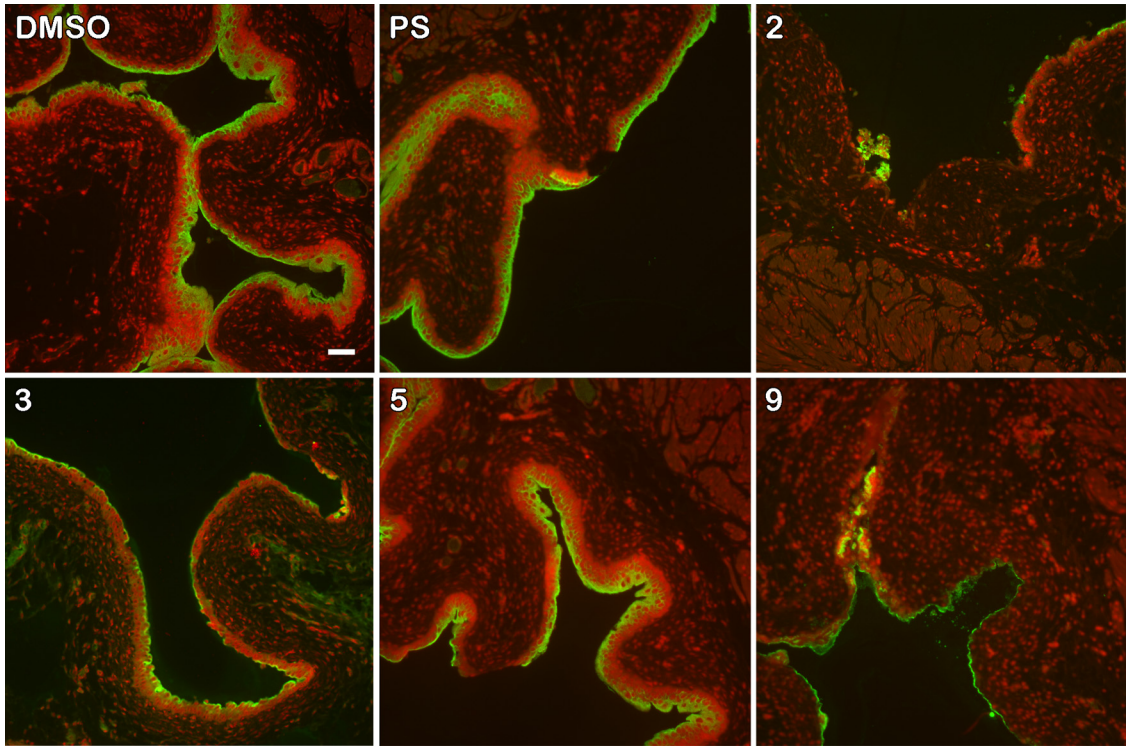


FIG 5 Immunofluorescence microscopy of treated bladder sections for uroplakin III. After transurethral bladder treatment with protamine sulfate (PS) (50 mg/ml), 0.8% DMSO (vehicle), or compound 2, 3, 5, or 9 (800 μM) as indicated, the bladders were harvested, fixed, sectioned, and processed using a dual-antibody scheme for uroplakin III (green) and Syto 61 nuclear stain (red) as detailed in Materials and Methods. DMSO-treated bladders appeared identical to untreated bladders (not shown). While PS treatment showed patchy mild exfoliation, compounds 2 and 9 were strongly exfoliative (i.e., had little UPIII staining), and compound 3 exerted a partial effect. Compound 5 treatment was nearly indistinguishable from vehicle-only (DMSO) treatment. Bar, 50 μm.

Cytokine analysis. Cytokine analysis was performed using the Bio-Plex Pro mouse cytokine Th17 panel A 6-plex group I kit (Bio-Rad) with magnetic beads following the manufacturer's instructions. Undiluted bladder homogenates were centrifuged at 10,000 × *g* for 10 min at 4°C, and cleared lysates were analyzed in duplicate wells (five mice in a group for the uninoculated group and PS- and DMSO-treated groups; six mice in a group for each group treated with compound 2, 3, 5, or 9). Data were analyzed with BioPlex Manager software according to the manufacturer's instructions.

RESULTS

Synthesis and characterization of a family of substituted imidazolium salts. Compounds 1 and 2 were synthesized according to a published procedure (32). To synthesize the remaining compounds, the imidazole derivatives were alkylated in two steps (Fig. 1). For compounds 3 to 8, commercially available imidazole, 4,5-dichloroimidazole, or benzimidazole was deprotonated by potassium hydroxide in acetonitrile followed by the addition of the respective alkyl halide ($X\text{-}CH_2\text{-}R^1$) and heating at reflux to generate the monosubstituted intermediates, which were isolated and characterized. To a solution of the intermediates in acetonitrile was added the second alkyl halide ($X\text{-}CH_2\text{-}R^2$), followed by heating at reflux to generate the corresponding imidazolium salts, compounds 3 to 8, which precipitated from the reaction mixtures. These salts were generated in 80 to 90% yields from the monosubstituted intermediates (with the exception of compound 3, which was generated in 50% yield) and required minimal purification. Compound 9 was synthesized by the same general procedure; however, the monosubstituted intermediate was not isolated. The starting imidazole derivative 2-((2-(2-methoxyethoxy)ethoxy) methyl)benzimidazole was first synthesized by the overnight reaction of *o*-phenylenediamine with 2-(2-(2-methoxyethoxy) ethoxy)acetic acid at 140°C. This imidazole derivative was then converted to the corresponding imidazolium salt, compound 9, via deprotonation and two sequential additions of the corresponding alkyl halides. All chemical intermediates were characterized by ^1H and $^{13}C\{^1H\}$ NMR spectroscopy, and compounds 3 to 9 were characterized by multiple spectroscopic techniques to verify their structures and purities. Additionally, the structures of compounds 3 to 6 and 8 were elucidated by X-ray crystallography. The structures of compounds 1 to 9 are shown in Fig. 2. Full details of the synthesis and characterization of the imidazolium salts (including crystallographic data links) are provided in the supplemental material.

***In vitro* cytotoxic effects of substituted imidazolium salts.** In evaluating the potential utility of the compound series as urinary exfoliants, we first assessed their cytotoxic effect on cultured 5637 bladder epithelial cells, using a standard cell viability (MTT) assay. We aimed to model exposure times relevant to *in vivo* application, based on dilution by continual arrival of urine into the bladder; short intervals between micturition events (in the murine host); and feasibility of potential treatment duration with a future, optimized agent in human patients. Therefore, treatment intervals of 15 and 60 min were chosen, and a broad range of compound concentrations were tested (0-800 μM). Among the 9 compounds, all demonstrated a dose-dependent and variably potent impact on 5637 uroepithelial cell viability after 15 min (see Fig. S2

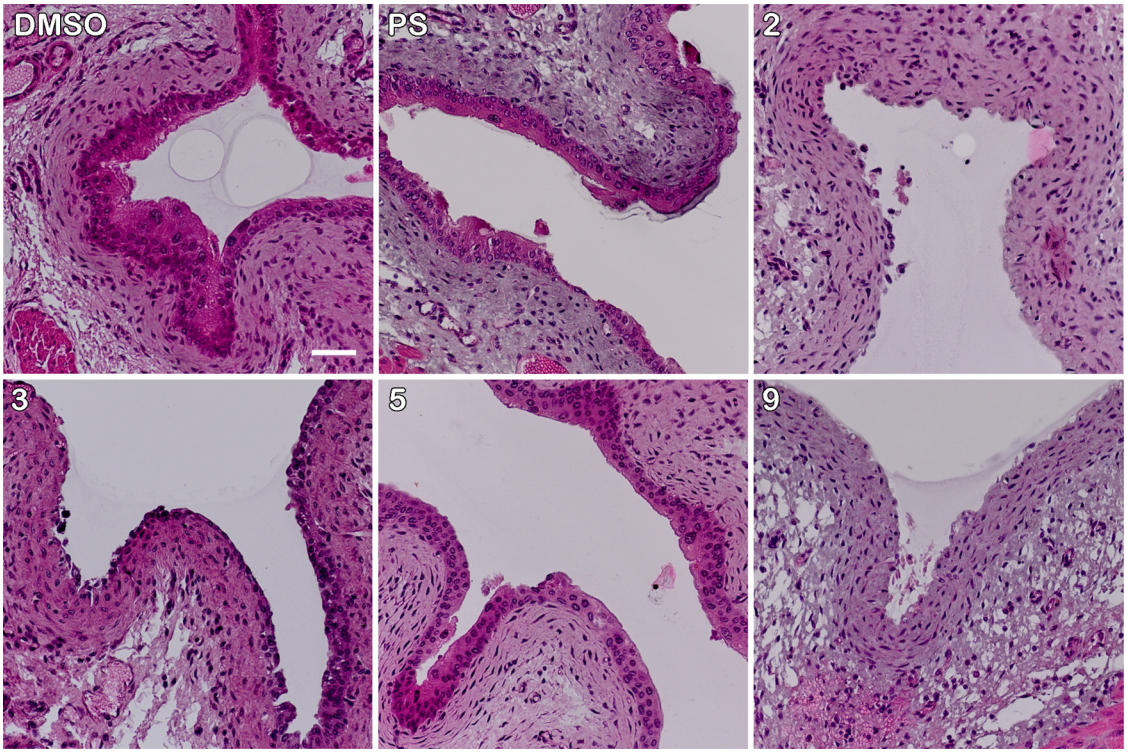


FIG 6 Histology of treated bladder sections. Bladders were treated *in vivo* with protamine sulfate (PS), 0.8% DMSO (vehicle), or compound 2, 3, 5, or 9 (800 μM) as indicated. Standard hematoxylin-and-eosin staining of fixed sections indicates scattered superficial epithelial loss with PS treatment, while DMSO treatment had no effect compared with untreated bladders (not shown). Treatment with compound 2 or 9 resulted in near-complete or complete exfoliation of the transitional epithelial cells, while compound 3 exhibited a partial effect. With this dosing and analysis schedule, evidence of inflammatory response (edema, cellular infiltrate) was not observed. Again, treatment with compound 5 had little effect on the histologic appearance of bladder sections. Bar, 50 μm.

in the supplemental material) or 60 min (Fig. 3) of treatment. Compounds 2 and 9 exhibited a marked cytotoxic effect at 50 μM, while the effects of compounds 1 and 7 were evident at 100 μM, and the effects of compounds 3 and 6 were evident at 200 μM. Compound 5 displayed the least potent activity, inducing significant cytotoxicity only at the highest concentration tested (800 μM).

***In vivo* exfoliative activity of selected imidazolium salts.** To correlate these *in vitro* findings with potential *in vivo* activity, we selected several compounds from the series for intravesical instillation in mice. Compounds 2 and 9 were selected for their robust *in vitro* activities, and compound 3 was selected for its moderate activity (median at 100 μM among the group); compound 5 was chosen as a comparator, since compound 5 displayed substantially less toxicity to cultured uroepithelial cells (Fig. 3). Each of these compounds, at the highest *in vitro*-tested dose (800 μM; chosen because *in vivo* contact time could not be ensured past 15 min of anesthesia), was applied to the bladder in a two-dose schedule over 16 h, and exfoliation of superficial bladder epithelial cells was assessed by multiple methods. By Western blotting of bladder homogenates for either uroplakin Ia or III (assessed independently), instillation of vehicle only (DMSO) caused no measurable exfoliation (Fig. 4). Protamine sulfate (50 mg/ml), the prototypic bladder epithelial exfoliant, elicited only ~25% exfoliation as measured by this method. In contrast, compounds 2, 3, and 9 induced near-complete exfoliation ($P \leq 0.02$ versus the value for vehicle only for these three compounds; Fig. 4). As predicted from our *in vitro* results, compound 5 had little effect on uroepithelial cell integrity (Fig. 4). Thus, trends among the compounds in *in vivo* epithelial exfoliation were reflected in the *in vitro* cytotoxicity results.

To further delineate integrity of the mammalian bladder epithelium upon exposure to these agents, we treated murine bladders as outlined above, and fixed the bladders for immunofluorescence analysis using a primary antibody to mouse uroplakin III (Fig. 5). Our results were consonant with those produced by Western blotting quantification. Specifically, treatment with DMSO only did not yield an appearance different from that of uninoculated bladders (uninoculated histology not shown). PS treatment resulted in moderate and patchy exfoliation, while application of compound 2, 3, or 9 at 800 μM caused near-complete exfoliation (Fig. 5). Of note, early uroplakin expression by underlying cells was evident by this time point (16 h), indicating initiation of epithelial differentiation pathways (11). Meanwhile, compound 5 (800 μM) had no discernible effect on the integrity of the facet cell layer.

Bladder tissue responses to imidazolium treatment. To discern inflammatory or other effects on bladder tissue upon exposure to imidazolium compounds, we inoculated mice as outlined above and fixed and stained sections of treated bladders with hematoxylin and eosin in standard fashion. The histology of DMSO-treated bladders was normal (Fig. 6), as was that of uninoculated bladders (not shown). PS treatment led to patchy epithelial loss, while compounds 2 and 9 yielded complete exfoliation of the epithelium. Compound 3 yielded incomplete exfoliation, while compound 5 had no histologically evident effects. Of note, treat-

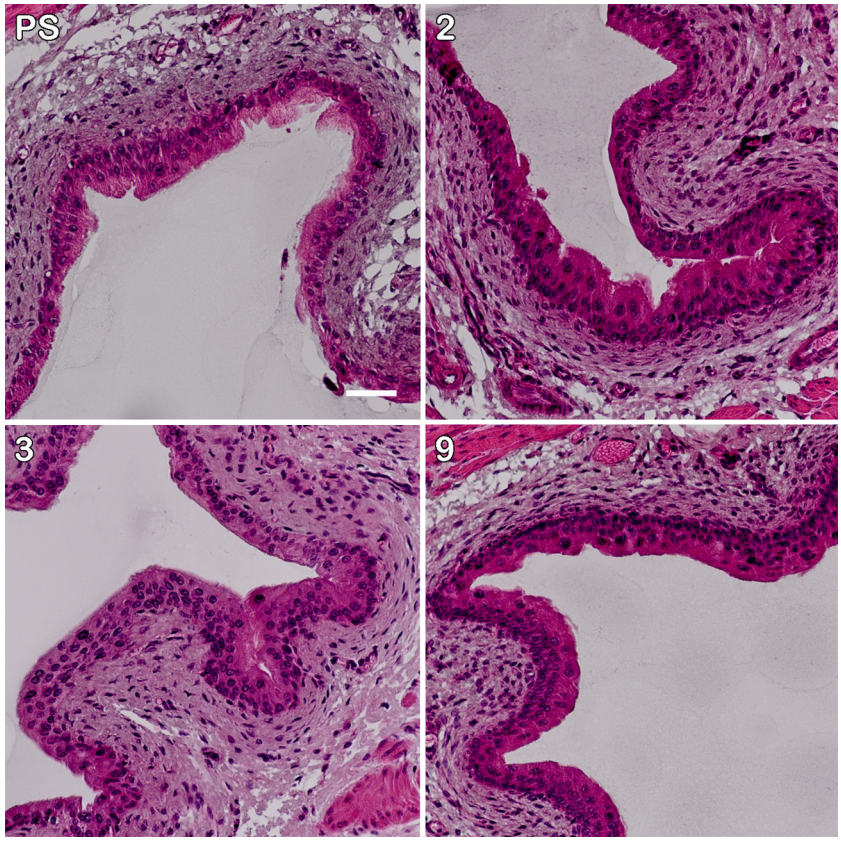


FIG 7 Recovery of bladder epithelium following exfoliation. Bladders were treated *in vivo* with protamine sulfate (PS) or compound 2, 3, or 9 (800 μM) as indicated. One week after inoculation, the mice were euthanized, and their bladders were processed for standard histology. Within this interval after treatment with any of these exfoliants, a histologically normal uroepithelium is regenerated atop the basement membrane, including the presence of multinucleate facet cells. Bar, 50 μm.

ment with the exfoliative imidazolium salts (compound 2, 3, or 9) elicited minimal to no cellular infiltrate or edema and no apparent cytotoxicity beyond the basement membrane (Fig. 6). In addition, levels of interleukin 1β (IL-1β), IL-6, IL-10, IL-17A, tumor necrosis factor alpha (TNF-α), or gamma interferon (IFN-γ) in bladder homogenates were not significantly changed after treatment with any of the compounds (data not shown). To assess epithelial recovery from exfoliation, we examined bladders harvested from mice 1 week following treatment as outlined above with the exfoliant PS or exfoliant compound 2, 3, or 9. All treated bladders exhibited a regenerated uroepithelium that was histologically normal in appearance, including restoration of the facet cell layer (Fig. 7).

DISCUSSION

There is a need in preclinical urologic studies for small-molecule agents that will efficiently achieve exfoliation of the urinary bladder. Protamine sulfate, the most widely used bladder epithelial exfoliant, is a relatively large molecule (~5 kDa) and, with many published application strategies, achieves incomplete epithelial exfoliation. Here, we present a novel series of imidazolium salts with different levels of potency in cytotoxicity to cultured uroepithelial cells and in exfoliative activity in the murine bladder. The compounds displaying the most potent *in vitro* cytotoxicity were also those with the most exfoliant activity *in vivo*; similar trends were observed across the compound series. Even the most potent exfoliators in this family remained safe, inducing no discernible systemic effects and allowing regeneration of the bladder epithelium within days. The chemical scaffold also enables conjugation and modification for a variety of experimental and potentially translatable applications.

Among these potential applications, there is recently burgeoning interest in bladder epithelial exfoliation as adjunctive therapy in chronic and recurrent urinary tract infection. As noted in the introduction, acute UPEC cystitis relies critically on rapid bacterial replication within the cytoplasm of superficial bladder epithelial cells, a niche that is protective against the phagocytic activity of arriving neutrophils. After resolution of bacteriuria and acute cystitis in mice, a small population of UPEC persists within uroepithelial cells. These bacteria resist immune clearance and systemic antimicrobial therapy and can reemerge to cause same-strain recurrent cystitis even if antimicrobials have symptomatically resolved the initial episode. More recent studies have suggested that a combined exfoliant/antimicrobial approach might help to eradicate these chronically resident bacteria (15). This approach, if translated to a population of women with recurrent cystitis, would markedly impact the morbidity of this recalcitrant condition. Preliminary work indicates that the exfoliants presented here exhibit antibacterial effect against UPEC at relatively high concentrations (e.g., >50 μM; data not shown). In addition to this potential

intrinsic antibacterial use, these exfoliants could be chemically modified with biocides (e.g., silver cation) or delivered in conjunction with existing antimicrobials to interrupt this cycle of recurrent infection or to prevent UTI, for instance, following urinary tract instrumentation.

More challenging is the clinical investigation and preclinical modeling of urologic chronic pelvic pain syndromes, including chronic bladder pain, interstitial cystitis, and chronic prostatitis (33). These conditions confer burdensome symptoms to many patients, and as the causes are poorly understood, directed and evidence-based management options are lacking. However, there is evidence that exfoliation of the superficial bladder epithelium might mitigate pain associated with these conditions (29). In part, this may be because the uroepithelial cells represent a primary source of proinflammatory cytokines and chemokines in bladder inflammatory responses. Thus, while one might at first glance expect that intentional uroepithelial exfoliation would be inflammatory, the approach may in fact curtail inflammation and provide analgesia (29). Our cytokine data also support the lack of significant inflammation associated with exfoliation.

A further application of urinary bladder exfoliation would be in the diagnosis and treatment of bladder cancer, specifically transitional cell carcinoma. Uroepithelial cancers can be diagnosed by cystoscopy and biopsy but also noninvasively by urine cytology. The sensitivity of cytology may be augmented with the use of either monoclonal antibodies to detect carcinoma antigens or fluorescent *in situ* hybridization probes for chromosomal abnormalities found in bladder carcinoma (reviewed in reference 34). In addition, postresection monitoring in bladder cancer patients may involve both cytology and other biomarker-based methods (e.g., detection of nuclear matrix protein 22). However, the utility of these newer approaches is not universally accepted, especially across distinct patient populations and clinical situations (35), and additional biomarkers and methodologies are under study. Topical administration of an exfoliant would provide a much richer sample for cytologic analysis, likely increasing the sensitivity of cell-based diagnostics. In addition, as mentioned above, the chemical scaffold presented here could, hypothetically, be modified to target the exfoliant more specifically to carcinoma cells. Overall, imidazolium salt-based uroepithelial exfoliants have a number of potential applications in both UTI and noninfectious conditions of the urinary tract.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants R01-DK082546 and P50-DK064540, and by the Akron Research Commercialization Corporation. Support from the Welch Foundation (A-0001) is also gratefully acknowledged. We acknowledge the National Science Foundation for funds used to purchase the Bruker APEX II SuperDuo X-ray diffractometer used in this research (CHE-0116041).

We thank E. Danka for technical advice.

REFERENCES

1. **Sun TT, Zhao H, Provet J, Aebi U, Wu XR.** 1996. Formation of asymmetric unit membrane during urothelial differentiation. Mol Biol Rep **23:**3–11. http://dx.doi.org/10.1007/BF00357068.
2. **Foxman B.** 2010. The epidemiology of urinary tract infection. Nat Rev Urol **7:**653–660. http://dx.doi.org/10.1038/nrurol.2010.190.
3. **Min G, Stolz M, Zhou G, Liang F, Sebbel P, Stoffler D, Glockshuber R, Sun TT, Aebi U, Kong XP.** 2002. Localization of uroplakin Ia, the urothelial receptor for bacterial adhesin FimH, on the six inner domains of the 16 nm urothelial plaque particle. J Mol Biol **317:**697–706. http://dx.doi.org/10.1006/jmbi.2002.5442.
4. **Wu XR, Sun TT, Medina JJ.** 1996. In vitro binding of type 1-fimbriated *Escherichia coli* to uroplakins 1a and 1b: relation to urinary tract infections. Proc Natl Acad Sci U S A **93:**9630–9635. http://dx.doi.org/10.1073/pnas.93.18.9630.
5. **Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ.** 2003. Intracellular bacterial biofilm-like pods in urinary tract infections. Science **301:**105–107. http://dx.doi.org/10.1126/science.1084550.
6. **Ulett GC, Totsika M, Schaale K, Carey AJ, Sweet MJ, Schembri MA.** 2013. Uropathogenic *Escherichia coli* virulence and innate immune responses during urinary tract infection. Curr Opin Microbiol **16:**100–107. http://dx.doi.org/10.1016/j.mib.2013.01.005.
7. **Mulvey MA, Lopez-Boado YS, Wilson CL, Roth R, Parks WC, Heuser J, Hultgren SJ.** 1998. Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. Science **282:**1494–1497. http://dx.doi.org/10.1126/science.282.5393.1494.
8. **Rosen DA, Hooton TM, Stamm WE, Humphrey PA, Hultgren SJ.** 2007. Detection of intracellular bacterial communities in human urinary tract infection. PLoS Med **4:**e329. http://dx.doi.org/10.1371/journal.pmed.0040329.
9. **Hunstad DA, Justice SS.** 2010. Intracellular lifestyles and immune evasion strategies of uropathogenic *Escherichia coli*. Annu Rev Microbiol **64:**203–221. http://dx.doi.org/10.1146/annurev.micro.112408.134258.
10. **Mysorekar IU, Hultgren SJ.** 2006. Mechanisms of uropathogenic *Escherichia coli* persistence and eradication from the urinary tract. Proc Natl Acad Sci U S A **103:**14170–14175. http://dx.doi.org/10.1073/pnas.0602136103.
11. **Mysorekar IU, Isaacson-Schmid M, Walker JN, Mills JC, Hultgren SJ.** 2009. Bone morphogenetic protein 4 signaling regulates epithelial renewal in the urinary tract in response to uropathogenic infection. Cell Host Microbe **5:**463–475. http://dx.doi.org/10.1016/j.chom.2009.04.005.
12. **Blango MG, Mulvey MA.** 2010. Persistence of uropathogenic *Escherichia coli* in the face of multiple antibiotics. Antimicrob Agents Chemother **54:**1855–1863. http://dx.doi.org/10.1128/AAC.00014-10.
13. **Mulvey MA, Schilling JD, Hultgren SJ.** 2001. Establishment of a persistent *Escherichia coli* reservoir during the acute phase of a bladder infection. Infect Immun **69:**4572–4579. http://dx.doi.org/10.1128/IAI.69.7.4572-4579.2001.
14. **Schilling JD, Lorenz RG, Hultgren SJ.** 2002. Effect of trimethoprim-sulfamethoxazole on recurrent bacteriuria and bacterial persistence in mice infected with uropathogenic *Escherichia coli*. Infect Immun **70:**7042–7049. http://dx.doi.org/10.1128/IAI.70.12.7042-7049.2002.
15. **Blango MG, Ott EM, Erman A, Veranic P, Mulvey MA.** 2014. Forced resurgence and targeting of intracellular uropathogenic *Escherichia coli* reservoirs. PLoS One **9:**e93327. http://dx.doi.org/10.1371/journal.pone.0093327.
16. **Russell AD, Hugo WB.** 1994. Antimicrobial activity and action of silver. Prog Med Chem **31:**351–370. http://dx.doi.org/10.1016/S0079-6468(08)70024-9.
17. **Holland CL, Singh AK, McMaster PR, Fang W.** 1984. Adverse reactions to protamine sulfate following cardiac surgery. Clin Cardiol **7:**157–162. http://dx.doi.org/10.1002/clc.4960070305.
18. **Lakin JD, Blocker TJ, Strong DM, Yocum MW.** 1978. Anaphylaxis to protamine sulfate mediated by a complement-dependent IgG antibody. J Allergy Clin Immunol **61:**102–107. http://dx.doi.org/10.1016/0091-6749(78)90232-4.
19. **Nybo M, Madsen JS.** 2008. Serious anaphylactic reactions due to protamine sulfate: a systematic literature review. Basic Clin Pharmacol Toxicol **103:**192–196. http://dx.doi.org/10.1111/j.1742-7843.2008.00274.x.
20. **Sanchez MB, Paolillo M, Chacon RS, Camejo M.** 1982. Protamine as a cause of generalised allergic reactions to NPH insulin. Lancet **i:**1243.
21. **Lavelle J, Meyers S, Ramage R, Doty D, Bastacky S, Apoddaca G, Zeidel M.** 2001. Protamine sulfate-induced cystitis: a model of selective cytodestruction of the urothelium. Urology **57**(6 Suppl 1)**:**113. http://dx.doi.org/10.1016/S0090-4295(01)01047-0.
22. **Niku SD, Stein PC, Scherz HC, Parsons CL.** 1994. A new method for cytodestruction of bladder epithelium using protamine sulfate and urea. J Urol **152:**1025–1028.
23. **Tzan CJ, Berg J, Lewis SA.** 1993. Effect of protamine sulfate on the permeability properties of the mammalian urinary bladder. J Membr Biol **133:**227–242.
24. **Chuang YC, Chancellor MB, Seki S, Yoshimura N, Tyagi P, Huang L,**

Lavelle JP, De Groat WC, Fraser MO. 2003. Intravesical protamine sulfate and potassium chloride as a model for bladder hyperactivity. Urology **61:**664–670. http://dx.doi.org/10.1016/S0090-4295(02)02280-X.
25. **Sinanoglu O, Dogan Ekici I, Ekici S.** 2014. Comparison of intravesical application of chondroitin sulphate and colchicine in rat protamine/lipopolysaccharide induced cystitis model. Urol J **11:**1296–1300.
26. **Soler R, Bruschini H, Freire MP, Alves MT, Srougi M, Ortiz V.** 2008. Urine is necessary to provoke bladder inflammation in protamine sulfate induced urothelial injury. J Urol **180:**1527–1531. http://dx.doi.org/10.1016/j.juro.2008.06.006.
27. **Bollini M, Casal JJ, Bruno AM.** 2008. Design, synthesis, and antitumor activity of new bis-aminomethylnaphthalenes. Bioorg Med Chem **16:**8003–8010. http://dx.doi.org/10.1016/j.bmc.2008.07.069.
28. **Sheldrick GM.** 2008. A short history of SHELX. Acta Crystallogr A **64:**112–122. http://dx.doi.org/10.1107/S0108767307043930.
29. **Stemler KM, Crock LW, Lai HH, Mills JC, Gereau RW, IV, Mysorekar IU.** 2013. Protamine sulfate induced bladder injury protects from distention induced bladder pain. J Urol **189:**343–351. http://dx.doi.org/10.1016/j.juro.2012.08.189.
30. **Hung CS, Dodson KW, Hultgren SJ.** 2009. A murine model of urinary tract infection. Nat Protoc **4:**1230–1243. http://dx.doi.org/10.1038/nprot.2009.116.
31. **Danka ES, Hunstad DA.** 2015. Cathelicidin augments epithelial receptivity and pathogenesis in experimental *Escherichia coli* cystitis. J Infect Dis **211:**1164–1173. http://dx.doi.org/10.1093/infdis/jiu577.
32. **Wright BD, Deblock MC, Wagers PO, Duah E, Robishaw NK, Shelton KL, Southerland MR, DeBord MA, Kersten KM, McDonald LM, Stiel JA, Panzner MJ, Tessier CA, Paruchuri S, Youngs WJ.** 13 February 2015. Anti-tumor activity of lipophilic imidazolium salts on select NSCLC cell lines. Med Chem Res http://dx.doi.org/10.1007/s00044-015-1330-z.
33. **Clemens JQ, Mullins C, Kusek JW, Kirkali Z, Mayer EA, Rodriguez LV, Klumpp DJ, Schaeffer AJ, Kreder KJ, Buchwald D, Andriole GL, Lucia MS, Landis JR, Clauw DJ.** 2014. The MAPP research network: a novel study of urologic chronic pelvic pain syndromes. BMC Urol **14:**57. http://dx.doi.org/10.1186/1471-2490-14-57.
34. **Mowatt G, Zhu S, Kilonzo M, Boachie C, Fraser C, Griffiths TR, N′Dow J, Nabi G, Cook J, Vale L.** 2010. Systematic review of the clinical effectiveness and cost-effectiveness of photodynamic diagnosis and urine biomarkers (FISH, ImmunoCyt, NMP22) and cytology for the detection and follow-up of bladder cancer. Health Technol Assess **14**(4)**:**1–331. http://dx.doi.org/10.3310/hta14040.
35. **Huber S, Schwentner C, Taeger D, Pesch B, Nasterlack M, Leng G, Mayer T, Gawrych K, Bonberg N, Pelster M, Johnen G, Bontrup H, Wellhausser H, Bierfreund HG, Wiens C, Bayer C, Eberle F, Scheuermann B, Kluckert M, Feil G, Bruning T, Stenzl A, UroScreen Study Group.** 2012. Nuclear matrix protein-22: a prospective evaluation in a population at risk for bladder cancer. Results from the UroScreen study. BJU Int **110:**699–708. http://dx.doi.org/10.1111/j.1464-410X.2011.10883.x.