Current Topics in Biochemical Research
Vol. 23, 2022

Original Article

Physiological reduction in urease activity in the urinary tract pathogen *Staphylococcus saprophyticus* by active-site inhibitors

Charles E. Deutch[*,#]
Microbion Research, 8931 W. Deanna Dr., Peoria, Arizona 85382, USA.

ABSTRACT

Urease is a key virulence factor for the urinary tract pathogen *Staphylococcus saprophyticus*. Previous studies indicated that dimethylsulfoxide, which resembles urea, could inhibit urease activity in a noncompetitive fashion but could not completely prevent the increase in pH associated with urease activity. The aims of this study are 1) to investigate the effects of boric acid, sodium fluoride, phenylmethylsulfonyl fluoride and dimethylglyoxime on the urease from *S. saprophyticus* and 2) to determine if these compounds in combination with dimethylsulfoxide could better inhibit bacterial growth and pH changes in artificial urine medium and human urine. Urease activity in extracts and whole cells was measured by the formation of ammonium ions. The effects of urease inhibition were determined by following bacterial growth, pH, and formation of insoluble crystals in cultures grown in artificial urine medium, urea broth, and human urine. Boric acid competitively inhibited urease activity in cell extracts up to 80% while sodium fluoride reduced activity up to 90% in an uncompetitive fashion. Phenylmethylsulfonyl fluoride inhibited activity about 80% but dimethylglyoxime only reduced activity by 50%. All of the compounds inhibited urease activity in whole cells grown in artificial urine medium. Boric acid and sodium fluoride in combination with dimethylsulfoxide blocked the color change that occurs in urea broth. When boric acid, sodium fluoride, and dimethylsulfoxide were added to *S. saprophyticus* cultures in artificial urine medium, there was a longer lag phase, a decrease in the rate at which the pH rose, and an inhibition of struvite crystal formation. These compounds also reduced bacterial growth and the pH increase in normal human urine. A combination of active-site inhibitors thus can reduce the physiological effects of urease activity and may be effective in treating patients with urinary tract infections that are resistant to antibiotics or herbal remedies.

KEYWORDS: boric acid, dimethylsulfoxide, dimethylglyoxime, phenylmethylsulfonyl fluoride sodium fluoride, *Staphylococcus saprophyticus*, urease, urinary tract infection.

INTRODUCTION

Urinary tract infections (UTIs) commonly occur in infants and small children, in adolescent and adult women, and in patients or older adults fitted with catheters [1-3]. The primary etiological agents are the Gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* and the Gram-positive bacteria *Staphylococcus saprophyticus*, *Enterococcus faecalis*, and *Staphylococcus aureus* [4]. Although most UTIs can be treated with antibiotics [5], resistant microorganisms are frequently recovered from

*Email id: charles.deutch@asu.edu
[#]Professor Emeritus, Arizona State University at the West Campus, 4701 W Thunderbird Rd., Phoenix, Arizona 85306 USA.

infected individuals [6] and recurrent infections are common [7].

Staphylococcus saprophyticus is a coagulase-negative novobiocin-resistant bacterium that is primarily associated with uncomplicated community-acquired UTIs in young women [8-12]. These infections are probably acquired from the gastrointestinal tract but increase with sexual activity and are highest in the late summer and fall. An essential virulence factor for *S. saprophyticus* is the enzyme urease (urea amidohydrolase, EC 3.5.1.5), which catalyzes the hydrolysis of urea to form ammonium ions and carbonic acid [13, 14]. The ammonium ions raise the urinary pH and can lead to the formation of struvite crystals, urinary stones, and the encrustation of urinary catheters [15-17]. The urease from *S. saprophyticus* shares some common features with other bacterial ureases but has some important differences [18]. It contains three types of subunits, but these are organized into an $(\alpha\beta\gamma)_4$ structure rather than an $(\alpha\beta\gamma)_3$ structure and there is only one Ni^{2+} ion in each larger catalytic subunit [19]. It also lacks any cysteine residues and has threonine rather than cysteine at the active site [19-20]. Formation of urease activity in *S. saprophyticus* is constitutive but may be affected by pH and the presence of other nitrogen sources [14, 19]. Although the enzyme can be inhibited by some common urease inhibitors such as acetohydroxamic acid and fluorofamide, it is not inhibited by omeprazole and similar compounds [21]. It is also resistant to inhibition by many herbal preparations that have been found to affect the ureases from jack beans, *Proteus mirabilis*, and *Helicobacter pylori* [22].

Dimethylsulfoxide (DMSO) structurally resembles urea and is commonly used as solvent for various nonpolar organic compounds and plant extracts. It can be used to treat interstitial cystitis/bladder pain syndrome, a poorly understood chronic condition characterized by urinary urgency and pain during voiding [23-25]. Treatment usually involves infusion of a 50% solution for several hours. In a recent project, DMSO was found to inhibit the ureases from *S. saprophyticus* and *P. mirabilis* [26]. Kinetic analysis indicated inhibition was of the noncompetitive type in which the V_{max} decreased and the K_m remained the same. DMSO also inhibited urease activity in whole cells. A 10% DMSO solution slowed the growth rate and reduced the yield in a rich medium (LB) or in LB medium supplemented with 100 mM urea. When the bacteria were grown in an artificial urine medium containing 10% DMSO, the growth rate again was reduced and the increase in pH associated with urease activity was delayed. While these results were promising, the inhibition of the urease-dependent pH increase associated with pathogenesis was incomplete. A series of other chemicals known to bind to the active site of ureases have now been tested as inhibitors of the *S. saprophyticus* enzyme individually and in combination with DMSO and a synergistic effect that leads to complete physiological inhibition of the pH change in human urine has been found.

MATERIALS AND METHODS

Bacterial strains and growth conditions

S. saprophyticus strain ATCC 15305 was obtained from the American Type Culture Collection (Manassas, VA, USA). Bacteria were maintained on Difco™ tryptic soy broth agar (Becton, Dickinson and Company, Sparks, MD, USA). Liquid P medium for *S. saprophyticus* was prepared as described by Gatermann *et al.* [14] and contained per liter: 10 g peptone, 5 g yeast extract, 1 g Na_2HPO_4, and 1 g D-glucose. The artificial urine medium (AUM) described by Minuth *et al.* [27] contained per liter: 0.65 g $CaCl_2.2\ H_2O$, 0.65 g $MgCl_2.6H_2O$, 4.6 g NaCl, 2.3 g Na_2SO_4, 2.8 g KH_2PO_4, 1.6 g KCl, 1.0 g NH_4Cl, 12 g urea, 1.1 g creatinine, and 10 g tryptic soy broth. The medium was sterilized by vacuum filtration through membrane filters (Genesee Scientific, El Cajon, CA, USA, PES membrane, 0.22 µm pores). Liquid cultures were routinely grown at 37 ^oC in 300 ml baffled nephelometer flasks containing less than 10% of the total volume of medium and shaken at 250 rev min^{-1}. Turbidities were measured in a Klett-Summerson colorimeter with a red (660 nm) filter. When inhibitors were added, the concentrations were 10% (v/v) DMSO, 25 mmol l^{-1} boric acid, and/or 25 mmol l^{-1} sodium fluoride. Dehydrated urea broth (Becton, Dickinson and Company, Sparks, MD, USA) was rehydrated and

filtered-sterilized as described above. Morning human urine was provided by laboratory volunteers and sterilized by vacuum filtration. Urea broth and human urine cultures (3 ml each) were grown in 14 ml sterile Falcon 352001 polystyrene tubes at 37 ^{o}C without shaking. Growth in these cultures was measured at 600 nm in a Shimadzu U-160 spectrophotometer.

Inhibition studies in artificial urine medium

To assess whether chemical inhibitors affect the increase in pH that normally occurs when *S. saprophyticus* is grown in artificial urine medium (AUM), an overnight culture was diluted 1/100 into 50 ml of artificial urine medium in 300 ml baffled nephelometer flasks without or with the inhibitors to be tested. The cultures were incubated with aeration at 37 ^{o}C and the turbidity was determined in a Klett-Summerson colorimeter every two hr. A 2.0 ml sample was removed at each time point, centrifuged at 10,000 *g* in a Bio-Lion XC-H165 centrifuge at room temperature for 5 min to remove the bacteria and any insoluble material, and the pH of the medium determined with a micro-combination electrode and Thermo Orion 720+ pH meter. The cell pellet was resuspended in 100 µl of sterile water and examined by phase-contrast microscopy with a Nikon Alphaphot microscope and a 100X oil immersion objective to look for the presence of insoluble salt crystals.

Preparation of cell extracts

S. saprophyticus was grown to late exponential phase (75 to 100 Klett Units) in a total of 900 ml of P medium at 37 ^{o}C with aeration. The bacteria were harvested by centrifugation at 10,000 *g* in a Bio-Lion XC-H165 centrifuge at room temperature, washed once with 0.85% (w/v) NaCl, and stored as a pellet at -20 ^{o}C. The cells were thawed and resuspended in 30 ml of urease resuspension buffer (50 mmol l^{-1} HEPES, 1 mmol l^{-1} Na_2EDTA, pH 7.5). The bacteria were combined with 1 mm glass beads and disrupted by 5 one-min cycles in a Bead-Beater® (Biospec Products, Inc., Bartlesville, OK, USA). After allowing most of the beads to settle, the remaining beads, unbroken cells, and debris were removed by centrifugation in a Bio-Lion XC-H165 centrifuge at room temperature for 5 min at 2,000 *g* and then for 10 min at 10,000 *g*. The supernatant fraction was saved as the cell extract and stored at -20 ^{o}C. Because the extract of *S. saprophyticus* was particularly active, it was diluted 1/3 with urease resuspension buffer before further use.

Preparation of whole cell suspensions

S. saprophyticus was grown to late exponential phase (75 to 100 Klett Units) in 10 ml of artificial urine medium at 37 ^{o}C with aeration. The bacteria were harvested by centrifugation at 10,000 *g* in a Bio-Lion XC-H165 centrifuge at room temperature, washed once with 0.85% (w/v) NaCl, and resuspended in 0.85% (w/v) NaCl to give a suspension equal to 100 Klett Units. The suspension was kept on ice until used in the urease assays.

Urease assays

Urease activity was determined using a colorimetric assay in which ammonium formation was measured by the phenol-hypochlorite method [28]. The absorbance of reactions was determined at 625 nm in a Shimadzu U-160 spectrophotometer and the ammonium concentrations calculated from an NH_4Cl standard curve. In a typical reaction, a sample of the *S. saprophyticus* cell extract (10 to 20 µl) or a whole bacteria cell suspension (200 µl) was added to urease assay buffer (50 mmol l^{-1} HEPES, 1 mmol l^{-1} Na_2EDTA, 25 mmol l^{-1} urea, pH 7.5), without or with a particular inhibitor to give a total volume of 2000 µl (2.0 ml). After 30 to 120 min at 37 ^{o}C, three replicate 100 µl samples were removed and added to 1.5 ml of Solution A (1.0 g phenol, 5 mg sodium nitroprusside [sodium nitroferricyanide (III)] in 100 ml water). 1.5 ml of Solution B (0.5 g NaOH, 870 µl sodium hypochlorite in 100 ml water) was immediately added and the solution rapidly mixed. After all of the samples were collected, they were incubated at 37 ^{o}C for 30 min and the absorbance at 625 nm determined.

Protein assays

Protein concentrations of the cell extracts and whole cell suspensions were determined by the Bradford bicinchoninic method [29] using bovine serum albumin as the standard. The absorbance of

reactions was determined at 562 nm in a Shimadzu U-160 spectrophotometer.

Statistical analysis

All of the data reported were within the linear range of urease activity. The urease assays were done in triplicate and varied by <10%. Results were normally expressed as a percent of the control reaction ± one standard deviation of the replicate assays. All growth and inhibition experiments were done at least twice. Representative results from a single experiment are shown unless otherwise noted.

RESULTS

Inhibition of urease activity in a cell extract of *S. saprophyticus* by single chemicals

Ureases from several bacteria including *Proteus mirabilis*, *Klebsiella aerogenes*, and *Helicobacter pylori* have been found to be inhibited by boric acid, which resembles the substrate urea [30-32]. The urease activity in a cell-free extract of *S. saprophyticus* was sensitive to inhibition by boric acid. There was a progressive decrease in activity with concentrations of boric acid up to 5 mmol l^{-1} (Figure 1(a)). Kinetic analysis using a Lineweaver-Burke plot indicated that inhibition was competitive ((Figure 1(b)). Ureases from *Klebsiella aerogenes*, *Staphylococcus epidermidis*, and *Sporosarcina pasteurii* can also be inhibited by sodium fluoride (NaF), which binds to the Ni^{2+} ion at the active site [33-35]. The urease activity in the cell-free extract from *S. saprophyticus* was very sensitive to inhibition by sodium fluoride (Figure 1(c)). Previous studies on the urease from *Klebsiella aerogenes* indicated that maximal inhibition by fluoride required pre-incubation with the inhibitor [33]. In the case of *S. saprophyticus*, however, preincubation of the extract for 30 min with NaF before adding urea as the substrate had no effect. Kinetic analysis indicated that inhibition in *S. saprophyticus* was mixed but basically of the uncompetitive type (Figure 1(d)).

Phenylmethylsulfonyl fluoride (PMSF) is a chemical that can react with serine or threonine residues at the active site of esterases and related enzymes, leading to the formation of an inactive covalent derivative [36, 37]. It has been shown to inhibit the urease from *S. saprophyticus* [20]. When the activity in a soluble extract from this bacterium was treated with increasing amounts of PMSF, there was a progressive decrease in activity with 95% inhibition at 2.5 mmol l^{-1} (Figure 2(a)). By contrast, dimethylglyoxime is a chemical that forms chelation complexes with Ni^{2+} ions [38]. When the urease activity in the soluble extract from *S. saprophyticus* was treated with increasing concentrations of this compound, there was only partial inhibition of activity (Figure 2(b)). About 50% of the initial activity was retained in the presence of 10 mmol l^{-1} dimethylglyoxime. To test whether incorporation of Ni^{2+} into the apoprotein can be inhibited by dimethylglyoxime, *S. saprophyticus* was grown in rich P medium or artificial urine medium containing increasing concentrations of dimethylglyoxime to exponential phase and the urease activity in whole cells determined. There was a decrease in the growth rate in both media as the concentration was increased to 3 mmol l^{-1} and growth was poor at higher concentrations. The presence of the dimethylglyoxime had only a modest effect on the specific activity in bacteria grown in the P medium, but reduced the activity to about one-third of the control activity in artificial urine medium (Figure 2(c)).

Inhibition of urease activity in a cell extract of *S. saprophyticus* by combinations of chemicals

These chemical inhibitors have different modes of action. To determine if they might act synergistically to reduce urease activity in *S. saprophyticus*, a soluble extract was treated with the single compounds as well as various combinations (Figure 3). Inhibition by boric acid and sodium fluoride was not additive to one another, but both compounds increased the inhibitory effects of phenylmethylsulfonyl fluoride (PMSF) and dimethylglyoxime (DMG).

Inhibition of urease activity in whole cells of *S. saprophyticus*

To determine if these active-site inhibitors might also affect urease activity in whole cells, exponential-phase cells grown in artificial urine medium were treated with increasing concentrations of boric acid, sodium fluoride, phenylmethylsulfonyl fluoride, and

dimethylglyoxime and the urease activity determined (Figure 4(a)). All of the compounds showed partial inhibition but were less effective than with cell-free extracts. Boric acid and sodium fluoride reduced activity to about 40% of the control level at concentrations of 10 mmol l^{-1}. PMSF decreased activity to about 20% of the control level at a concentration of 5 mmol l^{-1}. However, 10 mmol l^{-1} dimethylglyoxime only reduced activity to about 60% of the control level.

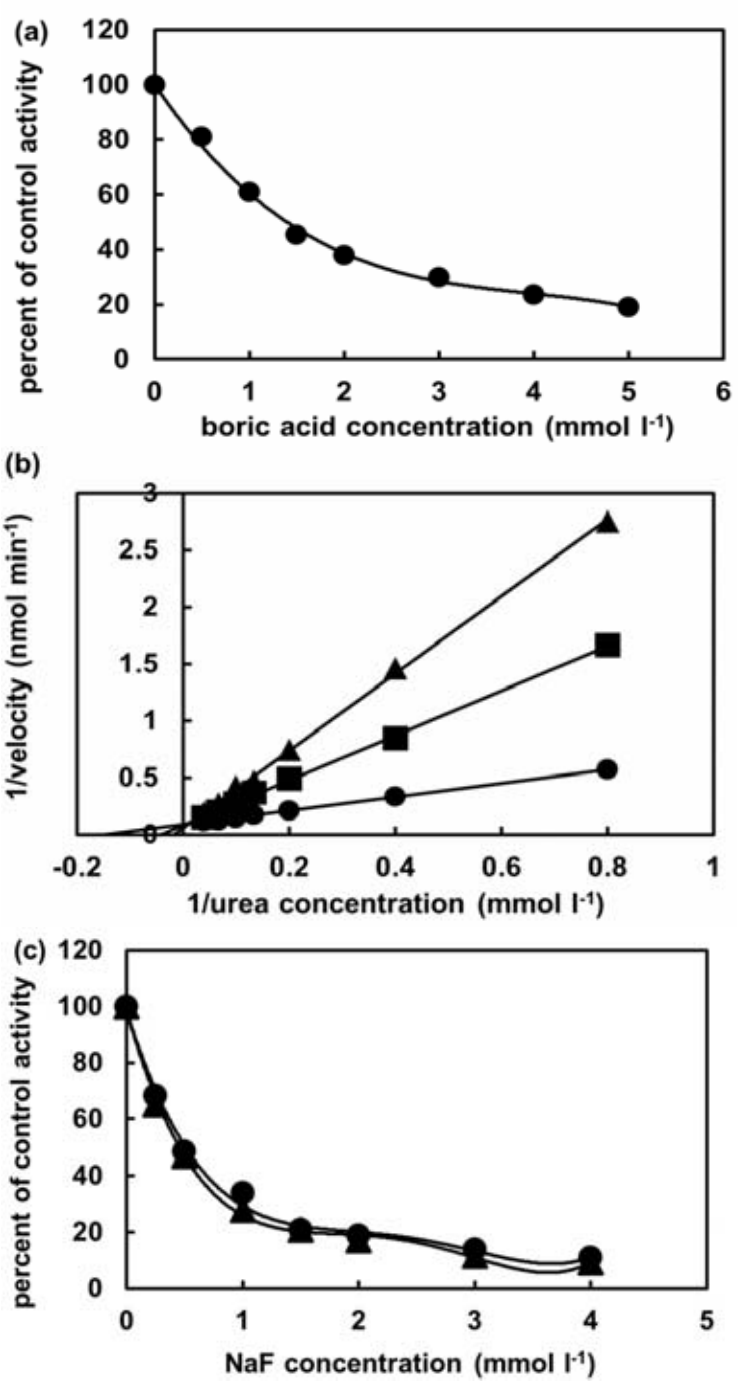

Figure 1

Figure 1 continued..

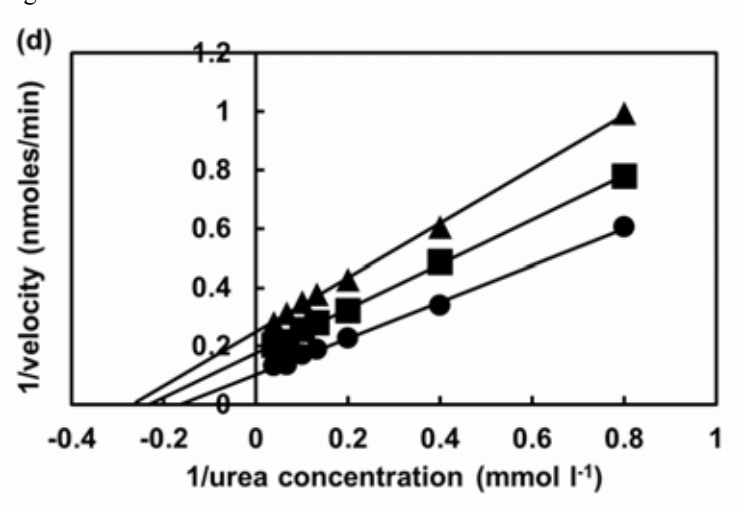

Figure 1. Inhibition of urease activity in a soluble extract of *Staphylococcus saprophyticus* by boric acid and sodium fluoride (NaF). (a) Effect of increasing concentrations of boric acid on urease activity. (b) Lineweaver-Burke plot of urease activity as a function of urea concentration in the presence of 0 boric acid (●), 0.5 mmol l^{-1} boric acid (■), or 1.0 mmol l^{-1} boric acid (▲). (c) Effect of increasing concentrations of sodium fluoride on urease activity. In one set of reactions, the extract was added simultaneously to urea and NaF (●). In a second set of reactions, the extract was treated with NaF for 30 min at 37 ^{o}C before addition of urea (▲). (d) Lineweaver-Burke plot of urease activity as a function of urea concentration in the presence of 0 NaF (●), 0.25 mmol l^{-1} NaF (■), or 0.5 mmol l^{-1} NaF (▲). No preincubation with NaF was employed. Individual points shows the means of three replicate assays for ammonium formation which varied by <10%. The specific activity of the control reactions was 329 nmol min^{-1} (mg protein)$^{-1}$.

PMSF is a broadly active esterase inhibitor, poorly soluble in water, relatively unstable, and toxic to most organisms [37]. It is thus not likely to be useful for the treatment of urinary tract infections associated with the urease from *S. saprophyticus*. Dimethylglyoxime slowed the growth of the bacteria at concentrations greater than 3 mmol l^{-1} and showed only partial urease inhibitory activity. It also is not likely to be useful as a physiological treatment for UTIs.

Dimethylsulfoxide (DSMO), a solvent that is structurally similar to urea and acts kinetically as a noncompetitive urease inhibitor, can effectively reduce the urease activity from *S. saprophyticus* in both cell extracts and whole cells [26]. The sensitivity of the urease in whole cells to inhibition by boric acid and sodium fluoride in combination with DMSO was determined (Figure 4(b)). DMSO at a concentration of 10% (v/v) was more inhibitory than 10 mmol l^{-1} boric acid or 10 mmol l^{-1} sodium fluoride alone. 10 mmol l^{-1} boric acid and 10 mmol l^{-1} sodium fluoride in combination with 10% (v/v) DMSO reduced the activity to less than 5% of the control level. The combination of all three inhibitors reduced activity to less than 3% of the control level.

Effect of chemical inhibitors on the behavior of *S. saprophyticus* in urea broth

Urease activity in *S. saprophyticus* and other bacteria is often detected in clinical laboratories using urea broth [39]. This is a phosphate-buffered medium containing 0.1 g l^{-1} yeast extract, 20 g l^{-1} urea (0.33 mole l^{-1}), and phenol red as pH indicator. The breakdown of urea to form of ammonium raises the pH and lead to formation of an intense pink color. To demonstrate the effects of boric acid, sodium fluoride, and DMSO on the growth of *S. saprophyticus* in urea broth, 10 mmol l^{-1} or 25 mmol l^{-1} boric acid, 10 mmol l^{-1} or 25 mmol l^{-1} sodium fluoride, and 10% (v/v) DMSO were added individually and in combinations to 3 ml portions of sterile urea broth. The cultures were inoculated with a 1/100 dilution of an overnight culture of *S. saprophyticus* and incubated at 37 ^{o}C for 24 hr and

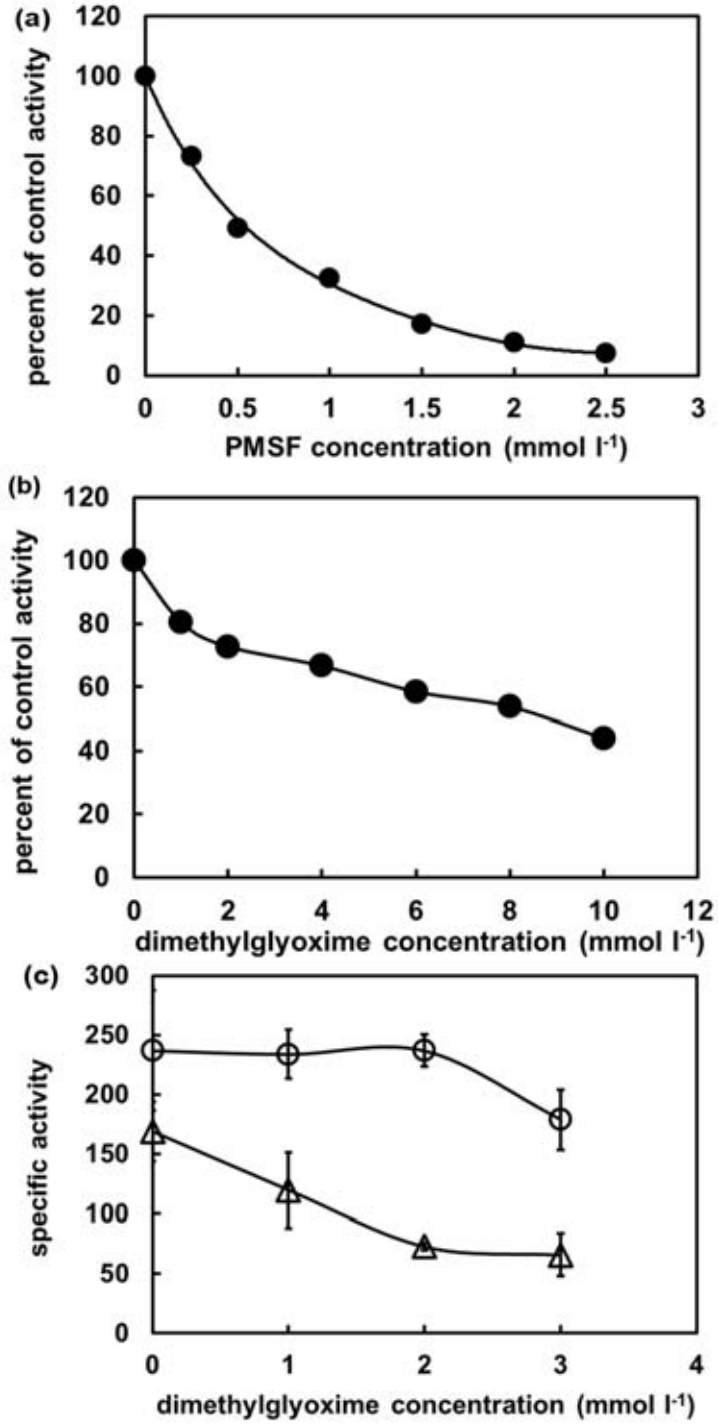

Figure 2. Inhibition urease activity in a soluble extract of *Staphylococcus saprophyticus* by phenylmethylsulfonyl fluoride (PMSF) and dimethylglyoxime. (a) Effect of increasing concentrations of PMSF on urease activity. (b) Effect of increasing concentrations of dimethylglyoxime on urease activity. Individual points shows the means of three replicate assays for ammonium formation which varied by <10%. The specific activity of the control reactions was 305 nmol min^{-1} (mg protein)$^{-1}$. (c) Specific activity in nmol min^{-1} (mg protein)$^{-1}$ of urease in whole cells of *S. saprophyticus* after growth to exponential phase in P medium (○) or artificial urine medium (△). The points show the average specific activities of two to four separate experiments ± one standard deviation.

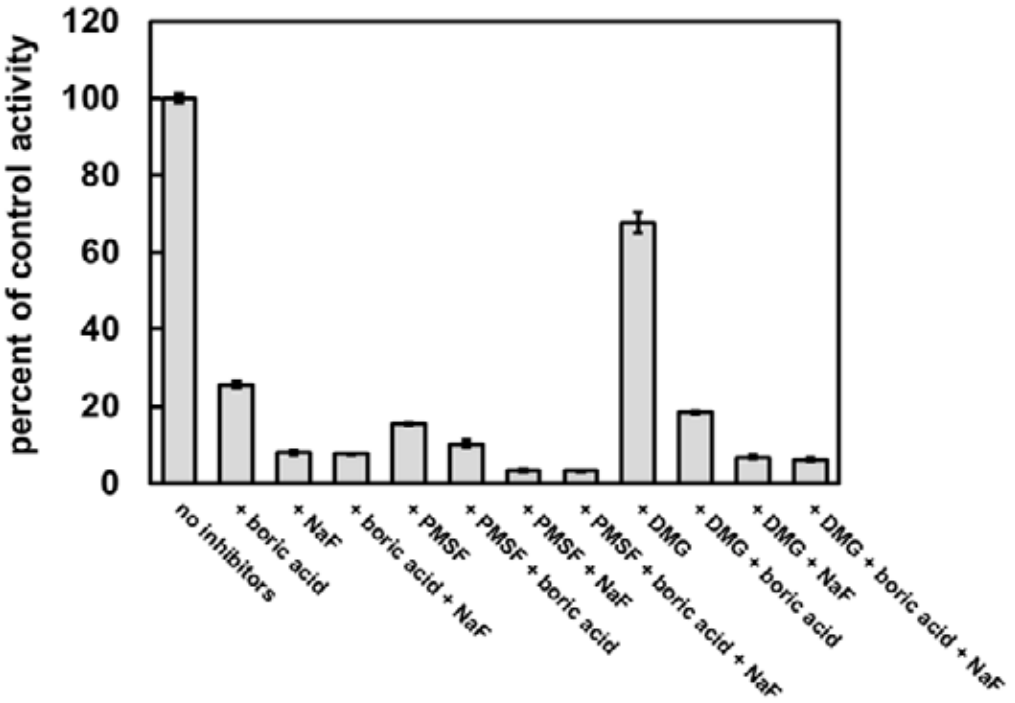

Figure 3. Reduction of urease activity in a soluble extract of *S. saprophyticus* by combinations of chemical inhibitors. A control reaction with a specific activity of 238 nmol min^{-1} (mg protein)$^{-1}$ was treated with 5 mmol l^{-1} boric acid, 5 mmol l^{-1} sodium fluoride, 2.5 mmol l^{-1} phenylmethylsulfonyl fluoride, and 10 mmol l^{-1} dimethylglyoxime individually and in different combinations. The bars show the mean $\pm$ one standard deviation of the three replicate assays in each case.

48 hr (Table 1). In the absence of any inhibitors, the medium changed from orange to pink in 24 hr. Addition of 10% (v/v) DMSO slowed the rate of color change but the culture did turn pink in 48 hr. Addition of 10 mmol l^{-1} boric acid slowed the rate of color change but had a more dramatic effect in the presence of 10% (v/v) DMSO; 25 mmol l^{-1} boric acid was a stronger inhibitor. Sodium fluoride at a concentration of 10 mmol l^{-1} or 25 mmol l^{-1} was a more effective inhibitor than boric acid and prevented any color change in both the presence and absence of 10% (v/v) DMSO.

Effect of chemical inhibitors on the growth and pH of cultures of *S. saprophyticus* in artificial urine

To quantitatively measure the effects of boric acid, sodium fluoride, and DMSO on the growth of *S. saprophyticus* and the increase in pH that occurs as a result of urease activity, the bacteria were grown in artificial urine medium and the turbidity and pH measured periodically. In artificial urine medium without added DMSO, 25 mmol l^{-1} boric acid caused an increase in the length of the lag phase following dilution of an overnight culture and a decrease in the growth rate (Figure 5(a)). Sodium fluoride (25 mmol l^{-1}) also increased the length of the lag phase but not as much as boric acid and the yield of cells in stationary phase was higher. The combination of 25 mmol l^{-1} boric acid and 25 mmol l^{-1} sodium fluoride gave a much longer lag phase and an even slower growth rate. The pH of the culture fluid followed the pattern of growth but eventually reached the same level of about 9.

In artificial urine medium containing 10% (v/v) DMSO, there was a further increase in the length of the lag phase, a decrease in the growth rate, and a delay in the pH increase (Figure 5(b)). In the presence of 10% (v/v) DMSO, 25 mmol l^{-1} boric acid, and 25 mmol l^{-1} sodium fluoride NaF, the lag phase lasted more than 24 hr. The bacteria eventually showed some growth, but the final pH was about 7.5 rather than 9. Microscope examination

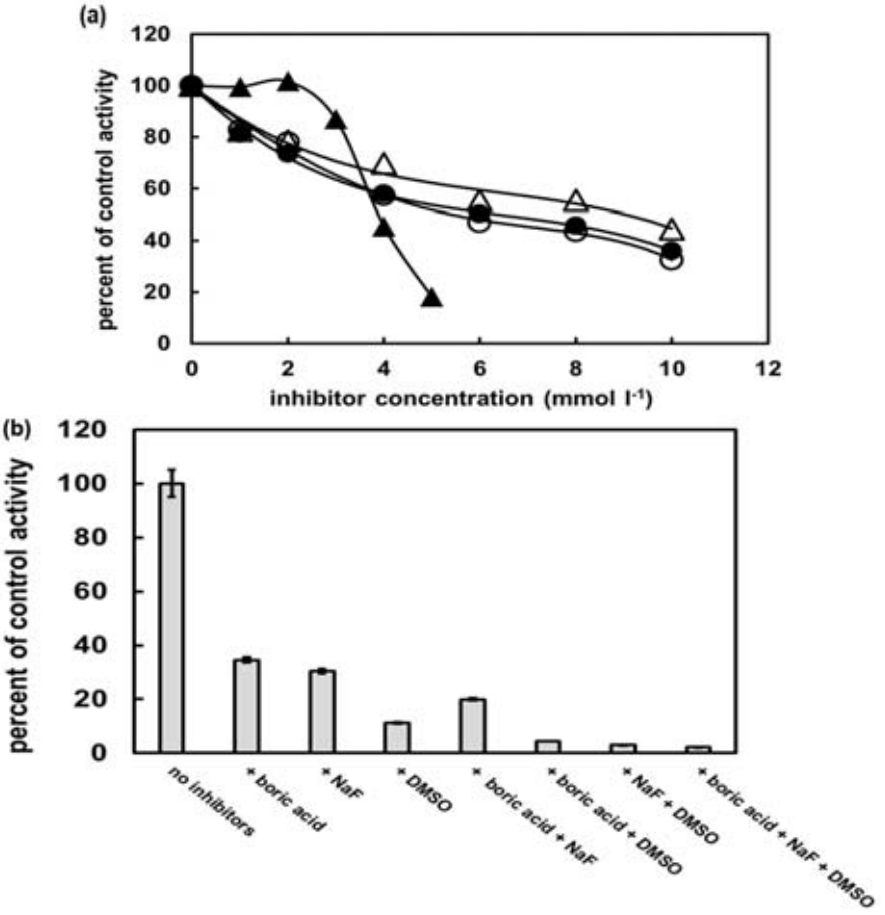

Figure 4. Inhibition of urease activity in exponential-phase whole cells of *Staphylococcus saprophyticus* grown in artificial urine medium. (a) Reduction of urease activity by increasing concentrations of boric acid (●), sodium fluoride (○), phenylmethylsulfonyl fluoride (▲), or dimethylglyoxime (△). The urease specific activities of the cell suspensions were 91.5 nmol min^{-1} (mg protein)$^{-1}$ for the boric acid reactions, 108.1 nmol min^{-1} (mg protein)$^{-1}$ for the sodium fluoride reactions, 113.6 nmol min^{-1} (mg protein)$^{-1}$ for the PMSF reactions, and 110.5 nmol min^{-1} (mg protein)$^{-1}$ for the dimethylglyoxime reactions. (b) Effects of combinations of inhibitors on urease activity in whole cells. The concentrations of the inhibitors in this experiment were 10 mmol l^{-1} for boric acid, 10 mmol l^{-1} for sodium fluoride, and 10% (v/v) for DMSO. The bars show the mean ± one standard deviation of the three replicate assays in each case. The specific activity of the control reaction was 113.3 nmol min^{-1} (mg protein)$^{-1}$.

of the cell suspensions after removal of the culture fluid for pH determinations indicated that large phase-bright rectangular crystals were present at the end of the growth period in the control cultures and the cultures treated with 25 mmol l^{-1} boric acid or 25 mmol l^{-1} sodium fluoride. 10% (v/v) DMSO alone had little effect on the number of crystals but there were no crystals in the cultures treated with DMSO, boric acid, and sodium fluoride.

Because DMSO, boric acid, and sodium fluoride might affect the activities of other enzymes or alter the overall metabolism of the bacteria, similar growth experiments were done with enriched P medium. In P medium without added DMSO, the presence of 25 mmol l^{-1} boric acid resulted in a somewhat longer lag phase, although it was not nearly as long as that seen in artificial urine medium (Figure 5(c)). Addition of 25 mmol l^{-1} sodium fluoride had little effect and the combination of boric acid and NaF was similar to boric acid alone. In P medium containing 10% (v/v) DMSO, there was a longer lag phase and a slower rate of growth but final yield of cells was the same (Figure 5(d)). The lag phase in the presence of 25 mmol l^{-1} boric

Table 1. Effect of chemical inhibitors on *S. saprophyticus* in urea broth.

Additions to urea broth	Color after 24 hr	Color after 48 hr
none	pink	pink
10 mmol l^{-1} boric acid	light pink	pink
10 mmol l^{-1} NaF	orange pink	pale pink
10 mmol l^{-1} boric acid + 10 mol l^{-1} NaF	orange	orange
25 mmol l^{-1} boric acid	orange pink	pink
25 mmol l^{-1} NaF	orange	orange
25 mmol l^{-1} boric acid + 25 mmol l^{-1} NaF	orange	orange
10% DMSO	pale pink	pink
10% DMSO + 10 mmol l^{-1} boric acid	orange pink	pale pink
10% DMSO + 10 mmol l^{-1} NaF	orange	orange
10% DMSO + 10 mmol l^{-1} boric acid + 10 mol l^{-1} NaF	orange	orange
10% DMSO + 25 mmol l^{-1} boric acid	orange pink	orange pink
10% DMSO + 25 mmol l^{-1} NaF	orange	orange
10% DMSO + 25 mmol l^{-1} boric acid + 25 mmol l^{-1} NaF	orange	orange

acid was again somewhat extended. 25 mmol l^{-1} NaF again had only a small effect and the growth curve in the presence of both compounds was similar to that in DMSO with boric acid.

Effect of chemical inhibitors on growth of *S. saprophyticus* in human urine

To determine if these active-site inhibitors might affect *S. saprophyticus* in human urine, small cultures of sterilized human urine were treated with 25 mmol l^{-1} boric acid, 25 mmol l^{-1} sodium fluoride, and 10% (v/v) DMSO individually and in combination for 48 hr (Table 2). 25 mmol l^{-1} boric acid alone caused a small reduction in the final turbidity (A_{600}) and pH. 25 mmol l^{-1} sodium fluoride had a more dramatic effect on the final turbidity but the final pH was similar to the control. In the presence of both compounds, both the final turbidity and the final pH decreased. 10% (v/v) DMSO alone reduced growth to about 2/3 of the control level but had only a minor effect of pH. In this case, 25 mmol l^{-1} boric acid had little effect but 25 mmol l^{-1} NaF reduced both the final turbidity and pH. The combination of 25 mmol l^{-1} boric acid, 25 mmol l^{-1} sodium fluoride, and 10% (v/v) DMSO almost completely inhibited growth and the corresponding increase in pH.

DISCUSSION

These experiments showed that the urease from *S. saprophyticus* could be inhibited competitively by boric acid and uncompetitively by sodium fluoride. The enzyme could also be inhibited by phenylmethylsulfonyl fluoride, which reacts with serine and threonine residues at active sites, and by

dimethylglyoxime, which binds to Ni^{2+} ions. Boric acid (10 mmol l^{-1}) and sodium fluoride (10 mmol l^{-1}), in combination with 10% (v/v) dimethylsulfoxide (DMSO), almost completely inhibited the urease activity in extracts and whole bacterial cells. These experiments extend the results of a previous project in which it was demonstrated that 10% (v/v) DMSO, a noncompetitive inhibitor

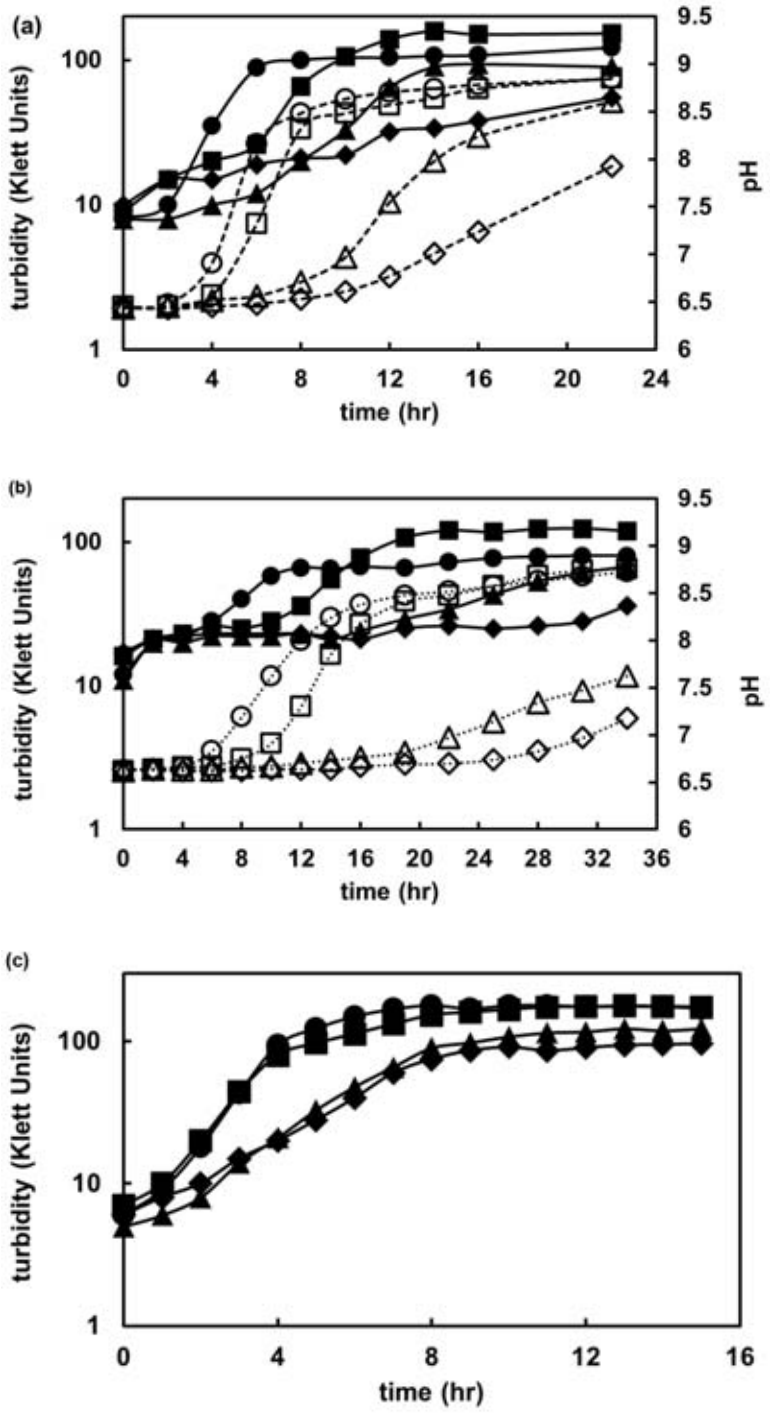

Figure 5

Figure 5 continued..

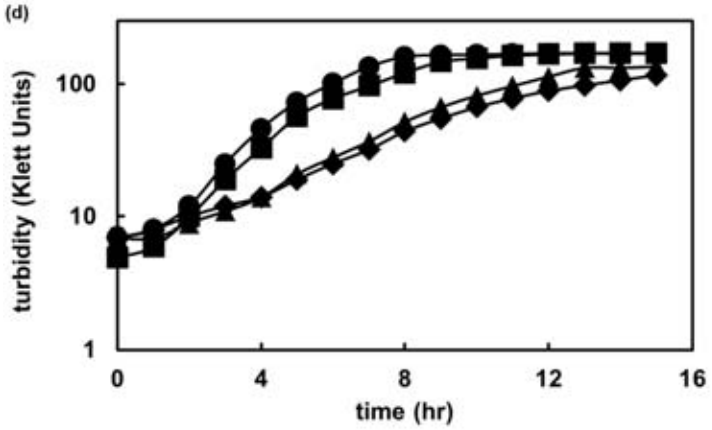

Figure 5. Effects of boric acid, sodium fluoride, and dimethylsulfoxide on the growth of *S. saprophyticus*. (a) Turbidity (filled symbols) and pH (open symbols) during the growth of *S. saprophyticus* in artificial urine medium without DMSO. No additional compounds (●, ○), 25 mmol l^{-1} boric acid (▲, △), 25 mmol l^{-1} NaF (■, □), or 25 mmol l^{-1} boric acid and 25 mmol l^{-1} NaF (◆, ◇) were added. (b) Turbidity (filled symbols) and pH (open symbols) during the growth of *S. saprophyticus* in artificial urine medium containing 10% (v/v) DMSO. No additional compounds (●, ○), 25 mmol l^{-1} boric acid (▲, △), 25 mmol l^{-1} NaF (■, □), or 25 mmol l^{-1} boric acid and 25 mmol l^{-1} NaF (◆, ◇) were added. (c) Turbidity during the growth of *S. saprophyticus* in P Medium without DMSO. No additional compounds (●), 25 mmol l^{-1} boric acid (▲), 25 mmol l^{-1} NaF (■) or 25 mmol l^{-1} boric acid and 25 mmol l^{-1} NaF (◆) were added. (d) Turbidity during the growth of *S. saprophyticus* in P medium containing 10% (v/v) DMSO. No additional compounds (●), 25 mmol l^{-1} boric acid (▲), 25 mmol l^{-1} NaF (■), or 25 mmol l^{-1} boric acid and 25 mmol l^{-1} NaF (◆) were added.

of the urease activity, slowed the growth of *S. saprophyticus* in LB medium or a defined *Staphylococcus* medium [26]. DMSO delayed but did not completely block the increase in pH in artificial urine medium. By combining 10% (v/v) DMSO with 25 mmol l^{-1} boric acid and 25 mmol l^{-1} sodium fluoride, it was possible to completely inhibit the color change that normally occurs in urea broth. It was also possible to reduce the growth of the bacteria in artificial urine medium and human urine and to prevent the increase in pH. With all three compounds, there was no precipitation of the salts in the medium as crystals. The combination of boric acid, sodium fluoride, and DMSO also caused a smaller increase in the lag phase and a decrease in the growth rate of *S. saprophyticus* in enriched P medium. Consequently, the long lag phase, reduced growth, and decrease in the corresponding pH seen in artificial culture medium and human urine reflects both the specific inhibition of urease activity and general metabolic effects. Formation of urease in *S. saprophyticus* is known to be consitutive [14] and control experiments indicated that the presence of DMSO, boric acid, or sodium fluoride in the culture medium did not reduce the urease specific activity in a major way.

A large number of chemicals including substrate analogues, hydroxamates, phosphoramides, polyphenolic compounds, and imidazoles can inhibit urease activity *in vitro* or in whole bacterial cells [40-42]. While some can prevent the change in pH associated with urease activity and decrease salt crystallization, but only acetohydroxamic acid has had limited clinical application [43]. These chemical inhibitors have usually been tested individually. The experiments described here are novel in that they show a synergistic effect of a combination of active-site inhibitors.

Instillation of 50% DMSO into the bladder has been used as a treatment for interstitial cystitis/bladder pain syndrome (IC/BPS, 23-25). DMSO has complex physiological effects on mammalian physiological systems [44-46], and while it is commercially available in the United States without a prescription, it is often used by patients

Table 2. Effect of chemical inhibitors on growth and pH of *S. saprophyticus* in human urine[a].

Additions to human urine	A(600) after 48 hr	pH after 48 hr
uninoculated urine	0	5.81 ± 0.08
none	0.926 ± 0.256	8.63 ± 0.10
25 mmol l^{-1} boric acid	0.737 ± 0.154	8.29 ± 0.12
25 mmol l^{-1} NaF	0.496 ± 0.100	8.49 ± 0.18
25 mmol l^{-1} boric acid + 25 mmol l^{-1} NaF	0.283 ± 0.160	6.60 ± 0.35
10% DMSO	0.669 ± 0.175	8.29 ± 0.10
10% DMSO + 25 mmol l^{-1} boric acid	0.674 ± 0.070	7.93 ± 0.14
10% DMSO + 25 mmol l^{-1} NaF	0.157 ± 0.085	6.50 ± 0.58
10% DMSO + 25 mmol l^{-1} boric acid + 25 mmol l^{-1} NaF	0.118 ± 0.040	6.12 ± 0.28

[a]Data show the means and standard deviations of five independent cultures.

without medical supervision. The addition of boric acid and sodium fluoride to DMSO may make it possible to reduce the concentrations used for physiological treatments to 10%. Another potential use of the boric acid/sodium fluoride/DMSO mixture may be as a "washout solution" for bladders or urinary catheters in patients in nursing homes and other facilities [47-49]. Catheters implanted for long periods of time tend to become clogged or encrusted, leading to persistent UTIs. Several types of catheter maintenance solutions have been used to improve patient outcomes, including physiological saline, mandelic acid or citric acid solutions, and cortisol solutions. Despite a relatively large number of studies, there is still no convincing evidence favoring a particular washout procedure. The next phase of this project might be to test the boric acid/sodium fluoride/DMSO solution in a laboratory bladder/catheter model [50] or in a mouse or similar animal model system [51] in which a urinary tract infection is created by introduction of *S. saprophyticus* or another urease-positive urinary tract pathogen.

CONCLUSIONS

The combination of 10% (v/v) DMSO, 25 mmol l^{-1} boric acid, and 25 mmol l^{-1} sodium fluoride can almost completely inhibit the urease activity in intact cells of *S. saprophyticus*. This can reduce growth in urine and media containing urea and prevent the increase in pH that results from urease activity. The inhibition is greater than that seen with DMSO alone or with chemicals such as acetohydroxamic acid, flurofamide, and phenolic compounds, and thus may have possible clinical applications.

CONFLICT OF INTEREST STATEMENT

The author declares there are no conflicts of interest.

REFERENCES

1. Foxman, B. 2014, Infect. Dis. Clin. North Am., 28(1), 1.
2. Geerlings, S. E. 2016, Microbiol. Spectr., 4(5), UTI-0002-2012.
3. Pietrucha-Dilanchian, P. and Hooton, T. M. 2016, Microbiol. Spectr., 4(6), UTI-0021-2015.
4. Flores-Mireles, A. L., Walker, J. N. Caparon, M. and Hultgren, S. J. 2015, Nat. Rev. Microbiol., 13(5), 269.
5. Wagenlehner, F. M., Wullt, B. and Perletti, G. 2011, Int. J. Antimicrob. Agents., 38 Suppl, 3.

6. Schito, G. C., Naber, K. G., Botto, H., Palou, J., Mazzei, T., Gualco, L. and Marchese, A. 2009, Int. J. Antimicrob. Agents., 34(5), 407.
7. Guglietta, A. 2017, Future Microbiol., 12, 239.
8. Latham, R. H., Running, K. and Stamm, W. E. 1983, JAMA., 250(22), 3063.
9. Hedman, P. and Ringertz, O. 1991, J. Infect., 23(2), 145.
10. Raz, R., Colodner, R. and Kunin, C. M. 2005, Clin. Infect. Dis., 40(6), 896 .
11. Eriksson, A., Giske, C. G. and Ternhag, A. 2013, APMIS, 121(1), 72.
12. Lo, D. S., Shieh, H. H., Barreira, E. R., Ragazzi, S. L. and Gilio, A. E. 2015, Pediatr. Infect. Dis. J., 34(9), 1023.
13. Mobley, H. L. T. and Hausinger, R. P. 1989, Microbiol. Rev., 53, 85.
14. Gatermann, S., John, J. and Marre, R. 1989, Infect. Immun., 57(9), 110.
15. Griffith, D. P., Musher, D. M. and Itin, C. 1976, Invest. Urol., 13(5), 346.
16. Hedelin, H. 2002, Int. J. Antimicrob. Agents., 19(6), 484.
17. Miano, R., Germani, S. and Vespasian, G. 2007, Urol. Int., 79(Suppl. 1), 32.
18. Kappaun, K., Piovesan, A. R., Carlini, C. R. and Ligabue-Braun, R. 2018, J. Adv. Res., 13, 3.
19. Schäfer, U. K. and Kaltwasser, H. 1994, Arch. Microbiol., 161(5), 393.
20. Jose, J., Schäfer, U. K. and Kaltwasser, H. 1994, Arch. Microbiol., 161(5), 384.
21. Loes, A. N., Ruyle, L., Arvizu, M., Gresko, K. E., Wilson, A. L. and Deutch, C. E. 2014, Lett. Appl. Microbiol., 58(1), 31.
22. Deutch, C. E. 2017, J. Appl. Microbiol., 122(5), 1380.
23. Rawls, W. F., Cox, L. and Rovner, E. S. 2017, Neurourol. Urodyn., 36(7), 1677.
24. Iyer, S., Lotsof, E., Zhou, Y., Tran, A., Botros, C., Sand, P., Goldberg, R., Tomezsko, J., Gafni-Kane, A. and Botros, S. 2017, Int. Urogyneco. J., 28(9), 1335.
25. Lim, V. N., Dwyer, P., Murray, C., Karmakar, D., Rosamilia, A. and Thomas, E. 2017, Int. Urogynechol. J., 28, 1085.
26. Deutch, C. E. 2020, J. Appl. Microbiol., 128(5), 1514.
27. Minuth, J. N., Musher, D. M. and Thorsteinsson, S. B. 1976, J. Infect. Dis., 133(1), 14.
28. Weatherburn, M. W. 1967, Anal. Chem., 39, 971.
29. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. 1985, Anal. Biochem., 150(1), 76.
30. Breitenbach, J. M. and Hausinger, R. P. 1988, Biochem. J., 250(3), 917.
31. Todd, M. J. and Hausinger, R. P. 1989, J. Biol. Chem., 264(27), 15835.
32. Tan, L., Li, C., Chen, H., Mo, Z, Zhou, J., Liu, Y., Ma, Z., Xu, Y., Yang, X., Xie, J. and Su, Z. 2017, Eur. J. Pharm. Sci., 110, 77.
33. Todd, M. J. and Hausinger, R. P. 2000, Biochemistry, 39(18), 5389.
34. Barboza-Silva, E., Castro, A. C. D. and Marquis, R. E. 2005, Oral Microbiol Immunol., 20(6), 323.
35. Benini, S., Cianci, M., Mazzei, L. and Ciurli, S. 2014, J. Biol. Inorg. Chem., 19, 1243.
36. Gold, A. M. and Fahrney, D. 1964, Biochemistry, 3, 783.
37. Sigma-Aldrich. Product information sheet for phenylmethylsulfonyl fluoride (P7626). https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product Information Sheet/1/p7626pis.pdf.
38. Benoit, S. L., Schmalstig, A. A., Glushka, J., Maier, S. E., Edison, A. S. and Maier, R. J. 2019, Sci. Rep., 9(1), 13851.
39. Stuart, C. A., Van Stratum, E. and Rustigian, R. 1945, J. Bacteriol., 49(5), 437.
40. Amtul, Z., Rahman, A. U., Siddiqui, R. A. and Choudhary, M. I. 2002, Curr. Med. Chem., 9(14), 1323.
41. Kosikowska, P. and Berlicki, L. 2011, Expert Opin. Ther. Pat., 21(6), 945.
42. Rego, Y. F., Queiroz, M. P., Brito, T. O., Carvalho, P. G., de Queiroz, V. T., de Fátima, A. and Macedo Jr., F. 2018, J. Adv. Res., 13, 69.

43. Williams, J. J., Rodman, J. S. and Peterson, C. M. 1984, New Eng. J. Med., 311(12), 760.
44. Swanson, B. N. 1985, Rev. Clin. Basic Pharm., 5(1-2), 1.
45. Jacob, S. W. and Herschler, R. 1986, Cryobiology, 23(1), 14.
46. Santos, N. C., Figueira-Coelho, J., Martins-Silva, J. and Saldanha, C. 2003, Biochem. Pharmacol., 65(7), 1035.
47. Getliffe, K. A. 1996, J. Adv. Nurs., 23(3), 548.
48. Moore, K. N., Hunter, K. F., McGinnis, R., Bacsu, C., Fader, M., Gray, M., Getliffe, K., Chobanuk, J., Puttagunta, L. and Voaklander, D. C. 2009, J. Wound Ostomy Continence Nurs., 36(1), 82.
49. Shepherd, A. J., Macay, W. G. and Hagen, S. 2017, Cochrane Database Syst. Rev., 3(3), CD004012.
50. King, J. B and Stickler, D. J. 1991, J. Hosp. Infect., 18(3), 179.
51. Hung, C. S., Dodson, K. W. and Hultgren S. J. 2009, Nat. Protoc., 4(8), 1230.