Nitazoxanide Inhibits Biofilm Formation by *Staphylococcus epidermidis* by Blocking Accumulation on Surfaces

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ABSTRACT

Coagulase negative species of *Staphylococcus* are often associated with opportunistic hospital acquired infections that arise from colonization of indwelling catheters. Here we show that the anti-parasitic drug nitazoxanide (NTZ) and its active metabolite tizoxanide (TIZ) are inhibitory to growth of *Staphylococcus epidermidis* and other staphylococci, including methicillin resistant *Staphylococcus aureus* strains, under aerobic and microaerobic conditions (MIC 8-16 µg/ml). At sub-MIC levels, NTZ and TIZ also inhibited biofilm production under static conditions by strains of *S. epidermidis* and *Staphylococcus haemolyticus* with an IC$_{50}$ of ~2.5 µg/ml (8 µM). The 5-nitro group was required for biological activity and a hydrophilic derivative of NTZ (AMIX) also inhibited biofilm formation. NTZ was not dispersive of existing biofilm, but did block further accumulation. Sub-MIC concentrations of NTZ had no effect on primary attachment to surfaces at either 4 or 37$^\circ$C. The inhibitory action of NTZ and TIZ, but not vancomycin, on biofilm production could be reversed by the addition of zinc salts (2.5 - 40 µM) but not by other metals, suggesting NTZ might target the zinc-dependent accumulation associated protein Aap that mediates accumulation on surfaces. However, neither NTZ nor TIZ formed chelation complexes with zinc salts based on spectrophotometric and NMR analyses and adding excess zinc to NTZ-grown bacteria (apo-Aap) did not restore the accumulation phenotype. Our studies suggest that sub-MIC levels of NTZ may affect the assembly or function of cell structures associated with the biofilm phenotype.
INTRODUCTION

Coagulase-negative staphylococci (CoNS) have emerged as important opportunistic hospital acquired pathogens that are the leading cause of catheter and indwelling device-associated infections (27, 31, 38). The ability of CoNS, including the archetypal species *Staphylococcus epidermidis*, to cause disease depends on their ability to adhere to polymer surfaces where they form thick, multilayered, cellular agglomerations known as biofilm (31, 38).

The principal component of *S. epidermidis* biofilm is poly-β-1,6-N-acetyl-D-glucosamine (PNAG, also known as PIA for polysaccharide intercellular adhesion), synthesized by the products of the ica genes (7, 17, 18). Recently, the cell-surface expressed accumulation-associated protein Aap has been shown to mediate PNAG-independent biofilm formation in some strains of *S. epidermidis* (4, 30), suggesting that proteinaceous matrix components are important for biofilm formation by certain strains. Biofilm contributes to persistence by limiting efficacy of antibiotics and host immune responses (2, 11, 29, 35). Bloodstream and urinary tract infections are ranked as 2nd and 3rd causes respectively of healthcare-associated deaths in the US in 2002 (35). More than 5 million central venous catheters are inserted annually in the U.S. and of the more than 200,000 healthcare-acquired bloodstream infections that occur annually, most are due to central venous catheter (14, 23, 35). These infections lead to increased morbidity, mortality, lengths of hospitalization, and total healthcare costs.

Many drugs and compounds have been tested as biofilm inhibitors and some are used to coat catheters (e.g. silver, minocycline, rifampin, platinum, nitrofurantoin, chlorhexidine, and sulfadiazine) (1, 10, 13, 20). Several randomized trials have shown benefits of using antibiotic(s)-impregnated catheters in hospitalized patients to reduce colonization and catheter-related bloodstream infections (CRBSI) and include: chlorhexidine-silver sulfadiazine...
impregnated catheters compared with non impregnated catheters; rifampin-minocycline coated catheters compared with non-coated catheters; and rifampin-minocycline impregnated catheters compared with chlorhexidine-silver sulfadiazine catheters (1, 20).

Nitazoxanide (NTZ) is a 5-nitrothiazole therapeutic (see Figure 1) that is used to treat a wide variety of parasitic and anaerobic bacterial infections (6) and is FDA approved for treatment of Cryptosporidium parvum and Giardia intestinalis infections in adults and children (8). The drug also shows efficacy against Clostridium difficile infections (22, 26). Mechanistic studies have shown that NTZ is a potent inhibitor of pyruvate: ferredoxin oxidoreductase (PFOR) (9) and therefore is active against all organisms (anaerobic bacteria and parasites) expressing this enzyme (28). Mechanistic studies revealed that the anionic form of the drug is biologically active and a proton abstraction mechanism has been proposed (9). Such a generic mechanism might account for the wide range of biological targets reported for this drug (9, 24, 25, 33).

One of the original communications on the spectrum of action of NTZ indicated that the drug was only active against Staphylococcus aureus under anaerobic conditions and the active metabolite tizoxanide (TIZ) of NTZ was not active against staphylococci (6). In the present study we explored the inhibitory nature of these inhibitors against strains of S. aureus and S. epidermidis. Our studies show that NTZ as well as TIZ are inhibitory to aerobic growth of staphylococcal species including MRSA strains of S. aureus (MIC 8-16 µg/ml). At sub-MIC levels NTZ blocks biofilm formation by S. epidermidis and this inhibition can be reversed in a dose dependent manner by zinc salts. While the function of accumulation associated protein (Aap) in biofilm formation is zinc dependent (4), our studies rule out simple chelation as a
mechanism of drug action. Rather, NTZ appears to affect assembly or function of surface
components associated with biofilm production.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in
Table 1. Unless otherwise specified, strains were grown in Trypticase soy broth or agar (TSB or
TSA, respectively) at 37°C with shaking for liquid cultures. All strains were stored at -80°C in
15% glycerol.

Identification of S. epidermidis isolates from catheter related bloodstream infections.
Infection-causing strains (ICS) of S. epidermidis were obtained from individuals with a long-
term intravenous catheter and a catheter-related bloodstream infection (CRBSI), defined by more
than one blood culture and a concomitant catheter tip culture with CoNS. Medical records for
these patients were reviewed to confirm that the patient exhibited clinical manifestations of a
systemic inflammatory response syndrome, the absence of another focus of infection, and
resolution of SIRS within 48 h of catheter removal.

Species identification was performed by one of two methods: either by using the API Staph
system (BioMerieux, Durham, NC), according to the manufacturer’s instructions, or by genetic
testing. The latter was performed by a combination of two methods: PCR identification of the S.
epidermidis specific gene serp0107 (16) and PCR restriction fragment length polymorphism
(RFLP) analysis of the Staphylococcus tuf gene (15). Genomic DNA was prepared by
suspending an individual bacterial colony in 20μl of lysis buffer containing 0.25% SDS and 0.05
N NaOH, incubating at 95°C for 5 min, and then adding 180μl of sterile dH2O. After
centrifugation for 1 min at 16,100 x g to sediment the debris, the supernatant was used as
template DNA for PCR. Samples were kept at -20°C for long-term storage. PCRs were carried out with a Mastercycler Gradient thermal cycler (Eppendorf, Westbury, NY). PCR amplification of *serp0107* was performed as previously described (16) using the primers listed in Table 1. The presence of a single 581-bp product on 1% agarose gel electrophoresis was consistent with a species identification of *S. epidermidis*. To confirm the identification, RFLP analysis of the *Staphylococcus tuf* gene PCR product was performed, using the method described by Kontos and colleagues (15). Briefly, PCR amplification of the *tuf* gene yielded a 370-bp amplicon that was confirmed by gel electrophoresis on a 2% agarose gel (15). The product was extracted using a QIAquick gel extraction kit (QIAGEN, Valencia, CA), according to the manufacturer’s instructions, and subjected to the BstZ17I, BseNI, and MseI restriction enzyme digestion. The resulting fragment(s) were resolved by electrophoresis through a 3% agarose gel containing ethidium bromide at 50V for 45 min and visualized under UV light. Digestion of the *tuf* gene amplicon of *S. epidermidis* produces two fragment using BstZ17I (243- and 127-bp), three fragment using BseNI (246-, 86-, and 38-bp), and no restriction using MseI (single 370-bp product). The presence of these fragments positively identified the strain as *S. epidermidis*. For both the *serp0107* amplification and *tuf* RFLP analysis, *S. epidermidis* strains ATCC 12228 and 9142 served as positive controls while *Staphylococcus haemolyticus* F16942, *S. aureus* 8325, and water alone served as negative controls. ICS *S. epidermidis* strains were identified when (a) the case met the clinical definition of a CRBSI, (b) concomitant blood and catheter culture isolates were both positively identified as *S. epidermidis*, and (c) the blood and catheter isolates had congruent antibiotic susceptibility patterns.

The study protocol was approved by the institutional review board of the University of Virginia Health System.
**Determination of MIC.** Minimal inhibitory concentration testing of NTZ was done in sterile round bottom 96 well microtiter polystyrene plates (Corning Inc., Corning, NY) by microdilution. Tizoxanide TIZ (deacetylated form of NTZ), denitro-TIZ (TIZ without 5-nitro group) and AMIX, a water soluble ethylamine derivative of NTZ (see Figure 1) were also tested. Bacteria were grown overnight and suspended in fresh TSB to an OD$_{600}$ of 0.01 and 100 µl were dispensed into wells with the first well containing 200 µl. NTZ and other antibiotics (DMSO control) were added to well one and serially diluted from 32 µg/ml. All compounds were tested in triplicate and plates were read visually or with a plate reader (Molecular Devices) at 8, 12 and at 24 h. MIC was determined as the first well in which no visible bacterial growth was noted relative to controls. The effects of accumulated biofilm was corrected for by transfer of 100 µl aliquots to another microplate. Drug effects on aerobic growth were determined in 125 ml flasks containing 25 ml of TSB medium and following inoculation (detailed above), flasks were shaken on a gyrorotory shaker at 200 rpms at 37°C. Final concentrations of NTZ were 0, 10 and 25 µg/ml and samples were removed at hourly intervals, diluted and turbidity was determined at 600 nm.

**Biofilm determinations.** Staphylococcal strains were cultured overnight in TSB at 37°C with shaking, diluted in fresh medium to an OD$_{600}$ of 0.01 and 100 µl was dispensed into sterile flat bottom polystyrene 96 well microtiter plates (Costar 3596, Corning Inc.). NTZ, TIZ, denitro-NTZ and AMIX were tested at different concentration (0, 5, 10, 15, 20 and 25 µg/ml) with DMSO as a control as described for MIC testing. Each compound and dilution was tested in triplicate. The plates were incubated overnight at 37°C in a humidified incubator, without shaking and both bacterial growth (turbidity) and biofilm accumulation were determined at 16 h.
Following recording of turbidities, the culture medium was aspirated and the wells were washed three times with distilled water, blotted on paper towels, and fixed with 75% ethanol for 10 minutes. To visualize biofilm material, 0.5% crystal violet was added to each well and after 5 minutes was removed and the wells washed three times with distilled water. Plates were read using a microplate reader at 570 nm (Molecular Devices). The dye was then extracted by adding 200 µl of 95% ethanol, and the absorbance was read again at 570 nm. All assays were performed in triplicate and the mean and standard deviation determined. Generally, the ethanol solubilized crystal violet was more reliable and these determinations are reported throughout.

**Catheter adherence model.** An overnight culture of *S. epidermidis* 9142 was used to inoculate fresh TSB medium to a final OD$_{600}$ of 0.01 in a volume of 3 ml in 15 ml screw capped tubes. Sterile sheets of polyurethane were divided into 1 cm square pieces and placed into tubes containing different concentrations of NTZ or controls containing DMSO. The tubes were incubated for 24 h with shaking. Each catheter portion was washed 3 times with sterile PBS, suspended in 4 ml sterile PBS and sonicated for 10 min in a sonic water bath. 10µl of the sonicated fluid was diluted in fresh PBS and plated onto TSA plates and incubated at 37°C overnight. Bacteria were enumerated (triplicate plates) and the mean and standard deviation were determined and reported as CFU/ml. Viability effects were corrected for by plating the supernatants from the 3 ml experiment for total bacterial counts.

**NTZ effect on biofilm dispersal.** The effect of NTZ on biofilm accumulation or dispersal of existing biofilms was determined in microtiter dishes in which *S. epidermidis* 9142 was allowed to grow for 8 h prior to drug treatment. In this experiment duplicate flat bottom plates were inoculated with 200 µl of a bacterial suspension diluted in fresh TSB to an OD$_{600}$ of 0.01 and incubated statically at 37°C. Three rows of each plate contained no drug, while subsequent
rows contained NTZ (2.5, 5, 10, and 15 µg/ml). At 8 h, one plate was developed for biofilm determination as described above, while the medium in the replicate plate was aspirated and replaced with fresh TSB, with the first three rows now containing NTZ at 5, 10 and 15 µg/ml and for the remaining wells, the same concentration of NTZ. These plates were developed at 24 h and the relative amount of biofilm for each set compared.

**Direct attachment assay.** To determine if NTZ directly inhibited attachment of bacteria to polystyrene, *S. epidermidis* strain 9142 was grown overnight in TSB medium with shaking in 125 ml flasks in the presence or absence of 15 µg/ml NTZ. In some experiments, overnight growth was diluted to an OD$_{600}$ of 0.1 in fresh TSB medium and grown exponentially (shaking or static and in the presence or absence of 15 µg/ml NTZ) for 3 h. In each condition, bacteria were harvested by centrifugation and suspended in PBS to an OD$_{600}$ of 0.01 and 200 µl was added to 6-well polystyrene microtiter plates (Nulcon, Nunc AIS. Denmark). After one hour incubation at 4 and 37°C, plates were washed with PBS (5x 5 ml) to remove non-adherent bacteria and the adherent bacteria were visualized microscopically (directly or following staining with crystal violet) with a Zeiss Axiovert 200 inverted microscope (Carl Zeiss Inc., Thornwood, NY). The number of adherent bacteria per field (multiple fields) was video recorded and analyzed using Image Pro Plus software (Media Cybernetics, Bethesda, MD). The means and SD were computed from at least 3 wells and 3 multiple fields and analyzed statistically (t-test).

**Effect of zinc on the action of NTZ.** To assess whether ZnSO$_4$ or ZnCl$_2$ could overcome the inhibitory action of NTZ on biofilm production, standard biofilm assays were established at an NTZ concentration of 12.5 µg/ml, which is sufficient to abolish biofilm formation by *S. epidermidis* strain 9142 (~ 2x IC$_{50}$). A 40 µM ZnSO$_4$ solution was serially diluted in wells containing NTZ (in triplicate) and biofilm production was assessed at 24 h with crystal violet. As
a control, ZnSO₄ was added to untreated bacteria. Additional controls included testing of 40 µM CaCl₂ and MgCl₂ in the same format. The inhibitory effect of EDTA on biofilm production and its reversibility by Zn²⁺ was also assessed as described for NTZ. The effect of Zn²⁺ on MIC of NTZ and AMIX was evaluated by microdilution in TSB at a fixed concentration of ZnCl₂ at 20 µM (in triplicate). Turbidities were measured at 8 and 24 h and mean and standard deviation reported.

**Spectrophotometric chelation assay.** To test direct binding of Zn²⁺ (Cl₂⁻ or SO₄²⁻) by NTZ in solution, we tested a range of concentrations of both NTZ and TIZ and scanned the solutions spectrophotometrically using an OLIS Cary-14 (OLIS Instruments Co., Bogart, Georgia). Absolute spectra were recorded over a UV/Vis range from 220 nm to 700 nm. Spectral shifts in the 418 nm range would be attributable to changes in resonance within the thiazole ring that would result if Zn²⁺ were coordinated by NTZ (9).

**¹H NMR.** Chelation experiments were also performed utilizing NTZ and TIZ in DMSO-d₆ (0.5 mL) with ZnCl₂ using a 300 MHz Varian MercuryPlus spectrometer. Specifically, three ratios of NTZ to Zn²⁺ were investigated (1:1, 1:2, 2:1) where NTZ was held constant (4 mg, 13 µmoles) while varying ZnCl₂ (1.8 mg, 13 µmoles or 3.5 mg, 26 µmoles) or NTZ (8 mg, 26 µmoles) with ZnCl₂ (1.8 mg, 13 µmoles). Three ratios of TIZ to Zn²⁺ were also investigated (1:1, 1:2, 2:1) where TIZ was held constant (4 mg, 15 µmoles) while varying ZnCl₂ (2.1 mg, 15 µmoles or 4.1 mg, 30 µmoles) or TIZ (8 mg, 30 µmoles) with ZnCl₂ (2.1 mg, 15 µmoles). ¹H NMR spectra were then obtained after mixing for 5 min, 24 h, 48 h and 72 h at room temperature. Any chelation event would be evidenced by shifting or broadening of the thiazole and benzene protons as well as loss of the amide proton.
RESULTS

MIC testing of *S. aureus* and *S. epidermidis* strains. The inhibitory effect of NTZ on staphylococcal strains was investigated first by determining MIC. Our studies showed that most strains tested were susceptible to NTZ and TIZ with an MIC of 8-16 µg/ml (see Figure 2A), indicating these strains are generally more susceptible to this drug than previously reported (6). In contrast to previous studies indicating the drug was active under anaerobic conditions (6), we found NTZ and the active metabolite TIZ inhibited the growth of *S. epidermidis* in liquid culture under vigorous aeration in a dose dependent manner with significant inhibition at 25 µg/ml (see Figure 2B). At lower drug concentrations (10 µg/ml), there was little effect on exponential growth rates. Under static (microaerobic) growth conditions in liquid culture, biofilm accumulation on the bottom of flasks was completely ablated by 10 µg/ml of NTZ, while final bacterial turbidities were only slightly decreased when compared with drug-free controls (data not presented). These observations suggest that sub inhibitory concentrations of NTZ may also affect the production of biofilm.

**NTZ inhibits biofilm production at sub-MIC levels.** To further explore the sub-MIC drug effects on biofilm accumulation, we screened several CoNS species for biofilm production. As seen in Figure 2C, NTZ inhibited biofilm production by *S. epidermidis* strain 9142 and *S. haemolyticus* strain 33208 in a dose dependent manner as determined by crystal violet staining. Biofilm inhibition was not due to growth inhibition (see Fig. 2A), although bacterial growth was decreased at 8 and 16 µg/ml drug concentrations. The IC$_{50}$ was determined to be 1-3 µg/ml (~ 3-10 µM). Two additional strains tested (Fig. 2C) were non-biofilm producing strains. While not depicted, the deacetylated form of NTZ (TIZ) gave equivalent results while the denitro form of the drug did not inhibit biofilm production and exhibited an MIC >32 µg/ml (data not presented).
Screening NTZ activity against *S. epidermidis* strains. To determine if the sub-MIC antibiofilm activity of NTZ was more generalized, we screened a collection of clinical *S. epidermidis* isolates obtained from patients with CRBSI (see Table 1 for details). As seen in Figure 3A, NTZ inhibited biofilm formation by all clinical isolates that produced biofilm in the absence of the drug in a dose dependent manner. Furthermore, NTZ inhibited biofilm formation by the *S. epidermidis* strain 5179-R1 (see Fig 3B), which generates a PNAG-independent, protein-rich biofilm that is dependent on the production of a proteolytically processed derivative of the Aap protein (30). For all strains tested, the IC$_{50}$ ranged between 1 and 5 µg/ml and the MIC was 16 µg/ml.

NTZ inhibits bacterial accumulation on catheters. Several studies have indicated that bacterial attachment and biofilm accumulation are distinct but interrelated activities (7). To test whether NTZ interfered with attachment, biofilm producing strains of *S. epidermidis* were scored for attachment to plastic squares, derived from catheter material, in the presence or absence of NTZ. As seen in Figure 4, NTZ inhibited attachment of *S. epidermidis* strain 9142 in a dose dependent manner and within the IC$_{50}$ concentration range. Since the attachment to catheter pieces was conducted over a 24 h period under growth permissive conditions, it is not possible to distinguish between primary attachment and attachment and subsequent accumulation.

NTZ does not inhibit primary attachment to plastic. To address primary attachment to plastic more directly, bacteria collected following overnight culture in TSB medium and either tested directly or following three hour outgrowth under shaking or static conditions. Adherent bacteria were enumerated after 1 h incubation at 4° and 37°C. Since temperature was not a variable, the results depicted in Table 2 are at 37°C. Bacterial attachment was not appreciably affected by the presence of NTZ during primary attachment (P = 0.157). Similarly, inclusion of
15 μg/ml NTZ during out growth for 3 h under static or shaken conditions did not significantly alter attachment efficiency. While there was a slight effect by NTZ under static conditions, the result was not considered significant, as static growth conditions or slight NTZ effect might have contributed to the count. Since zinc contributes to improved accumulation via Aap proteins, we tested whether 10 μM zinc sulfate or zinc chloride might improve attachment of bacteria in the presence of NTZ. Zinc salts (sulfate shown) had no effect on attachment (P = 0.49). Generally, zinc add-back to putative apo-Aap protein should result in bacterial clumping which was not observed in these experiments. These studies indicate that NTZ does not interfere with primary attachment which appears to be independent of mechanisms associated with biofilm formation.

Does NTZ affect biofilm production by colonized bacteria? We next investigated whether NTZ was inhibitory to biofilm production by established *S. epidermidis* communities. If NTZ is a specific inhibitor of accumulation, the drug should inhibit further deposition of biofilm in established communities. To test this hypothesis, we allowed *S. epidermidis* bacteria to establish biofilm in the absence of drug for 4 h, and following washings to remove non-adherent bacteria, NTZ (range of concentrations) was added in fresh TSB medium for an additional 12 h. As seen in Figure 5, the biofilm produced at 4 hr was ca 0.5 absorbance units by the crystal violet assay and by the end of the assay; the accumulated biofilm had increased to 3 absorbance units in the non-drug treated well. While biofilm also increased in the NTZ treated wells, there was a drug dependent effect on further accumulation of biofilm material. However, drug treatment did not eliminate the preformed biofilm indicating that NTZ was not dispersive.

Reversal of NTZ inhibition by zinc. Recent studies have shown that the surface proteins Aap of *S. epidermidis* and SasG of *S. aureus* promote intercellular adhesion of staphylococcal bacteria and that metal chelators like EDTA can inhibit biofilm production (4, 5, 13). Studies by
Conrady et al (4) have shown that Aap proteins contain G5 domain repeats that mediate associative accumulation of bacteria onto surfaces in a Zn^{2+}-dependent manner. These domains bind Zn^{2+}, which is hypothesized to enable Zn^{2+} zipper protein interactions to entwine protein ribbons in adherent bacterial aggregates on surfaces. To test whether NTZ affects the function of Aap proteins of *S. epidermidis*, we added increasing concentrations of ZnSO_{4} or ZnCl_{2} to the biofilm assays. As seen in Figure 6a, the addition of Zn^{2+} to the standard biofilm assay reversed the inhibitory action of NTZ in a dose dependent manner. While not depicted, EDTA also inhibited biofilm production and similar to NTZ, could be reversed by zinc (4, 13). The effects of Zn^{2+} were specific as addition of other metals (calcium and magnesium) did not reverse the inhibition (see Figure 6a). While NTZ seems to achieve the same phenotype as treatment with EDTA, we could not distinguish between NTZ acting directly as a Zn^{2+} chelator or if NTZ was bound to G5 domains.

**NTZ and TIZ do not chelate Zn^{2+}**. To address whether Zn^{2+} is directly chelated by NTZ (TIZ) and alters potency of the drug, we scanned putative NTZ/TIZ-Zn^{2+} coordination complexes for any spectral changes that might result from changes in ring resonance due to bound ligand. Based on NMR studies, the anionic form of NTZ exists in several resonance states that exist simultaneously for the amino thiazole moiety which contributes to the absorption maxima at 418 nm (9). Metal coordination would be predicted to alter resonance and this should manifest in a spectral shift. Spectral scans performed at different concentrations of NTZ and TIZ with Zn^{2+} did not reveal any changes in absorption spectrum (data not presented). To further explore NTZ metal interactions, putative NTZ and TIZ complexes with Zn^{2+} were analyzed by \textsuperscript{1}H NMR spectroscopy in DMSO-\textit{d6}. No chelation with ZnSO_{4} was evident from any ratios tested even after the 72 h time point with NTZ. All spectra remained identical and unchanged.
throughout the course of the experiment. Tizoxanide also displayed no chelation with ZnSO₄ under the experimental conditions. Taken together, these studies indicate that these drugs did not chelate zinc under these assay conditions.

**Effect of Zn²⁺ on bacterial growth.** To test the possibility that the action of Zn²⁺ on ablation of biofilm by NTZ might result from reversal of the growth inhibitory effect of the drug, bacterial turbidities at 16 h were determined at a fixed concentration of NTZ of 12.5 µg/ml. As seen in Figure 6b, Zn²⁺ enhances the growth of *S. epidermidis* strain 9142 in a dose dependent manner. MIC testing at a fixed concentration of Zn²⁺ (10 µM) showed a shift from 16 µg/ml to 32 µg/ml (data not presented). We also noted that Zn²⁺ supplementation of TSB medium improved bacterial growth as indicated by slightly higher final turbidities at 16 h (*A₆₀₀* of 0.65 for controls to 0.82 with Zn²⁺). To test whether enhanced growth might lead to breakthrough, we repeated MIC experiments over a shorter period. Zn²⁺ had no effect on the inhibitory action of NTZ up to 10 h, suggesting that zinc might affect other bacterial systems unrelated to biofilm production that promote breakthrough growth, thus contributing to biofilm production. It should be noted that similar studies with sub-MIC levels of vancomycin showed no effect by zinc (data not presented).

**Hydrophobicity effects.** NTZ is hydrophobic and sparingly soluble in water and its deacylated form (TIZ) is 97.5% bound to plasma proteins (34). It is possible therefore, that the action of the drug is nonspecific and results from binding onto surface proteins and ablating their action. To test this possibility we synthesized a more soluble derivative of NTZ (AMIX) by adding an ethylamine R group to the benzene ring of TIZ. The drug retained bioactivity with an MIC of 16-32 µg/ml. This derivative was soluble at 400 µg/ml in water (NTZ < 32 µg/ml) and as seen in Figure 7 exhibited a slightly higher IC₅₀ than NTZ (8 versus 4 µg/ml, respectively in this
experiment). The 2-fold difference in biofilm inhibition was less than the 15-fold difference in solubility. Taken together, these studies suggest that the action of NTZ in blocking accumulation and biofilm production is most likely specific.

**DISCUSSION**

We have determined that the antiparasitic drug nitazoxanide and its active metabolite tizoxanide are inhibitory to *in vitro* growth of various staphylococcal species, including MRSA strains of *S. aureus*. This contrasts with an earlier report that TIZ was not active against staphylococci and that the action of NTZ was limited to anaerobic growth conditions (6). In the current study we demonstrated that NTZ inhibited growth of *S. epidermidis* strain 9142 in highly aerated broth cultures at 25 µg/ml, but little inhibition was observed at concentrations at or below 10 µg/ml. We found NTZ to be slightly more active than TIZ by MIC tests which might be attributed to slight differences in solubility, while the de-nitro NTZ was biologically inert. Our studies further demonstrate that at sub-MIC concentrations (1 – 5 µg/ml), NTZ and TIZ inhibited production of biofilm in a static microtiter plate assay. Under static conditions in flasks, NTZ ablated biofilm production at 10 µg/ml with only a slight decrease in final bacterial turbidity and there was no discernable difference in primary attachment to plastic. In comparison with untreated bacteria, NTZ-treated bacteria did not clump and appeared more dispersed by microscopic examination. The anti-biofilm activity of NTZ could be reversed by zinc salts (IC$_{50}$ = 5 µM), but not by Ca$^{2+}$ or Mg$^{2+}$ and was growth dependent. The zinc effect seems to be unique to NTZ and TIZ as zinc had no effect on the sub-MIC action of vancomycin on growth or biofilm formation. The possibility that zinc inactivated NTZ or formed chelates could not be demonstrated by optical spectrophotometry or by NMR.
One possible candidate for the action of NTZ is the zinc-dependent associative adhesion protein (Aap) that has been extensively studied and recently shown to mediate intercellular adhesion of *S. epidermidis* on surfaces (4). In this regard, purified Aap protein monomers associated into aggregates in the presence of Zn$^{2+}$ and this association was mediated by G5 domains through formation of zinc zippers (4). The Aap target was initially considered because NTZ inhibited biofilm production by *S. epidermidis* strain 5179-R1, which only produces a proteinaceous biofilm composed of Aap (30). However, we found no evidence for chelation of Zn$^{2+}$ by NTZ, as is the case for the anti-biofilm action of EDTA and other metal chelators or by low pH (4, 13). Similarly NTZ does not appear to de-metal Aap surface proteins; since zinc add-back experiments to NTZ-grown bacteria did not restore spontaneous aggregation (clumping) of bacteria in solution that is attributed to Aap (4). While the absence of Aap function might suggest that NTZ affects *aap* gene expression or a later step in assembly of Aap, we could not rule out the possibility that these hydrophobic drugs might bind nonspecifically to surface components.

To address the possibility of nonspecific binding to surface proteins, polysaccharides or teichoic acids through hydrophobic or ionic interactions (38), we tested a more hydrophilic derivative of NTZ (AMIX) and found similar inhibitory anti-biofilm action. These studies lead us to suggest that NTZ affects either the regulation of biofilm gene expression (*aap* and *ica*) or some step in function required for the biofilm phenotype. The regulatory view is supported by the fact that accumulation mediated by Aap protein and polymerization and secretion of polysaccharide biofilm material by the Ica pathway are distinctly different. The possibility that a secretion step is targeted by NTZ is supported by previous studies that sub-MIC levels of NTZ affected secretion of VacA cytotoxin by *H. pylori* (37). In *E. coli*, NTZ reportedly ablates
assembly of fimbrial adhesins (AafA and Type I) (32). Studies are in progress to assess the
effects of NTZ on gene expression and on elaboration of surface proteins.
Coating catheters with antimicrobial agents has been shown to delay or prevent microbial
colonization and to extend their useful lifespan (1, 3, 13, 20). In simulated catheter experiments
under growth promoting conditions, we showed that NTZ blocked the attachment or more likely
the accumulation of bacteria onto catheter surfaces, but did not display any biofilm dispersive
activity. The prevention of further accumulation is consistent with the postulate that the drug
affects biofilm production in general. While NTZ has poor pharmacologic properties, it is likely
that less hydrophobic derivatives, such as AMIX, might prove more efficacious in treating
systemic infections. AMIX is less toxic (than NTZ) to staphylococci by MIC testing while
retaining antibiofilm activity. Moreover, the inhibitory action of AMIX was not reversed by
Zn$^{2+}$ salts, raising the possibility that the noted effects of Zn$^{2+}$ on NTZ potency might be related
to drug uptake or efflux activities, where differences in hydrophobicity would likely manifest.
The primary attachment to and accumulation of microorganisms on surfaces is an essential
step in the infection process and conceptually, targeting these requisite virulence determinants
might provide an additional strategy for limiting or reducing the severity of infections. Our
findings extend early observations of Dubreuil et al by demonstrating that NTZ and derivatives
are more potent against staphylococcal species including MRSA strains under aerobic as well as
microaerobic conditions than previously considered (6). Since we have synthesized derivatives
of NTZ in which the MIC and IC$_{50}$ are the same (~ 1 µg/ml), and assuming target is unchanged,
we suggest that NTZ must target an essential function where measured differences between MIC
and IC$_{50}$ can be explained by relative affinities of the drug for its target. Since the interactions of
staphylococci with surfaces is complex and involves many factors and surface materials, the
effects of NTZ on the phenotype of biofilm may not be direct.

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Azeredo. 2005. Effects of growth in the presence of subinhibitory concentrations of
dicloxacillin on Staphylococcus epidermidis and Staphylococcus haemolyticus biofilms.

dependent adhesion module is responsible for intercellular adhesion in staphylococcal


Table 1. Strains and primers used in this study.

<table>
<thead>
<tr>
<th>Strain or primer</th>
<th>Genotype/phenotype or sequence</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9142</td>
<td>Wild-type PIA producing strain</td>
<td>(19)</td>
</tr>
<tr>
<td>CAV1005</td>
<td>Native valve endocarditis clinical isolate</td>
<td>(36)</td>
</tr>
<tr>
<td>5179</td>
<td>CSF shunt infection isolate; biofilm- and PNAG negative strain; <em>icaA::IS257</em></td>
<td>(30)</td>
</tr>
<tr>
<td>5179-R1</td>
<td>5179 revertant that produces a proteinaceous biofilm dependent on a truncated 140-kDa isoform of Aag; PNAG negative; <em>icaA::IS257</em></td>
<td>(30)</td>
</tr>
<tr>
<td>ICS1</td>
<td>CRBSI</td>
<td>This study</td>
</tr>
<tr>
<td>ICS2</td>
<td>CRBSI</td>
<td>This study</td>
</tr>
<tr>
<td>ICS3</td>
<td>CRBSI</td>
<td>This study</td>
</tr>
<tr>
<td>ICS4</td>
<td>CRBSI</td>
<td>This study</td>
</tr>
<tr>
<td>ICS5</td>
<td>CRBSI</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCTC 8325</td>
<td>Reference strain</td>
<td>(12)</td>
</tr>
<tr>
<td><strong>Staphylococcus hominis subsp. novobiosepticus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F13532</td>
<td>Blood culture isolate</td>
<td>(2)</td>
</tr>
<tr>
<td><strong>Staphylococcus haemolyticus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S33208</td>
<td>Blood culture isolate</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Primer (5’ → 3’)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serp0107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGTAGCTTGTCATGGTGTCG</td>
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<td>(16)</td>
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<tr>
<td>serp0107R</td>
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<td></td>
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<tr>
<td>TTAGAGGTGTCACGTCHGAG</td>
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<td>(16)</td>
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<tr>
<td><strong>tuf</strong></td>
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<tr>
<td>GGGCGTGTGAGCGTGTGTCAATCA</td>
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<td>(21)</td>
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<tr>
<td>TStag765</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TACATTTCTACAATTCTTCTGTA</td>
<td></td>
<td>(21)</td>
</tr>
</tbody>
</table>

* CRBSI, catheter related bloodstream infection; I, inosine
Table 2. Primary attachment to plastic

<table>
<thead>
<tr>
<th>Growth Condition#</th>
<th>Microscopic Count (Mean ± SD)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Primary Attachment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- NTZ</td>
<td>314 ± 10</td>
<td>0.157</td>
</tr>
<tr>
<td>+ NTZ</td>
<td>300 ± 16</td>
<td></td>
</tr>
<tr>
<td>3h Growth (Shaken)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- NTZ</td>
<td>423 ± 36</td>
<td>0.263</td>
</tr>
<tr>
<td>+ NTZ</td>
<td>342 ± 40</td>
<td></td>
</tr>
<tr>
<td>3h Growth (Static)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- NTZ</td>
<td>324 ± 95</td>
<td>0.062</td>
</tr>
<tr>
<td>+ NTZ</td>
<td>243 ± 76</td>
<td></td>
</tr>
<tr>
<td>+ NTZ + 10 µM Zn²⁺</td>
<td>310 ± 7</td>
<td>0.49</td>
</tr>
</tbody>
</table>

# S. epidermidis strain 9142 was grown overnight in TSB with shaken, diluted to OD₆₀₀ of 0.01 in fresh medium and grown for 3 hr (with our without shaking). Bacteria were suspended in PBS to OD₆₀₀ of 0.1 and 1 ml was added to 6 well plates as described in the text. Nitazoxanide (NTZ) was added to wells at 15 µg/ml and ZnSO₄ was added at 10 µM. Adherent bacteria were enumerated microscopically and mean and SD computed from multiple fields of triplicates. Data were analyzed by one tail t-test. None of the listed P values was considered significant.
Figure legends

Figure 1. Chemical structures of NTZ and AMIX.

Figure 2. A. NTZ inhibition of *S. epidermidis* growth and biofilm production. A. MIC testing. Biofilm forming strain 9142 (■) and non-biofilm forming strains 5179 (♦) and CAV1005 (●) were tested for susceptibility to NTZ by microdilution and bacterial growth was measured by turbidity as described in the text. B. Aerobic growth. Bacteria were grown in TSB medium with shaking at 37°C in the presence of no NTZ (♦), 10 µg/ml (■) and 25 µg/ml (▲). C. Biofilm inhibition by NTZ. *Staphylococcus* strains were grown in the presence of different concentrations of NTZ in 96 well polystyrene plates and subjected to crystal violet staining after 24 h as described in the text. The biofilm positive strains (see legend) used are *S. epidermidis* 9142 and *S. haemolyticus* S33208, and the biofilm negative strains are *S. hominis* F13532 and *S. epidermidis* CAV1005. The IC_{50} for biofilm producing strains was 2.5 µg/ml.

Figure 3. Screening for antibiofilm activity. A. Clinical isolates confirmed as *S. epidermidis* were tested for biofilm production and for concentration dependent biofilm inhibition by NTZ at the indicated concentrations with DMSO serving as an additional control. ICS1 and ICS5 strains were biofilm positive. B. Concentration dependent inhibition of biofilm production of *S. epidermidis* strains 9142, icaA mutant strain 5179 and revertant strain 5179-R1 that produces a truncated Aap protein. The data presented represent the mean and standard deviation of three replicates.

Figure 4. Effect of NTZ on attachment to catheter. One centimeter square polyurethane pieces were incubated with indicated concentrations of NTZ and *S. epidermidis* strain 9142 and adherent bacteria were enumerated at 24h. Mean and standard deviation were determined from triplicates.
Figure 5. Biofilm dispersal by NTZ. *S. epidermidis* strain 9142 bacteria were grown under biofilm producing conditions for 4 h and following washing, fresh medium and the indicated concentrations of NTZ were added for an additional 16 h and the biofilm accumulation was determined with crystal violet. NTZ inhibited further biofilm formation in a concentration dependent manner, but did not disperse existing biofilm.

Figure 6. Effect of zinc on bacterial growth and biofilm production. A. Biofilm. Zinc sulfate (black bars), calcium chloride (hatched bars) and magnesium chloride (white bars) were added to TSB containing a fixed concentration of NTZ (12.5 µg/ml) (gray bars) at the indicated concentrations. Zinc chloride, but not the other metals reversed the biofilm inhibitory effect of NTZ with 50% reversal at ~5 µM. B. Growth. Effect of metals on bacterial growth at a fixed concentration of NTZ (12.5 µg/ml): ZnSO₄ (Black bars), ZnCl₂ (Dark gray bars), No metal (light gray bar), DMSO control (white bar) and no NTZ (dark grey bar).

Figure 7. Comparative antibiofilm activity of NTZ and AMIX. Concentration dependent inhibition of biofilm production by *S. epidermidis* strain 9142 by NTZ (●) and water soluble AMIX (■). All assays were performed in triplicate with mean and standard deviation presented.