Antimicrobial and antibiofilm efficacy of triclosan and DispersinB® combination

Rabih O. Darouiche1*, Mohammad D. Mansouri1, Purushottam V. Gawande2 and Srinivasa Madhyastha2

1Center for Prostheses Infections and Infectious Disease Section, Michael E. Debakey Veterans Affairs Medical Center and Baylor College of Medicine, Houston, TX, USA; 2Kane Biotech Inc., 5-1250 Waverley Street, Winnipeg, MB, Canada R3T 6C6

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Objectives: The objectives of this study were to examine: (i) synergy of the combination of triclosan and DispersinB® (DspB); (ii) in vitro efficacy and durability of triclosan + DspB-coated vascular catheters; and (iii) in vivo efficacy of triclosan + DspB-coated catheters compared with chlorhexidine–silver sulfadiazine (CH-SS)-coated and uncoated (control) vascular catheters in preventing colonization by Staphylococcus aureus.

Methods: We investigated the potential synergistic antimicrobial and antibiofilm activity of triclosan and DspB by biofilm assays. The in vitro antimicrobial efficacy of triclosan + DspB-coated catheters was determined by microbial colonization assays. Antimicrobial durability of the coated catheters was tested by soaking segments in bovine serum for 7 days and determining antimicrobial activity, and by a serial plate transfer method. The in vivo efficacy of triclosan + DspB-coated catheters compared with CH-SS-coated and uncoated catheters was assessed by subcutaneous implantation of segments in a rabbit model of S. aureus infection.

Results: The combination of triclosan and DspB showed synergistic antimicrobial and antibiofilm activity against S. aureus, Staphylococcus epidermidis and Escherichia coli, significantly reduced bacterial colonization (P<0.05) and generally demonstrated a prolonged superior antimicrobial activity against clinical pathogens compared with CH-SS-coated catheters. Triclosan + DspB-coated and CH-SS-coated catheters exhibited equal in vivo efficacy (P≤0.05) in reducing colonization by S. aureus compared with uncoated catheters.

Conclusions: Catheters coated with the triclosan + DspB combination showed synergistic, broad-spectrum and durable antimicrobial activity. Furthermore, the in vivo efficacy of catheters coated with this unique antimicrobial/antibiofilm composition prompts clinical evaluation of such an innovative approach.

Keywords: infection, catheter, bacteria

Introduction

Most cases of bloodstream infection arise from vascular catheters, and the vast majority of episodes of urinary tract infection are associated with bladder catheters. Over 250000 episodes of nosocomial bloodstream infection related to vascular catheters that occur each year in the USA are associated with a mortality rate between 12% and 25%, with a treatment cost of ~US$25,000 per survivor.1,2 Although associated with lower morbidity and mortality than vascular catheter-associated bloodstream infection, urinary tract infections occur more frequently and are the second most common source of nosocomial bacteraemia.3 These facts explain why there is a pressing need to effectively prevent catheter-related infections.

We chose to explore the efficacy of the unique combination of triclosan and DispersinB® (DspB) because: (i) triclosan, an antiseptic present in many household products, possesses a broad-spectrum antimicrobial activity by inhibiting the activity of the enoyl-acyl-carrier-protein (ACP) reductase enzyme involved in bacterial fatty acid biosynthesis;4,5 and DspB is an...
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antibiofilm enzyme, which has been shown to inhibit and disperse biofilms;\textsuperscript{8,9} (ii) triclosan has been reported to be safe for topical and surface-coating applications\textsuperscript{8} and DspB is a naturally occurring non-cytotoxic enzyme;\textsuperscript{9} and (iii) both triclosan and DspB have been shown to adhere to medical devices.\textsuperscript{7,9,10}

The objectives of this study were to examine: (i) the antimicrobial/antibiofilm synergistic effect of the combination of triclosan and DspB; (ii) the in vitro antimicrobial/antibiofilm efficacy and durability of activity of triclosan + DspB-coated vascular catheters; and (iii) the in vivo efficacy of triclosan + DspB-coated catheters in comparison with chlorhexidine–silver sulfadiazine (CH-SS)-coated and uncoated vascular catheters against colonization by Staphylococcus aureus.

Materials and methods

Chemicals and organisms

The chemicals, including media ingredients, were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO, USA) or BD Diagnostic System (Sparks, MD, USA). We tested antimicrobial-coated catheters against catheter-associated clinical isolates of S. aureus KB11, Staphylococcus epidermidis 1457, Escherichia coli P18 and Candida albicans KB17. All tested strains were maintained at −80 °C in tryptic soy broth (TSB) containing 15% glycerol and recovered onto tryptic soy agar (TSA). To evaluate the antimicrobial activity of triclosan + DspB-coated catheters in the presence of human blood, the zone of inhibition assay was performed using V agar containing 5% human blood. The microbiological evaluation of catheter segments explanted from animals was performed using TSA with 5% sheep blood. For inoculum preparation, an isolated colony was inoculated into TSB and incubated at 37 °C for 16 h.

Expression and purification of recombinant DspB protein

The DspB enzyme of Aggregatibacter actinomycetemcomitans CU1000 (formerly Actinobacillus actinomycetemcomitans) was expressed and purified from an overexpressing strain of E. coli, as previously described.\textsuperscript{11} Plasmid pRC1 carried a gene that encoded amino acids 31–381 of DspB protein from strain CU1000 fused to a 32 amino acid C-terminal tail that contained a hexahistidine metal-binding site and a thrombin protease cleavage site which could be used to cleave the C-terminal tail from the hybrid protein. This gene was located downstream from an isopropyl-\(\beta\)-d-thiogalactoside (IPTG)-inducible tac promoter.

A 2 L Erlenmeyer flask containing 500 mL ofuria–Bertani broth supplemented with kanamycin 30 mg/L was inoculated with 5 mL of an overnight culture of E. coli strain BL21(DE3)\textsuperscript{13} transformed with pRC1. The flask was incubated at 37 °C with agitation (200 rpm) until the optical density of the culture at 600 nm reached 0.6 (\(\sim\)3 h). IPTG was added to reach a final concentration of 0.2 mM in the culture broth, and the flask was incubated for an additional 5 h with agitation. The cells were harvested by centrifugation for 15 min at 6000 g and the cell pellet was stored at −80 °C.

The cell pellet was thawed at room temperature and resuspended in 20 mL of lysis buffer [20 mM Tris–HCl (pH 8.0)/500 mM NaCl/1 mM phenylmethylsulphonyl fluoride/0.1% Nonidet P-400] containing 2 mg/mL lysozyme. The cell suspension was then sonicated on ice for 30 s at 30% capacity with a 30% duty cycle by using a Branson Model 450 sonicator equipped with a microprobe. The cell debris was pelleted by centrifugation at 15,000 g for 20 min at 4 °C and the supernatant was loaded onto a 3 mL (bed volume) activated nickel affinity column (catalogue no. 154-0990; Pharmacia) according to the instructions supplied by the manufacturer. The column was washed with 50 mL of wash buffer [20 mM Tris (pH 7.5)/500 mM NaCl] containing 5 mM imidazole. Fractions of the eluate were collected and assayed for the presence of the hybrid protein by SDS–PAGE and Coomassie Blue staining.\textsuperscript{14} Fractions containing the protein were pooled and dialysed overnight against water by using a 10,000 kDa cut-off dialysis membrane. The purified protein was digested with 5 U of thrombin (Novagen) per milligram of thrombin cleavage capture kit (Novagen) used according to the instructions supplied with the kit. Undigested protein was removed by loading the sample onto a nickel affinity column as described above and washing the column with 10 mL of wash buffer containing 5 mM imidazole. Fractions containing the protein were pooled, dialysed against water and stored at −20 °C. The activity of DspB enzyme was \(\sim\)10\textsuperscript{3} U/mg protein.\textsuperscript{14} The purity of DspB enzyme was determined by SDS–PAGE analysis.

Biofilm assay

Biofilms were assayed by Crystal Violet staining as described previously.\textsuperscript{14} The overnight cultures were diluted to 5% in colony-forming antigen medium for Gram-negative bacteria and TSB for Gram-positive bacteria. The biofilm was grown in 96-well microtitre plates (Corning Inc., New York, USA). The plates were incubated at 37 °C for 16–18 h under stationary conditions and total growth was measured at 600 nm. Biofilm was measured by discarding the medium, rinsing the wells with 200 µL of water (three times) and staining the bound cells with Crystal Violet (0.4%, w/v) for 15 min, followed by washing with water (200 µL) to remove unbound stain. The dye was solubilized in 200 µL of 33% (v/v) acetic acid, and absorbance at 630 nm was determined using a microtitre plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). Six replicates were used for each sample and each experiment was performed three times.

Catheters

Sterile uncoated triple-lumen, 7-french (2.4 mm), polyurethane central venous catheters were purchased from Cook Critical Care (Bloomington, IN, USA). Catheters were coated by dipping in a solution that contained triclosan (10 mg/mL) and DspB (100 µg/mL) as described previously by Kaplan et al.\textsuperscript{7} and Hernandez-Richter et al.,\textsuperscript{10} and then gas sterilized with ethylene oxide. In addition, sterile polyurethane central venous catheters whose external surfaces are coated with CH-SS (ARROW\textsuperscript{\textregistered} Plus Blue) and sterile polyurethane central venous catheters whose internal and external surfaces are both coated with CH-SS (ARROW\textsuperscript{\textregistered} Plus Blue PLUS) were purchased from Arrow International (Reading, PA, USA).

The triclosan + DspB solution was prepared using an aqueous solvent system comprising 10% polyethylene glycol 400 and 10% ethanol. Catheter segments (1 cm) were dipped overnight in the triclosan + DspB solution at 4 °C to coat both internal and external catheter surfaces, following which they were left to dry for 8 h at room temperature. The dipping and drying procedure was repeated twice. The catheter segments were then left to dry for an additional 24 h.

Adhesion assay

A previously described method with a slight modification was used to examine the bacterial adherence to the surfaces of the catheters.\textsuperscript{15} Catheter segments were individually placed in 10 mL of TSB. Catheter segments were inoculated with 100 µL of inoculum

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Method on V agar. A modified Kirby–Bauer method was used to S. epidermidis 1, 4 and 7 days to perform the adhesion assay against 24 and 48 h, and the colonies were counted.

In vitro efficacy and durability of activity of coated catheters

The in vitro durability of coated catheters was examined by placing 1 cm long segments of uncoated control, triclosan+DspB-coated, ARROWg+ard Blue and ARROWg+ard Blue PLUS catheters in four separate 250 mL flasks containing 100 mL of TSB with 20% bovine serum at 37°C with agitation at 100 rpm. Half of the TSB containing 20% bovine serum was replaced by fresh medium daily in each group. Catheter segments were removed from the flask after 1, 4 and 7 days to perform the adhesion assay against S. aureus, S. epidermidis and C. albicans.

In vitro durability was also studied by the serial plate transfer method on V agar. A modified Kirby–Bauer method was used to evaluate the antimicrobial activity of catheter segments. Segments (1 cm long; 3 mm external diameter) of uncoated control and triclosan+DspB-coated catheters were pressed onto the centre of V agar plates containing 5% human blood that had been freshly inoculated with S. aureus. After incubating the agar plates at 37°C for 24 h, the zone of inhibition was determined by measuring the diameter of the clear zone perpendicular to the long axis of the catheter segment. The actual zones of inhibition were recorded by subtracting the external diameter of the catheter (3 mm) from each measurement. The catheter segments were transferred daily to fresh lawns on V agar. The diameter of the zones of inhibition was expressed as an average of three replicates.

In vivo antistaphylococcal efficacy of coated catheters

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine, Houston, TX, USA. A previously described rabbit model of bacterial infection of subcutaneously inserted devices was used. Twenty Female New Zealand White, specific pathogen-free rabbits (body weight 2–3 kg) were used in this study. Rabbits were anesthetized by intramuscular injection (0.5 mL/kg body weight) of a mixture of ketamine (37.5 mg/kg body weight) and acepromazine (1.70 mg/kg body weight). No prophylactic antibiotic was given preoperatively. The backs of the rabbits were shaved, cleansed with triclosan, 70% ethanol and then povidone–iodine, and then draped in a sterile fashion. Six 1 cm incisions were made (three on each side and ~4 cm apart and 4 cm lateral to the spine) and dissections were performed down to the plane between the panniculus carnosus and the fascia of the underlying muscles. Thirty 1 cm catheter segments from each of the four groups (triclosan+DspB-coated, ARROWg+ard Blue, ARROWg+ard Blue PLUS and uncoated catheters) were implanted subcutaneously in the back of a total of 20 rabbits (six implants from three different groups per rabbit). Based on the findings of a pilot trial that was initially performed to determine the optimal bacterial inoculum required for colonization of most uncoated catheter segments, a 50 μL aliquot of 2×10^6 cfu/mL (absolute inoculum of 10^8 cfu) of a clinical strain of S. aureus was inoculated onto the surface of the inserted catheter segments. The incisions were closed using 4-0 monofilament nylon suture material. The rabbits were monitored daily for signs of local infection, sepsis or major distress. Ketoprofen (2 mg/kg body weight) was given to rabbits intramuscularly after the surgical operation and on a daily basis as an anti-inflammatory/analgesic.

Microbiological assessment

Just prior to sacrificing, blood samples were collected from rabbits using sterile techniques and inoculated onto trypticase sheep agar plates containing 10% sheep blood. Rabbits were sacrificed humanely 1 week after surgery. To prevent contamination of the catheter segments during explantation by bacteria colonizing the surgical wound, another incision was made 2 cm medial to the surgical wound using an electrosurgical probe to remove the catheter segments. All retrieved catheter segments were quantitatively cultured by using the sonication technique, with a detectability limit of 20 cfu.

Statistical analysis

The frequencies of catheter colonization were compared between the different groups by using a two-tailed Fisher’s exact test (STATA, version 8.2; StataCorp, College Station, TX, USA). The mean bacterial colony counts retrieved from the explanted catheter segments were compared in a pair-wise fashion among the three groups by using the two-tailed Student’s t-test with unequal variance. P values of ≤0.05 indicated significant differences.

Results

Antibiofilm activity of DspB alone and in combination with triclosan

Triclosan in combination with DspB significantly (P<0.05) inhibited biofilm formation in S. aureus, S. epidermidis and E. coli as compared with control, triclosan alone or DspB alone, thereby indicating a synergistic antibiofilm activity by the combination of triclosan and DspB (Figure 1). Furthermore, S. epidermidis and E. coli biofilms were reduced to undetectable levels when triclosan was combined with DspB.

In vitro durability of the activity of triclosan+DspB-coated catheters

Colonization of triclosan+DspB-coated catheters by all tested organisms was significantly lower (P<0.05) than that of uncoated control catheters (Figure 2). Furthermore, triclosan+DspB-coated, ARROWg+ard Blue and ARROWg+ard Blue PLUS catheters were all significantly more effective (P<0.05) against colonization by S. aureus than control uncoated catheters after 1, 4 and 7 days of incubation in TSB with serum. In addition, while all coated catheters had significantly reduced colonization after 1 day of incubation, only triclosan+DspB-coated and ARROWg+ard Blue PLUS catheters significantly reduced colonization by S. epidermidis after 4 and 7 days (Figure 3; P<0.05). Although both triclosan+DspB-coated catheters and ARROWg+ard Blue PLUS catheters displayed significant protection against colonization, only triclosan+DspB-coated catheters were significantly less colonized by C. albicans after 4 days of incubation. Furthermore, triclosan+DspB-coated catheter segments displayed zones of inhibition >15 mm in diameter against S. aureus after 5 days and

(10^7–10^8 cfu/mL) and incubated in a water bath for 16 h at 37°C with agitation at 100 rpm. Catheter segments were then washed in saline three times and placed in 1 mL aliquots of saline. Adherent cells were removed by sonication at 42 kHz, 70 W output (Fisher Scientific, Pittsburgh, PA, USA; model no. FS20) for 30 s, followed by vortexing for 1 min. Cells were serially diluted in saline and then plated onto TSA plates. The plates were incubated at 37°C for 24 and 48 h, and the colonies were counted.
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Figure 1. Effect of triclosan and DspB alone and in combination on the adherence of S. aureus, S. epidermidis and E. coli as determined by a 96-well microtitre plate assay. Error bars are not visible where the standard deviations are less than the area occupied by a given symbol. Asterisks indicate a significant difference (P<0.05) between biofilm formation in the presence of triclosan+DspB combination and that in the presence of triclosan and DspB alone or control.

Figure 2. Effect of catheter coating on the adherence of S. aureus, S. epidermidis, E. coli and C. albicans to the catheter surface. Uncoated control and triclosan+DspB-coated catheter segments (1 cm length) were exposed to an inoculum of 1×10<sup>6</sup> cfu/mL for 24 h at 37°C. Error bars are not visible where the standard deviations are less than the area occupied by a given symbol. Asterisks indicate a significant difference (P<0.05) in the adherence of each organism to triclosan+DspB-coated catheters compared with uncoated control catheter.

Figure 3. In vitro durability of uncoated control, triclosan+DspB-coated, ARROWg+ard Blue (AGB) and ARROWg+ard Blue PLUS (AGBP) catheters in 20% bovine serum. The assay for catheter colonization was performed for 24 h in TSB at 37°C after soaking in TSB containing 20% bovine serum for 1, 4 and 7 days. Error bars are not visible where the standard deviations are less than the area occupied by a given symbol. Asterisks indicate significant differences (P<0.05) between uncoated control and coated catheters.

considerable enduring zones of >10 mm even after 8 days of serial plate transfer (Figure 4).

In vivo antistaphylococcal efficacy of catheters

All 20 rabbits tolerated surgery well and exhibited no evidence of sepsis or failure to thrive. Overall, 1/30 (3.3%) triclosan+DspB-coated catheters, 4/30 (13.3%) ARROWg+ard Blue catheters, 1/30 (3.3%) ARROWg+ard Blue PLUS catheters and 29/30 (96.7%) uncoated control catheters became colonized with S. aureus. Pair-wise comparison of all coated catheter segments against control uncoated catheter segments indicated that coated catheter segments are significantly less likely to become colonized by S. aureus (P<0.001). However, no significant differences were observed when catheter colonization was compared among triclosan+DspB-coated,
enterococci and venous catheters, respectively; whereas Gram-negative bacilli, urinary catheter-associated infections, respectively. The antimicrobial activity for Gram-negative bacilli (E. coli).

Discussion

Biofilm-embedded bacteria and yeast are tenacious and 100–1000 times more resistant to antimicrobial treatment. Thus, inhibition of biofilm formation and/or dispersal of pre-formed biofilms may make biofilm-embedded bacteria and yeast more susceptible to antimicrobial agents. Accordingly, we evaluated the effect of combining the antimicrobial triclosan with the antibiofilm enzyme DspB against catheter-associated bacteria and yeast. The triclosan + DspB combination displayed synergistic efficacy against S. aureus, S. epidermidis and E. coli. These findings are in agreement with previous reports describing the synergistic activity of the combination of antibiofilm enzyme DspB with antibiotics (cefamandole nafate or ampicillin) and non-antibiotic compounds such as SDS.

Staphylococci, Gram-negative bacilli and Candida account for ~75%, 20% and 10% of infections associated with central venous catheters, respectively; whereas Gram-negative bacilli, enterococci and Candida account for 67%, 25% and 10% of urinary catheter-associated infections, respectively. The results of this study showed that catheters coated with triclosan + DspB were significantly protected against colonization by Gram-positive cocci (S. aureus and S. epidermidis), Gram-negative bacilli (E. coli) and C. albicans. The retention of antimicrobial activity for >8 days by triclosan + DspB-coated catheters indicates that the antibiotic/antimicrobial agents on these catheters may have a slow release mechanism, thereby generating a durable anti-infective activity. Our results are consistent with a previous study showing a lack of antimicrobial durability of ARROWg + arid catheters against C. albicans.

A major finding of this study was that catheters coated with triclosan + DspB were as effective as commercially available CH-SS-coated catheters against S. aureus colonization in vivo. However, S. aureus colonization of triclosan + DspB-coated catheters, ARROWg + arid Blue and ARROWg + arid Blue PLUS was significantly lower than that of uncoated control catheters (P < 0.05). The superior in vitro antimicrobial activity and comparable in vivo performance of triclosan + DspB-coated catheters versus the CH-SS-coated catheters may be attributed, at least in part, to the relatively long durability of triclosan + DspB-coated catheters against both bacteria and yeast, as compared with ARROWg + arid catheters.

Recently, DspB was reported to inhibit and disperse biofilm formation by depolymerizing a polysaccharide, β-1,6-N-acetyl-D-glucosamine (PGA), which is essential for biofilm formation of E. coli and some staphylococci. The in vitro synergistic activity of triclosan + DspB antimicrobial–antibiofilm combination could be due to the fact that DspB makes biofilm-embedded bacteria more susceptible to the antimicrobial action of triclosan.

In summary, triclosan + DspB coating of both internal and external surfaces of catheters possesses in vitro broad-spectrum antimicrobial activity against catheter-associated pathogens and provides a strong in vivo antimicrobial efficacy against colonization by S. aureus. These promising results encourage the clinical evaluation of this novel approach. This combination of clinically safe triclosan and naturally occurring non-cytotoxic DspB could be incorporated onto the surfaces of various medical devices for potential prevention of device-related infections.

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Transparency declarations

The proprietary combination of triclosan and DispersinB® method is owned by Kane Biotech Inc., Winnipeg, MB, Canada. R. O. D. has served as a consultant for Kane Biotech Inc. P. V. G. and S. M. are employees of Kane Biotech Inc. and both own stocks and have options as well. M. D. M.: none to declare.

References

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