In Vitro Antimicrobial and Antibiofilm Activity of DispersinB®-Triclosan Wound Gel against Chronic Wound-associated Bacteria

Purushottam V. Gawande, Nandadeva Yakandawala, Karen LoVetri and Srinivasa Madhyastha*

Kane Biotech Inc, 5-1250 Waverley Street, Winnipeg, MB, R3T 6C6, Canada

Abstract: Since the traditional methods of treatment have proven ineffective against chronic wounds involving biofilms, the present study evaluates the *in vitro* efficacy of a novel wound gel comprising an antibiofilm DispersinB® enzyme and a broad-spectrum antimicrobial triclosan against chronic wound-associated bacteria. The antimicrobial and antibiofilm activity of DispersinB®-triclosan wound gel was tested against chronic wound-associated bacteria such as *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Staphylococcus epidermidis,* coagulase-negative Staphylococci, *Acinetobacter baumannii* and *Klebsiella pneumoniae* using a quantitative culture method and a biofilm assay. The DispersinB®-triclosan wound gel showed more enduring antimicrobial activity against the test organisms compared to commercial wound gels Silver-Sept™ and IODOSORB. When DispersinB®-triclosan wound gel was tested against preformed biofilm, it showed 2-4 $\log_{10}$ reduction in the biofilm embedded bacterial cells. Furthermore, DispersinB®-triclosan wound gel compared to IODOSORB was significantly more effective in inhibiting biofilm in MRSA and *A. baumannii* ($P < 0.05$). This study indicates the potential application of a broad-spectrum DispersinB®-triclosan wound gel with both antibiofilm and antimicrobial activity in biofilm-based chronic wound management.

Keywords: DispersinB®, triclosan, chronic wounds, biofilms.

INTRODUCTION

Chronic human infections including chronic wounds or non-healing wounds constitute 60-80% of all human infectious diseases [1]. Chronic wounds including diabetic foot ulcer, venous leg ulcer and pressure ulcers are often resistant to natural healing and require long term medical care. The cost of chronic infections represents a major portion of the health care budget and these costs continue to grow at an exponential rate. In chronic wounds, the biofilm mode of growth is characterized by adherence to biotic or abiotic surfaces, slow development of overt symptoms, and lack of resolution by the host defense and resistance to antibiotic therapy [2]. Furthermore, recent studies by Singh and Barbul [3] have demonstrated biofilm as a potential reason why chronic wounds do not heal. In addition, James *et al*. [4] have reported biofilms in over 60% of bacterial infections associated with chronic wounds such as diabetic foot ulcers, venous leg ulcers and pressure ulcers. Also, Wolcott and Rhoads [5] observed that the chronic wound treatments that specifically target biofilms transformed non-healable wounds into healable wounds. They also showed that the use of antibiotics declined approximately 25% during the four-year study period. Thus, the use of suitable topical agents that inhibit biofilm formation or disrupt preformed biofilm should be integral to the management of chronic wound infections.

Antiseptic wound dressings are currently the most common clinical strategy employed to address wound infection with limited success against chronic wounds involving biofilms. Although systemic antibiotic administration has shown some efficacy against wound infections, the growing concern regarding bacteria that are resistant to antibiotic therapy shows the need for developing alternative and possibly better ways to treat chronic wound-associated infections involving biofilms. Realizing the need for a wound care product with both the antibioflm and antimicrobial activity, we formulated a novel DispersinB® antibiofilm enzyme based wound gel containing a broad-spectrum antimicrobial triclosan. DispersinB® is a naturally occurring enzyme produced by an oral bacterium *Aggregatibacter actinomycetemcomitans*, which is associated with the juvenile periodontitis [6]. DispersinB® is active against poly-*N*-acetylglucosamine (PNAG), which is a major polysaccharide in biofilms of wound-associated bacteria such as *Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae* and *Acinetobacter baumannii.* It has been shown to inhibit biofilm formation and disperse preformed biofilm without affecting the growth of these bacteria [7]. However, DispersinB® in combination with an antimicrobial has been shown to make the bacteria more susceptible to antimicrobial killing either by inhibiting biofilm formation or by disrupting preformed biofilm [8, 9]. Triclosan, an antimicrobial present in many household products, has a broad spectrum antimicrobial activity due to its inhibitory effect on the activity of enoyl-acyl-carrier-protein (ACP) reductase enzyme involved in bacterial fatty acid biosynthesis [10, 11].

In this study, we evaluated the *in vitro* efficacy of DispersinB®-triclosan wound gel against chronic wound-associated bacteria such as *S. aureus, S. epidermidis, K. pneumoniae* and *A. baumannii* based on the antimicrobial

*Address correspondence to this author at the Kane Biotech Inc, 5-1250 Waverley Street, Winnipeg, MB, R3T 6C6, Canada; Tel: + 1 204 478 5600; Fax: +1 204 453 1314; E-mail: srim@kanebiotech.com

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activity in comparison with that of commercial Silver-Sept™ and IODOSORB wound gels, and also the antibiofilm activity of both the heat-treated and untreated DispersinB®-triclosan wound gels. Additionally, the antibiofilm activity of DispersinB®-triclosan wound gel was compared with that of IODOSORB alone as it showed a better antimicrobial activity than Silver-Sept™.

MATERIAL AND METHODOLOGY

Reagents, Microorganisms and Culture Conditions

DispersinB® enzyme was purified from a recombinant *Escherichia coli* strain as previously described [6]. The enzyme had a specific activity of ~ 10^3 units mg^{-1} of protein. All the chemicals (including media ingredients) were of analytical grade and purchased from Sigma-Aldrich (St. Louis, USA) or BD Diagnostic Systems (Sparks, USA). The commercial Silver-Sept™ and IODOSORB wound gels were purchased from Health Products for You (1 Hillview Drive West, New Fairfield, CT, USA) and Source Medical (11620-181 Street, Edmonton, AB, Canada), respectively. The cultures of *S. aureus* KB11*,* methicillin-resistant *S. aureus* (MRSA) 64791, *S. epidermidis* 1457, *K. pneumoniae* P30 and *A. baumannii* 63270 were provided by Dr. George G. Zhanel (University of Manitoba, Winnipeg, MB, Canada). The clinical wound isolates of *S. aureus* Gav 16a, and coagulase-negative Staphylococcus (CoNS) 42 were obtained from Dr. Randolph D. Wolcott (Southwest Regional Wound Care Center, Lubbock, TX, USA). All the strains were maintained at -80°C in 15% glycerol and recovered onto tryptic soy agar (TSA). For inoculum preparation, an isolated colony was inoculated into tryptic soy broth (TSB) and incubated at 37°C for 16-18 h.

DispersinB®-Triclosan Gel

The aqueous based wound gel was formulated by combining 100 mg ml^{-1} polyethylene glycol 400 (PEG 400) and 100 mg ml^{-1} ethanol with 10 mg ml^{-1} triclosan, 200 µg ml^{-1} DispersinB® and 15 mg ml^{-1} sodium alginate. The aqueous triclosan solution was prepared in ethanol and PEG 400 by mixing at 60°C for 6 h to produce a stable white colloidal suspension [10]. After cooling triclosan solution to room temperature, DispersinB® and sodium alginate were added and mixed till it formed uniform gel.

Quantitative Culture Method

Bacterial suspensions were prepared by inoculating 3 ml TSB with 5 X 10^8 CFUs of the bacterial cultures. One gram each of topical wound gels was added to the bacterial suspensions separately and incubated at 37°C [12]. Samples (0.1 ml) were removed at 24 h, 48 h, and 72 h; diluted 10-times to reduce antimicrobial carryover; plated onto TSA and incubated for 48 hours at 37°C. Colonies were counted and expressed as CFU ml^{-1}. For the purpose of this study, low bactericidal activity was considered to be less than 1 $\log_{10}$ CFU ml^{-1} reduction, moderate activity between 1 and 3 $\log_{10}$ CFU ml^{-1}, and high bactericidal activity as greater than 3 $\log_{10}$ CFU ml^{-1}.

Biofilm Assay

Biofilms were grown in 1.5-ml polypropylene micro-centrifuge tubes [13]. Tubes were filled with 200 µl of inoculum (diluted 1:100 in fresh broth) and incubated at 37°C for 24 h. For biofilm assays, the DispersinB®-triclosan wound gel was diluted 100 times in fresh TSB to reduce the viscosity of gels. A portion of the diluted wound gel was heat-treated at 65°C for 30 min. The heat treatment at 65°C denatures DispersinB® and thus it looses its activity, but it does not affect the activity of triclosan. The planktonic growth was aspirated; biofilm was washed once and treated with heat-treated and untreated wound gel dilutions separately. After 3 h incubation at 37°C, the cells were vortexed for 1 min, pelleted and rinsed with sterile phosphate buffered saline (PBS) two times to remove the wound gel. Cell pellets were resuspended in 200 µl of PBS containing 20 µg ml^{-1} of DispersinB® and incubated for 5 min at 37°C to disaggregate the cells. Tubes were vortexed briefly and the number of CFU ml^{-1} was determined by dilution plating on TSA. The results were calculated as averages and standard deviations from three experiments. Statistical analysis was performed with Student's *t*-test. *P* values of < 0.05 were considered statistically significant.

RESULTS

In Vitro Antimicrobial and Antibiofilm Activity of DispersinB®-Triclosan, Silver-Sept™ and IODOSORB Wound Gels

The *in vitro* antimicrobial activity of DispersinB®-triclosan wound gel in comparison with that of commercial Silver-Sept™ and IODOSORB was tested against chronic wound-associated bacteria including clinical isolates using a quantitative culture method. While DispersinB®-triclosan and IODOSORB reduced viable counts to undetectable level of all the test organisms over a period of 72 h incubation, Silver-Sept™ showed appreciable antibacterial activity only against CoNS, *S. epidermidis,* and *A. baumannii* (Table **1**). Furthermore, Silver-Sept™ was moderately effective against *S. aureus* and *K. pneumoniae* reducing only 1-2 $\log_{10}$ CFU ml^{-1}, but not active against MRSA.

The antibiofilm activity of 100-fold diluted heat-treated and untreated DispersinB®-triclosan wound gels against chronic wound-associated bacteria was determined using a biofilm assay. The antibiofilm activity of heat-treated wound gel was compared with untreated wound gel to evaluate the role of DispersinB® in the wound gel formulation. All the strains formed good biofilms under the given experimental conditions, showing more than 8 $\log_{10}$ CFU ml^{-1} biofilm. The untreated wound gel inhibited significantly more biofilm in all the test organisms compared to heat-treated wound gel (Fig. **1**; $P < 0.05$). The heat treated wound gel showed different levels of biofilm inhibition among the bacterial species tested due to the difference in the susceptibility towards triclosan.

Comparison of *In Vitro* Antibiofilm Activity of DispersinB®-Triclosan and IODOSORB Wound Gels

Since DispersinB®-triclosan and IODOSORB wound gels were more effective in inhibiting wound associated bacteria compared to Silver-Sept™ showing a minimal inhibitory effect, they were chosen for testing their antibiofilm activity. The antibiofilm activity of 100-times diluted DispersinB®-triclosan wound gel and IODOSORB against MRSA and *A.*

Table 1. Comparison of Antimicrobial Activity of DispersinB®-triclosan, Silver-Sept™ and IODOSORB Gels Against Chronic Wound-associated Bacteria

Pathogen	Time (h)	Control	DispersinB®-Triclosan	Silver-Sept™	IODOSORB
S. aureus Gav 16a	24	1.0×10^9	N.G.	5.63×10^9	N.G.
	48	1.03×10^9	N.G.	3.27×10^7	N.G.
	72	1.37×10^9	N.G.	1.33×10^8	N.G.
Methicillin-resistant *S. aureus* (MRSA) 64791	24	1.21×10^9	N.G.	4.63×10^8	N.G.
	48	1.47×10^9	N.G.	1.01×10^9	N.G.
	72	1.14×10^9	N.G.	1.34×10^9	N.G.
S. aureus KB11	24	1.21×10^9	N.G.	5.27×10^6	N.G.
	48	1.28×10^9	N.G.	2.93×10^8	N.G.
	72	9.67×10^8	N.G.	5.2×10^8	N.G.
Coagulase-negative Staphylococcus 42	24	8.3×10^8	N.G.	1.0×10^6	N.G.
	48	1.42×10^9	N.G.	N.G	N.G.
	72	1.22×10^9	N.G.	N.G.	N.G.
S. epidermidis 1457	24	9.1×10^8	N.G.	N.G.	N.G.
	48	9.9×10^8	N.G.	N.G	N.G.
	72	1.03×10^9	N.G.	N.G.	N.G.
A. baumannii 63270	24	3.5×10^9	N.G.	3.0×10^3	N.G.
	48	3.5×10^9	N.G.	8.3×10^2	N.G.
	72	3.3×10^9	N.G.	N.G.	N.G.
K. pneumoniae P30	24	9.93×10^8	N.G.	6.47×10^5	N.G.
	48	1.04×10^9	N.G.	2.73×10^8	N.G.
	72	8.77×10^8	N.G.	8.23×10^8	N.G.

N.G.: No Growth.

baumannii biofilms was compared using a biofilm assay. While commercial wound gel IODOSORB reduced only 1 $\log_{10}$ CFU ml^{-1} of biofilm in test organisms, DispersinB®-triclosan wound gel showed 4 and >5 $\log_{10}$ CFU ml^{-1} reduction in MRSA and *A. baumannii* biofilms, respectively compared to the control (Fig. **2**). Furthermore, DispersinB®-triclosan wound gel inhibited significantly more biofilm in MRSA and *A. baumannii* compared to IODOSORB ($P < 0.05$).

DISCUSSION

Biofilm formation is an effective protection strategy adopted by bacteria to promote survival within hostile environments such as that of chronic wounds. It is well recognized that infections involving biofilms are difficult to eradicate, as sessile bacteria employ mechanisms that raise survival and resistance to antimicrobials up to 1000 times compared to their planktonic counterparts [14]. In this paper, we report the *in vitro* antimicrobial and antibiofilm efficacy of a wound gel formulation comprising an antibiofilm enzyme DispersinB® that inhibits as well as disrupts bacterial biofilms and a broad-spectrum antimicrobial triclosan. While Kaplan *et al*. [7] demonstrated the antibiofilm activity of DispersinB® against *S. aureus*, *S. epidermidis*, *K. pneumoniae,* and *Acinetobacter* spp., Darouiche *et al.* [8] reported the synergistic *in vitro* and *in vivo* antimicrobial and antibiofilm efficacy of DispersinB® and triclosan combination against central venous catheter associated pathogens such as *S. aureus*, *S. epidermidis* and *Candida albicans*. The synergy between these two compounds could be attributed to DispersinB® making the bacterial biofilm more susceptible to antimicrobial triclosan killing either by inhibiting biofilm formation or by disrupting preformed biofilms.

While DispersinB®-triclosan wound gel and IODOSORB containing cadexomer iodine exhibited a sustained antibacterial activity over a 72 h incubation period against all the chronic wound-associated bacteria, silver containing wound gel Silver-Sept™ was moderately active against *S. aureus* and not active against MRSA, which accounts for 93.5% of venous leg ulcer infections and 40% of diabetic foot ulcer infections [15, 16]. Our results are consistent with

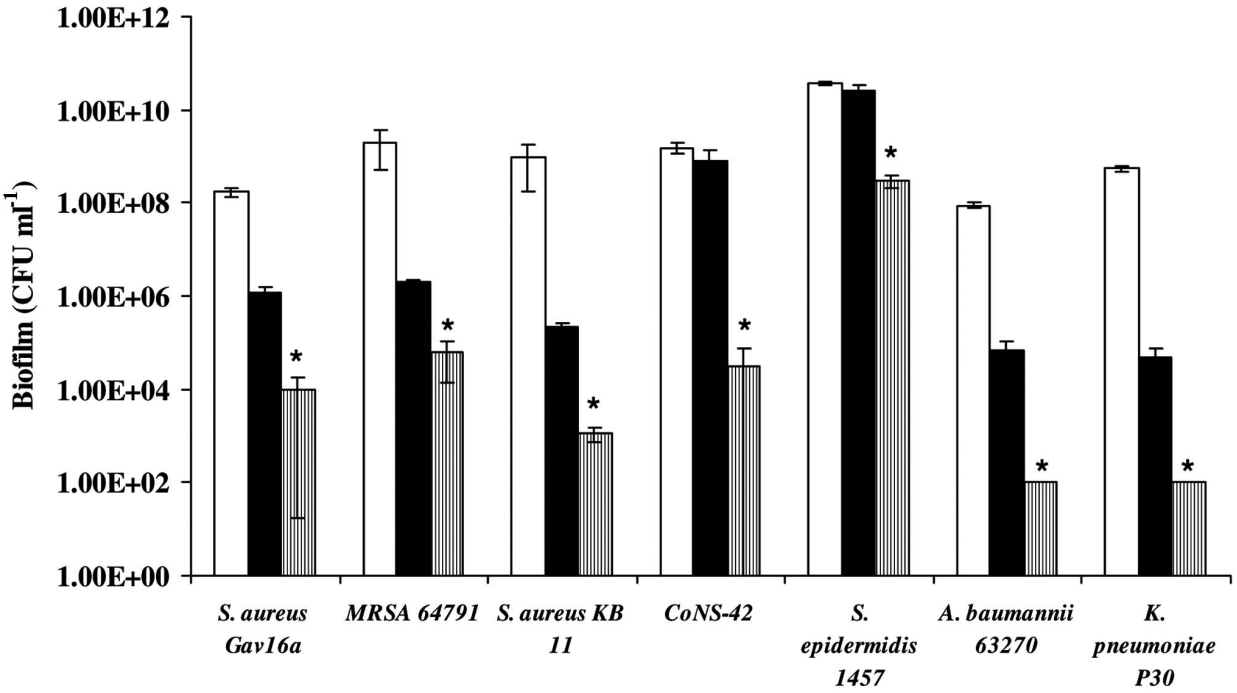

Fig. (1). Effect of control ( ), 100-times diluted heat-treated DispersinB®-triclosan wound gel ( ), and 100-times diluted untreated DispersinB®-triclosan gel ( ) on biofilm. Error bars are not visible where the standard deviations are less than the area occupied by a given symbol. Asterisks indicate a significant difference ($P < 0.05$) between biofilm in the presence of untreated wound gel and heat-treated wound gel, and control.

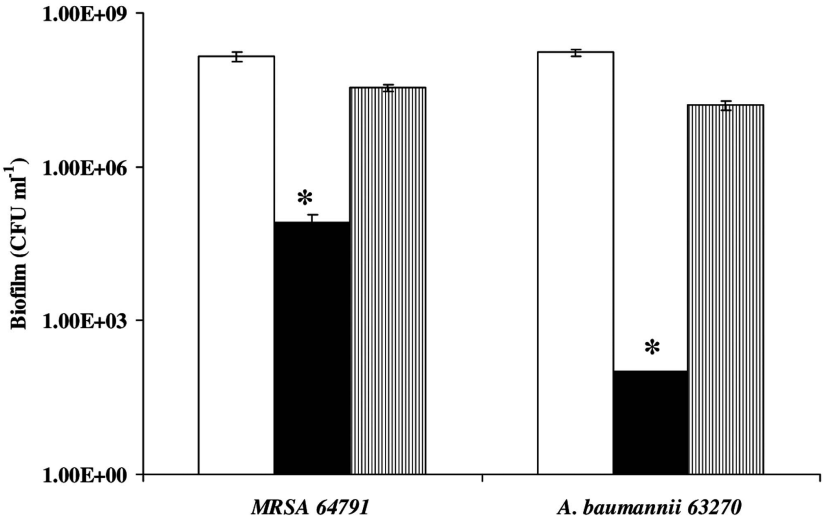

Fig. (2). Effect of control ( ), 100-times diluted DispersinB®-triclosan wound gel ( ), and 100-times diluted IODOSORB gel ( ) against biofilm of chronic wound-associated bacteria. Error bars are not visible where the standard deviations are less than the area occupied by a given symbol. Asterisks indicate a significant difference ($P < 0.05$) between biofilm in the presence of DispersinB®-triclosan wound gel and IODOSORB, and control.

the previous study showing poor bactericidal activity of silver containing wound dressings [12]. Furthermore, when the antibiofilm activity of heat-treated DispersinB®-triclosan wound gel was compared with that of untreated against chronic wound-associated bacteria, the untreated wound gel was significantly more effective in inhibiting biofilm compared to heat-treated wound gel in all the test organisms ($P < 0.05$) indicating the enhancing effect of DispersinB® on the antimicrobial activity of triclosan. Based on the observation that the antimicrobial activity of IODOSORB is superior to Silver-Sept™ and equivalent to that of DispersinB®-triclosan wound gel, IODOSORB was chosen for further studies to compare its antibiofilm activity with that of DispersinB®-triclosan wound gel against MRSA and *A. baumannii*. DispersinB®-triclosan gel reduced 2 -5 $\log_{10}$ CFU ml^{-1} more biofilm in MRSA and *A. baumannii* compared to that by IODOSORB. The superior biofilm killing activity of DispersinB®-triclosan could again be attributed to the fact that DispersinB® depolymerizes PNAG matrix in the biofilm and thereby making the bacterial biofilm more susceptible to the antimicrobial action of triclosan [7].

A major finding of this study was the antimicrobial and antibiofilm activity of DispersinB®-triclosan wound gel formulation and its superior inhibitory effect on biofilm in wound associated bacteria compared to commercially available wound gels. Since DispersinB® is an antibiofilm enzyme and does not have antimicrobial activity; it is highly unlikely to cause bacterial resistance [7]. Other advantage to using multiple, compatible agents in combination include lowering the probability that resistance will emerge and increase the spectrum of microbicidal activity [17]. This DispersinB®-triclosan wound gel formulation comprising naturally occurring biocompatible DispersinB® enzyme and clinically safe triclosan could meet the unmet clinical need for prevention and treatment of chronic wounds involving biofilms [9, 18]. Furthermore, this wound gel with both the antimicrobial and antibiofilm activities has potential applications in wound and skin care products such as wound dressings, wound sprays, bandage, creams, lotions and ointments.

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CONFLICT OF INTEREST

The proprietary combination of DispersinB® and triclosan is patented and owned by Kane Biotech., Inc. All the authors are employees of Kane biotech Inc. and own stocks and have options as well.

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