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Potential for repurposing the personal care product preservatives bronopol and bronidox as broad-spectrum antibiofilm agents for topical application

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Running title: Bronopol and bronidox as antibiofilm agents

Abstract

Objectives: Bacterial biofilms represent a major impediment to healing in chronic wounds, and are largely refractory to antibacterial agents currently used in wound management. From a repurposing screen of compounds considered safe for topical application in humans, we report the identification of bronopol and bronidox as broad-spectrum antibiofilm agents and potential candidates for reducing biofilm burden in chronic wounds.

Methods: Antibiofilm activity was assessed by viable counting against single-species biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in the Calgary Biofilm Device (CBD) and against mixed-species biofilms of the two organisms growing on nitrocellulose disks.

Results: The personal care product preservatives, bronopol and bronidox, exhibited broad-spectrum antibiofilm activity that encompassed the two major wound pathogens, *S. aureus* and *Ps. aeruginosa*. When impregnated into gauze dressings at their existing maximum authorised concentrations for safe use and placed onto an established mixed-species biofilm, bronopol and bronidox completely eradicated *Ps. aeruginosa* and achieved a ~5 $\log_{10}$ reduction in the *S. aureus* population. The antibiofilm action of bronopol and bronidox was attributed to their ability to kill slow- or non-growing bacteria found in biofilms, and both compounds exhibited synergistic antibiofilm effects in combination with established wound-treatment agents.

Conclusions: Bronopol and bronidox kill bacteria regardless of growth state, a property that endows them with broad-spectrum antibiofilm activity. Since this effect is observed at concentrations authorised for use on human skin, these compounds represent promising candidates for the treatment of chronic wounds.

Introduction

Biofilms are spatially-structured communities of microorganisms encased within a self-produced polymeric matrix, and are a feature of >80% of bacterial infections in humans. [1] Infections involving a substantial biofilm component are extremely difficult to treat, since the nature of this structure acts to thwart the very processes that are ordinarily relied upon to achieve resolution of a bacterial infection; the extracellular matrix physically shields the biofilm residents from components of the host's immune system, whilst the slow-or non-growing (SONG) status of biofilm-associated bacteria renders them refractory to killing by most antibacterial drugs. [1,2]

A common biofilm-associated disease that is particularly challenging to manage is the chronic wound. Biofilms are a near universal feature of such wounds, [3] and their presence is associated with persistent inflammation and a failure to heal. [4, 5] It is estimated that over 1 million adults in the UK are affected by chronic wounds, with treatment of this condition costing the NHS £3.2 billion each year. [6] Grave complications are associated with chronic wounds that are not adequately managed and persist; in patients with comorbidities such as diabetes, lower extremity amputation may be required, [7] and the five year mortality following such an intervention stands at 50%. [8] With our ageing population and an increasing incidence of diabetes and obesity, the number of individuals affected by chronic wounds is set to rise. [9, 10] To help address the current difficulties that this condition presents, it will be vital to identify agents that are capable of reducing or eradicating the biofilm burden associated with the wound bed, thereby removing a major impediment to healing.

In recent studies, we have reported several potent antibiofilm compounds with potential for topical treatment of human skin infections involving a biofilm component. [11, 12] Our approach to identifying such agents has involved evaluating the antibiofilm activity of compounds that are already in human use for other purposes, [11, 12] and which are therefore considered safe for topical application. Such a repurposing strategy, which takes as its starting point chemicals with established safety profiles, potentially offers an accelerated route by which new antibiofilm agents could reach the clinic. An important limitation of the antibiofilm compounds we have identified to date is their narrow spectrum of activity, which is

exclusively directed towards Gram-positive bacteria such as *Staphylococcus aureus*. In view of the fact that chronic wound biofilms often comprise multiple bacterial species, including Gram-negative genera, broad-spectrum activity would represent a highly desirable property in a candidate antibiofilm compound for use in wound management.

Here we report the identification and characterization of two related compounds, bronopol (2-Bromo-2-nitro-1,3-propanediol) and bronidox (5-Bromo-5-nitro-1,3-dioxane)(Figure 1), that exhibit broad spectrum antibiofilm activity, and which therefore represent promising candidates for topical reduction/ removal of the chronic wound biofilm.

Materials and Methods

General aspects. S. aureus SH1000 [13, 14] and *Ps. aeruginosa* PAO1 [15] were routinely propagated using cation-adjusted Mueller-Hinton broth (MHB) and agar (MHA) (both from Sigma Aldrich, Poole, UK) at 37°C. Antibiotics, antiseptics and other chemicals were also from Sigma Aldrich, whilst commercial wound treatment agents were from Williams Medical (Rhymney, UK).

Evaluation of antibacterial activity against planktonic cultures. MICs were determined according to the Clinical and Laboratory Standards Institute broth microdilution guidelines. [16] Time-kill studies were performed with both exponential- and non-growing (stationary phase) cultures of *S. aureus* SH1000, essentially as previously described. [12]

Evaluation of antibiofilm activity and mode of action. Minimum Biofilm Eradication Concentrations (MBECs), defined as the lowest concentration of antibacterial agent required to completely sterilize the biofilm, were determined using the Calgary Biofilm Device (CBD; Nunc A/S, Roskilde, Denmark). [17]

The cellulose disk biofilm model [18] was modified to assess the activity of bronopol and bronidox against larger, dual-species biofilms. Briefly, 25mm mixed cellulose disks (Millipore, Watford, UK), preconditioned overnight with 4% human plasma (Sera Laboratories, West Sussex, UK), were inoculated with saturated cultures of *P. aeruginosa* PAO1 (1.8 mL) and *S. aureus* SH1000 (200 µL) and placed on Brain Heart Infusion agar (BHIA). After 48 hours' incubation at 37°C, the disks were transferred to fresh BHIA and challenged either with sterile gauze saturated with bronopol and bronidox at their maximum authorised concentrations (0.1% w/v), or with a commercial wound treatment agent. After a further 24 hours' incubation at 37°C, adherent cells were released from the disks [18] and bacteria enumerated by plating onto MHA (to achieve a total count) and Mannitol Salt Agar (Sigma Aldrich, Poole, UK) (for enumeration of staphylococci). [19]

The antibiofilm mode of action of bronopol and bronidox was investigated essentially as described. [12, 20] Briefly, *S. aureus* SH1000 biofilms were grown in MHB in 96-well microtitre plates, exposed to bronopol and bronidox at 16X MBEC for varying lengths of time, and biofilm matrix material quantified using the fluorescent stain, SyproRuby®(Invitrogen, UK). In parallel experiments, bacteria were recovered from biofilms treated with bronopol and bronidox and enumerated by plating onto MHA. [12]

To detect synergistic interactions between bronopol/ bronidox and established wound agents, a checkerboard assay [21] was employed to determine fractional biofilm eradication concentration (FBEC) indices.

Results and discussion

Ongoing screening of compounds found in personal care products against biofilms of the most prevalent Gram-positive and Gram-negative pathogens occurring in chronic wounds (*S. aureus* and *Ps. aeruginosa*, respectively [22]) identified the related bromine-containing preservative agents bronopol and bronidox (Figure 1) as broad-spectrum antibiofilm agents. Against biofilms of both *Ps. aeruginosa* PAO1 and *S. aureus* SH1000 grown on the Calgary Biofilm Device (CBD), bronidox exhibited MBECs of 128 mg/L, whilst bronopol showed greater potency against *Ps. aeruginosa* biofilms (MBEC of 64 mg/L) compared with *S. aureus* biofilms (MBEC of 256 mg/L). Bronopol and bronidox also demonstrated antibacterial activity against planktonic cultures of *Ps. aeruginosa*, *S. aureus* and a range of other common wound pathogens (*Acinetobacter baumannii, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Klebsiella penumoniae*), with MICs in the range 4-32 mg/L (bronopol) and 8-64 mg/L (bronidox) (Supplementary Table S1).

Whilst the CBD provides a convenient high-throughput approach to assay antibiofilm activity, the biofilms formed are generally small (~1×10^5 cfu) and immature, and likely substantially less robust in the face of antimicrobial challenge compared with those occurring in chronic wounds. With a view to assessing the antibiofilm activity of bronopol and bronidox against biofilms more representative of those found in wounds, we established larger, dual-species biofilms of *S. aureus* and *Ps. aeruginosa* on nitrocellulose disks. [18] These biofilms were challenged with bronopol and bronidox impregnated into sterile gauze at their maximum authorised concentrations (MACs) for safe human use (0.1% [23]). Over 24 hours, both bronopol- and bronidox-containing dressings reduced the *Ps. aeruginosa* population in the biofilm from approximately 10^8 cfu/mL to below the limit of detection and achieved a ~5 $\log_{10}$ reduction in the size of the *S. aureus* population (Figure 2A). We compared the antibiofilm activity of bronopol and bronidox in this biofilm model against commercial wound dressings and wound irrigation solutions; in terms of reducing biofilm viability, bronopol and bronidox substantially outperformed Inadine (povidone iodine; Systagenix), Aquacel Ag+ (silver; Convatec) and Prontosan (PHMB; B. Braun) (Figure 2B).

In previous work to characterize agents capable of eradicating established biofilms of Gram-positive pathogens, we distinguished two mechanistic classes of antibiofilm compound. One class primarily mediates destructuring of the biofilm matrix, [12] whilst the other involves killing

of bacteria within the biofilm, including SONG cells. [11] To establish which mechanistic class bronopol and bronidox belong to, we challenged *S. aureus* biofilms independently with both agents at 16X MBEC and monitored effects on biofilm matrix integrity and bacterial viability. Over a 6-hour challenge, bronopol and bronidox effectively sterilized the bacterial population in the biofilm, with either no (bronidox) or only ~40% (bronopol) reduction in biofilm matrix material (Figure 3), suggesting that these compounds exert their antibiofilm action predominantly by killing bacteria in the biofilm. We subsequently confirmed that bronopol and bronidox are capable of directly killing bacteria - including SONG cells – by performing time-kill studies with these compounds and both actively-growing (exponential phase) and non-growing (stationary phase) planktonic cultures of *S. aureus*. Over a 24-hour period, bronopol and bronidox at 4X MIC exhibited potent bactericidal activity in these experiments, sterilizing both growing and non-growing cultures (Figure 3B, C). By contrast, the bactericidal comparator antibiotic vancomycin demonstrated negligible killing of stationary phase cells. The ability of bronopol and bronidox to kill bacteria regardless of growth state therefore appears to underlie their antibiofilm ability.

In view of the challenges associated with clinical management of biofilm-associated infections, it has been suggested that treatment efficacy might be improved by combining therapeutic approaches. [5, 24, 25] We assessed *in vitro* whether the antibiofilm activity of bronopol and bronidox could be further enhanced by combining them with existing wound treatment agents. In combination with all three established wound agents tested (silver nitrate, chlorhexidine, and cetrimide at ¼ X MBEC), bronopol and bronidox exhibited synergistic activity (FBEC of <0.5) against single-species biofilms of *S. aureus* and *Ps. aeruginosa* in the CBD (Table 1). The most synergistic combination was bronopol with chlorhexidine, yielding FBEC indices of 0.15 (*S. aureus*) and 0.25 (*Ps. aeruginosa*). Thus, bronopol and bronidox might profitably be used in conjunction with established wound treatment agents to further increase their antibiofilm effect.

Finally, we examined the likelihood that resistance to bronopol and bronidox would develop were these agents to be deployed for the treatment of wound infection. *S. aureus* cultures were exposed to a range of sub-inhibitory concentrations of bronopol/ bronidox for 15 days, passaging once a day. No significant increase in MIC was detected for either compound over the course of the passage experiment (*data not shown*), indicating a low potential for

selection of resistance, and implying that the therapeutic potential of these compounds should not rapidly become compromised by resistance.

Conclusions

To date, very few selectively toxic small molecules have been reported that are able to eradicate mature biofilms of both Gram-positive and Gram-negative bacteria. [26] We have described here the ability of the closely-related preservative agents bronopol and bronidox to deliver broad spectrum antibiofilm activity in multiple *in vitro* biofilm models. Since these compounds have a ~40 year history of safe use as components of personal care products, and exert potent antibiofilm effects at concentrations that are authorised for topical use, they represent promising candidates for repurposing in the treatment of chronic wound infection. The observation that their activity against biofilms is potentiated in the presence of established antibacterial wound-treatment agents suggests that bronopol and bronidox might also represent a useful adjunct to existing wound therapies to enhance their efficacy.

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Transparency declarations

None to declare

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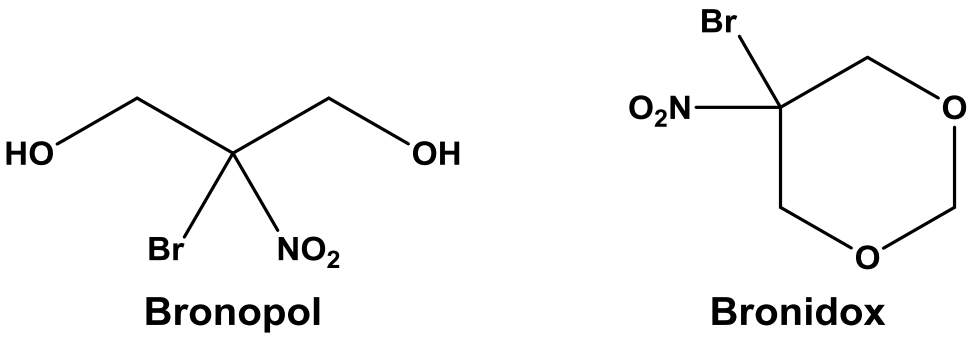


Figure 1. Chemical structures of bronopol and bronidox. Bronidox is the formaldehyde acetal of bronopol.

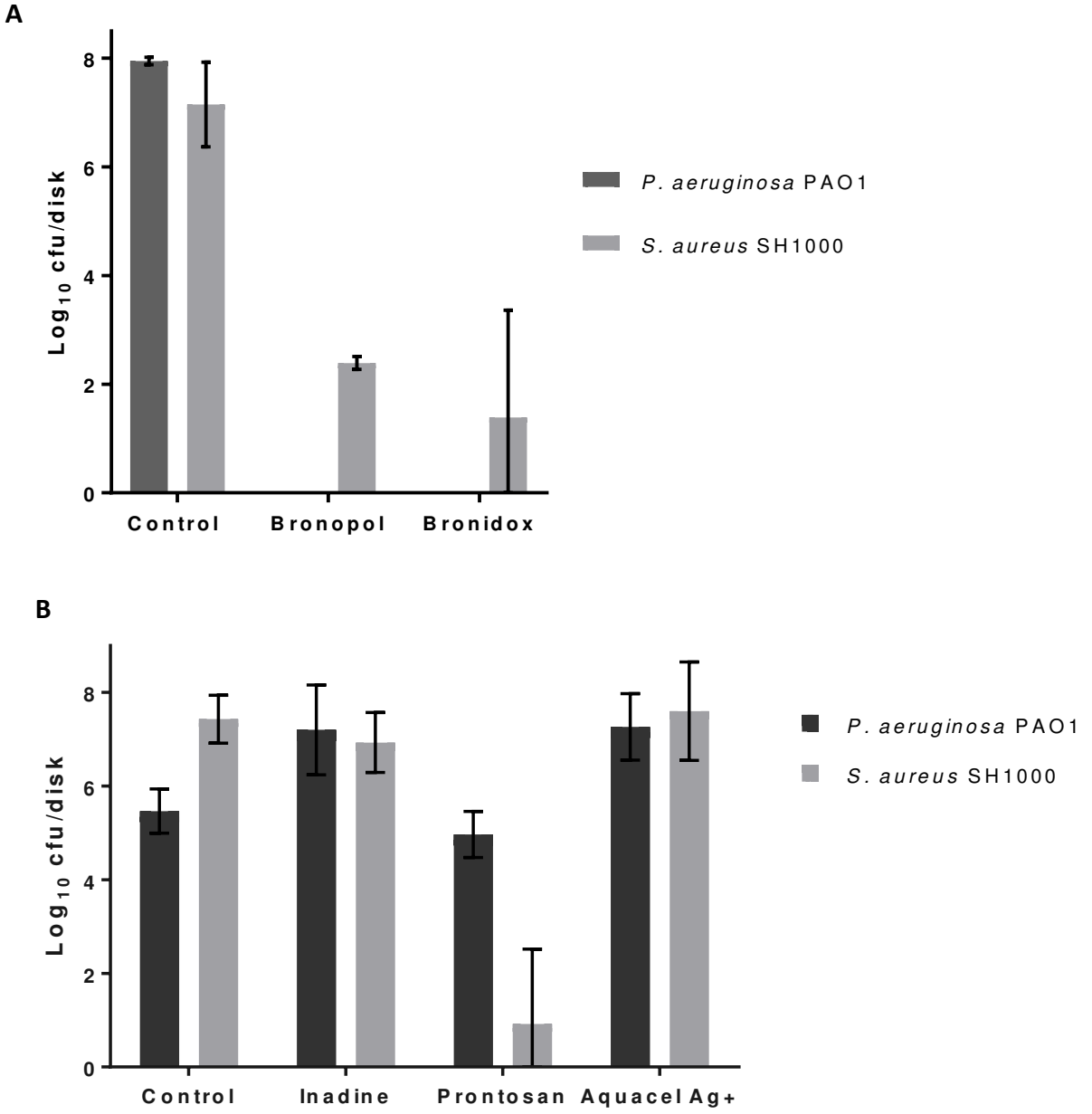
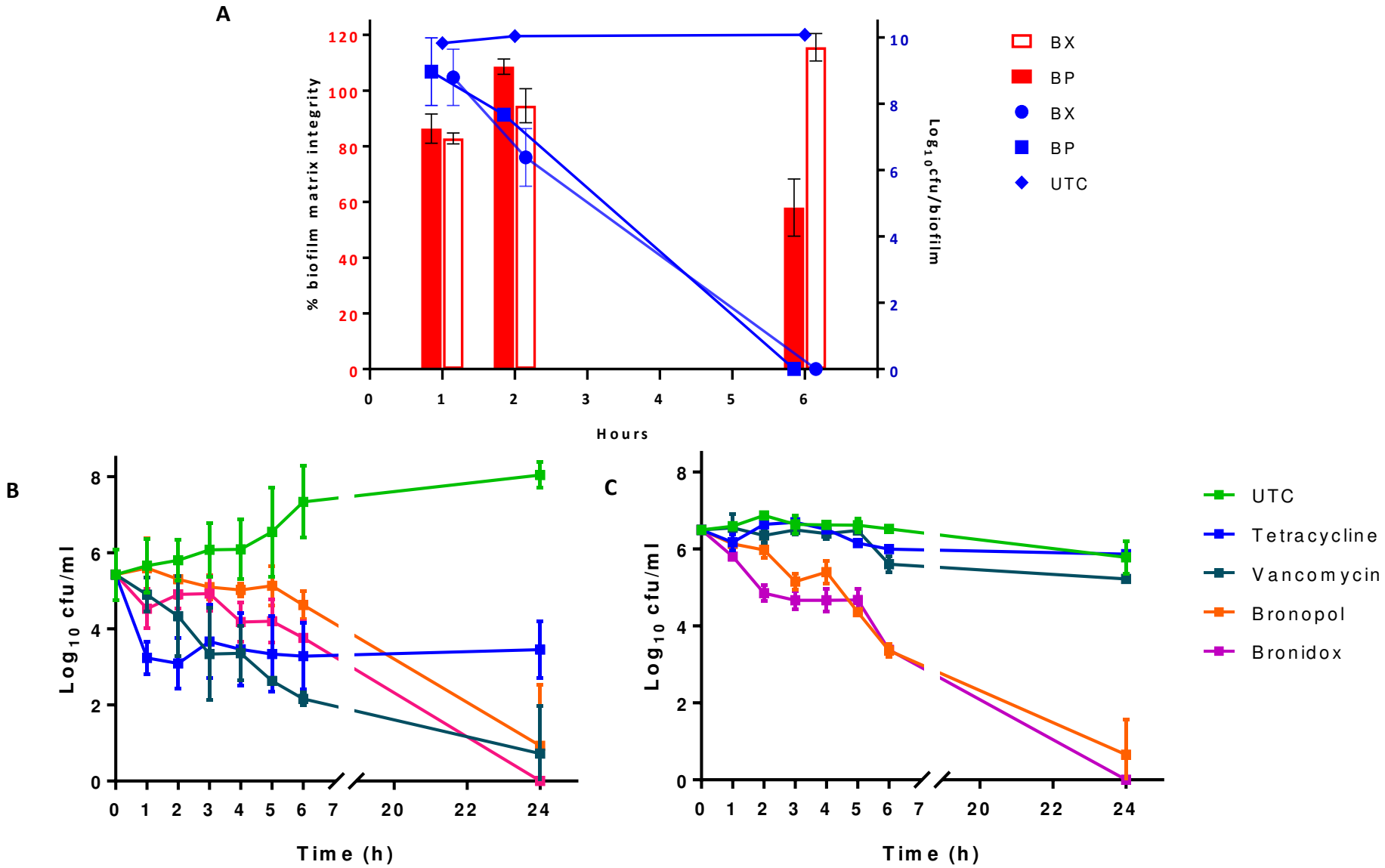


Figure 2. Antibiofilm activity of bronopol and bronidox at their maximum authorised concentrations (0.1% w/v) against a dual-species biofilm comprising *Ps. aeruginosa* and *S. aureus* (A), compared with the antibiofilm activity of wound dressings and irrigation solutions that are currently in clinical use (B). Results are means of at least three independent determinations and error bars show standard deviations.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UTC
Tetracycline
Vancomycin
Bronopol
Bronidox

Figure 3. Antibacterial and antibiofilm properties of bronopol and bronidox. (A) Effect of the compounds at 16X MBEC on cell viability and matrix integrity of the *S. aureus* SH1000 biofilm over six hours. Red bars represent % matrix integrity relative to the untreated control, whilst blue data points show Log_{10} cfu/ biofilm. (B) Viability of *S. aureus* SH1000 exponential phase cultures following exposure to bronopol, bronidox and comparator agents at 4X MIC. (C) Viability of *S. aureus* SH1000 stationary phase cultures following exposure to bronopol, bronidox and comparator agents at 4X MIC. Results are means of at least three independent determinations and error bars show standard deviations.

		Silver Nitrate	Cetrimide	Chlorhexidine
Bronidox	*S. aureus*	0.25	0.5	0.5
	Ps. aeruginosa	0.5	0.375	0.5
Bronopol	*S. aureus*	0.5	0.25	0.15
	Ps. aeruginosa	0.25	0.375	0.25

Table 1. Fractional biofilm eradication (FBEC) indices for bronopol and bronidox in combination with established wound agents against single species biofilms of *S. aureus* and *Ps. aeruginosa.* An FBEC index of ≤ 0.5 is indicative of synergism. Each FBEC index was determined multiple times, and representative values are shown.

Supplementary Table

Strain	Reference/ source	MIC (mg/L)	
		Bronopol	**Bronidox**
***Acinetobacter baumannii* 033**	Clinical isolate	4	8
***Acinetobacter baumannii* 097**	Clinical isolate	4	8
***Enterobacter cloacae* 052**	Clinical isolate	16	16
***Enterobacter cloacae* 067**	Clinical isolate	8	16
***Enterococcus faecalis* ATCC 29212**	ATCC	32	8
***Escherichia coli* 32**	Clinical isolate	8	16
***Escherichia coli* 52**	Clinical isolate	8	16
***Klebsiella pneumoniae* 052**	Clinical isolate	16	16
***Klebsiella pneumoniae* 062**	Clinical isolate	16	16
***Pseudomonas aeruginosa* NCTC 10332**	NCTC	8	64
***Pseudomonas aeruginosa* PA14**	[1]	16	64
***Staphylococcus aureus* UAMS-1**	[2]	16	8
***Staphylococcus aureus* USA300 JE2**	[3]	16	8
***Staphylococcus epidermidis* RP62A**	ATCC 35984	8	8

Table S1. Antibacterial activity of bronopol and bronidox against common pathogens. MICs were determined on a minimum of three independent occasions to ensure reproducibility.

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