In vivo activity of anprocide alone, and *in vitro* activity in combination with conventional antibiotics against *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms

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The alarming spread of multiple drug resistance in *Staphylococcus aureus*, combined with the frequent occurrence of *S. aureus* and *Staphylococcus epidermidis* in biofilm-type infections, indicates a growing need for new therapies. The experimental steroidal amide anprocide [3 β -acetoxy-17 β -(L-prolyl)amino-5 α -androstane] significantly reduced c.f.u. ml⁻¹ per suture ($P < 0.0001$) in a murine model of topical *S. aureus* infection. In checkerboard assays with planktonic-grown *S. aureus* and *S. epidermidis*, anprocide was synergistic with bacitracin, oxacillin, clindamycin or ceftriaxone. Anprocide was also synergistic in combination with bacitracin or oxacillin against some isolates of biofilm-grown *S. aureus* and *S. epidermidis*.

INTRODUCTION

Antibiotic-resistant *Staphylococcus aureus* is a major public health problem. Meticillin-resistant *S. aureus* (MRSA) is increasing in both nosocomial and community-acquired infections (Lipsky *et al.*, 2007). A 2004 National Nosocomial Infections Surveillance System Report (NNIS System, 2004) indicated that nearly 60 % of intensive care unit *S. aureus* isolates were resistant to meticillin, oxacillin or nafcillin. There were 94 360 invasive MRSA infections in the US in 2005, which were associated with death in 18 650 cases (Klevens *et al.*, 2007).

In the US, one million nosocomial infections each year are related to infections caused by biofilms on implanted devices (Schierholz & Beuth, 2001). Mortality for septicaemias associated with vascular devices ranges from 20 to 40 % (Stamm, 1978), and intravenous catheters are the most common cause of nosocomial septicaemia (Maki, 1992). *S. aureus* and *Staphylococcus epidermidis* are the most common infectious agents associated with foreign device infections (Moreillon & Que, 2004; Waldvogel & Bisno, 2000; Zimmerli *et al.*, 2004), and are found in biofilms in a wide range of other diseases, including endocarditis and osteomyelitis (Donlan & Costerton,

2002). Bacterial biofilms are generally quite resistant to antibiotic treatment (Donlan & Costerton, 2002).

The steroidal amide anprocide [3 β -acetoxy-17 β -(L-prolyl)-amino-5 α -androstane] was synthesized in 1967 (Pettit *et al.*, 1967). In 2000, its activity against 179 Gram-positive clinical isolates was reported (Pettit *et al.*, 2000). Anprocide was bactericidal for the majority of MRSA, vancomycin-resistant *Enterococcus* spp., penicillin-resistant *Streptococcus pneumoniae*, invasive *Streptococcus pneumoniae*, group A *Streptococcus* and *Rhodococcus* spp. isolates, and has activity against biofilms of *S. epidermidis* ATCC 35984 (Pettit *et al.*, 2005). The maximum tolerated dose in mice is >400 mg kg⁻¹ i.p. (Pettit *et al.*, 2000). In the present report, the efficacy of anprocide in a murine model of topical *S. aureus* infection was investigated, as were possible synergistic interactions with approved antibiotics against planktonic- and biofilm-grown *S. aureus* and *S. epidermidis*.

METHODS

Murine model. The animal study was performed at the Southern Research Institute, Frederick, MD, USA, with strict adherence to ethical standards. The mouse surgical wound infected with *S. aureus* model was used (Gisby & Bryant, 2000). The murine model of skin wound infection represents the secondary skin infections that may occur following damage by accidental trauma, surgery and burns, or as a result of superinfection of an underlying skin disease (Gisby & Bryant, 2000). Mouse virulent *S. aureus* ATCC 14154 was grown for 12 h at 38 °C in brain heart infusion broth. Just prior to surgery, sterile silk sutures were cut into 1 cm lengths, soaked in the undiluted

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Abbreviations: AB, Alamar blue; FIC, fractional inhibitory concentration; MRSA, meticillin-resistant *S. aureus*.

broth culture for 30 min and excess liquid was removed by blotting with sterile filter paper. Fifty HSD:ICR mice (five groups, 10 animals each) were anaesthetized and the skin on their backs was prepared for aseptic surgery by clipping, cleansing with iodine scrub and rinsing with alcohol. Mice were kept warm during surgery on a recirculating hot water blanket. Sutures were placed just under the skin on the dorsum of each mouse using sterile instruments. Sutures were knotted in the subcutaneous tissue to keep them in place. A half-thickness skin wound was made over the sutures using the side of an 18 gauge needle as a scalpel. Treatment was initiated 4 h after surgery. Anprocide was reconstituted in MeOH/saline and placed over the wound site twice daily at 12.5 or 25 mg kg⁻¹ per day. Enrofloxacin (Baytril) was given subcutaneously once a day at 10 mg kg⁻¹ per day. After 7 days of treatment, mice were euthanized with i.p. pentobarbital solution. Two groups of mice were untreated and served as controls: one had infected sutures and the other sterile sutures. Sutures were aseptically removed, vortexed in PBS, and *Staphylococcus* c.f.u. per suture were determined by dilution plating. The Mann-Whitney nonparametric *t*-test was used to determine statistical significance.

Planktonic chequerboard assay. Anprocide was tested alone and in combination with seven antibiotics against clinical isolates and type strains of *S. aureus* and *S. epidermidis*. Nonduplicate clinical isolates (from sterile sites) and antibiotic resistance information were obtained from the Arizona Department of Health Services and Phoenix Children's Hospital (identified to species level using the Vitek II system). Reference strains were obtained from the American Type Culture Collection (ATCC). For *S. aureus* planktonic chequerboard assays, nine methicillin-resistant clinical isolates and ATCC 29213 were used. For *S. epidermidis* planktonic chequerboard assays, seven clinical isolates and ATCC 35984, ATCC 49461 and ATCC 12228 were used. Bacitracin, vancomycin, nitrofurazone, enrofloxacin and clindamycin were obtained from ICN. Ceftriaxone and oxacillin were from Sigma. Nitrofurazone, enrofloxacin and clindamycin were dissolved in sterile DMSO and the remaining antibiotics were dissolved in sterile H₂O. Anprocide was synthesized in our laboratory as previously described (Pettit *et al.*, 1967), and prior to each assay, dissolved in MeOH. Planktonic susceptibility testing of *S. aureus* and *S. epidermidis* was performed by the reference broth microdilution assay outlined by the Clinical and Laboratory Standards Institute (CLSI) (NCCLS, 2000), using round-bottom, polystyrene, tissue culture treated microtitre plates and cation-adjusted Mueller-Hinton II broth. Turbidity controls containing the highest concentrations of DMSO or MeOH were identical in appearance to turbidity controls lacking solvent. The MIC was defined as the lowest concentration of drug that inhibited all visible growth of the test organism (optically clear). Planktonic chequerboard assays were performed using a well-established method (Eliopoulos & Moellering, 1996). Drug interaction was classified as synergistic, indifferent or antagonistic on the basis of the fractional inhibitory concentration (FIC) index. The FIC index is the sum of the FIC of each of the drugs, which in turn is defined as the MIC of each drug when used in combination divided by the MIC of the drug when used alone. The interaction was defined as synergistic if the FIC index was less than or equal to 0.5, indifferent if the FIC index was greater than 0.5 and less than or equal to 4.0, and antagonistic if the FIC index was greater than 4.0 (Barchiesi *et al.*, 2004).

Biofilm chequerboard assay. Four biofilm-positive isolates (confirmed with Congo red-stained phase-contrast micrographs) were used for each species (Pettit *et al.*, 2005; R. K. Pettit, C. A. Weber & G. R. Pettit, unpublished): *S. aureus* ATCC 29213 and clinical isolates (from sterile sites) 520009, 520016 and 520020 (Arizona Department of Health Services, Phoenix, AZ, USA), and *S. epidermidis* ATCC 12228, ATCC 35984 (RP62A) and two clinical isolates, S67166 (central venous catheter) and X64787 (endotracheal tube) (Phoenix

Children's Hospital, Phoenix, AZ, USA). *S. epidermidis* ATCC 12228 is sometimes referred to as biofilm-negative, perhaps because the biofilms formed are not as robust as those of biofilm-positive strain ATCC 35984. However, *S. epidermidis* ATCC 12228 has been shown to produce biofilms in many studies (Greco *et al.*, 2007; Henriques *et al.*, 2005; Okajima *et al.*, 2006; Pettit *et al.*, 2005), and may provide another example of biofilm formation via *ica*-independent means (Greco *et al.*, 2007; Kogan *et al.*, 2006; Rohde *et al.*, 2005). The method used to prepare and drug treat biofilms was exactly as previously described (Pettit *et al.*, 2005; R. K. Pettit, C. A. Weber & G. R. Pettit, unpublished), except that drug combinations instead of individual drugs were used. Addition of Alamar blue (AB) and calculation of per cent reduction was exactly as previously described (Pettit *et al.*, 2005). FIC indices were calculated as described above.

RESULTS AND DISCUSSION

With the continuing increase in resistance of *Staphylococcus* to available antimicrobials, there is a pressing need for novel compounds and effective drug combinations. In a murine surgical wound model, anprocide caused a 5000–10 000-fold reduction in c.f.u. per suture (Table 1), with no weight loss or other adverse clinical effects noted. There was some difficulty in keeping the anprocide in contact with the wound. As such, anprocide may be even more efficacious when in an ointment or cream formulation or on an adhesive bandage.

Antimicrobial combinations may increase the rate of microbial killing, shorten the duration of therapy, avoid the emergence of drug resistance, expand the spectrum of activity and decrease drug-related toxicities by permitting use of lower doses (Barchiesi *et al.*, 2001). Anprocide was synergistic in combination with bacitracin, oxacillin, clindamycin and ceftriaxone for some isolates of *S. aureus*, and synergistic with bacitracin, oxacillin, clindamycin, ceftriaxone and vancomycin for some isolates of *S. epidermidis* (Table 2). None of the tested combinations were antagonistic.

S. aureus and *S. epidermidis* are the most common infectious agents associated with foreign device infections (Moreillon & Que, 2004; Waldvogel & Bisno, 2000; Zimmerli *et al.*, 2004), and are found in biofilms in a wide range of other diseases, including endocarditis and

Table 1. Efficacy of anprocide in a murine model of topical *S. aureus* infection

Treatment	Dose (mg kg ⁻¹ per day)	Log c.f.u. per suture (mean log ± SD)	P-value vs no therapy
Anprocide	12.5	2.90 ± 0.33	<0.0001
Anprocide	25	2.66 ± 0.15	<0.0001
Baytril	10	1.33 ± 0.86	<0.0001
Untreated		6.54 ± 0.37	
No infection, no treatment		0	

Table 2. *In vitro* activities of anprocide in combination with conventional antibiotics against planktonic-grown *S. aureus* and *S. epidermidis*

Drug used in combination with anprocide	Percentage of isolates where results were synergistic (FIC index ≤ 0.5)*	
	<i>S. aureus</i> (<i>n</i> =10 isolates)	<i>S. epidermidis</i> (<i>n</i> =10 isolates)
Nitrofurazone	0	0
Bacitracin	40	30
Oxacillin	10	50
Clindamycin	90	10
Ceftriaxone	40	10
Enrofloxacin	0	0
Vancomycin	0	10

*Results for remaining strains were indifferent (FIC index >0.5 –4.0).

osteomyelitis (Donlan & Costerton, 2002). Two drug combinations that were particularly effective against either *S. aureus* or *S. epidermidis* grown planktonically were evaluated against biofilms using the microplate AB biofilm susceptibility assay (Pettit *et al.*, 2005; R. K. Pettit, C. A. Weber & G. R. Pettit, unpublished). The AB method has excellent correlation with c.f.u. ml⁻¹ (Pettit *et al.*, 2005); as such, the AB biofilm susceptibility assay provides a simple way of assessing biofilm viability.

When *S. aureus* was grown planktonically, the combination of anprocide and bacitracin was synergistic for clinical isolate 520020 and indifferent for ATCC 29213 and clinical isolates 520009 and 520016. When *S. epidermidis* was grown planktonically, the combination of anprocide and bacitracin was synergistic for ATCC 35984 and clinical isolate X64787, and indifferent for ATCC 12228 and clinical isolate S67166. When grown as biofilms, the combination of anprocide and bacitracin was synergistic for all four isolates of *S. epidermidis* and 2/4 (except for one replicate with ATCC 29213) isolates of *S. aureus* (Table 3). The combination of anprocide and oxacillin was synergistic

for *S. aureus* clinical isolate 520016 when grown planktonically, and indifferent for ATCC 29213 and clinical isolates 520009 and 520020 when grown planktonically. The combination of anprocide and oxacillin was synergistic for *S. epidermidis* clinical isolates S67166 and X64787 when grown planktonically, and indifferent for ATCC 12228 and ATCC 35984 when grown planktonically. For biofilms of *S. aureus*, the combination of anprocide and oxacillin was indifferent (except for one replicate with clinical isolate 520016) (Table 3). For biofilms of *S. epidermidis*, the combination of anprocide and oxacillin was synergistic in just over half of the replicate tests (Table 3). Although clindamycin and anprocide were synergistic for 9/10 *S. aureus* isolates grown planktonically (Table 2), the combination was indifferent when evaluated against the four isolates of *S. aureus* grown as biofilms (data not shown).

As demonstrated, the AB biofilm susceptibility method works well for drug combination studies as well as single drug studies using *S. aureus* and *S. epidermidis*. We previously reported (Pettit *et al.*, 2005) that none of eight FDA-approved antibiotics inhibited *S. epidermidis* ATCC 35984 biofilms at $<512 \mu\text{g ml}^{-1}$ [most, including bacitracin and oxacillin, had minimum biofilm inhibitory concentrations (MBICs) $>4096 \mu\text{g ml}^{-1}$]. As shown in the current study, there was a dramatic reduction in the MBICs of bacitracin and oxacillin in the presence of anprocide (e.g. for *S. epidermidis*, the MBIC of bacitracin = $16\,384 \mu\text{g ml}^{-1}$, while the MBIC of bacitracin + anprocide = $4\text{--}8 \mu\text{g ml}^{-1}$), suggesting that these drugs have different mechanisms/targets which are mutually beneficial to the antibacterial action. The mechanism by which one drug enhances the activity of the other was not investigated here. Given that anprocide is a steroid, it may be membrane-active, and could facilitate the uptake of bacitracin and oxacillin.

Anprocide should be pursued as a possible treatment for Gram-positive infections. Anprocide is unrelated to systemically administered agents, well tolerated at high doses (Pettit *et al.*, 2000), has a spontaneous mutation rate in the expected range for a compound in early preclinical

Table 3. Effect of anprocide in combination with bacitracin or oxacillin on *S. aureus* and *S. epidermidis* biofilms

Strain	Range of FIC index anprocide/bacitracin	No. replicates exhibiting synergy*/no. replicates	Range of FIC index anprocide/oxacillin	No. replicates exhibiting synergy*/no. replicates
<i>S. aureus</i> ATCC 29213	0.14–1.00	2/3	2.00–3.00	0/2
<i>S. aureus</i> 520009	0.14–0.15	2/2	2.00	0/2
<i>S. aureus</i> 520016	0.51–0.56	0/2	0.37–0.53	1/2
<i>S. aureus</i> 520020	2.00	0/2	2.00	0/2
<i>S. epidermidis</i> ATCC 35984	0.25–0.50	4/4	0.50–0.53	2/4
<i>S. epidermidis</i> ATCC 12228	0.09–0.50	4/4	0.28–1.00	3/4
<i>S. epidermidis</i> S67166	0.25–0.50	4/4	0.50–1.00	2/4
<i>S. epidermidis</i> X64787	0.25–0.50	4/4	0.50–1.00	1/2

*Remaining replicates exhibited indifference.

development (Pettit *et al.*, 2000), is active at pH 6–8 (Pettit *et al.*, 2000), bactericidal (Pettit *et al.*, 2000), and has now been shown to have topical *in vivo* activity, and *in vitro* activity against biofilms alone (Pettit *et al.*, 2005), and in combination with bacitracin.

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