The rapid rise in antimicrobial resistance in bacteria has generated an increased demand for the development of novel therapies to treat contemporary infections, especially those caused by methicillin-resistant Staphylococcus aureus (MRSA). However, antimicrobial development has been largely abandoned by the pharmaceutical industry. We recently isolated the previously described thiopeptide antibiotic nosiheptide from a marine actinomycete strain and evaluated its activity against contemporary clinically relevant bacterial pathogens. Nosiheptide exhibited extremely potent activity against all contemporary MRSA strains tested including multiple drug-resistant clinical isolates, with MIC values ≤ 0.25 mg l⁻¹. Nosiheptide was also highly active against Enterococcus spp. and the contemporary hypervirulent BI/NAP1/027 strain of Clostridium difficile but was inactive against most Gram-negative strains tested. Time-kill analysis revealed nosiheptide to be rapidly bactericidal against MRSA in 4–44 min, found to be non-cytotoxic against mammalian cells at > > 100 × MIC, and its anti-MRSA activity was not inhibited by 20% human serum. Notably, nosiheptide exhibited a significantly prolonged post-antibiotic effect against both healthcare- and community-associated MRSA compared with vancomycin. Nosiheptide also demonstrated in vivo activity in a murine model of MRSA infection, and therefore represents a promising antibiotic for the treatment of serious infections caused by contemporary strains of MRSA.

**Keywords:** contemporary MRSA; marine actinomycete; nosiheptide; thiopeptide

**INTRODUCTION**

Multidrug resistant hospital-associated (HA-) and highly virulent community-associated (CA-) methicillin-resistant *Staphylococcus aureus* (MRSA) have shown increased tolerance or resistance in recent years to vancomycin, linezolid and daptomycin with accompanying reduction in clinical antimicrobial efficacy. The development of novel agents without cross-resistance to current antimicrobials against MRSA infections is desperately needed.¹

In our screen of marine-derived actinomycete extract libraries for anti-MRSA activity, we identified a potent fraction derived from strain CNT-373, a *Streptomyces* species isolated from a marine sediment collected in Fiji. The 1221.16-mol wt active component was purified and identified by NMR as the thiopeptide antibiotic nosiheptide. Thiopeptide antibiotics encompass a large family of compounds that include thiostrepton and nocathiacin and are comprised of sulfur- and nitrogen-rich heterocycles linked to non-natural amino acids.²,³ Nosiheptide, also referred to historically as multhiomycin, was originally isolated in 1970 and shown to be structurally similar to thiostrepton.⁴ Several thiopeptide antibiotics including nosiheptide block protein synthesis by the inhibition of elongation factors Tu and G.⁶ Historically, nosiheptide has been used as a growth-promoting additive in animal feed⁷ but was never developed further as a human therapeutic.

Although the discovery of new chemical scaffolds provides the potential for novel drugs with new mechanisms of action, the re-investigation of previously discovered antibacterial scaffolds also provides a valuable source of compounds with therapeutic potential. Surprisingly, despite the structural identification and description of Gram-positive antibacterial activity, no detailed characterization of nosiheptide activity against contemporary drug-resistant strains such as MRSA has been undertaken. Here, we investigate nosiheptide activity *in vitro* against a panel of contemporary MRSA and other Gram-positive clinical isolates. MRSA killing kinetics and post-antibiotic effects (PAE) are characterized, and nosiheptide *in vivo*
activity is demonstrated. Our results indicate that 're-discovered' natural product antibiotics may harbor meaningful therapeutic activity against contemporary multidrug resistant pathogens, and therefore warrant further consideration and preclinical development in hopes of expanding our current limited pharmacological arsenal.

MATERIALS AND METHODS

Strain isolation and identification

Strain CNT-373 was isolated from a marine sediment sample collected at a depth of 5 m off Nacula Island, Fiji. The sediment was dried overnight in a laminar flow hood and then stamped on agar plates containing medium A1 (10 g starch, 4 g peptone, 16 g agar and 1 l seawater) supplemented with 100 mg l\(^{-1}\) cyclohexamide to reduce fungal growth. The strain was identified as a *Streptomyces* sp. based on 16S rRNA gene sequence analysis (http://eztaxon-e.ezbiocloud.net/ezt_ideny) and shares greatest similarity (98.3%) with the type strain *Streptomyces althioticus*. This low level of sequence identity suggests it may be a new species. The 16S rRNA sequence has been deposited in GenBank under accession number JQ946086.

Fermentation and extraction

A 2-ml-frozen glycerol stock of strain CNT-373 was used to inoculate 25 ml of liquid A1 medium and shaken at 230 r.p.m. and 27 °C. After 5 days, the seed culture was used to inoculate a 1-l culture in growth medium A1bfe (10 g starch, 4 g yeast extract, 2 g peptone, 16 g agar and 1 l seawater) supplemented with 1 g CaCO\(_3\), 100 mg KBr and 40 mg Fe \(_2\)(SO\(_4\))\(_3\) \(_{10}\)H\(_2\)O. After 3 days of shaking, 25-ml aliquots were used to inoculate 18 Fernbach flasks (2.8 l) each containing 1 l medium A1bfe \(_{10}\)/C\(_0\) \(_{15}\) \(_{500}\) ml). The combined ethyl acetate extracts were dried over anhydrous sodium sulfate, decanted and concentrated to yield 2.4 g of crude material.

Isolation of nosiheptide

The crude extract was dissolved in methanol and dichloromethane, adsorbed onto silica gel (2.5 g), and fractionated on a short column of silica gel (2.5 cm, 4H\(_2\)O). After 3 days of shaking, 25-ml aliquots were used to inoculate 18 Fernbach flasks (2.8 l) each containing 1 l medium A1bfe \(_{10}\)/C\(_0\) \(_{15}\) \(_{500}\) ml). The combined ethyl acetate extracts were dried over anhydrous sodium sulfate, decanted and concentrated to dryness to yield 2.4 g of crude material.

Spectroscopic analysis of nosiheptide

\(^1\)H, \(^{13}\)C and 2D NMR spectroscopic data were obtained on a Varian Inova 500-MHz spectrometer in a solvent mixture of methanol-d\(_4\) and CDCl\(_3\) (3:1) to facilitate solubility. Offline processing was conducted using topspin NMR software by Bruker BioSpin 2011 (INMR, http://www.inmr.net). The NMR data (Table 1) are in good agreement with previously published NMR data for nosiheptide,\(^8\) with the resultant nosiheptide structure shown (Figure 1). High-resolution ESI-TOF mass spectra were provided by the MS facility at the Department of Chemistry and Biochemistry at the University of California San Diego, CA: HR-ESI-TOF-MS [M + H]\(^+\) m/z 1222.1565 (calcd for C\(_{31}\)H\(_{36}\)N\(_{10}\)O\(_{8}\)S\(_{2}\), 1222.1551, \(\Delta\)1.13 p.p.m.). Low resolution LC-MS data were measured using a Hewlett-Packard HP1100 integrated LC/MS system with a reversed-phase C\(_18\) column (Phenomenex Luna, 4.6 mm \(\times\) 100 mm, 5 mm, Phenomenex, Torrance, CA, USA) at a flow rate of 0.7 ml min\(^{-1}\).

Bacterial strains and susceptibility testing

Details on the strains used in this study are in Table 2. MRSA Sanger 252 USA 200 strain was obtained through the Network of Antimicrobial Resistance in *S. aureus* (NARSA) program supported under NIAID/NIH contract no. HHSN272200700055C. Susceptibility testing was performed in duplicate using cation-adjusted Mueller–Hinton broth (CA-MHB) and Mueller–Hinton agar according to Clinical and Laboratory Standards Institute methods.\(^6\) The MIC for *Clostridium difficile* was determined by the agar dilution reference method according to CLSI guidelines.\(^7,8,11\) Susceptibility of nosiheptide against MRSA strain TCH1316 was also determined in the presence of 20% activated-pooled human serum (freshly collected from consenting healthy donors) and 80% MHB.

Table 1 \(^1\)H and \(^{13}\)C NMR spectral data of nosiheptide (δ in p.p.m., \(J\) in Hz)

|     | 1H | 13C | 2D |
|-----|----|-----|-----|
| Ind CO | 180.69 | But 3 | 128.14 | 6.32 \((q, J = 6.84)\) |
| Glu CO | 173.52 | Pyr 4 | 127.17 | 7.64 \((s)\) |
| Thrz(3) | 170.18 | Thrz(5) | 126.52 | 8.28 \((s)\) |
| Thrz(4) | 169.44 | Thrz(1) | 125.82 | 8.47 \((s)\) |
| Thrz(5) | 168.83 | Ind 6 | 125.28 | 7.41 \((t, J = 7.57)\) |
| Thrz(2) | 166.41 | Ind 3a | 124.36 |
| Deala CO | 165.99 | Thrz(2) | 123.91 | 7.91 \((s)\) |
| Thrz(1) | 164.85 | Ind 5 | 122.24 | 7.12 \((d, J = 6.95)\) |
| Thrz(3) | 161.43 | Thrz(4) | 120.38 | 7.66 \((s)\) |
| Thrz(2) | 160.84 | Ind 3 | 118.90 |
| Thrz(1) | 160.57 | Ind 7 | 115.31 | 7.79 \((d, J = 7.04)\) |
| Thrz(5) | 158.59 | Deala 3 | 104.03 | 6.60 \((s, J = 1.89)\), 5.58 \((Z, d, J = 1.89)\) |
| Thrz(4) | 155.00 | Thr 3 | 67.45 | 4.04 \((m)\) |
| Pyr 3 | 150.30 | Glu 4 | 67.18 | 4.14 \((d, J = 11.54)\) |
| Thrz(1) | 149.81 | Ind 4' | 66.60 | 5.85 \((d, J = 11.25)\), 5.02 \((d, Br, J = 10.79)\) |
| Thrz(5) | 149.76 | Thr 2 | 56.75 | 4.36 \((d, J = 7.79)\) |
| Thrz(3) | 148.45 | Cys 2 | 50.17 | 5.98 \((d, Br = 9.00)\) |
| Thrz(2) | 147.86 | Glu 2 | 45.85 | 5.76 \((t, J = 10.19)\) |
| Pyr 6 | 143.99 | Glu 3 | 36.72 | 2.35 \((s, 1.80 \(R \(m)\)\) |
| Ind 7a | 137.44 | Cys 3 | 29.71 | 3.79 \((dd, J = 3.15, 12.06)\), 3.71 \((dd, J = 4.50, 12.00)\) |
| Pyr 2 | 134.46 | Thr CH\(_3\) | 17.52 | 0.95 \((s)\) |
| Deala 2 | 132.94 | But CH\(_3\) | 14.58 | 1.68 \((d, J = 7.00)\) |
| Ind 2a | 130.64 | Ind CH\(_3\) | 11.96 | 2.54 \((s)\) |
| Pyr 5 | 130.44 | Ind NH | 10.83 \((s, Br)\) |
| But 2 | 129.41 | Deala NH | 9.99 \((s)\) |
| Ind 4 | 128.73 | But NH | 9.76 \((s)\) |
| Glu NH | 7.97 \((d, Br, J = 7.29)\) |
| Glu OH | not observed |
| Cys NH | 7.82 \((s)\) |
| Thr NH | 7.50 \((s)\) |
| Thr OH | 7.48 \((s)\) |

Figure 1 Structure of marine-derived nosiheptide.\(^8\) A full color version of this figure is available at The Journal of Antibiotics journal online.

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by adding resazurin and assessing the conversion to resorufin exactly as previously described.12 This method provides a visual readout of color change from the blue indicator resazurin to the pink resorufin, a sign of bacterial growth.

**Time-kill studies**

Time-kill studies were done essentially as previously described in this laboratory.13 Briefly, nosiheptide was added at multiples of the MIC in CA-MHB. MRSA strains or vancomycin-resistant *Enterococcus faecium* were added at a starting inoculum of 5 × 10^5 cfu ml^-1, and the cultures were incubated in a 37 °C shaking incubator (New Brunswick Scientific, Enfield, CT, USA). Samples were taken at specified timepoints for enumeration of viable bacteria by plating serial dilutions on T odd–Hewitt agar plates.

**Post-antibiotic effect**

PAE was assessed using the viable plate count method as previously described.13 For these studies, either nosiheptide or vancomycin was added at 10 × their respective MICs to 14 ml Falcon tubes containing CA-MHB. CA-MRSA strain TCH1516 or HA-MRSA strain Sanger 252 was added at 5 × 10^5 cfu ml^-1, and the tubes were incubated under shaking conditions at 37 °C for 1 h. The bacteria were then pelleted and washed in 4 ml of antibiotic-free CA-MHB. The pellets were resuspended in 4 ml CA-MHB and incubated at 37 °C in a shaking incubator. Surviving bacteria were assessed at specified timepoints as for the time-kill assay. The PAE was determined as previously described.13,14

**Mammalian cell cytotoxicity**

Mammalian cell cytotoxicity was assessed as previously described.13 Briefly, 2 × 10^4 HeLa cells (American Type Culture Collection no. CCL-2, ATCC, Manassas, VA, USA) were seeded per well of sterile 96-well tissue culture-treated plates (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA). After 24 h, the medium (RPMI containing 10% heat-inactivated fetal bovine serum) was replaced with fresh medium containing increasing concentrations of nosiheptide up to 128 mg l^-1, and the plates were incubated at 37 °C in 5% CO₂. Cell viability was assayed at 72 h by measuring the reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) using the CellTiter 96 Aqueous nonradioactive cell proliferation assay according to the manufacturer's instructions (Promega, Madison, WI, USA).

**In vivo testing**

A murine model of i.p. infection was used to test *in vivo* efficacy of nosiheptide.15 Eight-week-old female CD1 mice (Charles River, Wilmington, MA, USA) were injected i.p. with 1–2 × 10^6 cfu of the HA-MRSA strain Sanger 252 in 4% hog gastric mucin. The mice were treated with nosiheptide (20 mg kg^-1) i.p. at 1 and 8 h after bacterial inoculation and were monitored for mortality and clinical status twice daily thereafter for 5 days. Moribund mice were humanely killed as were mice at the end of the study. These experiments were reviewed and approved by the Animal Subjects Committee of UCSD.

### Table 2 Nosiheptide MIC

| Strain            | Details                                      | MIC (mg l^-1) |
|-------------------|----------------------------------------------|---------------|
| TCH1516 USA 300 CA-MRSA (ATCC) | 0.06 (0.06)^a                            |               |
| Sanger 252 USA 200 HA-MRSA (NARSA) | 0.03                                         |               |
| ATCC 33591 HA-MRSA | 0.06                                         |               |
| A5937 Bloodstream MRSA, progenitor to A5940 | 0.125                                       |               |
| A5940 VISA Trait  | 0.125                                       |               |
| A6300 VISA Trait  | 0.125                                       |               |
| A6298 VISA Trait  | 0.125                                       |               |
| SA853 Progenitor to Si853b | 0.125                                       |               |
| SA853b Progenitor to Si853b | 0.125                                       |               |
| HIPS836 N J VISA | 0.125                                       |               |
| VRSA PA vanA vancomycin-resistant *S. aureus* | 0.06                                       |               |
| VRSA Mi vanA vancomycin-resistant *S. aureus* | 0.125                                       |               |
| A781 Progenitor to A7819, A7819erm | 0.06                                       |               |
| A781LineR Progenitor to SA355 | 0.125                                       |               |
| A781erm LineR Erithromycin-resistant variant (plasmid) | 0.06                                       |               |
| SA354 Progenitor to SA355 | 0.125                                       |               |
| SA355 LineR Lincld isolate | 0.125                                       |               |
| 0616 (Dap) Endocarditis MSSA, progenitor to 0701 | 0.125                                       |               |
| 0701 (Dap) Daptomycin-resistant MSSA | 0.06                                       |               |
| MSSA ATCC 29213 | 0.125                                       |               |
| RN9120 VISA selected *in vitro* | 0.25                                        |               |
| Staphylococcus epidermidis ATCC 12228 | 0.5                                        |               |
| VRE-CUS VRE bloodstream left-sided endocarditis | 0.125                                       |               |
| VRE-WMC VRE bloodstream infection BI/NAP1/027 Hypervirulent Contemporary | 0.125 |               |
| Clostridium difficile BI Clostridium difficile | 0.008                                       |               |
| Moraxella catarrhalis ATCC 25238 | 0.5                                        |               |
| Eseudomonas coli 1035 Clinical respiratory tract isolate | > 4                                           |               |
| Pseudomonas aeruginosa ATCC 27853 | > 4                                        |               |
| Acinetobacter baumannii ATCC 19606 | > 4                                        |               |
| Enterobacter cloacae ATCC 13047 | > 4                                        |               |

Abbreviations: ATCC, American Type Culture Collection; VRE-CUS, vancomycin-resistant *Enterococcus faecium*. ^aMIC in 20% human serum.

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Statistical analysis
The data from the survival studies were analyzed with GraphPad Prism (GraphPad Software, Incorporated, La Jolla, CA, USA) using log-rank analysis (Mantel–Cox test). Data were considered significant at \( P<0.05 \).

RESULTS
In vitro antimicrobial activity
Nosiheptide exhibited strong activity against all contemporary MRSA and MSSA strains tested including multidrug-resistant clinical isolates (Table 2), with MIC values \( \leq 0.25 \text{mg} \cdot \text{L}^{-1} \). Notably, clinical MRSA isolates previously demonstrated to have developed resistance to front-line antibiotics including daptomycin, linezolid and vancomycin remained highly sensitive to nosiheptide. This activity against MRSA was also completely unaffected by the presence of 20% human serum (Table 2). Nosiheptide was also highly active against Enterococcus faecium isolate of vancomycin-resistant MRSA (ribotype 027 strain).

Time-kill kinetics
In vitro time-kill analysis was used to assess nosiheptide killing kinetics. Nosiheptide was rapidly bactericidal against MRSA in a concentration- and time-dependent manner (Figure 2a), with a nearly 2-log kill noted within 6 h at both 10 \( \times \) and 20 \( \times \) MIC. A 2-log kill of MRSA was also noted for nosiheptide against MRSA resistant to other antibiotics, such as linezolid or erythromycin (Figure 2b). Although nosiheptide demonstrated bactericidal activity against MRSA at 10 \( \times \) and 20 \( \times \) the MIC, it was bacteriostatic against a clinical bloodstream isolate of vancomycin-resistant Enterococcus faecium up to 64 \( \times \) MIC (Figure 2c). The MRSA killing kinetics of nosiheptide also compared favorably with vancomycin kinetics at 10 \( \times \) and 20 \( \times \) their respective MICs (Figure 2d).

Prolonged PAE and lack of cytotoxicity
Given its favorable MRSA killing kinetics, nosiheptide was assessed for its PAE. Notably, nosiheptide exhibited prolonged PAEs against both HA- and CA-MRSA compared with the most commonly used systemic antibiotic, vancomycin, at 10 \( \times \) their respective MICs (Figures 3a and b). The PAE for nosiheptide was calculated to exceed 9 h for both HA- and CA-MRSA. We also tested nosiheptide for in vitro evidence of mammalian cell cytotoxicity as a prelude to our in vivo experiments. No evidence for nosiheptide cytotoxicity was observed when incubated for 72 h with the cervical carcinoma HeLa cell line at up to 128 mg l\(^{-1}\), which is \( \sim \)1000-fold above the MIC against MRSA. We also found that nosiheptide activity against USA300 MRSA was not inhibited in 20% human serum (Table 2).

In vivo activity
Owing to its potent anti-MRSA activities in vitro, nosiheptide was tested in a murine model of i.p. MRSA infection. Mice were infected with HA-MRSA strain Sanger 252, followed by nosiheptide treatment (20 mg kg\(^{-1}\), i.p.) at 1 and 8 h post infection. Although all mice became lethargic and exhibited piloerection within \( \sim \)4 h of infection, nosiheptide provided significant (\( P<0.03 \)) protection against mortality (Figure 4). Ten out of 10 of the nosiheptide-treated mice remained alive on day 3, whereas 6/10 of the controls died on day 1. By the end of the study, only one mouse in the nosiheptide group had died. These results provide evidence of significant in vivo activity for nosiheptide.

DISCUSSION
In our search for new anti-MRSA antibiotics from marine-derived microorganisms, we identified an actinomycete strain that produced a metabolite shown to be nosiheptide. Although the structure of this
Nosiheptide activity against contemporary MRSA
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Figure 3 PAE of nosiheptide on contemporary MRSA strains. CA-MRSA strain TCH-1516 (a) or HA-MRSA strain Sanger 252 (b) were incubated for 1 h with nosiheptide or vancomycin at 10 × MIC. The antibiotics were removed, and the bacteria were washed and allowed to recover in antibiotic-free media. The curves show the rates of growth following antibiotic treatment. The data represent the mean ± s.d. of one representative assay repeated in duplicate.

Figure 4 Nosiheptide protection in a murine model of i.p. MRSA infection. Plot showing survival of mice (n=10 per group) infected i.p. with HA-MRSA strain Sanger 252, followed by i.p. treatment with either nosiheptide (20 mg kg⁻¹, filled circles) or vehicle control (filled squares) at 1 h or 8 h after infection (represented by upward arrows on the x axis). N=10 mice per treatment group (P<0.03 by log-rank analysis).

Nosiheptide exhibited favorable MRSA killing kinetics, a very prolonged PAE, and was active in a murine model of i.p. infection. Although in vivo evidence of nosiheptide activity has not been published, Benazet et al.¹⁶ report that nosiheptide protected mice from S. aureus mortality only when the compound was administered at the site of infection. However, no data were presented to support this claim. We report in the current paper nosiheptide activity when the compound is administered 1 and 8 h after MRSA injection at the site of infection as well (Figure 4). Our data are consistent with the claims of Benazet et al.¹⁶ and point to the fact that nosiheptide is efficacious in vivo but may either be metabolized or not distributed (due to localized precipitation or lack of absorption) when injected. A closely related compound, glycothiohexide α, was once shown to be quite active against MRSA and vancomycin-resistant E. faecium.¹⁷–¹⁹ However, despite its in vitro potency, this compound had minimal activity in a S. aureus model of i.p. infection when administered s.c. It is unknown whether in the case of glycothiohexide α, pharmacokinetic and/or possible metabolic factors abrogated potential efficacy. In pilot studies, we also found that nosiheptide exhibited significantly reduced activity when injected s.c. (data not shown) following i.p. MRSA inoculation and are currently embarking upon collaborative studies of liposomal and other lipophilic drug formulations to optimize nosiheptide delivery in vivo.

In sum, nosiheptide represents a promising antibiotic scaffold for further development owing to its potent anti-MRSA and other Gram-positive activity, lack of inhibition by human serum, lack of mammalian cell cytotoxicity and demonstrated tolerability when delivered orally to animals.² Many of the synthesis/manufacturing issues have already been addressed, as nosiheptide is produced in mass quantities by manufacturers worldwide. Cumulatively, these properties provide nosiheptide an advantage over other early-stage antibacterials, many of which are later found to be cytotoxic or serum-inhibited, or are fraught with difficulties in scale-up of production.

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