

Class II HMG-CoA Reductase Inhibitors targeting Methicillin-Resistant *Staphylococcus pseudintermedius*

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ABSTRACT

Staphylococcus pseudintermedius is a component of the normal flora of companion animals that contributes to opportunistic infections in dogs. Clinical isolates of *S. pseudintermedius* (chiefly methicillin-resistant *S. pseudintermedius* (MRSP)) have been identified that exhibit resistance to nearly all antibiotic classes. There is a need for new antibiotics that target novel pathways within resistant pathogens such as MRSP. A possible novel antibacterial target in Gram-positive cocci is class II HMG-CoA reductase (HMGR), a key enzyme present in the mevalonate pathway that is essential for bacterial survival. In this study, we examined novel synthetic compounds that are potent inhibitors of bacterial HMGRs. The compounds inhibited growth, in low micromolar concentration, of clinical isolates of methicillin-sensitive *S. pseudintermedius* (MSSP) and MRSP via the broth microdilution assay. The MTS assay confirmed the most potent compound (6) was not toxic to different mammalian cell lines (up to 128 μ M). A time-kill assay revealed this compound rapidly eradicates a high inoculum of MRSP within two hours. This study provides evidence that compound 6 is a promising agent that warrants further investigation as a novel treatment option for MRSP infections.

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Introduction

Staphylococcus pseudintermedius is a normal inhabitant of the skin and mucosa and can be isolated from the nares, mouth, pharynx, forehead, groin and anus of healthy dogs and cats (Rubin and Chirino-Trejo, 2011). Though it is an opportunistic pathogen, *S. pseudintermedius* is a leading cause of skin and ear infections and post-operative wound infections in dogs and cats (Weese and Duijkeren 2010; Mohammad *et al.*, 2015). This bacterial pathogen has also been linked to severe infections in companion animals including urinary tract infections, complicated skin infections, surgical site infections, and otitis (Weese *et al.*, 2012).

The challenge of treating infections caused by *S. pseudinter-*

medius has become more troublesome with the isolation of strains displaying resistance to numerous antibacterial classes including β -lactams, fluoroquinolones, lincosamides, macrolides, aminoglycosides, tetracyclines, sulfonamides, and chloramphenicol (Perreten *et al.* 2010; Weese and Duijkeren 2010; Eckholm *et al.*, 2013). Of these strains, methicillin-resistant *S. pseudintermedius* (MRSP) isolates are particularly troubling. Resistance to methicillin and other β -lactam antibiotics is mediated by the *mecA* gene that encodes a modified penicillin binding protein (PBP), similar to methicillin-resistant *S. aureus* (MRSA) (van Duijkeren *et al.*, 2011). Usually, β -lactam antibiotics attach to the PBP of *S. pseudintermedius* and interfere with proper formation of the bacterial cell wall. MRSP isolates are frequently not only resistant to β -lactam antibiotics, but also to several other classes of antimicrobial drugs. Thus treatment of MRSP infections is an emerging challenge in veterinary medicine because of limited therapeutic options (Wettstein *et al.*, 2008). Hence, there is a need for antibacterial agents capable of inhibiting previously unexploited drug tar-

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gets to treat infections caused by methicillin-sensitive and methicillin-resistant *S. pseudintermedius*. One such target is the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) enzyme.

There are two distinct classes of HMGR. Class I HMGR is present in all eukaryotes and some archaea while Class II HMGR is present only in prokaryotes (Bochar *et al.*, 1999). HMGR catalyzes the first committed reaction of the mevalonate pathway for biosynthesis of isoprenoids (Edwards and Ericsson, 1998). While most bacteria synthesize isopentenyl diphosphate by the alternative non-mevalonate 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP) pathway, isoprenoids in *S. aureus* are synthesized through a mevalonate pathway with a Class II HMGR (Wilding *et al.*, 2000). Clearly HMGR represents a potential target for antibacterial agents directed against multidrug-resistant strains including MRSP. In spite of the attractive features offered by HMGR as a potential therapeutic target for antibiotic drug development, a very limited number of inhibitors have been reported (Hedl and Rodwell, 2004). With this point in mind, the present study aimed to identify small molecule inhibitors (designed to target bacterial HMGR) that possess potent antibacterial activity against *S. pseudintermedius* and exhibit limited toxicity to mammalian cells.

Materials and methods

Methicillin-resistant *Staphylococcus aureus* USA300 (MRSA) and *Pseudomonas aeruginosa* ATCC 15442 were obtained from BEI Resources and the American Tissue Culture

Collection, respectively. Fifteen isolates of *Staphylococcus pseudintermedius* (6 methicillin-sensitive *S. pseudintermedius* (MSSP) and 9 methicillin-resistant *S. pseudintermedius* (MRSP)) were included in the study (Table 1). The specimens were obtained from patients admitted to the Purdue University small animal hospital for treatment and confirmed by the Indiana Animal Disease Diagnostic Laboratory. Clinical specimens were inoculated onto 5% sheep blood agar and incubated at 35 °C for 18–24 hours. Standard methods including examination of colony morphology and hemolysis and biochemical tests such as tube coagulase, Voges-Proskauer (VP) and fermentation tests for maltose, trehalose and lactose, were used to identify isolates (Murugaiyan *et al.*, 2014) and (Versalovic, 2011). Antimicrobial susceptibility was determined via the broth microdilution assay using the SensiTitre (Trek). Isolates demonstrating resistance to oxacillin (minimum inhibitory concentration (MIC) greater than or equal to 0.5 mg/ml), a surrogate for methicillin, were screened for *mecA* by PCR, as previously described (Vannuffel *et al.*, 1995). A *mecA* positive result was assigned to samples with a visible 310 bp band on a 1.5% agarose gel.

Clindamycin hydrochloride monohydrate was purchased from Tokyo Chemical Industry (Portland, OR, USA) and rifampicin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The compounds presented in this study are derivatives of 5-(N-(4-butylphenyl) sulfa moyl)-2-hydroxybenzoic acid 1 (N-bsha) and were synthesized by Dr. Mark Lipton's research group at Purdue University. The compounds were confirmed to inhibit the bacterial HMGR enzyme by Dr. Cynthia Stauf-

Table 1. Clinical isolates of *Staphylococcus pseudintermedius* (SP) used in this study

	Isolate Name	Species	Breed	Age	Origin	Resistance Phenotype ^a
Methicillin sensitive <i>S. pseudintermedius</i> (MSSP)	SP1	Canine	Mixed	9 years	Urine	PEN and AMP
	SP2	Canine	Cocker Spaniel	10.5 years	Urine	PEN, AMP, CLIN, ENRO, ERYTH, GEN, MARBO, TMP-SMX
	SP4	Canine	Scottish Terrier	13.5 years	Urine	PEN, AMP, TMP-SMX
	SP6	Canine	Labrador Retriever	6 years	Ear	None
	SP7	Canine	Cocker Spaniel	10 years	Ear	AMK, PEN, AMP, CLIN, ENR, ERM, GEN, MARB, TMP-SMX
	SP10	Canine	Mixed	14.5 years	Urine	PEN, AMP, CLIN, ERM, TMP-SMX
Methicillin resistant <i>S. pseudintermedius</i> (MRSP)	SP3	Canine	English Bulldog	8 months	Orthopedic implant	AMP, PEN, AMO, CEF, ERM, IMI, OXA, TIC
	SP5	Canine	Mixed	10.5 years	Urine	AMK, AMP, PEN, AMO, CEF, CHL, CLIN, ENR, ERM, GEN, IMI, MARB, OXA, TIC, TMP-SMX
	SP8	Canine	Maltese	10 years	Urine	AMP, PEN, AMO, CEF, ERM, CLIN, IMI, OXA, TIC, CHL
	SP9	Canine	Mixed	4 years	Skin	AMK, AMP, PEN, AMO, CEF, CHL, CLIN, ENR, ERM, GEN, IMI, MARB, OXA, TIC, TMP-SMX
	SP28	canine West Highland	White	15 years	urine	AMK, CEF, CHL, CLIN, ENR, ERM, GEN, IMI, MARB, OXA, TIC, TMP-SMX
	SP38	Canine	Bull terrier	8 years	implant	CEF, CHL, CLIN, ENR, ERM, GEN, IMI, MARB, OXA, TIC, TMP-SMX
	SP39	Canine	English bulldog	6 years	Ear	AMK, CEF, ENR, ERM, GEN, IMI, MARB, OXA, TIC
	SP40	Canine	Chihuahua	14 years	Ear	CEF, ENR, ERM, CLIN, IMI, MARB, OXA, TIC
	SP41	Canine	Yorkshire terrier	14 years	Urine	AMK, CEF, CHL, CLIN, ENR, ERM, GEN, IMI, MARB, OXA, TIC, TMP-SMX

Abbreviations: PEN: penicillin, AMP: ampicillin, AMK: amikacin, CEF: cefpodxime, CLIN: clindamycin, GEN: gentamicin, CHL: chloramphenicol, ENR: enrofloxacin, MARB: marbofloxacin, ERM: erythromycin, BAC: bacitracin, NEO: neomycin, TOB: tobramycin, CIP: ciprofloxacin, OXY: Ox-tetracycline, TEM-SMX: trimethoprim-sulfamethoxazole

facher's research group at Purdue University (data not published). All antibiotics and compounds were dissolved in dimethyl sulfoxide (DMSO) to obtain a stock 10 mM solution.

Murine macrophages (J774A.1), human keratinocytes (HaCat), and human ileocecal colorectal (HRT-18) cell lines and fetal horse serum were purchased from the American Tissue Culture Collection (Manassas, VA, USA). Trypticase soy agar (TSA), Trypticase soy broth (TSB) were purchased from Becton, Dickinson and Company (Cockeysville, MD, USA). Phosphate buffered saline (PBS), Mueller-Hinton broth (MHB) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The MTS reagent for toxicity assessment was purchased commercially (Promega, Madison, WI, USA).

Primary screening of class II HMGR inhibitors against clinical isolates of MRSA and Pseudomonas aeruginosa

The MIC and minimum bactericidal concentration (MBC) of the 15 compounds was initially determined against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2007). The most potent compound (6) was subsequently tested against clinical isolates of *S. pseudintermedius*, as described below.

The MIC and MBC of compound 6 against clinical isolates of MSSP and MRSP

The MIC of compound 6, rifampicin, and clindamycin against nine isolates of MRSP and six isolates MSSP was determined using the broth microdilution method in accordance with the recommendations contained in the CLSI guidelines (Mohammad et al., 2015). Bacteria were prepared in phosphate-buffered saline (PBS) to achieve a McFarland standard of 0.5. The solution was subsequently diluted 1:300 in Mueller-Hinton broth (MHB) for MRSP and MSSP to reach a starting inoculum of 1×10^5 colony-forming units (CFU/mL). Bacteria were then transferred to a 96-well microtiter plate. Compound 6, rifampicin, and clindamycin were added (in triplicate) to wells in the first row of the microtiter plate and serially diluted two-fold. The plate was incubated at 37°C for 18-20 hours before the MIC was recorded as the lowest concentration where no bacterial growth was observed. The MBC was determined by plating 5 μ L from wells on the 96-well microtiter plate where no growth was observed, onto TSA plates. The plates were incubated at 37 °C for 18-20 hours before the MBC was determined. The MBC was classified as the concentration where $\geq 99\%$ reduction in bacterial cell count was observed.

Time-kill kinetic analysis

MRSP SP3 cells in late logarithmic growth phase were diluted to $\sim 1 \times 10^6$ colony-forming units (CFU/mL) and exposed to concentrations equal to $3 \times \text{MIC}$ or $5 \times \text{MIC}$ (in triplicate) of compound 6, clindamycin, or rifampicin in TSB. Samples (20 μ L) were collected after 0, 2, 4, 6, 8, 10, and 12 hours of incubation at 37°C and then serially diluted in PBS. Bacteria were transferred to TSA plates and incubated at 37°C for 18-20 hours before viable CFU/mL was determined as previously described (Mohammad et al., 2015).

In vitro cytotoxicity analysis

HMGR inhibitor compound 6 assayed against murine macrophage cells (J774A.1)

Compound 6 was assayed at concentrations of 16, 32, 64,

and 128 μ M against a murine macrophage-like cell line (J774A.1) to determine the potential toxic effect in vitro. Briefly, J774A.1 cells were seeded at a density of 1.56×10^4 per well in a tissue culture 96-well plate (CytoOne, CC7682-7596) in DMEM media supplemented with 10% fetal bovine serum (FBS), and incubated at 37°C in a 5% CO₂ atmosphere for 24 hours. The cells were treated with HMGR inhibitor at for 24 hours. Untreated cells were used as a negative control. After incubation, the cells were washed three times with PBS and before addition of the reagent, MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium. Cells were incubated further for four hours at 37 °C with 5% CO₂. Corrected absorbance readings (actual absorbance readings for each treatment subtracted from background absorbance) were taken using a kinetic ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was expressed as percent absorbance in comparison to the negative control (untreated cells) as reported elsewhere (Bahnsen et al., 2013; Mohamed et al., 2014).

Compound 6 assayed against a human keratinocyte cell line (HaCat)

Human keratinocyte (HaCat) cells were seeded at a density of 10,000 cells per well in a 96-well tissue culture plate (CytoOne, CC7682-7596) in DMEM media containing 10% fetal bovine serum (FBS) and incubated overnight at 37°C. Then cells were treated with compound 6 at concentrations of 16, 32, 64, and 128 μ M for 24 hours. Treated cells were washed three times with PBS and DMEM media containing MTS assay reagent was added to each well. After four hours of incubation at 37 °C, absorbance was measured using ELISA microplate reader. Percent cell viability of cells treated with compound 6 were calculated relative to the untreated cells according to (Thangamani et al., 2015).

Compound 6 assayed against a human ileocecal colorectal cell line (HRT-18)

Human ileocecal colorectal cell line (HRT-18) cells were seeded at a density of 10,000 cells per well in a 96-well tissue culture plate (CytoOne, CC7682-7596) in RPMI-1640 supplemented with 10% fetal horse serum and incubated overnight at 37°C. Cells were treated with compound 6 at concentrations of 16, 32, 64, and 128 μ M for 24 hours. Treated cells were washed three times with PBS before addition of DMEM media containing MTS assay reagent. After four hours of incubation at 37°C, absorbance was measured using an ELISA microplate reader. The percent cell viability of cells treated with compound 6 was calculated relative to the untreated cells.

Statistical analysis

Statistical analysis was completed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Statistical significance was determined using the two-tailed Student's t-test. P values of < 0.05 were considered significant. Data are presented as mean \pm SD.

Results

Antibacterial activity of 15 class II HMGR inhibitors tested against S. aureus and P. aeruginosa

The antibacterial activity of 15 synthesized class II HMGR inhibitors were initially evaluated against one Gram-positive (MRSA USA300) and one Gram-negative bacterial pathogen, *Pseudomonas aeruginosa* ATCC 15442. As presented in Table

2, five compounds 5, 6, 9, 10, and 12 were capable of inhibiting MRSA USA300 growth at concentrations ranging from 16 μ M to 64 μ M. Interestingly, none of the compounds examined possessed antibacterial activity against *P. aeruginosa* ATCC 15442 which has the alternative MEP/DOXP isoprenoid biosynthesis pathway (MIC > 128 μ M). This indicates the antibacterial activity of the designed inhibitors may be restricted to Gram-positive pathogens.

From the initial screening results, compound 6 emerged as the most potent inhibitor of *S. aureus* growth. Thus we moved next to examine compound 6 for its ability to inhibit growth of important multidrug-resistant strains of MRSP and MSSP

Table 2. The MIC and MBC of HMG-CoA reductase inhibitors tested against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*.

Compound No	MRSA MIC/MBC	<i>Pseudomonas aeruginosa</i>
1	>128/ND	>128/ND
2	>128/ND	>128/ND
3	>128/ND	>128/ND
4	128/128	>128/ND
5	64/64	>128/ND
6	16/16	>128/ND
7	128/128	>128/ND
8	>128/ND	>128/ND
9	64/64	>128/ND
10	64/64	>128/ND
11	>128/ND	>128/ND
12	32/32	>128/ND
13	>128/ND	>128/ND
14	>128/ND	>128/ND
15	>128/ND	>128/ND

Antibacterial activity of compound 6 against *Staphylococcus pseudintermedius*

The antibacterial activity of compound 6 was tested against a panel of clinical isolates of methicillin-sensitive and methicillin-resistant *S. pseudintermedius* (Table 3). Against isolates of MSSP, compound 6 inhibited growth consistently at a concentration of 8 μ M. When 6 was evaluated against MRSP, there was a one-fold increase in the MIC observed (MIC was 16 μ M against most isolates). This MIC value correlates well

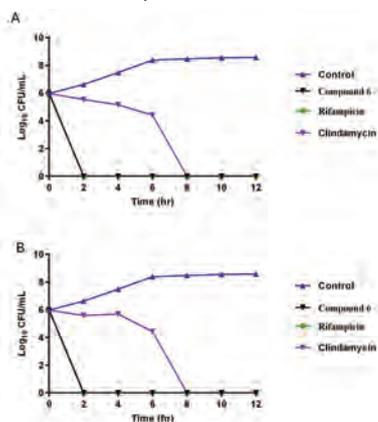


Fig. 1. Time-kill assay of compound 6, rifampicin, and clindamycin against MRSP. Compound 6, rifampicin and clindamycin were incubated with MRSP SP3 (~9.6 × 10⁵ CFU/ml) and samples were collected every two hours to enumerate viable CFU/mL. Panel A) 3 × MIC of test agent and Panel B) 5 × MIC of test agent. The error bars represent standard deviation values obtained from triplicate samples used for compound/antibiotic studied.

with the MIC noted for compound 6 against MRSA. Control antibiotics rifampicin and clindamycin inhibited growth of most *S. pseudintermedius* isolates at a concentration of ≤ 0.5 μ M and 1 μ M, respectively. Interestingly, compound 6 retained its potent antibacterial activity against four isolates of *S. pseudintermedius* exhibiting resistance to clindamycin (SP2, SP28, SP40, and SP41) indicating cross-resistance between 6 and clindamycin is unlikely to occur. Compound 6 showed bactericidal activity against both MRSP and MSSP strains with a minimum bactericidal concentration (MBC) ranging from 16 to 32 μ M. This behavior is similar to the antibiotics rifampicin and clindamycin.

Table 3. MIC and MBC of compound 6, rifampicin, and clindamycin against MSSP and MRSP clinical isolates.

Isolate Name	Compound 6 MIC/MBC (μ M)	Rifampicin MIC/MBC (μ M)	Clindamycin MIC/MBC (μ M)
Methicillin sensitive <i>S. pseudintermedius</i> (MSSP)			
SP1	8/16	≤0.5/≤0.5	1/1
SP2	16/16	≤0.5/≤0.5	128/>>128
SP4	8/16	≤0.5/≤0.5	0.5/>>128
SP6	8/32	≤0.5/≤0.5	1/1
SP7	8/8	≤0.5/≤0.5	1/1
SP10	8/16	≤0.5/≤0.5	0.5/>>128
Methicillin resistant <i>S. pseudintermedius</i> (MRSP)			
SP3	16/16	≤0.5/2	1/1
SP5	16/16	≤0.5/≤0.5	1/>>128
SP8	16/32	≤0.5/≤0.5	0.5/1
SP9	16/16	≤0.5/≤0.5	0.5/>>128
SP28	16/16	≤0.5/≤0.5	>128/ND
SP38	16/16	≤0.5/≤0.5	0.125/0.125
SP39	8/16	≤0.5/≤0.5	2/4
SP40	16/16	≤0.5/≤0.5	>128/ND
SP41	16/16	≤0.5/≤0.5	>128/ND

Time-kill assay of compound 6 versus MRSP

Preliminary data from the MBC study indicated compound 6 is bactericidal against *S. pseudintermedius*. In order to confirm this result, a time-kill assay was conducted with compound 6, rifampicin, and clindamycin against MRSP SP3. As depicted in Fig. 1, compound 6 mimics the behavior of rifampicin as both agents completely eradicate a high inoculum of MRSP within two hours. No re-growth of bacteria is observed over the remaining ten hour sampling period. In contrast, clindamycin requires eight hours to achieve the same result.

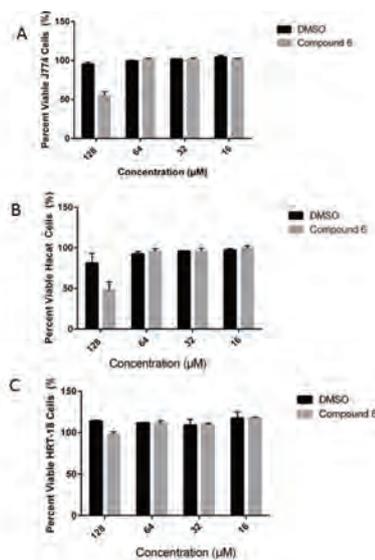


Fig. 2. Toxicity examination of compound 6 against multiple mammalian cell lines. Compound 6 was examined at 16, 32, 64, and 128 μ M against three different cell lines A) Murine macrophage cell line (J774.1), B) human keratinocyte cells (HaCaT), and C) human colorectal cells (HRT-18) using the MTS assay. An unpaired t-test ($P < 0.05$) was used to evaluate toxicity of cells exposed to compound 6 compared to cells treated with DMSO.

Irrespective of the concentration tested ($3\times$ or $5\times$ MIC), the same result is observed indicating the compound and drugs do not exhibit a concentration-dependent effect. The time-kill assay confirms the result obtained with the MBC study, proving that compound 6 is in fact a bactericidal agent.

Toxicity analysis of compound 6 against mammalian cells

Confirmation of compound 6's potent antibacterial activity led us to next investigate whether this compound is toxic to mammalian tissues. Compounds that exhibit toxicity to host (mammalian) tissues possess limited therapeutic utility as antibacterial agents. Thus we evaluated compound 6 for potential toxicity to three different cell lines (Fig. 2). Against murine macrophage cells (J774), compound 6 was found to be non-toxic at the highest concentration tested ($128\ \mu\text{M}$). The same result was obtained for compound 6 examined against human keratinocytes (Fig. 5B) and human colorectal cells (Fig. 5C). This represents a greater than 16-fold difference between the MIC of compound 6 against *S. pseudintermedius* and the highest concentration tested for toxicity against all three mammalian cell lines. Collectively, this demonstrates compound 6 exhibits limited toxicity to mammalian cells.

Discussion

Staphylococcus pseudintermedius infections are an emerging issue in veterinary medicine; until recently, most diseases observed in small animal veterinary services were amenable to treatment with a variety of antimicrobials (Beco et al., 2013). Be that as it may, the quick development and worldwide spread of multidrug-resistant *S. pseudintermedius* (to be specific MRSP) in the previous ten years has exhibited a noteworthy challenge to veterinary specialists (Jones et al., 2007; Ruscher et al., 2009). Clinical isolates have been recognized that display resistance to various antibiotic classes, reducing the treatment alternatives available to veterinarians. This underscores the need to develop new antimicrobials to battle this significant medicinal challenge.

Most antibiotics target the same essential processes in bacteria such as cell wall synthesis (β -lactams and glycopeptides), protein synthesis (oxazolidinones, tetracyclines, and aminoglycosides), and DNA (quinolones) or RNA synthesis (rifampicin) (Payne et al., 2007). Astoundingly, more than 40% of antibiotics target cell wall synthesis. However, studies have identified bacteria, such as *S. aureus*, encode for multiple essential genes that represent a potential reservoir of novel antibacterial targets (Silver, 2007; Zoraghi et al., 2011; Haag et al., 2012). One of these targets is the mevalonate pathway which is essential for the survival of Gram-positive cocci. One gene of interest in this pathway is the HMG-CoA reductase gene. This target differs from human and animal HMG-CoA reductase in catalytically important residues and in regions of the proteins that participate in oligomerization (Istvan, 2001).

To date, no chemical compounds have been designed thus far that specifically target bacterial HMG-CoA reductase. Thus the present study aimed to examine 15 derivatives of 5-(N-(4-butylphenyl) sulfamoyl)-2-hydroxybenzoic acid 1 (N-bsha) which inhibit the HMGR coenzyme of *S. aureus* at low concentration ($5\ \mu\text{M}$). We initially screened the antibacterial activity of the 15 compounds against one Gram-positive (MRSA USA300) and one Gram-negative (*Pseudomonas aeruginosa* ATCC 15442). We found five class II HMGR inhibitors were capable of inhibiting MRSA USA300 growth with a minimum inhibitory concentration (MIC) value ranging from $16\ \mu\text{M}$ to $64\ \mu\text{M}$. None of the compounds were active against *P. aeruginosa* ATCC 15442. Of the compounds screened, compound 6 was the most potent inhibitor of MRSA USA300 growth (MIC =

μM). As *S. pseudintermedius* and *S. aureus* have comparable hereditary and phenotypic traits, we hypothesized that compound 6 would exhibit potent antibacterial activity against both MSSP and MRSP. Therefore, the MIC and MBC of compound 6 was tested against nine clinical isolates of MRSP and six clinical isolates of MSSP. As predicted, compound 6 exhibited potent antibacterial activity against clinical isolates of MSSP and MRSP (MIC ranged from 8 – $16\ \mu\text{M}$). Though the compound was not as potent as clindamycin (MIC ranged from 0.5 to >128 against both MSSP and MRSP), which is a first-line treatment option for treatment of *S. pseudintermedius* pyoderma infections (Beco et al., 2013), compound 6 was more effective than this drug against four strains of *S. pseudintermedius* exhibiting resistance to clindamycin.

Interestingly, compound 6 maintained its antibacterial activity against six MSSP and nine MRSP isolates which were found to be resistant to clindamycin and other antibiotics; this indicates there is no cross-resistance present between these antibiotics and the compound 6. This promotes the concept that this compound 6 has the potential to be used as new antibacterial agents, especially against *S. pseudintermedius* infections resistant to treatment with other antibiotics.

We were interested to examine if compound 6 is a bacteriostatic or bactericidal agent. It has been suggested that bactericidal antimicrobials have several advantages over their bacteriostatic counterparts, including helping patients recoup more quickly from infection, decreasing the potential development of bacterial resistance to these antibiotics, and restricting the spread of infection (French, 2006). Initial analysis revealed compound 6 was bactericidal as its MBC was identical to or two-fold higher than its MIC values against both MSSP and MRSP isolates. To confirm this result, a time-kill assay was conducted. This assay revealed that compound 6 is a rapid bactericidal agent as it eliminated MRSP within two hours. This result was similar to the result obtained with rifampicin, an antibiotic frequently used in combination with other antibiotics to treat pyoderma infections (Beco et al., 2013). Thus one potential advantage of compound 6's rapid bactericidal activity is it may help to resolve an infection more quickly.

To confirm the compound 6 selectively targets bacterial HMG-CoA reductase and not the mammalian homolog, the toxicity of compound 6 was tested against different cell lines. Against three different cell lines (J774, HaCaT and HRT-18) compound 6 was not toxic at concentrations up to $128\ \mu\text{M}$ (an 8 to 16-fold difference from the compound's MIC values against *S. pseudintermedius*). This indicates compound 6 exhibits limited toxicity to mammalian tissues and warrants further investigation as a novel antibacterial agent for treatment of infections caused by *S. pseudintermedius*.

Conclusion

In this study we have demonstrated novel HMGR coenzyme inhibitors, synthesized by our research group, are potent inhibitors of staphylococcal growth. One of these inhibitors (compound 6) exhibits potent antibacterial activity against clinical isolates of methicillin-sensitive and methicillin-resistant *S. pseudintermedius*. This compound is capable of inhibiting bacterial growth at low micromolar concentrations even against isolates exhibiting resistance to the drug of choice (clindamycin) for treatment of canine pyoderma infections. This compound is bactericidal and exhibits superior killing kinetics to clindamycin as it completely eliminates a high inoculum of MRSP within two hours. Compound 6 appears to be the most suitable HMGR coenzyme inhibitor to examine in further studies involving *S. pseudintermedius* as it is not toxic to mammalian cells at a concentration that is 8 or 16-fold higher than its MIC. Finally, this study provides a basis for ad-

ditional analysis and expansion of HMGR inhibitors novel treatment options for infections caused by multidrug-resistant pathogens that possess the mevalonate pathway

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