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Title page:

Discovery of a quorum sensing inhibitor of drug resistant staphylococcal infections by structure-based virtual screening

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Running Title Page

Running title: Hamamelitannin inhibits MRSA infections

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List of non-standard abbreviations:

Available Chemicals Database (ACD),

Agr autoinducing peptide (AIP)

Colony forming unit (CFU)

Luria broth (LB)

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Methicillin-resistant *Staphylococcus aureus* (MRSA)

Methicillin-sensitive *Staphylococcus aureus* (MSSA)

Methicillin-resistant *Staphylococcus epidermidis* (MRSE)

Methicillin-sensitive *Staphylococcus epidermidis* (MSSE)

Minimal inhibitory concentration (MIC)

Optical density (OD)

Phosphate buffered saline (PBS)

Quorum sensing (QS)

RNAIII activating protein (RAP)

RNAIII inhibiting peptide (RIP)

Staphylococcus aureus (S. aureus)

Staphylococcus epidermidis (S. epidermidis)

Staphylococcus quorum sensing (SQS)

Target of RAP (TRAP)

Tryptic soy broth (TSB)

Tumor necrosis factor (TNF)

Vancomycin- Intermediate resistant *S. aureus* (VISA)

Vancomycin- Intermediate resistant *S. epidermidis* (VISE)

Vancomycin-resistant *S. aureus* (VRSA)

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ABSTRACT

Staphylococci are a major health threat due to increasing resistance to antibiotics. An alternative to antibiotic treatment is preventing virulence by inhibition of bacterial cell-to-cell communication using the quorum sensing inhibitor RNAIII-inhibiting peptide (RIP). In this work we identified hamamelitannin as a nonpeptide analog of RIP by virtual screening of a RIP-based pharmacophore against a database of commercially available small-molecule compounds. Hamamelitannin is a natural product found in the bark of witch hazel, has no effect on staphylococcal growth *in vitro*, but like RIP, it does inhibit the quorum-sensing regulator RNAIII. In a rat graft model hamamelitannin prevented device-associated infections *in vivo*, including infections caused by methicillin resistant *S. aureus* and *S. epidermidis* (MRSA, MRSE) strains. These findings suggest that hamamelitannin may be used as a suppressor to staphylococcal infections.

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INTRODUCTION

Staphylococcus aureus and *S. epidermidis* are among the most important pathogens of nosocomial infections, causing over 70,000 deaths per year in the US. Nearly all *S. aureus* strains are resistant to penicillin and many are resistant to methicillin-related drugs (MRSA strains). Lately cases of intermediate or complete resistance to vancomycin, for many years the only uniformly effective treatment, have emerged (VISA/VRSA strains) (Furuya and Lowy, 2006; Lowy, 1998; Lowy, 2003). Staphylococci are also a common cause of infections related to bacterial biofilm formation on implanted devices. Infections may result in longer hospitalization time, need of surgery and can even cause death (Costerton et al., 1999). Biofilms are highly resistant to antibiotic treatment (Costerton et al., 2005; Costerton et al., 1999; Donlan and Costerton, 2002; Stewart and Costerton, 2001; Stoodley et al., 2002). The spread of drug-resistant strains of staphylococci and the ineffectiveness of treatments in cases of biofilm-related infections underscores the necessity to find new modes of prevention and effective alternatives to antibiotic treatment. A novel way would be to interfere with bacterial cell-to-cell communication that leads to virulence.

S. aureus cause disease through the production of virulence factors

S. aureus are part of our normal flora, but can cause fatal diseases due to the expression of multiple virulence factors. These include adhesins, exotoxins, enterotoxins, hemolysins, leukocidin, as well as proteases that enable the bacteria to spread within the host (Balaban and Rasooly, 2000; Hong-Geller and Gupta, 2003; Lowy, 1998). Strains defective in their ability to form a biofilm or produce toxins show diminished virulence (Gov et al., 2004), suggesting that a

novel approach for therapy development would be to interfere with the production of virulence factors.

Regulation of virulence through quorum sensing mechanisms

Quorum sensing (QS) refers to the molecular mechanism of regulation of gene expression in response to fluctuations in cell density (March and Bentley, 2004). Bacteria produce and release QS signaling molecules called autoinducers. The concentration of the autoinducers increases as a function of cell density, leading to distinct patterns of gene expression often regulated by phosphorylation. Two quorum sensing systems that act in tandem have been described in staphylococci (SQS1 and SQS2) (Korem et al., 2005) and in *Pseudomonas aeruginosa* (Waters and Bassler, 2005). SQS1 consists of the autoinducer RNAIII-Activating Protein (RAP) and its target molecule TRAP (Balaban et al., 2001; Balaban et al., 1998; Balaban and Novick, 1995; Gov et al., 2004; Korem et al., 2005; Korem et al., 2003). SQS1 induces the activation of SQS2 (Balaban et al., 2001), which encompasses the products of the *agr* system and include the autoinducer peptide (AIP), its receptor AgrC (Lina et al., 1998), and a regulatory mRNA molecule (RNAIII) that induces toxin production (Gustafsson et al., 2004).

RAP is a 277-amino acid residue protein that activates the *agr* system by inducing the phosphorylation of TRAP. RAP is an ortholog of the 50S ribosomal protein L2 that is secreted by *S. aureus* (Balaban et al., 2001; Balaban et al., 1998; Balaban and Novick, 1995; Gov et al., 2004; Korem et al., 2005; Korem et al., 2003). This suggests that RAP has an extra-ribosomal activity in *S. aureus*. When RAP activity is inhibited by anti-RAP or anti-TRAP antibodies (Balaban et al., 1998; Yang et al., 2005), by RAP-binding peptides (Yang et al., 2003), or by the RNAIII-inhibiting peptide (RIP), virulence is inhibited (Balaban et al., 2000; Balaban et al.,

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2003a; Balaban et al., 2003b; Balaban et al., 2005; Cirioni et al., 2003; Cirioni et al., 2006; Giacometti et al., 2003; Gov et al., 2001; Vieira-da-Motta et al., 2001).

TRAP is a membrane-associated 167-amino acid residue protein that is highly conserved amongst staphylococci. TRAP is hypothesized to be a sensor that is part of an unorthodox two-component signaling system. When TRAP is not expressed or not phosphorylated, the bacteria do not adhere, do not form a biofilm, do not express toxins, and do not cause disease. TRAP expression is constitutive, but its phosphorylation is regulated by RAP and reaches peak levels in the mid-exponential phase of growth (Balaban et al., 2001; Gov et al., 2004; Korem et al., 2005), followed by activation of *agr* and induction of SQS2 components. How TRAP regulates *agr* and/or virulence is under investigation (Shaw et al., 2007; Tsang et al., 2007; Adhikari et al., 2007) but seems to involve activation of the *ctsR* operon and ClpP production (Kiran et al in preparation) that regulate both DNA repair genes in addition to *agr* and virulence genes (Michel et al., 2006).

Inhibition of staphylococcal virulence by RIP

Virulence can be inhibited by the heptapeptide RIP (Balaban et al., 2000; Balaban et al., 2003a; Balaban et al., 2003b; Balaban and Rasooly, 2000; Cirioni et al., 2003; Cirioni et al., 2006; Giacometti et al., 2003; Gov et al., 2001; Lowy, 2003). RIP interferes with SQS1, thereby turning off downstream SQS2 as well, by competing with RAP to block TRAP phosphorylation and *agr* expression (Gov et al., 2004). This was also demonstrated in vitro, where RAP upregulated and RIP downregulated TRAP phosphorylation in vitro, in the absence of other cellular component (Kim, personal communication). The sequence of RIP (YSPWTNF-NH2) is similar to the sequence of residues 4-9 of RAP (YKPITN). This suggests that RIP is structurally

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similar to a segment of RAP and that RAP probably acts as an agonist and RIP as an antagonist to the same receptor (TRAP). Synthetic linear RIP has already been shown to prevent numerous types of *S. aureus* and *S. epidermidis* infections *in vivo*, including medical device associated infections (tested against MRSA, MRSE, VISA and VISE) (Balaban et al., 2001; Balaban et al., 2003b; Balaban et al., 2005; Cirioni et al., 2003; Cirioni et al., 2006; Giacometti et al., 2003; Gov et al., 2001; Korem et al., 2005; Lowy, 2003). These findings indicate that RIP can suppress virulence of any staphylococcal strain (Gov et al., 2004).

In this work, hamamelitannin has been discovered as a nonpeptide analog of RIP that effectively prevents biofilm formation and RNAIII *in vitro* as well as device-associated infections *in vivo*.

MATERIAL AND METHODS

Bacteria: *In vivo* studies were carried out using a clinical isolate of methicillin-resistant *S. epidermidis* (MRSE) and methicillin-resistant *S. aureus* ATCC 43300 (MRSA). *In vitro* studies were carried out using *S. aureus* lab strain 8325-4, RN6390 containing *agr* P3-*blaZ* fusion plasmid pRN6683 (Novick et al., 1995). *S. epidermidis* clinical isolate strain MH (Robinson, 2005). Bacteria were grown in Luria broth (LB) or tryptic soy broth (TSB) at 37°C with shaking.

Model Building of the RIP peptide

A model of the three-dimensional structure of the heptapeptide RIP (YSPWTNF-NH2) was built by homology to the crystal structure of residues 6-12 (YRPYTPS) of ribosomal protein L2 within the crystal structure of the 50S ribosomal subunit from *Deinococcus radiodurans* (Harms

et al., 2001). Program O (Jones et al., 1991) was used for this purpose on an Octane workstation (Silicon Graphics Inc.).

In silico Screening for RIP analogs

Screening for small molecule nonpeptide analogs of RIP was carried out by a computer search with the ISIS software (Integrated Scientific Information System) from Elsevier MDL against the Available Chemicals Database (ACD), a library of 300,000 commercially available small molecule compounds. The principal modules of the ISIS software used in this work were ISIS/Host, ISIS/Base and ISIS/Draw. The screening was carried out on a PC under the Microsoft Windows 2000 operating system. Use of the ISIS software package required access to program ORACLE. The model of RIP served as the basis for the search. Our first approach was to carry out similarity searches with the RIP models against the ACD. As this search yielded only peptides it was abandoned. Next we turned to a search of the ACD based on a pharmacophore approach, in which queries were defined by a set of distance ranges between aromatic rings (the midpoint of the Tyr, Phe and Trp rings was used) and hydrogen bond donors or acceptors, based on the RIP model. Compounds with a molecular weight in excess of 1,000 Da and compounds deemed unsuitable for prophylaxis or therapy, such as dyes and fluorescent compounds, were eliminated from the list of candidate compounds. The coordinates of the top hits were converted from the internal MOL format to PDB format by program BABEL (OpenEye Scientific Software). The structures of the top hits were superimposed on the RIP model and viewed either with program SwissPDBViewer on a PC or with program O (Jones et al., 1991) on a Silicon Graphics Octane workstation.

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RIP and hamamelitannin

RIP was synthesized in its amide form (YSPWTNF-NH2) (Neosystem, France, >98% purity), was dissolved in water and stored at -70^{o}C until use.

Hamamelitannin (2',5-di-O-galloyl-D-hamamelose) (Chromadex CA) was dissolved in water and stored at -70^{o}C until use. Sample was tested by reverse phase chromatography to confirm activity at >99% purity.

Hamamelitannin derivative used as a control: 2-0-acetyl-1,3,5-tris-0-(2-methoxibezoyl)-alpha-D-ribofuranose (Compound 2) (Sigma-Aldrich) was dissolved in DMSO and stored at -70^{o}C until use.

Antibacterial activity assay

S. aureus strain 8325-4 were grown overnight in LB, diluted 1:100 in LB and grown to the early exponential phase of growth ($OD_{595\ nm}$= 0.2). 100 µl LB containing 1000 freshly prepared bacteria were applied to sterile polystyrene 96-well plates (Falcon) together with RIP or hamamelitannin (0-125µg in 5µl water). Bacteria were grown for 24 hours at 37ºC without shaking, and the optical density was determined at 595nm. Ampicillin (Sigma Aldrich) was used a control at 0.01-10µg

Bacterial attachment *in vitro*

Bacteria were grown overnight in LB, diluted 1:100 in LB and grown for about two more hrs to the early exponential phase of growth ($OD_{595\ nm}$ = 0.2). To test for cell attachment, 0.1 ml (equivalent to about $6x10^{7}$ bacteria) were placed in polystyrene 96 well plates (Falcon) with 5 µl water, RIP, hamamelitannin or Compound 2 or control 3% (final) DMSO. Cells were grown for

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3 hrs without shaking at 37^{o}C. (To ensure that the same number of cells was applied to the wells and to test for bacterial growth at the end of incubation time, cell density was determined before and at the end of the experiment by measuring the optical density at 595 nm). At the end of the 3 hr incubation, unbound cells were removed and gently washed x2 with PBS. Cells were air dried, fixed with 100% ethanol, dried and stained for 2 min with filtered 0.4% gentian violet diluted in 12% ethanol. Stain was removed, and wells were gently washed x5 with PBS. 100 µl 1% SDS was added to solubilize stained cells and the 96 well plate was read at OD 595 nm in an ELISA plate reader.

RNAIII and δ hemolysin production *in vitro*

Northern blotting and α β–lactamase transcriptional fusion was used as described (Korem et al., 2003) for the detection of *agr* activity, using the *agr* P3-*blaZ* fusion plasmid, pRN6683 in lab strain RN6390 (Ji et al., 1995), These *S. aureus* fusion cells (in their early exponential phase, 2x10^{7} CFU in 30µl LB) were grown for 2.5 hrs at 37^{o}C with increasing amounts of hamamelitannin or RIP or for 60 min with or without 5µg recombinant RAP (in 5µl buffer containing 50 mM Tris pH 8.0/150 mM NaCl/1 mM DTT/10% glycerol). β-lactamase activity was measured by adding the substrate nitrocefin (Calbiochem) (40µl of nitrocefin 132 µg/ml in 0.1M sodium phosphate buffer pH 5.8). OD was determined using a microtiter plate reader (Bio-Tek KC4) at 490/630 nm using the kinetic analysis mode and results expressed as mean or maximum slope (V mean or V max).

For testing RNAIII production by Northern blotting, cells (*S. aureus* lab strain 8325-4, MRSA USA300, and clinical *S. epidermidis* isolate strain MH were freshly grown with shaking to the early exponential phase (3x10^{8} CFU in 1ml LB). Cells were grown for 6 hrs in the

presence of 12µl water or 300µg hamamelitannin that were added at time 0 and 3 hrs. The cells were harvested by centrifugation at 8000g for 10 min. The cell pellet and supernatants (see below) were collected. From the cell pellet RNA was isolated and RNAIII detected by northern blotting as described (Korem et al., 2003). As a loading control and to ensure that hamamelitannin is not a general transcriptional regulator, northern blots were also incubated with radiolabeled *traP* probe as described (Balaban et al., 2001).

For testing hemolysin production, the supernatants of MRSA+/- hamamelitannin were filtered through 0.22 micron filter, concentrated to 10x by evaporation, and 5µl applied on 20% SDS PAGE, western blotted and δ hemolysin detected using rabbit anti-δ hemolysin antibodies as described (Balaban and Novick, 1995). Equal loading was confirmed by ponceau S (Diasys Europe).

Rat graft *in vivo* infection

Sterile collagen-sealed double velour knitted polyethylene terephthalate (Dacron) grafts were utilized as medical devices in these experiments. Adult male Wistar rats (n=10) were randomized in control groups (no graft contamination), contaminated groups that did not receive any prophylaxis and treated groups that received hamamelitannin-coated grafts (local prophylaxis) or that received uncoated grafts but were challenged with bacteria + hamamelitannin. Rats were anesthetized with ether, the hair on the back shaved and the skin cleansed with 10% povidone-iodine solution. One subcutaneous pocket was made on each side of the median line by a 1.5 cm incision. Aseptically, 1-cm^2 sterile Dacron grafts were implanted into the pockets. Before implantation, the Dacron graft segments were soaked for 1 hr in different concentrations of hamamelitannin or saline. The pockets were closed by means of skin clips and saline (1 ml)

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containing the staphylococcal strains at a concentration of 2×10^7 CFU/ml (+/– hamamelitannin) (grown in standard conditions to the mid exponential phase of growth) were inoculated on to the graft surface using a tuberculin syringe to create a subcutaneous fluid-filled pocket. The animals were returned to individual cages and thoroughly examined daily. All grafts were explanted 7 days following implantation. The explanted grafts were placed in sterile tubes, washed in sterile saline solution, placed in tubes containing 10 ml of phosphate-buffered saline solution and sonicated for 5 min to remove the adherent bacteria. Quantitation of viable bacteria was performed by serial dilutions (0.1 ml) of the bacterial suspension in 10 mM sodium HEPES buffer, pH 7.2, and culturing each dilution on blood agar plates. Colony-forming units (CFUs) were determined the next day. To summarize, in experiment 1 bacteria were preincubated with hamamelitannin for 30 min at room temperature (0, 0.5, 10, 20, 30 and 50 µg hamamelitannin/$2x10^7$ bacteria in 150 µl saline) and mixture used for challenge. In experiment 2 dacron grafts were soaked for 1 hr with hamamelitannin at concentrations of 0.5, 10, 20, 30 and 50 mg/L prior to implantation and challenge.

Statistical analysis

Quantitative culture results from all groups are presented as mean ± standard deviation and the statistical comparisons between groups made using analysis of variance (ANOVA) on the log-transformed data with Tukey-Kramer Honestly Significant Difference Test. Significance was accepted when the *P* value was ≤0.05.

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RESULTS

Model building of the RIP peptide

Short peptides such as RIP do not have a fixed conformation in solution. However, the active conformation of RIP can be deduced from the corresponding sequence segment in RAP, since RIP competes with RAP (Korem et al., 2003) and the sequence of RIP (YSPWTNF) is similar to the sequence of residues 4-10 of RAP (YKPITNG). Consequently, we hypothesized that the structure of RIP is very similar to the corresponding segment in RAP. Building a model of RIP based on homology to RAP was thus entirely feasible. Since a crystal structure or a solution NMR structure of RAP is not available, we resorted to another source for homology model building of RIP, the crystal structure of ribosomal protein L2 from *Dienococcus radiodurans* (L2 Dr) which is available (PDB code 1NKW) (Harms et al., 2001). This protein has 61.9% sequence identity to RAP in 278 overlapping residues, ensuring a close structural relationship between L2 Dr and RAP. The amino acid sequence of RIP and the corresponding segments in RAP and L2 Dr are YSPWTNF, YKPITNG and YRPYTPS, respectively. Positions 1, 3, and 5 in RIP are entirely conserved, and in position 4 the sequence differences are conservative, i.e. an aromatic or aliphatic residue. RIP homologs with conservative amino acid replacements in positions 2 and 4 have been shown to retain their inhibitory activity (Gov et al., 2001; Vieira-da-Motta et al., 2001). A model of RIP was built based on the crystal structure of L2-Dr (PDB code 1NKW (Harms et al., 2001)). This homology-built model of RIP was subjected to energy minimization with program CNS (Brunger et al., 1998) (Figure 1).

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Definition of a pharmacophore for a RIP analog

The basis for the pharmacophore design was the RIP model. The pharmacophore was defined in terms of distances in the RIP model between pairs of aromatic moieties, distances between aromatic moieties and hydrogen donors or acceptors and distances between pairs of hydrogen bond donors/acceptors. Different pharmacophores were used in the search for a suitable RIP analog. The search results were filtered to eliminate compounds that are obviously to be avoided, such as dyes (like Chlorazol Fast Pink and Direct Black). Figure 2 shows the pharmacophore that led to the discovery of hamamelitannin as a small-molecule nonpeptide RIP analog. This was the top-ranking compound in the search with this pharmacophore (Figure 3).

Effects of hamamelitannin on bacterial growth, RNAIII production and cell attachment in vitro

The effects of hamamelitannin in vitro were initially tested on available lab strains and later confirmed on drug resistant strains. The effects of hamamelitannin that are shown below were essentially identical on any staphylococcal strain tested so far.

Hamamelitannin does not affect bacterial growth *in vitro*

To test if hamamelitannin has antibacterial activity, 1000 Colony-Forming Units (CFU) of *S. aureus* were grown for 24 hrs with 0-125µg hamamelitannin in a final volume of 100 µL (up to 2.5mM). As shown in Figure 4, even at highest concentration, hamamelitannin or RIP had no effect on bacterial growth. RIP and hamamelitannin were also tested for their effect on growth of multiple strains of *S. aureus* and *S. epidermidis,* and no effect on growth was ever observed in vitro (tested on MRSA, VISA, MRSE, and VISE). In this context it is noteworthy that the

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minimal inhibitory concentration (MIC) of antibiotics like ampicillin against *S. aureus* 8325-4 is 0.1 μg/ml (0.2 μM). Thus, hamamelitannin at a concentration as high as 12,500 times the MIC of ampicillin does not inhibit cell growth. In conclusion, hamamelitannin (or RIP) cannot be considered a conventional antibiotic.

Hamamelitannin competes with RAP and inhibits RNAIII and δ hemolysin production *in vitro*

To test if hamamelitannin is a quorum sensing inhibitor and thus suppresses *agr* activity, $2x10^7$ cells containing *rnaiii::blaZ* fusion construct (reporter cells) were incubated with increasing amounts (0-50μg) of hamamelitannin or RIP. RNAIII levels were measured as β-lactamase activity as a reporter gene product by the addition of nitrocefin as substrate. As shown in Figure 5A, both hamamelitannin and RIP inhibit RNAIII production in a concentration dependent manner, and are most effective at concentrations > 7μg / 10^7 bacteria (~5 nM / 10^3 bacteria). Reporter cells were also grown in the presence of 5μg recombinant RAP and Hamamelitannin, (25 and 50μg) and tested for RNAIII production 60 min later. As shown in Fig. 5B, recombinant RAP significantly ($p<0.05$) upregulated RNAIII production and 50μg Hamamelitannin significantly ($P< 0.01$) competed with RAP and downregulated RNAIII production. Of note is the fact that native RAP was also expected to be present as it is continuously produced by the cells (Korem et al., 2003). To test for the effect of Hamamelitannin on RNAIII production in other strains, *S. aureus* MRSA strain USA300 and clinical isolate *S. epidermidis* strain MH were grown with Hamamelitannin for 6 hrs and RNAIII tested by northern blotting. As shown in Fig. 5C, Hamamelitannin reduced RNAIII production in all strains tested. Hamamelitannin had no effect on the transcription of *traP* that is known to be constitutively expressed (Balaban et al.,

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2001) and used here as a control. Additionally, the effect of hamamelitannin on hemolysin production was tested by western blotting as described (Balaban and Novick 1995), as shown in Fig. 5C; the amount of δ hemolysin produced in the presence of hamamelitannin was reduced.

Hamamelitannin inhibits cell attachment *in vitro*

To test for the effect of hamamelitannin on bacterial attachment *in vitro*, *S. aureus* cells were incubated with 0-50 µg hamamelitannin or RIP in polystyrene plates for 3 hrs at 37^{o} C. Adherent bacteria were stained and OD determined. As shown in Figure 6A, hamamelitannin (or RIP) reduced cell attachment in a concentration-dependent manner and was most effective when ~10^{7} bacteria were grown in 4µg hamamelitannin or RIP (~8 nM per 10^{3} bacteria). Similar results were obtained with MRSA and with *S. epidermidis* (data not shown). Hamamelitannin derivative 2-0-acetyl-1,3,5-tris-0-(2-methoxibezoyl)-alpha-D-ribofuranose (Compound 2) had no effect on bacterial attachment, suggesting that the effects we observed of hamamelitannin on cell adhesion were specific. Hamamelitannin also inhibits attachment of *S. epidermidis*, as shown in Figure 6B. Of note is that attachment experiments were carried out over a short period of time (several hours) instead of biofilm studies carried out for days because over time the amount of RAP expressed by the cell (Korem et al., 2003) can compete out the inhibitory effect of RIP or hamamelitannin, unless an immune response had reduced the number of bacteria in the intervening time frame.

Coating with hamamelitannin prevents device-associated infections *in vivo*

To measure the amount of hamamelitannin necessary to prevent device-associated infections, bacteria ($2x10^{7}$ MRSA or MRSE) were pre-incubated with increasing amounts of

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hamamelitannin for 30 min at room temperature. Grafts were implanted and rats were challenged with the pre-incubated bacteria. Seven days later the graft was removed and bacterial load determined. As shown in Figure 7A, while bacterial load in the control untreated group was ~10^7 CFU/ml, bacterial load on the graft decreased with increasing dose of hamamelitannin. No bacteria was found when either bacteria (MRSA or MRSE) were pre-incubated with >20µg hamamelitannin, comparable to results obtained previously with RIP (Balaban et al., 2005).

In a parallel experiment grafts were soaked for 1 hr in increasing hamamelitannin concentrations. The grafts were subsequently implanted into the animal, and bacteria injected onto the graft. Seven days later the graft was removed and bacteria on the graft counted. As shown in Figure 7B for both MRSA and MRSE a significant ($p<0.05$) decrease in bacterial load was found when the grafts were presoaked with increasing concentrations of hamamelitannin while untreated control groups demonstrated evidence of graft infections with quantitative culture results showing ~10^7 CFU/ml. Grafts soaked in 30mg/L hamamelitannin showed no sign of bacterial load.

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DISCUSSION

In this work we have demonstrated the potential of a new way to inhibit staphylococcal infections. Instead of killing the bacteria, as is done with antibiotics, staphylococci are rendered harmless by inhibiting their quorum sensing mechanisms. We have previously shown that the peptide RIP acts as an inhibitor of quorum sensing (summarized in Balaban et al., 2005). In this work we have shown that hamamelitannin can prevent staphylococcal infections in a way analogous to RIP.

Hamamelitannin (2,5-di-O-galloyl-D-hamamelose) is the ester of D-hamamelose (2-hydroxymethyl-D-ribose) with two molecules of gallic acid (Figure 3). Since gallic acid contains three phenolic functional groups, hamamelitannin is considered a polyphenol, which have been shown to have multiple activities (see below). Hamamelitannin belongs to the family of tannins, which are plant polyphenols that are used in tanning animal hides into leather.

Hamamelitannin is a natural product found in the bark and the leaves of *Hamamelis virginiana* (witch hazel), a deciduous shrub native to damp woods in eastern North America and Canada. The concentration of hamamelitannin in the bark is 5% and in the leaves is less than 0.04% (w/w) (Wang et al., 2003). Witch hazel extracts were used by Native Americans for pain relief, colds and fever, and they are nowadays used in skin care products and in dermatological treatment of sun burn, irritated skin, atopic eczema (Korting et al., 1995) as well as to promote wound healing via anti-inflammatory effects (Korting et al., 1993). Hamamelitannin also was shown to inhibit tumor necrosis factor α - mediated endothelial cell death at concentrations less than 100 μM (Habtemariam, 2002). Hamamelitannin, at a minimum concentration of 50 μM,

was also found to have a high protective activity against cell damage induced by peroxides (Masaki et al., 1995a) or UVB radiation (Masaki et al., 1995b). In addition, some antibacterial properties of witch hazel have been reported, where aqueous extracts of the bark or the leaves inhibited the growth of *E. coli, S. aureus, B. subtilis and E. faecalis* (Brantner and Grein, 1994). In contrast, we have determined that hamamelitannin has no effect on bacterial growth *in vitro* even at concentrations as high as 2.5 mM per 1000 bacteria, 13,000 times the MIC of ampicillin to the same *S. aureus* strain (0.2 μM per 1000 bacteria). Hamamelitannin derivative 2-0-acetyl-1,3,5-tris-0-(2-methoxibezoyl)-alpha-D-ribofuranose (Compound 2) had no effect on bacterial attachment, suggesting that the effect of hamamelitannin was specific. Of note is that hamamelitannin that was purchased from Chromadex at >93% purity was re-purified by high pressure liquid chromatography (HPLC, C18 reverse phase Thermo Hypersil Gold) and shown to be as active at >99% purity.

It has been suggested (Otto et al., 1998) that RIP is an amphipathic peptide and thus may work by being a detergent. This is unlikely because neither RIP nor hamamelitannin have any impact on growth even at concentrations as high as 2.5 mM per 1000 bacteria, while a detergent activity would impact growth. Detergents would also exhibit toxicity against eukaryotic cells, which was not found in animals treated either with RIP or with hamamelitannin.

Hamamelitannin inhibits staphylococcal virulence by acting as a quorum sensing inhibitor. This was demonstrated by inhibition of RNAIII production, which is part of the *agr* quorum sensing system. Its effect on RNAIII is similar to that of RIP, and the minimal effective

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concentration of hamamelitannin and RIP on RNAIII production in vitro was <10nM per 1000 bacteria.

Hamamelitannin (and RIP) also inhibit cell attachment in vitro at minimal effective concentration of <10nM per 1000 bacteria. This is interesting because the accepted view has been that *agr* upregulates the expression of genes encoding for toxins and downregulates the expression of genes encoding for cell surface proteins like Protein A and various adhesion molecules, leading to phase variation (Novick et al 1993). It was thus expected that any molecule that inhibits the *agr* would cause an increase in cell adhesion, and therefore disease (Vuong et al., 2003; Kong et al., 2006; Otto 2004, 2007, 2008). However, as shown by many in vivo studies carried out around the world (see below), *agr* inhibitors do in fact suppress diseases. While so many reports indicate that the anti-*agr* is a viable approach, one must consider the possibility that differences in technical approaches, types of disease (chronic or acute), or differences in strains may lead to the different views sometimes held. So far, inhibitors of *agr* were shown to suppress diseases like endocarditis (Cheung et al., 1994; Xiong et al., 2004), pneumonia (Heyer et al., 2002), Cellulitis/abscess/sepsis (Balaban et al 1998; Mayville et al 1999; Wright et al 2005; Gov et al 2001; Vieira-da-Motta et al, 2001; Park et al 2007) , mastitis, keratitis, sepsis, arthritis, osteomyelitis (Balaban et al., 2000), device associated infections (Balaban et al., 2003, 2005, 2007; Cirioni et al., 2003, 2006; Giacometti et al., 2003, 2005; Ghiselli et al., 2004, 2006; Dell'Acqua et al., 2004), and wound infections (Wolcott 2008).

In contrast to the multiple in vivo reports that show that inhibition of *agr* is a viable therapeutic approach, many reports show that when *agr* is directly inhibited, biofilm formation increases in vitro (see e,g, review by Kong et al., 2006). The fact that the in vitro reports do not

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always mirror the in vivo findings may be due to difference in environmental conditions. In addition, the techniques used in biofilm studies in vitro vary and may lead to differences in results (as reviewed by Yarwood and Schlievert 2003). Interestingly, microarray analyses on *agr* mutants also do not show a distinct switch in gene expression, and while indeed protein A is upregulated in *agr* mutants, adhesion molecules are not distinctly upregulated, suggesting that phase variation is not strictly regulated by *agr* (Korem et al., 2005; Dunman et al., 2001; Beenken et al., 2004).

Unlike direct *agr* inhibitors that suppress disease in vivo but enhance biofilm formation in vitro, both RIP and hamamelitannin downregulate both *agr* expression and biofilm formation. Our working hypothesis is that this is because both molecules are expected to affect cellular processes upstream of *agr*. For example, RIP has been shown to downregulate TRAP phosphorylation, leading to upregulation of ctsR/clpC, leading to repression of clpP, which in turn leads to downregulation of virulence, oxidative stress and DNA repair (Derre et al., 1999; Michel et al., 2006; Frees et al., 2004, 2005). Such cells are highly compromised in the host and as shown by the multiple in vivo studies, are nonpathogenic.

Most importantly, Hamamelitannin is an excellent inhibitor of device-associated infections *in vivo*. Inhibition of infection is concentration dependent. Grafts pre-soaked with 30 mg/L hamamelitannin, showed no signs of infection even though the animals were challenged with a high bacterial load of $2x10^7$ CFU. These results are similar to those observed previously with RIP (e.g. Balaban et al., 2005). Device-associated infections are prevented by merely soaking a graft in the hamamelitannin solutions, suggesting that hamamelitannin can be used to coat medical devices to prevent staphylococcal infections, including those caused by drug resistant

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strains MRSA and MRSE. These findings may have important and far-reaching benefits for the prevention and treatment of *S. aureus* and *S. epidermidis* infections.

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REFERENCES

Adhikari RP, Arvidson S and Novick RP (2007) A Nonsense Mutation in agrA Accounts for the Defect in agr Expression and the Avirulence of Staphylococcus aureus 8325-4 traP:kan. *Infect Immun* 75(9):4534-4540.

Balaban N, Collins LV, Cullor JS, Hume EB, Medina-Acosta E, Vieira da Motta O, O'Callaghan R, Rossitto PV, Shirtliff ME, Serafim da Silveira L, Tarkowski A and Torres JV (2000) Prevention of diseases caused by Staphylococcus aureus using the peptide RIP. *Peptides* 21(9):1301-1311.

Balaban N, Giacometti A, Cirioni O, Gov Y, Ghiselli R, Mocchegiani F, Viticchi C, Del Prete MS, Saba V, Scalise G and Dell'Acqua G (2003a) Use of the quorum-sensing inhibitor RNAIII-inhibiting peptide to prevent biofilm formation in vivo by drug-resistant Staphylococcus epidermidis. *J infect dis* 187:625-630.

Balaban N, Goldkorn T, Gov Y, Hirshberg M, Koyfman N, Matthews HR, Nhan RT, Singh B and Uziel O (2001) Regulation of Staphylococcus aureus Pathogenesis via Target of RNAIII-activating Protein (TRAP). *J Biol Chem* 276:2658-2667.

Balaban N, Goldkorn T, Nhan RT, Dang LB, Scott S, Ridgley RM, Rasooly A, Wright SC, Larrick JW, Rasooly R and Carlson JR (1998) Autoinducer of virulence as a target for vaccine and therapy against Staphylococcus aureus. *Science* 280:438-440.

Balaban N, Gov Y, Bitler A and Boelaert JR (2003b) Prevention of Staphylococcus aureus biofilm on dialysis catheters and adherence to human cells. *Kidney International* 63:340-345.

Balaban N and Novick RP (1995) Translation of RNAIII, the Staphylococcus aureus agr regulatory RNA molecule, can be activated by a 3'-end deletion. *FEMS Microbiol Lett.* 133:155-161.

Balaban N and Rasooly A (2000) Staphylococcal enterotoxins. *Int J Food Microbiol* 61:1-10.

Balaban N, Stoodley P, Fux CA, Wilson S, Costerton JW and Dell'Acqua G (2005) Prevention of staphylococcal biofilm-associated infections by the quorum sensing inhibitor RIP. *Clin Orthop Relat Res.* 437:48-54.

Beenken KE, Dunman PM, McAleese F, Macapagal D, Murphy E, Projan SJ, Blevins JS and Smeltzer MS (2004) Global gene expression in Staphylococcus aureus biofilms. *J Bacteriol.* 186:4665-4684

Brantner A and Grein E (1994) Antibacterial activity of plant extracts used externally in traditional medicine. *J Ethnopharmacol* 44:35-40.

Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T and Warren GL

(1998) Crystallography&NMR system: A new software suite for macromolecular structure determination. *Acta Crystallographica* 54:905-921.

Cheung AL, Eberhardt KJ, Chung E, Yeaman MR, Sullam PM, Ramos M, and Bayer AS (1994) Diminished virulence of a sar-/agr- mutant of Staphylococcus aureus in the rabbit model of endocarditis. *J Clin Invest.* 94:1815-1822.

Cirioni O, Giacometti A, Ghiselli R, Dell'Acqua G, Gov Y, Kamysz W, Lukasiak J, Mocchegiani F, Orlando F, D'Amato G, Balaban N, Saba V and Scalise G (2003) Prophylactic Efficacy of Topical Temporin A and RNAIII-Inhibiting Peptide in a Subcutaneous Rat Pouch Model of Graft Infection Attributable to Staphylococci With Intermediate Resistance to Glycopeptides. *Circulation* 108:767-771.

Cirioni O, Giacometti A, Ghiselli R, Dell'Acqua G, Orlando F, Mocchegiani F, Silvestri C, Licci A, Saba V, Scalise G and Balaban N (2006) RNAIII-inhibiting peptide significantly reduces bacterial load and enhances the effect of antibiotics in the treatment of central venous catheter-associated Staphylococcus aureus infections. *The J infec dis.* 193:180-186.

Costerton JW, Montanaro L and Arciola CR (2005) Biofilm in implant infections: its production and regulation. *Int j artificial organs.* 28:1062-1068.

Costerton JW, Stewart PS and Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284:1318-1322.

Derré I, Rapoport G and Msadek T. CtsR, a novel regulator of stress and heat shock response, controls clp and molecular chaperone gene expression in gram-positive bacteria. *Mol Microbiol.* 31:117-31.

Donlan RM and Costerton JW (2002) Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. *Clin Microbiol.* 15:167-193.

Dunman PM, Murphy E, Haney S, Palacios D, Tucker-Kellogg G, Wu S, Brown EL, Zagursky RJ, Shlaes D and Projan SJ (2001) Transcription profiling-based identification of Staphylococcus aureus genes regulated by the agr and/or sarA loci. *J Bacteriol.* 183:7341-53.

Frees D, Chastanet A, Qazi S, Sørensen K, Hill P, Msadek T and Ingmer H (2004) Clp ATPases are required for stress tolerance, intracellular replication and biofilm formation in Staphylococcus aureus. *Mol Microbiol.* 54:1445-62.

Frees D, Sørensen K and Ingmer H (2005) Global virulence regulation in Staphylococcus aureus: pinpointing the roles of ClpP and ClpX in the sar/agr regulatory network. *Infect Immun.* 73:8100-8108.

Furuya EY and Lowy FD (2006) Antimicrobial-resistant bacteria in the community setting. *Nat Rev Micro*. 4:36-45.

Ghiselli R, Giacometti A, Cirioni O, Dell'Acqua G, Mocchegiani F, Orlando F, D'Amato G, Rocchi M, Scalise G and Saba V (2004) RNAIII-inhibiting peptide and/or nisin inhibit experimental vascular graft infection with methicillin-susceptible and methicillin-resistant Staphylococcus epidermidis. *Eur J Vasc Endovasc Surg.* 27:603-607.

Ghiselli R, Giacometti A, Cirioni O, Dell'Acqua G, Bergnach C, Orlando F, Mocchegiani F, Silvestri C, Skerlavaj B, Licci A, Balaban N, Zanetti M, Scalise G and Saba V (2006) RNAIII-inhibiting peptide in combination with the cathelicidin BMAP-28 reduces lethality in mouse models of staphylococcal sepsis. *Shock* 26:296-301

Giacometti A, Cirioni O, Gov Y, Ghiselli R, Del Prete MS, Mocchegiani F, Saba V, Orlando F, Scalise G, Balaban N and Dell'Acqua G (2003) RNA III Inhibiting Peptide Inhibits In Vivo Biofilm Formation by Drug-Resistant Staphylococcus aureus. *Antimicrob Agents Chemother*. 47(6):1979-1983.

Giacometti A, Cirioni O, Ghiselli R, Dell'Acqua G, Orlando F, D'Amato G, Mocchegiani F, Silvestri C, Del Prete MS, Rocchi M, Balaban N, Saba V and Scalise G (2005) RNAIII-inhibiting peptide improves efficacy of clinically used antibiotics in a murine model of staphylococcal sepsis. *Peptides* 26:169-75.

Gov Y, Bitler A, Dell'Acqua G, Torres JV and Balaban N (2001) RNAIII inhibiting peptide (RIP), a global inhibitor of Staphylococcus aureus pathogenesis: structure and function analysis. *Peptides* 22:1609-1620.

Gov Y, Borovok I, Korem M, Singh VK, Jayaswal RK, Wilkinson BJ, Rich SM and Balaban N (2004) Quorum Sensing in Staphylococci Is Regulated via Phosphorylation of Three Conserved Histidine Residues. *J Biol Chem*. 279:14665-14672.

Gustafsson E, Nilsson P, Karlsson S and Arvidson S (2004) Characterizing the dynamics of the quorum-sensing system in Staphylococcus aureus. *J Mole Microbiol Biotec.* 8:232-242.

Habtemariam S (2002) Hamamelitannin from Hamamelis virginiana inhibits the tumour necrosis factor-alpha (TNF)-induced endothelial cell death in vitro. *Toxicon* 40:83-88.

Harms J, Schluenzen F, Zarivach R, Bashan A, Gat S, Agmon I, Bartels H, Franceschi F and Yonath A (2001) High resolution structure of the large ribosomal subunit from a mesophilic eubacterium. *Cell* 107:679-688.

Heyer G, Saba S, Adamo R, Rush W, Soong G, Cheung A and Prince A (2002) Staphylococcus aureus agr and sarA functions are required for invasive infection but not inflammatory responses in the lung. *Infect Immun.* 70:127-33.

Hong-Geller E and Gupta G (2003) Therapeutic approaches to superantigen-based diseases: a review. *J molecular recognition* 16:91-101.

Ji G, Beavis RC and Novick RP (1995) Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proc Nati Acad Sci USA* 92:12055-12059.

Jones TA, Zou JY, Cowan SW and Kjeldgaard (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta crystallographica* 47:110-119.

Kong KF, Vuong C and Otto M (2006) Staphylococcus quorum sensing in biofilm formation and infection. *Int J Med Microbiol.* 296:133-139.

Korem M, Gov Y, Kiran MD and Balaban N (2005) Transcriptional Profiling of Target of RNAIII-Activating Protein, a Master Regulator of Staphylococcal Virulence. *Infect Immun*. 73:6220-6228.

Korem M, Sheoran AS, Gov Y, Tzipori S, Borovok I and Balaban N (2003) Characterization of RAP, a quorum sensing activator of Staphylococcus aureus. *FEMS Microbiol Lett* 223:167-175.

Korting HC, Schafer-Korting M, Hart H, Laux P and Schmid M (1993) Anti-inflammatory activity of hamamelis distillate applied topically to the skin. Influence of vehicle and dose. *Eur J Clinical Pharmacol*. 44:315-318.

Korting HC, Schafer-Korting M, Klovekorn W, Klovekorn G, Martin C and Laux P (1995) Comparative efficacy of hamamelis distillate and hydrocortisone cream in atopic eczema. *Eur J Clinical Pharmacol*. 48:461-465.

Lina G, Jarraud S, Ji G, Greenland T, Pedraza A, Etienne J, Novick RP and Vandenesch F (1998) Transmembrane topology and histidine protein kinase activity of AgrC, the agr signal receptor in Staphylococcus aureus. *Mol Microbiol*. 28:655-652.

Lowy FD (1998) Staphylococcus aureus Infections. *N Engl J Med*. 339:520-532.

Lowy FD (2003) Antimicrobial resistance: the example of Staphylococcus aureus. *J clini invest*. 111:1265-1273.

March JC and Bentley WE (2004) Quorum sensing and bacterial cross-talk in biotechnology. *Curr Opi Biotechnol*. 15:495-502.

Masaki H, Atsumi T and Sakurai H (1995a) Peroxyl radical scavenging activities of hamamelitannin in chemical and biological systems. *Free Radical Res*. 22:419-430.

Masaki H, Atsumi T and Sakurai H (1995b) Protective activity of hamamelitannin on cell damage of murine skin fibroblasts induced by UVB irradiation. *J Dermat Sci*. 10:25-34.

Michel A, Agerer F, Hauck CR, Herrmann M, Ullrich J, Hacker J and Ohlsen K (2006) Global regulatory impact of ClpP protease of Staphylococcus aureus on regulons involved in virulence, oxidative stress response, autolysis, and DNA repair. *J Bacteriol*. 188:5783-5796.

Mayville P, Ji G, Beavis R, Yang H, Goger M, Novick RP and Muir TW (1999) Structure-activity analysis of synthetic autoinducing thiolactone peptides from Staphylococcus aureus responsible for virulence. *Proc Natl Acad Sci U S A.* 96:1218-1223

Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B and Moghazeh S (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J.* 12:3967-3975.

Novick RP, Projan SJ, Kornblum J, Ross HF, Ji G, Kreiswirth B, Vandenesch F and Moghazeh S (1995) The agr P2 operon: an autocatalytic sensory transduction system in Staphylococcus aureus. *Mol Gen Genet.* 248:446-458.

Otto M (2004) Quorum-sensing control in Staphylococci -- a target for antimicrobial drug therapy? *FEMS Microbiol Lett.* 241:135-141.

Otto M (2007) Antibodies to block Staph virulence. *Chem Biol.* 14:1093-1094.

Otto M (2008) Targeted Immunotherapy for Staphylococcal Infections: Focus on Anti-MSCRAMM Antibodies. *BioDrugs.* 22:27-36.

Otto M, Sussmuth R, Jung G and Gotz F (1998) Structure of the pheromone peptide of the Staphylococcus epidermidis agr system. *FEBS Lett.* 424:89-94.

Park J, Jagasia R, Kaufmann GF, Mathison JC, Ruiz DI, Moss JA, Meijler MM, Ulevitch RJ and Janda KD (2007) Infection control by antibody disruption of bacterial quorum sensing signaling. *Chem Biol.* 14:1119-1127.

Robinson, DH (2005) Pleomorphic mammalian tumor-derived bacteria self-organize as multicellular mammalian eukaryotic-like organisms: morphogenetic properties in vitro, possible origins, and possible roles in mammalian 'tumor ecologies'. *Med Hypotheses* 64:177-185

Shaw LN, Jonnson I-M, Singh VK, Tarkowski A and Stewart GC (2007) Inactivation of traP has no effect on the Agr quorum sensing system or virulence of Staphylococcus aureus. *Infect Immun*.75:4519-4527.

Stewart PS and Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. *The Lancet* 358:135-138.

Stoodley P, Sauer K, Davies DG and Costerton JW (2002) Biofilms as complex differentiated communities. *Ann Rev Microbiol.* 56:187-209.

Tsang LH, Daily ST, Weiss EC and Smeltzer MS (2007) Mutation of traP in Staphylococcus aureus has no impact on expression of agr or biofilm formation. *Infect Immun*. 75:4528-4533.

Vieira-da-Motta O, Ribeiro PD, Dias da Silva W and Medina-Acosta E (2001) RNAIII inhibiting peptide (RIP) inhibits agr-regulated toxin production. *Peptides* 22:1621-1627.

Vuong C, Gerke C, Somerville GA, Fischer ER and Otto M. (2003) Quorum-sensing control of biofilm factors in Staphylococcus epidermidis. *J Infect Dis.* 188:706-718.

Wang H, Provan GJ and Helliwell K (2003) Determination of hamamelitannin, catechins and gallic acid in witch hazel bark, twig and leaf by HPLC. *J Pharmaceutical Biomedical Anal.* 33:539-544.

Waters CM and Bassler BL (2005) Quorum sensing: Cell-to-Cell Communication in Bacteria. *Ann Rev Cell Developmental Biol* 21:319-346.

Wolcott RD (2008) Clinical wound healing using signal inhibitors. In: Control of Biofilm Infections by Signal Manipulation. Balaban N, Ed. Springer. Series on Biofilms. Vol 2:

157-170.

Wright JS 3rd, Jin R and Novick RP (2005) Transient interference with staphylococcal quorum sensing blocks abscess formation. *Proc Natl Acad Sci U S A.* 102:1691- 1696.

Xiong YQ, Bayer AS, Yeaman MR, Van Wamel W, Manna AC and Cheung AL (2004) Impacts of sarA and agr in Staphylococcus aureus strain Newman on fibronectin-binding protein A gene expression and fibronectin adherence capacity in vitro and in experimental infective endocarditis. *Infect Immun.* 72:1832-1836

Yang G, Cheng H, Liu C, Xue Y, Gao Y, Liu N, Gao B, Wang D, Li S, Shen B and Shao N (2003) Inhibition of Staphylococcus aureus pathogenesis in vitro and in vivo by RAP-binding peptides. *Peptides* 24:1823-1828.

Yang G, Gao Y, Dong J, Liu C, Xue Y, Fan M, Shen B and Shao N (2005) A novel peptide screened by phage display can mimic TRAP antigen epitope against Staphylococcus aureus infections. *J Biol Chem.* 280:27431-27435 .

Yarwood JM and Schlievert PM (2003) Quorum sensing in Staphylococcus infections. *J Clin Invest.* 112:1620-1625.

FOOTNOTE:

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Legends for figures:

Figure 1: Homology-built model of RIP. Ball-and-stick representation of the RIP model. Note the amphiphilic nature of this peptide. Distances between the midpoints of the three aromatic rings as well as distances of hydrogen bond donors or acceptors to each of the three aromatic rings were utilized to define pharmacophores.

Figure 2: Definition of the pharmacophore that lead to the discovery of hamamelitannin as a RIP small-molecule nonpeptide analog. N,O denotes hydrogen bond donor or acceptor with either nitrogen or oxygen atom. The numbers above or below the straight lines are distance criteria used in the search in Å.

Figure 3: Structure of hamamelitannin (2,5-di-O-galloyl-D-hamamelose).

Figure 4: Hamamelitannin (Hama) has no effect on bacterial growth in vitro: *S. aureus* (1000 cells) were grown overnight at 37^{o} C with increasing amounts of hamamelitannin or RIP. Bacterial density was determined at OD 595 nm.

Figure 5A. Hamamelitannin (Hama) inhibits RNAIII production: $2x10^{7}$ early exponential *S. aureus* cells containing *rnaiii::blaZ* fusion construct were grown for 2.5 hrs with increasing amounts of hamamelitannin or RIP. RNAIII levels were determined as β-lactamase activity (reporter gene product) and denoted as V_{max}.

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Figure 5B. Hamamelitannin competes with RAP: *S. aureus* fusion cells (in their early exponential phase, $2x10^7$ CFU in 30µl LB) were grown for 1 hr at 37^oC with 5µg recombinant RAP+/- 25µg or 50µg hamamelitannin, RNAIII levels were determined for 10 min after substrate addition as β-lactamase activity and denoted as V_{mean}.

Figure 5C. RNAIII production *by* Northern blotting and hemolysin production by western blotting: 1ml cells (early exponential ~10^8 CFU *S. aureus* lab strain 8325-4, MRSA USA300, and clinical *S. epidermidis* isolate strain MH) were grown for 6 hrs in the presence of buffer control or 300µg/ml hamamelitannin added at time 0 and 3 hrs. The cells were harvested by centrifugation and cell pellet and supernatants were collected. From the cell pellet RNA was isolated and RNAIII and *traP* (as a control) were detected by northern blotting using radiolabeled specific probes.

For testing hemolysin production, the supernatants of MRSA+/- hamamelitannin were applied on 20% SDS PAGE, western blotted and δ hemolysin detected using rabbit anti-δ hemolysin antibodies. Equal loading was confirmed by staining.

Figure 6: Hamamelitannin (Hama) inhibits cell attachment *in vitro*: *S. aureus* 8325-4 (**A**) or *S. epidermidis* clinical isolate MH (**B**) were placed in polystyrene plates and incubated with increasing amounts of hamamelitannin or RIP for 3 hrs at 37^oC without shaking. Attached cells were stained and OD_{595nm} determined.

Figure 7: Hamamelitannin inhibits infections in vivo: A: Bacteria ($2x10^7$ CFU) were incubated with hamamelitannin for 30 min before challenge. Seven days later the

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graft was removed and the number of bacteria determined. *no detectable bacteria, suggesting <10 CFU/ml.

B: Grafts were pre-soaked with hamamelitannin, implanted, animals challenged with MRSE or MRSA ($2x10^7$ CFU), and seven days later grafts were removed and number of bacteria determined. *no detectable bacteria, suggesting <10 CFU/ml.

Figure 1

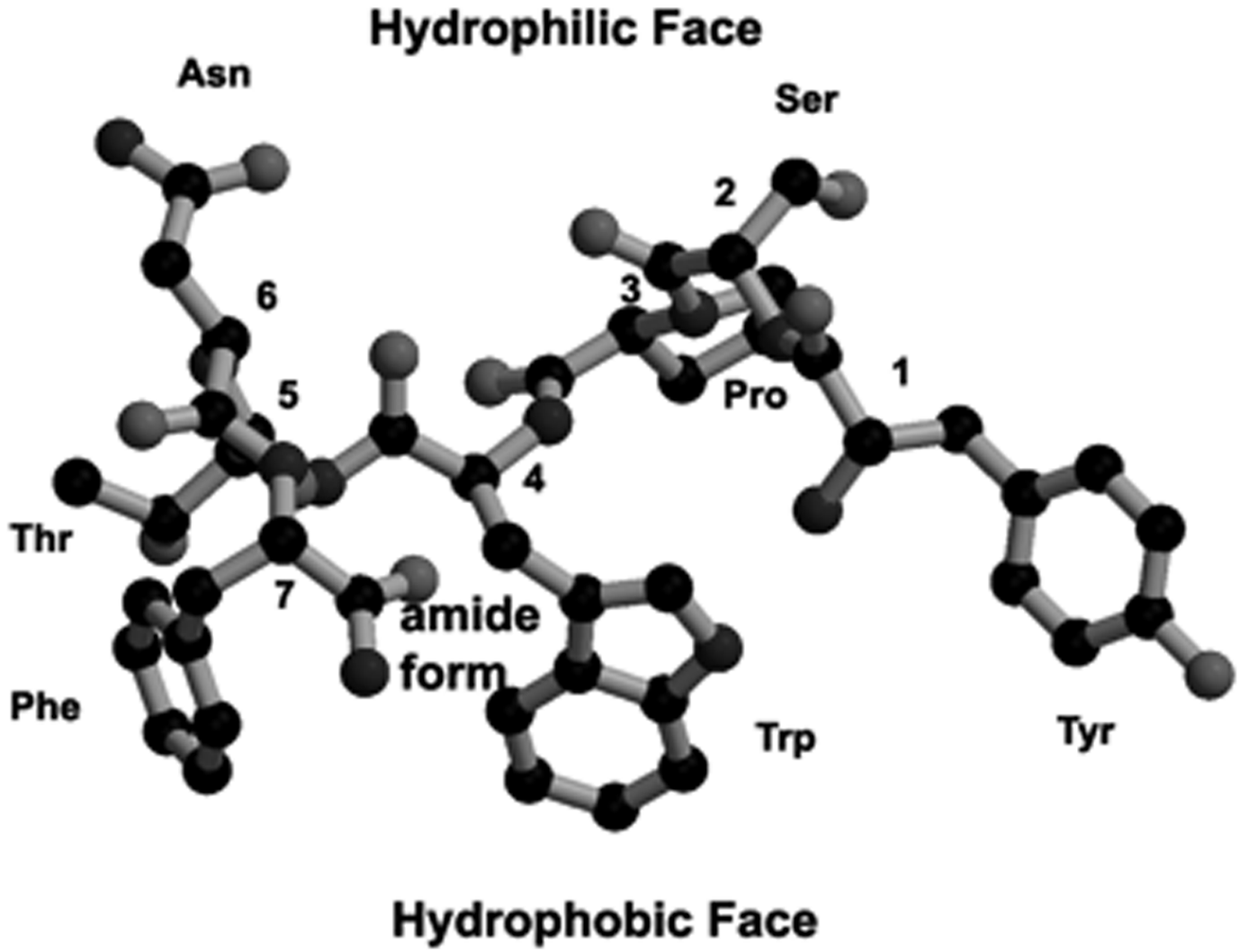

Figure 2

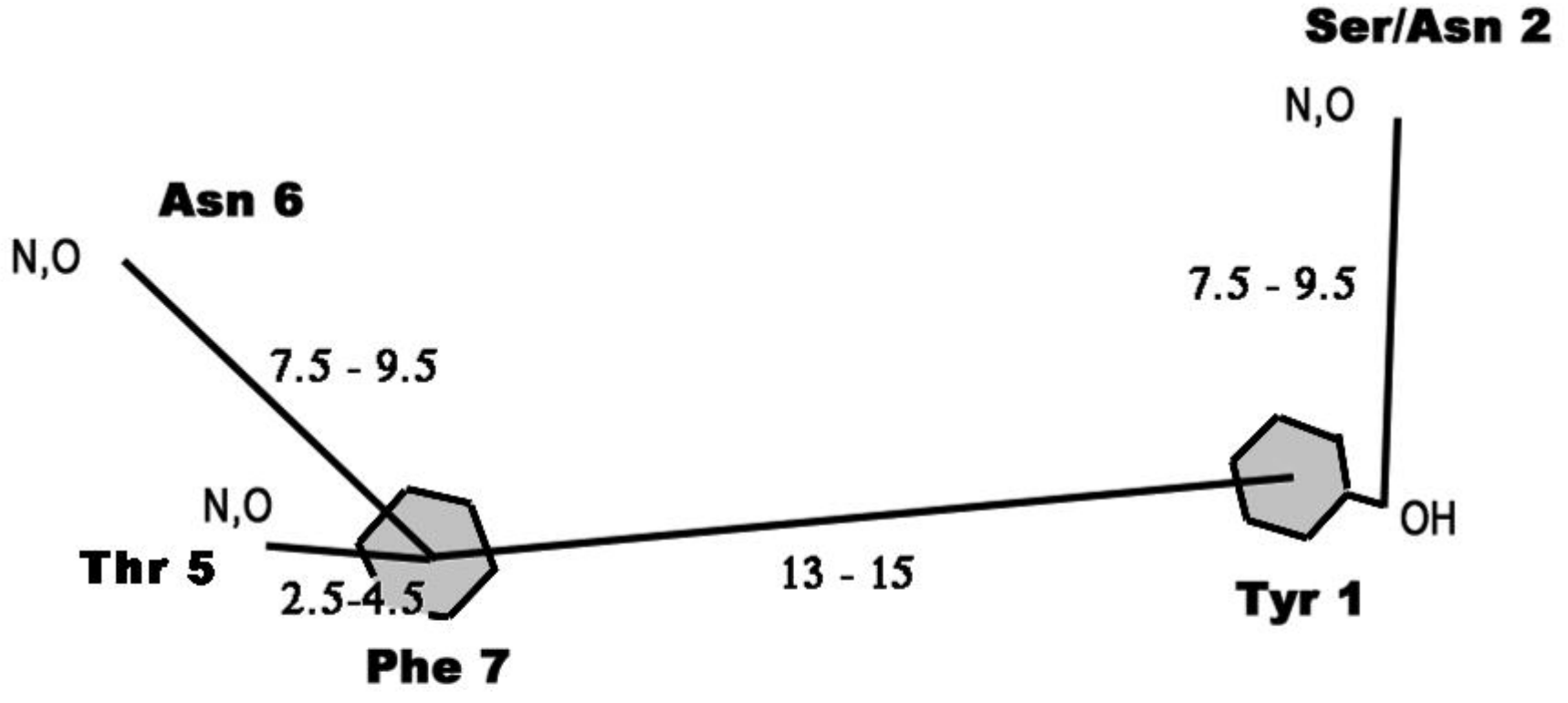

Figure 3

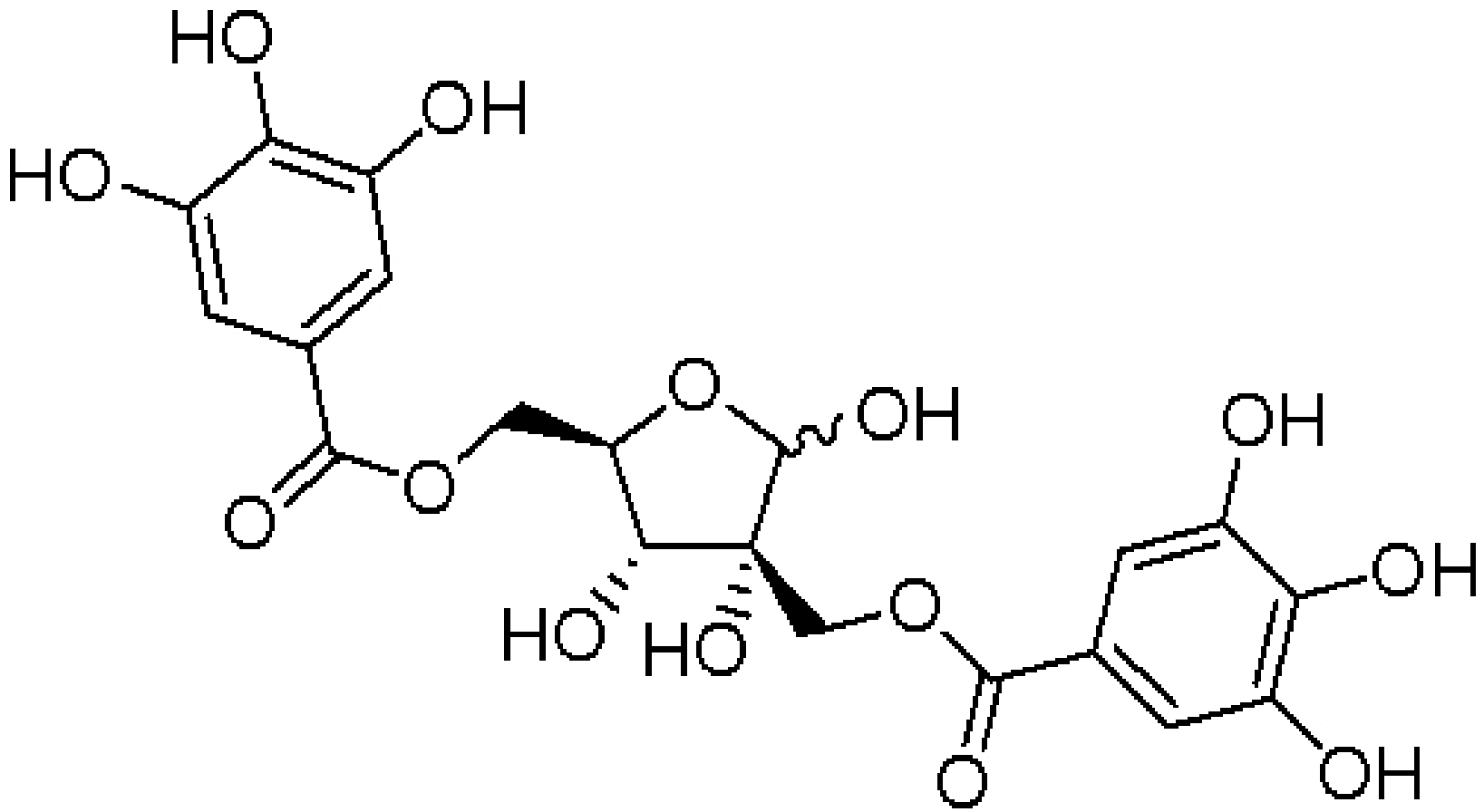

Figure 4

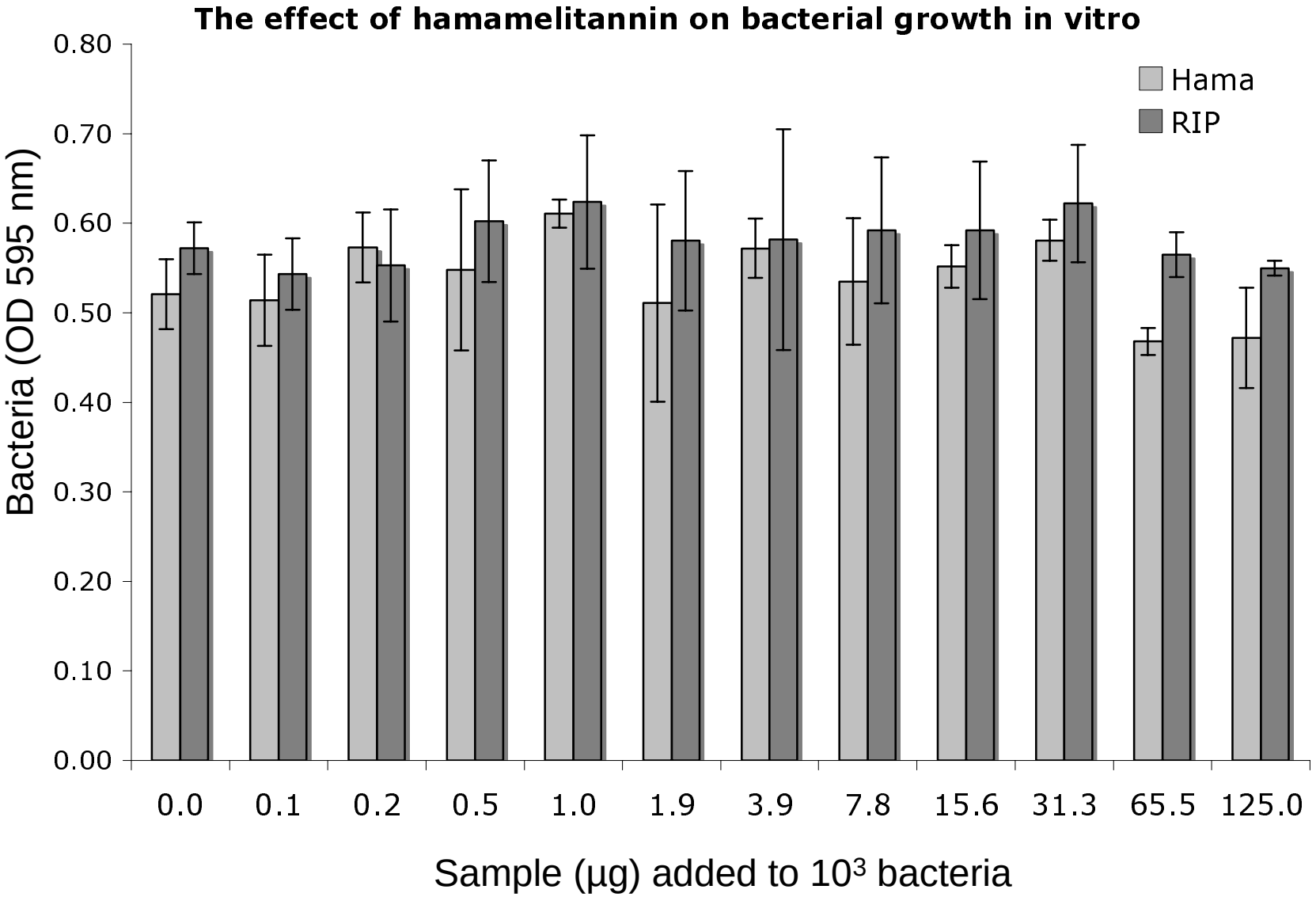

Figure 5A

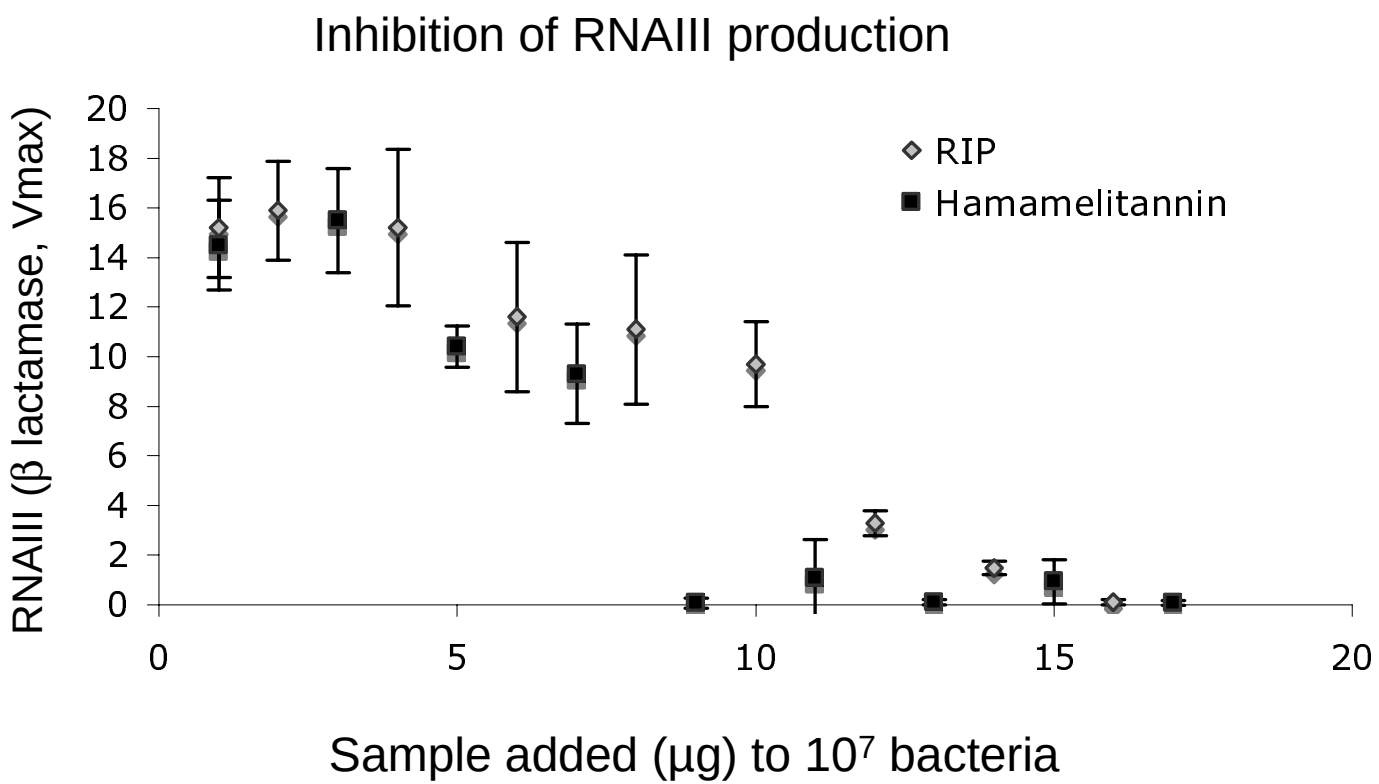

Figure 5B

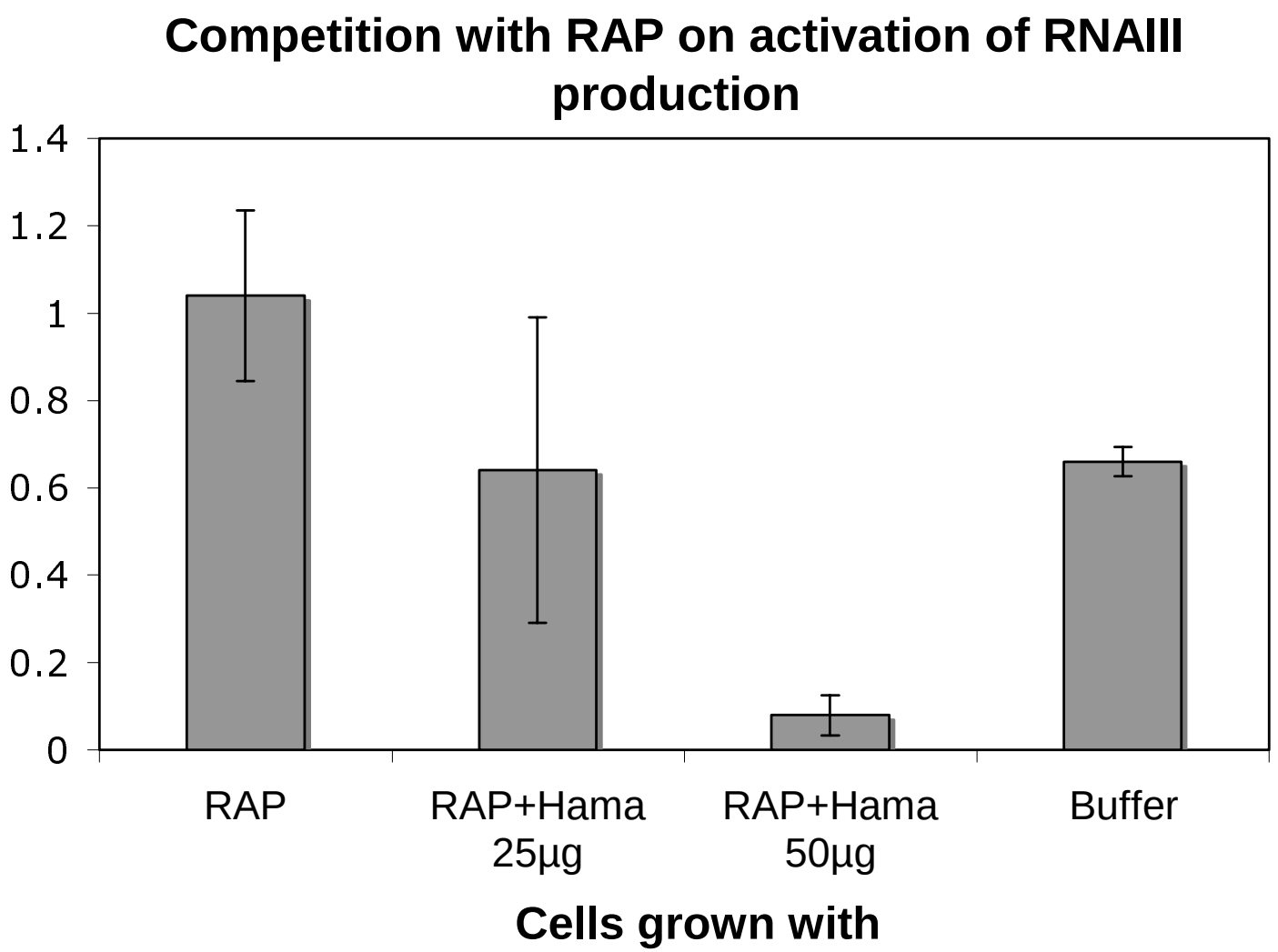

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Figure 5C

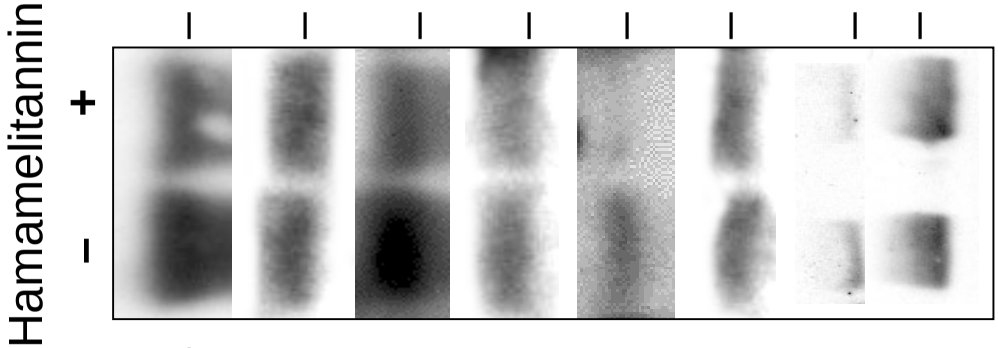

Figure 6A

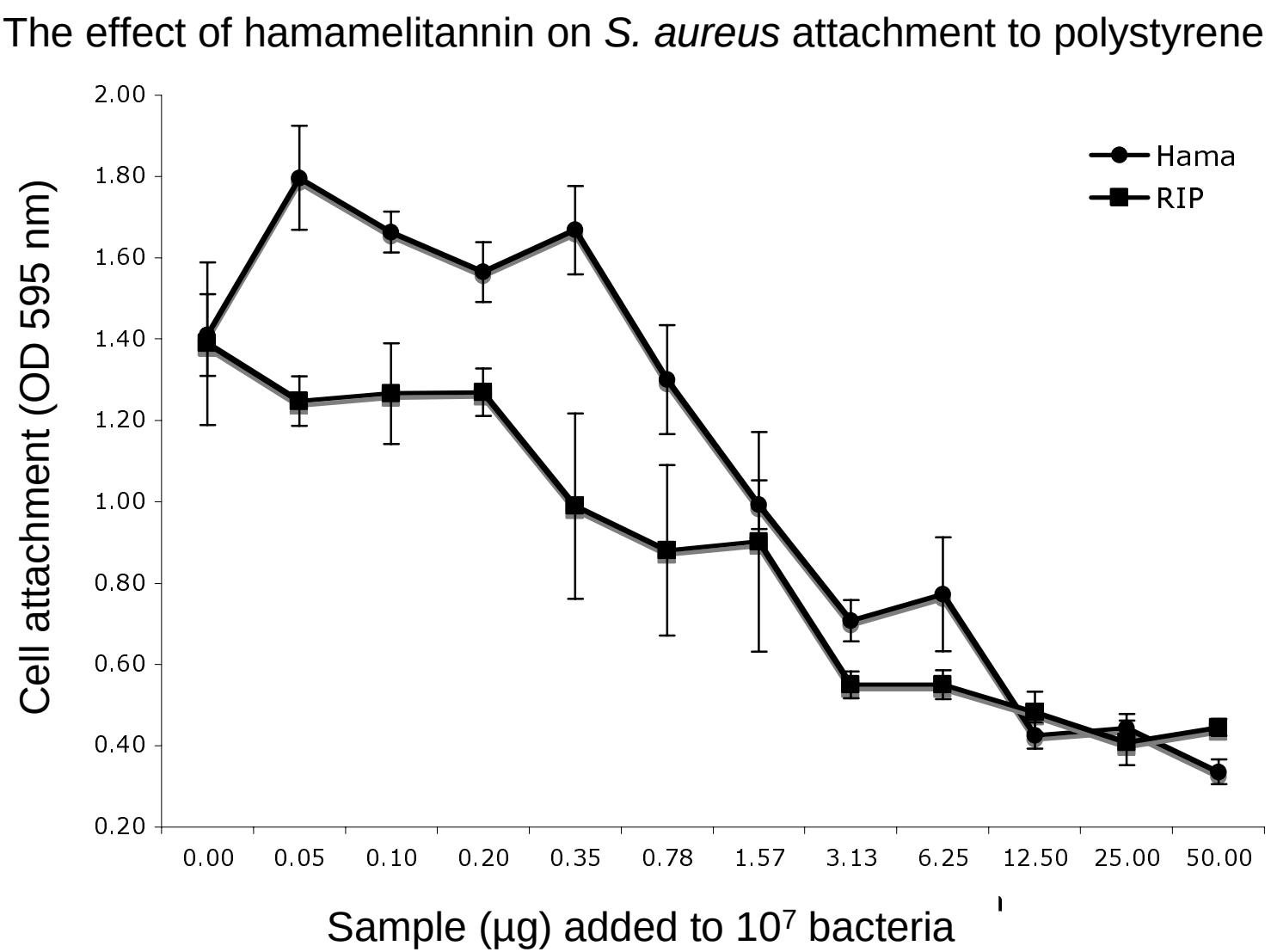

Figure 6B

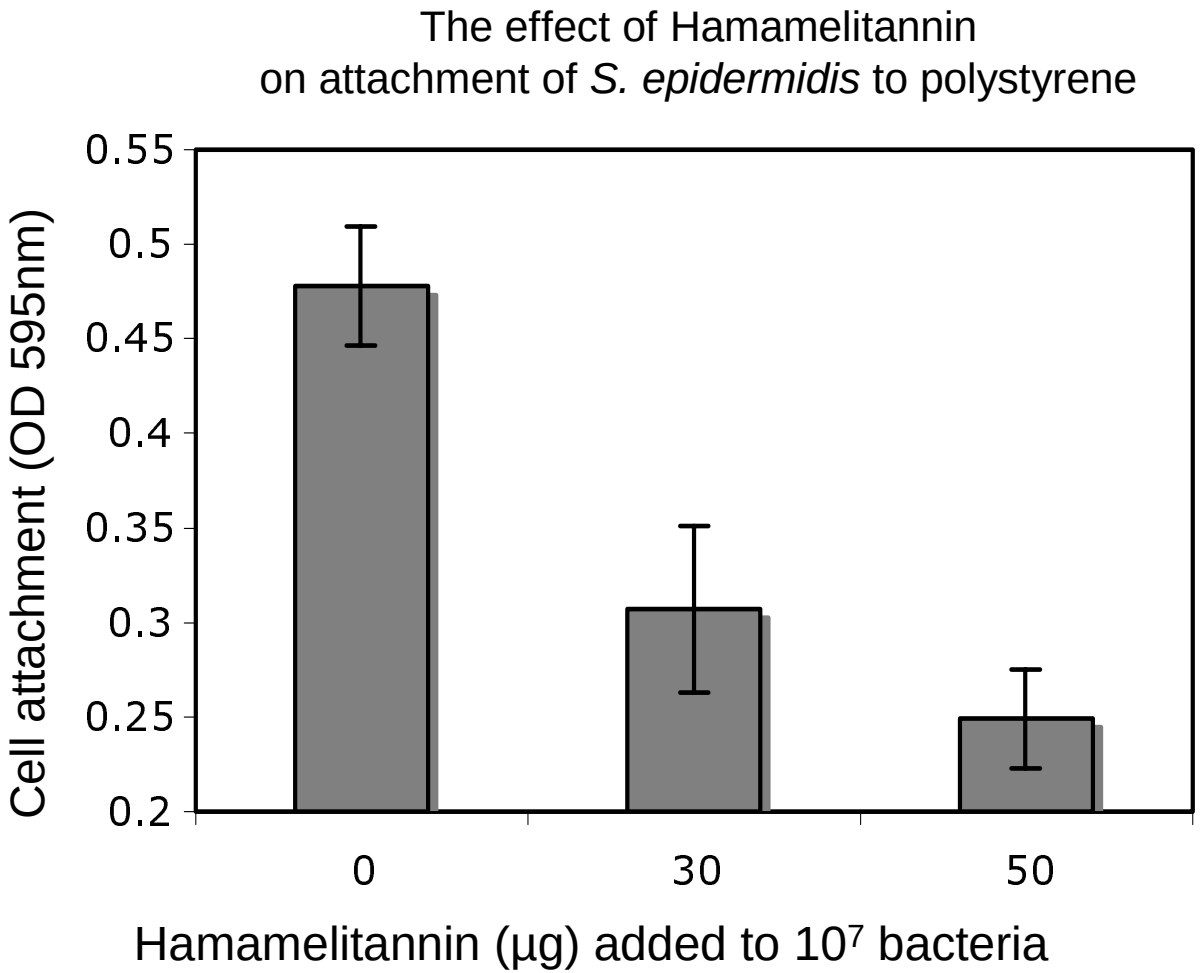

Figure 7A

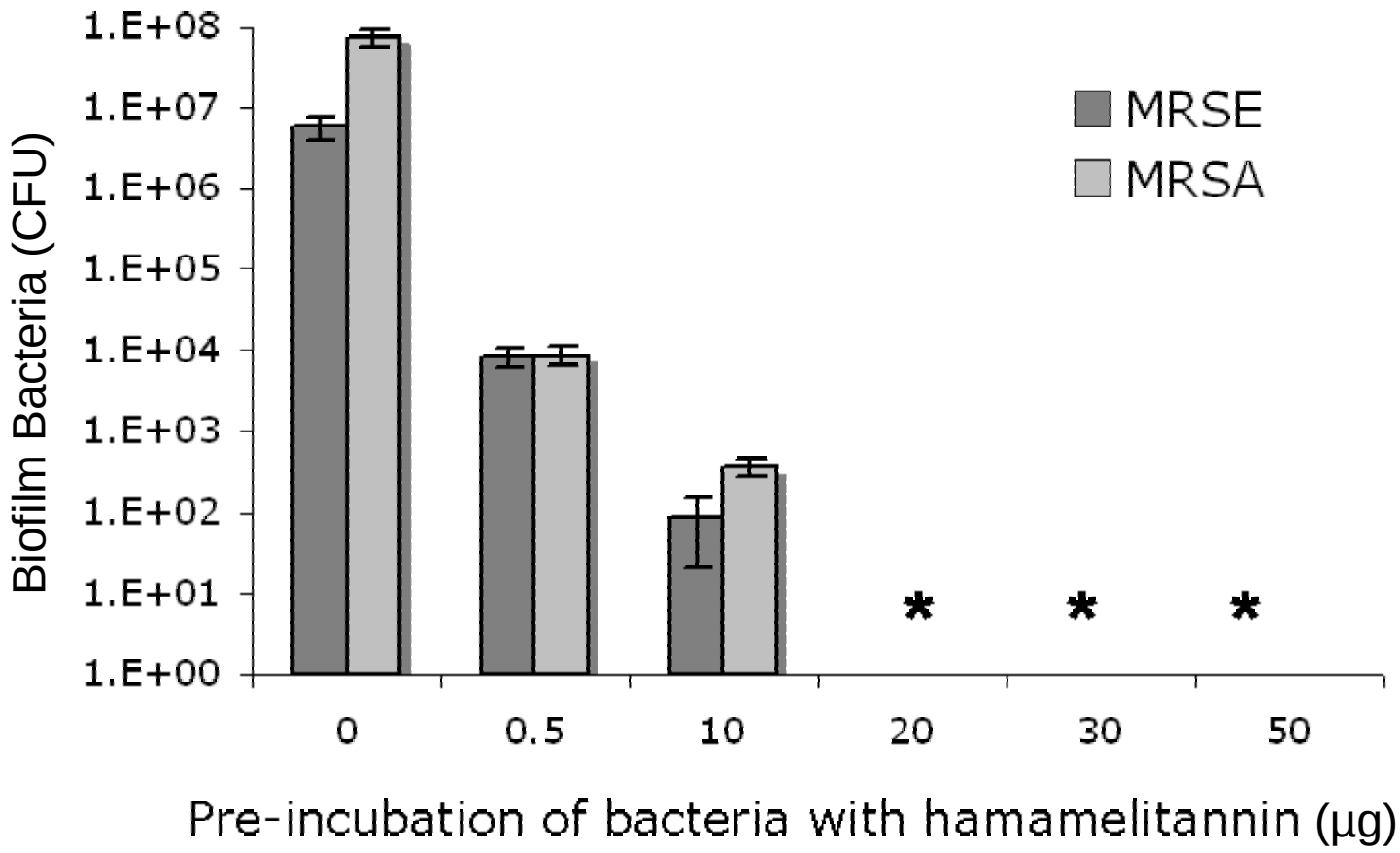

Figure 7B