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Discovery of a Quorum-Sensing Inhibitor of Drug-Resistant Staphylococcal Infections by Structure-Based Virtual Screening

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ABSTRACT

Staphylococci are a major health threat because of 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 2′,5-di-*O*-galloyl-D-hamamelose (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 *Hamamelis virginiana* (witch hazel), and it 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 *Staphylococcus aureus* and *Staphylococcus epidermidis* strains. These findings suggest that hamamelitannin may be used as a suppressor to staphylococcal infections.

Staphylococcus aureus and *Staphylococcus epidermidis* are among the most important pathogens of nosocomial infections, causing more than 70,000 deaths/year in the United States. Nearly all *S. aureus* strains are resistant to penicillin, and many are resistant to methicillin-related drugs (MRSA strains). Cases of intermediate or complete resistance to vancomycin, for many years the only uniformly effective treatment, have emerged (vancomycin-intermediate resistant/-resistant *S. aureus* strains) (Lowy, 1998, 2003; Furuya and Lowy, 2006). Staphylococci are also a common cause of infections related to bacterial biofilm formation on implanted devices. Infections may result in longer hospitalization time, or need for surgery, and they can even cause death (Costerton et al., 1999). Biofilms are highly resistant to antibiotic treatment (Costerton et al., 1999, 2005; Stewart and Costerton, 2001; Donlan and Costerton, 2002; Stoodley et al., 2002). The spread of drug-resistant strains of staphylococci and the ineffectiveness of treatments in cases of biofilm-related infections underscore 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 they can cause fatal diseases as a result of the expression of multiple virulence factors. These factors include adhesins, exotoxins, enterotoxins, hemolysins, and leukocidin, as well as proteases that enable the bacteria to spread within the

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ABBREVIATIONS: MRSA, methicillin-resistant *Staphylococcus aureus*; VISA, vancomycin-intermediate resistant *Staphylococcus aureus*; QS, quorum sensing; SQS, *Staphylococcus* quorum sensing; RAP, RNAIII-activating protein; TRAP, target of RNAIII-activating protein; RIP, RNAIII-inhibiting peptide; MRSE, methicillin-resistant *Staphylococcus epidermidis*; LB, Luria broth; ISIS, Integrated Scientific Information System; ACD, Available Chemicals Database; OD, optical density; CFU, colony-forming unit; L2 Dr, ribosomal protein L2 from *Dienococcus radiodurans*; PDB, Protein Data Bank; MIC, minimal inhibitory concentration; compound 2, 2-0-acetyl-1,3,5-tris-0-(2-methoxibezoyl)-α-D-ribofuranose; Hama, hamamelitannin; PET, polyethylene terephthalate.

host (Lowy, 1998; Balaban and Rasooly, 2000; Hong-Geller and Gupta, 2003). 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 and Novick, 1995; Balaban et al., 1998, 2001; Korem et al., 2003, 2005; Gov et al., 2004). SQS1 induces the activation of SQS2 (Balaban et al., 2001), which encompasses the products of the *agr* system and includes the autoinducer peptide, 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 and Novick, 1995; Balaban et al., 1998, 2001; Korem et al., 2003, 2005; Gov et al., 2004). This suggests that RAP has an extraribosomal 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, 2003a,b, 2005; Gov et al., 2001; Vieira-da-Motta et al., 2001; Cirioni et al., 2003, 2006; Giacometti et al., 2003).

TRAP is a membrane-associated 167-amino acid residue protein that is highly conserved among 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 (Adhikari et al., 2007; Shaw et al., 2007; Tsang et al., 2007), but it seems to involve activation of the *ctsR* operon and ClpP production (M. D. Kiran, unpublished data) that regulate 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 and Rasooly, 2000; Balaban et al., 2000, 2003a,b; Gov et al., 2001; Cirioni et al., 2003, 2006; Giacometti et al., 2003; 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 up-regulated and RIP down-regulated TRAP phosphorylation in vitro, in the absence of other cellular component (K. Kim, personal communication). The sequence of RIP (YSPWTNF-NH2) is similar to the sequence of residues 4 to 9 of RAP (YKPITN). This suggests that RIP is structurally 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 methicillin-resistant *S. aureus* ATCC 43300 (MRSA), methicillin-resistant *S. epidermidis* (MRSE), VISA, and vancomycin-intermediate resistant *S. epidermidis*] (Balaban et al., 2001, 2003b, 2005; Gov et al., 2001; Cirioni et al., 2003, 2006; Giacometti et al., 2003; Lowy, 2003; Korem et al., 2005). These findings indicate that RIP can suppress virulence of any staphylococcal strain (Gov et al., 2004).

In this work, 2′,5-di-*O*-galloyl-D-hamamelose (hamamelitannin; Hama) has been discovered as a nonpeptide analog of RIP that effectively prevents biofilm formation and RNAIII production in vitro as well as device-associated infections in vivo.

Materials and Methods

Bacteria. In vivo studies were carried out using a clinical isolate of MRSE and 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 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 to 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 (SGI, Mountain View, CA).

In Silico Screening for RIP Analogs. Screening for small-molecule nonpeptide analogs of RIP was carried out by a computer search with the Integrated Scientific Information System (ISIS) software from Elsevier MDL (Hayward, CA) 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 (Microsoft, Redmond, WA). 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. Because 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 mass in excess of 1000 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, Santa Fe, NM). The structures of the top hits were superimposed on the RIP model, and they were viewed either with program SwissPDBViewer on a PC or with program O (Jones et al., 1991) on an SGI Octane workstation.

RIP and Hamamelitannin. RIP was synthesized in its amide form (YSPWTNF-NH2) (>98% purity; Neosystem, Strasbourg, France), dissolved in water, and stored at −70°C until use.

Hamamelitannin (ChromaDex, Santa Anna, CA) was dissolved in water and stored at −70°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)-α-D-ribofuranose (compound 2) (Sigma-Aldrich, St. Louis, MO), was dissolved in dimethyl sulfoxide and stored at −70°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). Then, 100 μl of LB containing 1000 freshly prepared bacteria was applied to sterile polystyrene 96-well plates (Falcon; BD Biosciences Discovery Labware, Bedford, MA) together with RIP or hamamelitannin (0–125 μg in 5 μl of water). Bacteria were grown for 24 h at 37°C without shaking, and the optical density was determined at 595 nm. Ampicillin (Sigma-Aldrich) was used a control at 0.01 to 10 μg

Bacterial Attachment in Vitro. Bacteria were grown overnight in LB, diluted 1:100 in LB, and grown for approximately 2 h more to the early exponential phase of growth ($OD_{595\ nm}$ = 0.2). To test for cell attachment, 0.1 ml (equivalent to approximately 6×10^7 bacteria) was placed in polystyrene 96-well plates (Falcon; BD Biosciences Discovery Labware) with 5 μl of water, RIP, hamamelitannin, or compound 2 or control 3% (final) dimethyl sulfoxide. Cells were grown for 3 h without shaking at 37°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-h incubation, unbound cells were removed, and they were gently washed two times with phosphate-buffered saline. Cells were air-dried, fixed with 100% ethanol, dried, and then stained for 2 min with filtered 0.4% gentian violet diluted in 12% ethanol. Stain was removed, and wells were gently washed five times with phosphate-buffered saline. Then, 100 μl of 1% SDS was added to solubilize stained cells, and the 96-well plate was read at $OD_{595\ nm}$ in an enzyme-linked immunosorbent assay plate reader.

RNAIII and δ-Hemolysin Production in Vitro. Northern blotting and β-lactamase transcriptional fusion was used as described previously (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, 2×10^7 colony-forming units (CFUs) in 30 μl of LB] were grown for 2.5 h at 37°C with increasing amounts of hamamelitannin or RIP or for 60 min with or without 5 μg of recombinant RAP (in 5 μl of buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, and 10% glycerol). β-Lactamase activity was measured by adding the substrate nitrocefin (Calbiochem, San Diego, CA) (40 μl of nitrocefin; 132 μg/ml in 0.1 M sodium phosphate buffer, pH 5.8). OD was determined using a microtiter plate reader (KC4; Bio-Tek Instruments, Winooski, VT) at 490/630 nm using the kinetic analysis mode, and results are 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 (3×10^8 CFUs in 1 ml of LB). Cells were grown for 6 h in the presence of 12 μl of water or 300 μg of hamamelitannin that was added at time 0 and 3 h. The cells were harvested by centrifugation at 8000*g* for 10 min. The cell pellet and supernatants (see below) were collected. From the cell pellet, RNA was isolated and RNAIII was detected by Northern blotting as described previously (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 previously (Balaban et al., 2001).

For testing hemolysin production, the supernatants of MRSA ± hamamelitannin were filtered through a 0.22-μm filter, and then they were concentrated to 10× by evaporation. Next, 5 μl was applied on 20% SDS-polyacrylamide gel electrophoresis and Western blotted. δ-Hemolysin was detected using rabbit anti-δ-hemolysin antibodies as described previously (Balaban and Novick, 1995). Equal loading was confirmed by ponceau S (Diasys Europe, Wokingham, UK).

Rat Graft in Vivo Infection. Sterile collagen-sealed double velour-knitted polyethylene terephthalate (PET; Dacron) grafts were used 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 was shaved, and the skin was cleansed with 10% povidone-iodine solution. One subcutaneous pocket was made on each side of the median line by a 1.5-cm incision. Sterile PET grafts (1 cm^2) were implanted aseptically into the pockets. Before implantation, the PET graft segments were soaked for 1 h in different concentrations of hamamelitannin or saline. The pockets were closed by means of skin clips and saline (1 ml) containing the staphylococcal strains at a concentration of 2×10^7 CFUs/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 they were thoroughly examined daily. All grafts were explanted 7 days after 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. 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 of hamamelitannin/2×10^7 bacteria in 150 μl of saline), and the mixture was used for challenge. In experiment 2, PET grafts were soaked for 1 h with hamamelitannin at concentrations of 0.5, 10, 20, 30, and 50 mg/l before implantation and challenge.

Statistical Analysis. Quantitative culture results from all groups are presented as mean ± S.D., and the statistical comparisons between groups were made using analysis of variance on the log-transformed data with Tukey-Kramer honestly significant difference test. Significance was accepted when the P value was ≤0.05.

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, because RIP competes with RAP (Korem et al., 2003) and the sequence of RIP (YSPWTNF) is similar to the sequence of residues 4 to 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. Because 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 *D. 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. Posi-

tions 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 (Brünger et al., 1998) (Fig. 1).

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 (e.g., 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 (Fig. 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 Did Not Affect Bacterial Growth in Vitro. To test whether hamamelitannin has antibacterial activity, 1000 CFUs of *S. aureus* were grown for 24 h with 0 to 125 μg of hamamelitannin in a final volume of 100 μl (up to 2.5 mM). As shown in Fig. 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 vancomycin-intermediate resistant *S. epidermidis*). In this context, it is noteworthy that the minimal inhibitory concentration (MIC) of antibiotics such as 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 Competed with RAP and Inhibited RNAIII and δ-Hemolysin Production in Vitro. To test whether hamamelitannin is a quorum-sensing inhibitor and thus suppresses *agr* activity, 2×10^7 cells containing *rnaiii*::*blaZ* fusion construct (reporter cells) were incubated with increasing amounts (0–50 μg) 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 Fig. 5A, both hamamelitannin and RIP inhibit RNAIII production in a concentration-dependent manner, and they are most effective at concentrations >7 μg/10^7 bacteria (~5 nM/10^3 bacteria). Reporter cells were also grown in the presence of 5 μg of recombinant RAP and 25 and 50 μg of hamamelitannin, and then they were tested for RNAIII production 60 min later. As shown in Fig. 5B, recombinant RAP significantly ($P < 0.05$) up-regulated RNAIII production, and 50 μg of hamamelitannin significantly ($P < 0.01$) competed with RAP and down-regulated RNAIII production. Of note is 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 h, and RNAIII was 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., 2001)

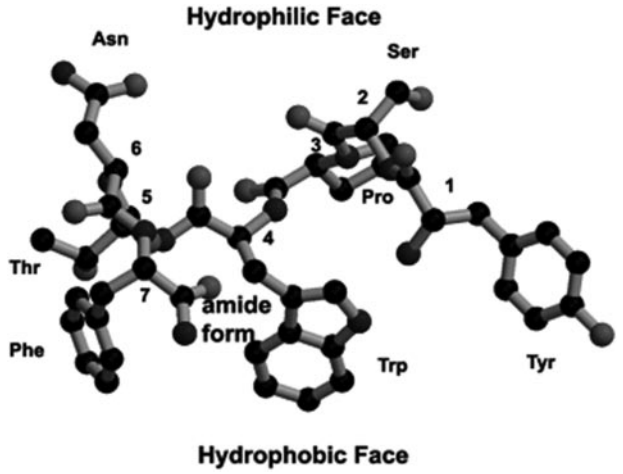

Fig. 1. Homology-built model of RIP. Ball-and-stick representation of the RIP model. Note the amphiphilic nature of this peptide. Distances between the midpoints of the three aromatic rings as well as distances of hydrogen bond donors or acceptors to each of the three aromatic rings were used to define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

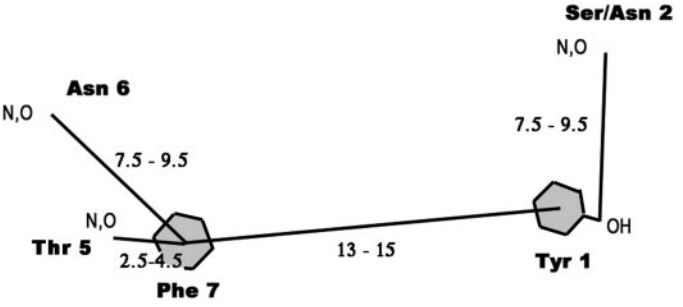

Fig. 2. Definition of the pharmacophore that lead to the discovery of hamamelitannin as a RIP small-molecule nonpeptide analog. N,O denotes hydrogen bond donor or acceptor with either nitrogen or oxygen atom. The numbers above or below the straight lines are distance criteria used in the search in angstroms.

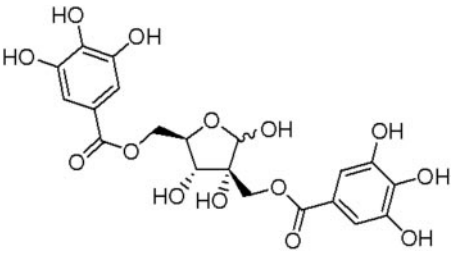

Fig. 3. Structure of hamamelitannin.

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

Hamamelitannin Inhibited Cell Attachment in Vitro. To test for the effect of hamamelitannin on bacterial attachment in vitro, *S. aureus* cells were incubated with 0 to 50 μg of hamamelitannin or RIP in polystyrene plates for 3 h at 37°C. Adherent bacteria were stained, and OD was determined. As shown in Fig. 6A, hamamelitannin (or RIP) reduced cell attachment in a concentration-dependent manner, and it was most effective when ~10^7 bacteria were grown in 4 μg of hamamelitannin or RIP (~8 nM/10^3 bacteria). Similar results were obtained with MRSA and with *S. epidermidis* (data not shown). Hamamelitannin derivative 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 Fig. 6B. Of note is that attachment experiments were carried out over a short period (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 Prevented Device-Associated Infections in Vivo. To measure the amount of hamamelitannin necessary to prevent device-associated infections, bacteria (2×10^7 MRSA or MRSE) were preincubated with increasing amounts of hamamelitannin for 30 min at room temperature. Grafts were implanted, and rats were challenged with the preincubated bacteria. Seven days later, the graft was removed and bacterial load was determined. As shown in Fig. 7A, although bacterial load in the control untreated group was ~10^7 CFUs/ml, bacterial load on the graft decreased with increasing dose of hamamelitannin. No bacteria were found when either bacteria (MRSA or MRSE) was preincubated with >20 μg of hamamelitannin, comparable with results obtained previously with RIP (Balaban et al., 2005).

In a parallel experiment, grafts were soaked for 1 h in

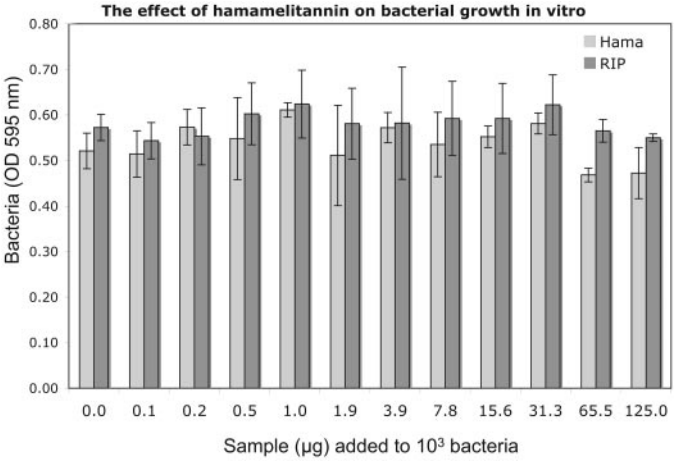

Fig. 4. Hama has no effect on bacterial growth in vitro: *S. aureus* (1000 cells) were grown overnight at 37°C with increasing amounts of hamamelitannin or RIP. Bacterial density was determined at $OD_{595\ nm}$.

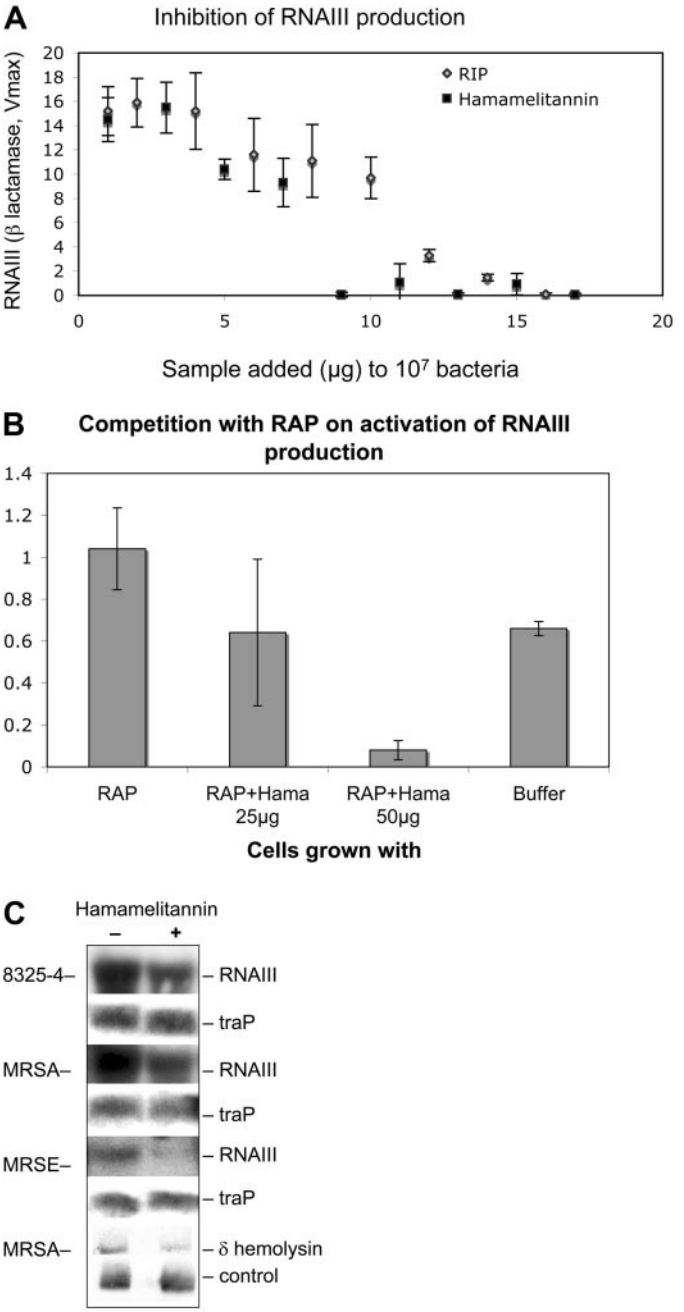

Fig. 5. A, Hama inhibits RNAIII production: 2×10^7 early exponential *S. aureus* cells containing *rnaiii*::*blaZ* fusion construct were grown for 2.5 h with increasing amounts of hamamelitannin or RIP. RNAIII levels were determined as β-lactamase activity (reporter gene product) and denoted as V_{max}. B, hamamelitannin competes with RAP: *S. aureus* fusion cells (in their early exponential phase, 2×10^7 CFUs in 30 μl of LB) were grown for 1 h at 37°C with 5 μg of recombinant RAP ± 25 or 50 μg of hamamelitannin, and RNAIII levels were determined for 10 min after substrate addition as β-lactamase activity and denoted as V_{mean}. C, RNAIII production by Northern blotting and hemolysin production by Western blotting: 1 ml of cells (early exponential ~10^8 CFUs of *S. aureus* lab strain 8325-4, MRSA USA300, and clinical *S. epidermidis* isolate strain MH) was grown for 6 h in the presence of buffer control or 300 μg/ml hamamelitannin added at time 0 and 3 h. 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.

increasing hamamelitannin concentrations. The grafts were subsequently implanted into the animal, and bacteria were injected onto the graft. Seven days later, the graft was removed, and bacteria on the graft were counted. As shown in Fig. 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, whereas untreated control groups demonstrated evidence of graft infections, with quantitative culture results showing ~10^7 CFUs/ml. Grafts soaked in 30 mg/l hamamelitannin showed no sign of bacterial load.

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 is the ester of D-hamamelose (2-hydroxymethyl-D-ribose) with two molecules of gallic acid (Fig. 3). Because gallic acid contains three phenolic functional groups, hamamelitannin is considered a polyphenol, and polyphenols 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 it 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. They are currently used in skin care products and in dermatological treatment of sunburn, irritated skin, and 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, also was 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

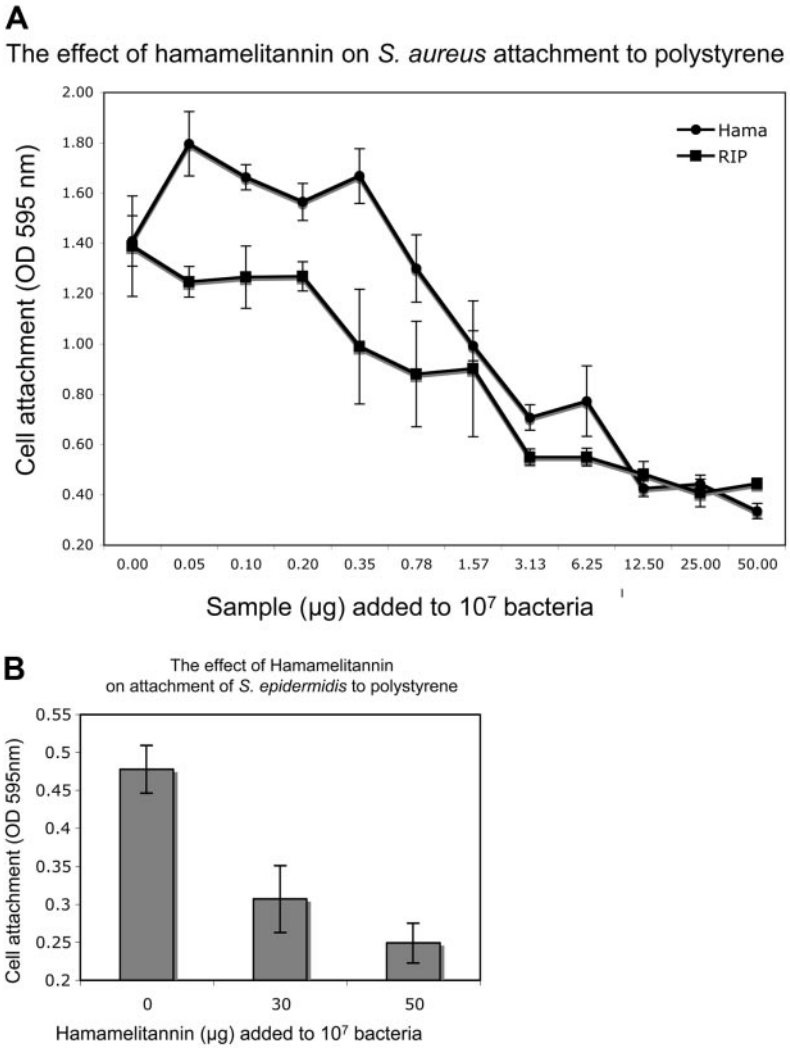

Fig. 6. Hama inhibits cell attachment in vitro: *S. aureus* 8325-4 (A) or *S. epidermidis* clinical isolate MH (B) was placed in polystyrene plates and incubated with increasing amounts of hamamelitannin or RIP for 3 h at 37°C without shaking. Attached cells were stained, and $OD_{595\ nm}$ was determined.

inhibited the growth of *Escherichia coli*, *S. aureus*, *Bacillus subtilis*, and *Enterococcus 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/1000 bacteria, 13,000 times the MIC of ampicillin to the same *S. aureus* strain (0.2 μM/ 1000 bacteria). Hamamelitannin derivative 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 repurified by high-pressure liquid chromatography (C18 reverse phase, Thermo Hypersil Gold; Thermo Fisher Scientific, Waltham, MA), and it was shown to be as active at >99% purity.

It has been suggested (Otto et al., 1998) that RIP is an amphipathic peptide; thus, it 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/1000 bacteria, whereas a detergent activity would affect 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 concentration of hamamelitannin and RIP on RNAIII production in vitro was <10 nM/1000 bacteria.

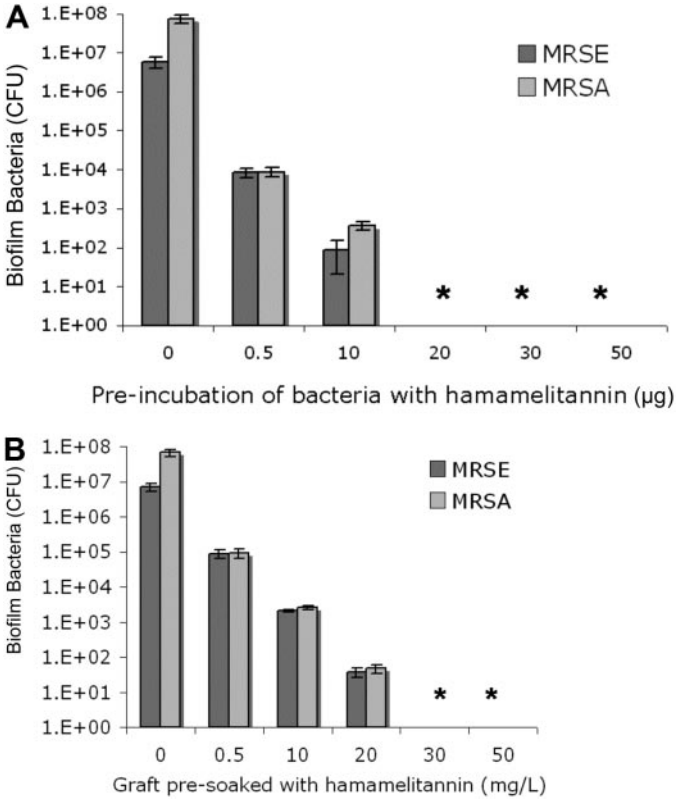

Fig. 7. Hamamelitannin inhibits infections in vivo. A, bacteria (2×10^7 CFUs) were incubated with hamamelitannin for 30 min before challenge. Seven days later, the graft was removed, and the number of bacteria was determined. Asterisk (*) indicates no detectable bacteria, suggesting <10 CFU/ml. B, grafts were presoaked with hamamelitannin, and then they were implanted and animals were challenged with MRSE or MRSA (2×10^7 CFUs). Seven days later, grafts were removed, and the number of bacteria was determined. Asterisk (*) indicates no detectable bacteria, suggesting <10 CFUs/ml.

Hamamelitannin (and RIP) also inhibit cell attachment in vitro at a minimal effective concentration of <10 nM/1000 bacteria. This is interesting because the accepted view has been that *agr* up-regulates the expression of genes encoding for toxins and that it down-regulates the expression of genes encoding for cell surface proteins such as 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. Although 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 such as 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; Gov et al., 2001; Vieira-da-Motta et al., 2001; Wright et al., 2005; 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; Dell'acqua et al., 2004; Ghiselli et al., 2004, 2006); 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 (for review, see Kong et al., 2006). That the in vitro reports do not 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 they may lead to differences in results (for review, see Yarwood and Schlievert 2003). It is noteworthy that microarray analyses on *agr* mutants also do not show a distinct switch in gene expression, and although protein A is indeed up-regulated in *agr* mutants, adhesion molecules are not distinctly up-regulated, suggesting that phase variation is not strictly regulated by *agr* (Dunman et al., 2001; Beenken et al., 2004; Korem et al., 2005).

Unlike direct *agr* inhibitors that suppress disease in vivo but enhance biofilm formation in vitro, both RIP and hamamelitannin down-regulate *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 down-regulate TRAP phosphorylation, leading to up-regulation of ctsR/clpC, leading to repression of clpP, which in turn leads to down-regulation of virulence, oxidative stress, and DNA repair (Derré et al., 1999; Frees et al., 2004, 2005; Michel et al., 2006). Such cells are highly compromised in the host, and as shown by the multiple in vivo studies, they are nonpathogenic.

Most importantly, hamamelitannin is an excellent inhibitor of device-associated infections in vivo. Inhibition of infection is concentration-dependent. Grafts presoaked with 30 mg/l hamamelitannin showed no signs of infection, even though the animals were challenged with a high bacterial

load of 2×10^7 CFUs. 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 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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