Supporting Information

Sustained Release of a Synthetic Autoinducing Peptide Mimetic Blocks Bacterial Communication and Virulence In Vivo

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SUPPORTING INFORMATION

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Materials and methods.

Reagents, bacterial strains, and general methods. Poly (DL-lactide-co-glycolide) (PLG; lactide:glycolide, 50:50; 30–60 kDa), N,N-dimethylformamide (DMF), tetrahydrofuran (THF), dimethylsulphoxide (DMSO), chloramphenicol, and the protein ApoB were purchased from Sigma-Aldrich and used as according to accompanying instructions. Phosphate-buffered saline (PBS, 10x solution) was purchased from Dot Scientific and diluted 10x into water and titrated to pH 7.4 before use. Deionization of distilled water was performed using a Milli-Q system, yielding 18.2 MΩ water. The USA300 LAC methicillin-resistant Staphylococcus aureus (MRSA) strain was used for growth experiments. The USA300 LAC MRSA reporter strain AH1677[1] was used for reporter assays and general in vivo murine dermonecrosis assays. The S. aureus wild-type lab strain RN6390B[2] and the Δagr RN9222[3] strain were used only to examine agr dependence for abscess formation in the dermonecrosis model. All strains were cultured with brain heart infusion (BHI, Teknova) medium and incubated at 37 °C with shaking at 200 rpm, with AH1677 cultures supplemented with 10 µg/mL chloramphenicol.

Instrumentation and related considerations. Top-down scanning electron micrographs were acquired using a LEO-1550 VP field-emission SEM instrument operated with an accelerating voltage of 1.0 kV. Microparticle diameters were measured using ImageJ version 1.52a software. Solution fluorescence and absorbance in bacterial cultures was measured using a BioTek Synergy 2 plate reader using Gen5 software. RP-HPLC was performed on a Shimadzu system with a SCL-10Avp controller, a DGU-20A5 degasser, a LC-20AT solvent delivery unit, a SIL-10AF autosampler, a CO-20A column oven with a manual injector, a SPD-M20A UV-Vis diode array detector, and analytical Kromasil Eternity C18 column (4.6 mm by 250 mm, with a 5 mm particle size and 100 Å pore size). Solvent A was water with 0.1% trifluoroacetic acid and solvent B was acetonitrile with 0.1% trifluoroacetic acid. A linear gradient was used to analyze peptides and solutions, beginning at 10% solvent B, and ramping to 95% solvent B over 27 min at a flow rate of 1 mL/min.

Fluorescence-based bacterial reporter assay. Compound activity screening was performed as previously described using S. aureus AH1677.[4] GraphPad Prism 7 software was used for statistical analysis of the resulting data. Supernatants from microparticle release experiments were subjected to this reporter assay with the following modifications: supernatant was serially diluted into PBS, and 20 µL aliquots of each dilution were transferred to a black 96-well microtiter plate and incubated with 180 µL of 1:50 dilution of an overnight culture with fresh BHI medium. Each plate contained additional controls for data processing: a PBS control (20 µL) and a tr AIP-III D2A control (100 nM final concentration, 2 µL of 10 µM tr AIP-III D2A stock solution in DMSO and 18 µl PBS).

Ex vivo tissue sequestration assay. In 1.5 mL microcentrifuge tubes, 1 mM DMSO stock solutions of compounds were diluted in PBS to 10,000x their respective IC₅₀ values in a total volume of 500 µL, along with a PBS control without any compound. A mouse tissue sample was bath sonicated for 5 minutes (and, if appropriate, incubated at 80 °C for 15 min then cooled in ice bath for 5 min) and transferred into each microcentrifuge tube. The tubes were vortexed, and 10 µL aliquots were stored in a chemical resistant microtiter plate in a -20 °C freezer. The microcentrifuge tubes were incubated at 37 °C with shaking at 200 rpm until the next time point was reached, at which point the tubes were removed, vortexed, and additional 10 µL aliquots were taken. This process was repeated for all the time points. After the final time point, 2 µL of each aliquot or a dilution (including 5- and 10-fold dilutions) of each aliquot was transferred to a black 96-well microtiter plate and tested using the fluorescence reporter assay detailed above.
**ApoB peptide sequestration assay.** In 1.5 mL microcentrifuge tubes, 1 mM DMSO stocks of peptides were diluted to 50 µM in 0.5 mL water either with or without 10 µg/mL added ApoB. This quantity of ApoB was selected to compare to past reports studying AIP interactions with ApoB as well as physiological levels of ApoB in human serum. Tubes were shaken for 3 hr at 37 °C. Solutions were then transferred to 10k mol. wt. cutoff dialysis cassettes (Thermo Fisher), incubated in 25 mL of 5% DMSO in water, and shaken gently for 2 h at room temperature to dialyze compound. Thereafter, two 10 mL portions of dialysate were transferred to falcon tubes and lyophilized. Lyophilized samples were reconstituted in 250 µL of 20% acetonitrile in water and analyzed by analytical RP-HPLC using the solvents and gradients described above to quantify the amount of peptide.

**Fabrication of tr AIP-III D2A-loaded PLG microparticles.** A 0.8 mM solution of tr AIP-III D2A in DMF was diluted with THF to produce a THF:DMF solvent system (3:1, v/v) resulting in a final tr AIP-III D2A concentration of 0.2 mM. A 60 mg/mL solution of PLG in THF:DMF (3:1, v/v) was prepared and allowed to stir until the polymer was dissolved. Electro-spraying of the microparticles was conducted on a custom-built electro-spraying device with a digital syringe pump (Harvard Bioscience Co.) at a flow rate of 1.0 mL/hr. A 15 cm working distance separated a blunt 20G needle and the 10 x 10 cm grounded aluminum foil sample collector. The produced microparticles were harvested from the surface of the aluminum foil using an eyelash applicator brush, followed by immersion of the brush in water and sonicating to create a microparticle suspension. The suspension was then lyophilized to produce dry microparticles.

**Characterization of peptide loading of PLG microparticles.** Microparticles were dissolved in DMSO (20 mg/mL) and then precipitated into water by mixing 100 µL of the DMSO solution into 900 µL of water. The suspension was centrifuged at 5,000 g for 5 min, and the resulting supernatant was analyzed by analytical RP-HPLC to determine the amount of peptide in the microparticles.

**Characterization of peptide loaded PLG microparticle release profiles.** PLG microparticles (10 mg) were immersed in 1 mL of PBS. The particles were sonicated to suspend them in solution and allowed to incubate while rotating at room temperature. At predetermined time points, the microparticles were centrifuged at 5,000 g for 5 min and the supernatant was removed and replaced. Each supernatant sample was immediately frozen and stored at -20 °C until analysis. At time of analysis, supernatant samples were thawed and centrifuged at 16,000 g for 5 min to remove any solid polymer debris that may have been isolated with the supernatant. The concentrations of the peptides released at each timepoint were measured using the microparticle-modified bacterial reporter assay described above. After 21 days of incubation in buffer, the remaining polymer solids were characterized to determine the amount of unreleased peptide using the dissolution and analytical RP-HPLC method described above.

**Murine abscess infection model.** The infection assay protocol was modified from that of Sully et al. Briefly, cultures of the appropriate S. aureus strain were grown overnight in BHI medium and diluted in sterile PBS to an OD600 corresponding to 2x10⁸ CFU/mL. For experiments on peptide in solution, DMSO (1%, control) or peptide in DMSO solution were added to 2x10⁸ CFU/mL culture to reach an appropriate desired concentration (50 µM or 500 nM). For PLG microparticle experiments, 2x10⁸ CFU/mL culture was added to lyophilized microparticle tubes to reach a concentration of 20 mg/mL particles. Cultures were vortexed to mix and then 50 µL were injected subcutaneously into the shaved flank of age- and sex-matched C57BL/6 mice. Body weight was recorded at time of injection and monitored over the course of the experiment (see Figure S7 for all body weight data collected from all experiments; no consistent differences between treated and untreated animals were observed over the time course of these
experiments). Images of lesions were taken on days 1, 3, 5, and 7, and analyzed using ImageJ to determine lesion area. GraphPad Prism 7 was used to obtain statistical information about the abscess sizes.

All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at UW–Madison (Approval #: V005384; PI: Prof. Jonathan McAnulty) and conformed to the NIH Guide for the Care and Use of Laboratory Animals, 8th edition (2011).

**Statistical information.** All statistical tests and sigmoidal regression fits were performed using GraphPad Prism (GraphPad Software, version 7.0). For reporter assay experiments, data were generated by at least three independent experiments unless noted otherwise. Separate biological replicates were the result of three technical replicates. Sequestration of compound by ApoB was evaluated with two-tailed unpaired t-tests. Statistical comparisons of *in vivo* experiments were evaluated using two-tailed Mann-Whitney tests or one-way Kruskal-Wallis tests with Dunn’s correction for multiple comparisons. The criterion used to accept statistical significance was a p value of less than 0.05.
Figure S1: A schematic of ex vivo experimental system to examine compound activity incubated at 10,000-fold IC$_{50}$ in presence of mouse tissue.
Figure S2: Activity of S. aureus agr-I fluorescence reporter strain when exposed to supernatant of AIP-III D4A incubated in PBS in the presence (blue line) or absence (red line) of mouse tissue sample. Both a full aliquot (A) and a 5-fold dilution (B) were assayed. The values shown are the average and SEM of three (n=3) technical replicates. Some error bars are obscured due to the size of the data point.
Figure S3: Activity of AIP-III D4A in *S. aureus* agr-I fluorescent reporter strain when exposed to either heat denatured mouse tissue (blue) or a control tissue (red). Both a full aliquot (A) and a 10-fold dilution (B) were assayed. The values shown are the average and SEM of three (n=3) technical replicates. Some error bars are obscured due to the size of the data point.
Figure S4: Examination of interactions of AIP-III D4A and tr AIP-III D2A with 10 µg/ml ApoB as analyzed via analytical RP-HPLC. Peaks were analyzed by both peak area (A, B) and peak height (C, D). The peak area for AIP-III D4A (A) was substantially reduced (unpaired t-test, t=46.80, df=2, ***p<0.001) in the presence of ApoB, while there was no significant effect on the peak area corresponding to tr AIP-III D2A (B) when ApoB was added (unpaired t-test, t=0.5398, df=2). Similar results were obtained when analyzing via peak height. The peak height for AIP-III D4A (C) was substantially reduced (unpaired t-test, t=78, df=2, ***p<0.001) in the presence of ApoB, while there was no significant effect on the peak height corresponding to tr AIP-III D2A (D) when ApoB was added (unpaired t-test, t=0.551, df=2). Peak areas and heights were normalized to the average peak height of controls lacking ApoB. Analytical RP-HPLC analysis performed in duplicate (n=2). Data represented as mean and SEM.
Figure S5: *S. aureus* agr activity and growth curves in the presence of tr AIP-III D2A. Time-courses for treatment of the AH1677 reporter strain (A,B) or the parent USA300 LAC strain (C) with 50 µM tr AIP-III D2A (blue) or DMSO vehicle control (red) over 24 hours. YFP fluorescence values (A) and OD\textsubscript{600} values (B) for AH1677 demonstrate the effects of compound treatment on agr activity and growth, respectively, compared to the vehicle control. Comparison of the growth curves for AH1677 (B) to USA300 LAC (C) on treatment with vehicle (red) suggest that the lag in growth observed for AH1677 is caused by production of YFP protein upon agr activation. Data represented as mean and standard deviation for each group of three (n=3) independent technical and biological replicates.
Figure S6: Infection abscess analysis of mice inoculated with wild-type and Δagr S. aureus. (A) Observed abscess size for mice receiving either a wild-type strain of S. aureus (RN6390B, n=6) or an Δagr mutant of this strain (RN9222, n=6). Values shown represent averages and SEM. Representative images of mice from (B) wild-type infected group and (C) Δagr infected group. Ruler indicates centimeters.
**Figure S7**: Mouse body weight changes during *in vivo* experiments. Introduction of tr AIP-III D2A in bolus or microparticle format *in vivo* using subcutaneous injection in the mouse abscess model shows no clear, consistent, or large negative effect on mouse body weight compared to controls. (A) Dose escalation and *in vivo* safety of tr AIP-III D2A experiment: bolus delivery of 2.5 nmol (1x, 50 µM) tr AIP-III D2A, 5 nmol (2x, 100 µM) tr AIP-III D2A, or vehicle (1% DMSO in PBS), n=8 mice per condition. (B) Delivery of 2.5 nmol (50 µM) tr AIP-III D2A bolus or vehicle (1% DMSO in PBS) mixed with *Staphylococcus aureus* agr-I AH1677, n=10 mice per condition. (C) Delivery of 1 mg tr AIP-III D2A-loaded microparticles (n=10 mice) or empty microparticles (n=9 mice) with AH1677. (D) Delivery of 1 mg tr AIP-III D2A-loaded microparticles, 25 pmol tr AIP-III D2A in bolus, or vehicle (1% DMSO in PBS) with AH1677, n=8 mice per condition. Data represents mean and SEM for each group.
Images of mouse lesions from day 7 of abscess experiments.

2.5 nmol tr AIP-III D2A vs. DMSO vehicle control experiment:

Control - Day 7  2.5 nmol tr-AIP III D2A - Day 7

White bar = 1 cm.
1 mg tr AIP-III D2A particles vs. empty (unloaded) PLG particles experiment:

Unloaded Microparticles - Day 7

tr AIP-III D2A Loaded Microparticles - Day 7

White bar = 1 cm.
1 mg tr AIP-III D2A particles vs. 25 pmol tr AIP-III D2A vs. DMSO vehicle experiment:

White bar = 1 cm.
Figure S8: PLG microparticle size histogram as measured by SEM. The average particle diameter was 1.48 ± 0.38 µm. Particle diameters measured by the minor axis of elliptically shaped particles. The histogram shows measurements for (n=450) microparticles (150 particles were counted from three independent samples).
Figure S9: HPLC standard curve for tr AIP III D2A. Serially diluted tr AIP-III D2A was analyzed by RP-HPLC to determine relationship between peak height and concentration.

\[
y = 12.18x - 11.31 \\
R^2 = 0.999
\]
Discussion of peptide quantification.

We utilized Equation S1, the four-parameter logistic regression model, to model the dose-response activity of our inhibitory compounds in *S. aureus agr* reporter assays. By rearranging the variables into Equation S2, using the observed activity level of samples with unknown concentrations in our reporter assays, we can then estimate the concentration of the compound in samples from our other assays (i.e., compound release from microparticles). Activity values between 20–80% were used to estimate compound concentration.

- **Y** = *agr* activity level
- **X** = Compound concentration in nanomolar
- **T** = Top (upper) plateau of activity in model
- **B** = Bottom (lower) plateau of activity in model
- **H** = Hill slope
- **IC$_{50}$** = Inhibitory Concentration at 50% activity

**Equation S1:**

$$ Y = B + \frac{T - B}{1 + \left(\frac{X}{IC_{50}}\right)^H} $$

**Equation S2:**

$$ X = IC_{50} \left(\frac{T - B}{Y - B} - 1\right)^{1/H} $$
Figure S10: Dose-response inhibition curve for tr AIP-III D2A following the microparticle-modified S. aureus agr fluorescence reporter assay. The values shown are the average and SEM of three (n=3) independent technical and biological replicates. The following parameters were observed for this dose response analysis: T=106.1, B=4.293, IC\textsubscript{50}=0.3353, H=1.648. These values were used in Equation S2 to quantify concentration of compound released in microparticle characterization experiments.
Figure S11: Activity of supernatants isolated over time in release experiments from tr AIP-III D2A-loaded PLG microparticles. Compound activity measured using the *S. aureus* agr-I fluorescence reporter. Supernatants were diluted 10-fold into reporter strain culture for each timepoint. The values shown are the average and single standard deviation of three (n=3) independent technical and material replicates.
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