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Modulation of Quorum Sensing in a Gram-Positive Pathogen by Linear Molecularly Imprinted Polymers with Anti-infective Properties

Anfal Motib, Antonio Guerreiro,* Firas Al-Bayati, Elena Piletska, Irfan Manzoor, Sulman Shafeeq, Anagha Kadam, Oscar Kuipers, Luisa Hiller, Todd Cowen, Sergey Piletsky, Peter W. Andrew, and Hasan Yesilkaya*

Abstract: We describe the development, characterization, and biological testing of a new type of linear molecularly imprinted polymer (LMIP) designed to act as an anti-infective by blocking the quorum sensing (QS) mechanism and so abrogating the virulence of the pathogen *Streptococcus pneumoniae*. The LMIP is prepared (polymerized) in presence of a template molecule, but unlike in traditional molecular imprinting approaches, no cross-linker is used. This results in soluble low-molecular-weight oligomers that can act as a therapeutic agent in vitro and in vivo. The LMIP was characterized by mass spectrometry to determine its monomer composition. Fragments identified were then aligned along the peptide template by computer modeling to predict the possible monomer sequence of the LMIP. These findings provide a proof of principle that LMIPs can be used to block QS, thus setting the stage for the development of LMIPs a novel drug-discovery platform and class of materials to target Gram-positive pathogens.

Antibiotic resistance is one of the top threats to global health.^[1] There is an urgent need to develop antimicrobial agents that are effective and less prone to microbial resistance. Interfering with bacterial communication systems is considered an effective strategy to develop anti-infective agents.^[1] Bacteria communicate with each other to coordinate their behaviour through the production and detection of signalling molecules, a mechanism called quorum sensing (QS).^[2] A range of phenotypes are modulated by QS systems,

including growth, biofilm formation, and virulence expression.^[3,4] Since QS systems are absent in mammals, this minimises the risks of host-toxicity of therapies which target microbial QS.^[5] The QS systems of Gram-negative bacteria use small molecules (homoserine lactones), while Gram-positive bacteria utilize processed peptides.^[2] Conventional methods to manipulate QS systems include the use of lactonases and amidases (hydrolyzing the homoserine lactone) or removal of the signalling molecule using affinity ligands like peptides and antibodies.^[6–8] A degree of success in the modulation of QS by these two methods has been reported in in vitro and ex vivo models, although work is needed to improve the stability of therapeutic agents based on biomolecules.^[9,10] Alternatives include synthetic cross-linked adsorbents like molecularly imprinted (and non-imprinted) polymers, which are not biodegradable. These have been used to remove homoserine lactones from solution and prevent QS.^[11,12] Linear random co-polymers have been tested, but less successfully due to lower affinity towards the signalling molecule.^[13] Unfortunately cross-linked polymers have limitations as drug candidates since it is impossible to elucidate their random structure. Affinity ligands made with linear molecularly imprinted polymers (LMIPs)^[14] can overcome some of these limitations since they are smaller in size and can conceivably be sequenced. These are ideal attributes that should allow for their use as drug candidates or drug-discovery tools.

The TprA receptor and its signalling peptide PhrA^[15] were chosen as the QS target for two reasons. First, this pair is widespread across bacilli and streptococci and is thus relevant to human pathogens.^[16] Second, we present data that demonstrate that TprA/PhrA system is a major virulence determinant. It is important for pneumococcal growth on galactose and mannose as well as on mucin (Figure S1 in the Supporting Information), the primary carbon sources for pneumococcus in vivo.^[17,18] PhrA peptide expression is induced by galactose and mannose (Table S1 in the Supporting Information). Independent deletion mutation of TprA and PhrA both abrogates pneumococcal virulence in a mouse model of pneumonia and septicemia (Figure S2), as well as in a chinchilla model of otitis (Figure S3). Therefore, it was envisaged that interfering with the binding of PhrA by TprA would block the phenotypic manifestations of the TprA/PhrA QS system. In order to prepare a modulator of this system, the inducer (signalling) peptide (PhrA10) was used to create the LMIP (LMIP-PhrA). PhrA10 is the active signal peptide with capability to induce PhrA expression,^[15] and the C-terminal end of this peptide (SNGLDVGKAD) was used as a template

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for LMIP preparation. Unlike the binary functional monomer/cross-linker compositions commonly used for production of MIPs, a mixture of different monomers with various functionalities was used to prepare the LMIP and so avoid the formation of homopolymers (which would be incapable of selective binding). It is hypothesized that in order to obtain good affinity and selectivity, the sequence of monomers along the polymer backbone should be directed pre-polymerization by self-assembly around the template. Two monomers capable of establishing ionic interactions were used: acrylic acid and *N*-(3-aminopropyl)methacrylamide. Acrylamide was used since it can establish hydrogen bonds, and *N*-*tert*-butylacrylamide was used to target hydrophobic areas on the template. To perform solid-phase imprinting, PhrA10 was immobilized at the surface of glass beads as the solid-phase (SP) according to an established method (see the Supporting Information).^[19] These were then placed in contact with the aqueous monomer mixture and polymerization initiated.

After polymerization and affinity purification on SP, 9 mg of polymer were obtained, which is equivalent to 0.15 mg gram⁻¹ of SP used. As determined by mass-spectrometry, the molecular weight of the polymer was on average 1006 Da (Figure S4 b). The following identifiable fragments were observed in the SIR mode: m/z 73 (acrylic acid, $M + 1$), m/z 214 (triplet of acrylamide, $3M + 1$), m/z 270 (one molecule of *N*-*tert*-butylacrylamide and doublet of acrylamide); m/z 593 (three acrylamide and three molecules of butyl acrylamide). The MRM fragmentation of both m/z 740 and the largest ion (m/z 1006) generated a m/z 381.4 daughter, possibly a triplet of butyl acrylamide moieties, which was also observed in the SIR mode (Figure S4). There were losses of 28 Da associated with m/z 381.4, 739.6 and 1005.9 ions. Another recurrent pattern was the loss of a neutral fragment (358 Da) during the fragmentation of m/z 739.6 and m/z 1005.9, which could correspond to two moieties of acrylamide and three moieties of acrylic acid. Accordingly, the polymer consisted mainly of sequences containing three acrylamide, three butyl acrylamide, and two or three acrylic acid blocks. This method mimics in essence a single biopanning cycle. In addition to serving as the template during imprinting, immobilised PhrA10 also works at the washing/elution stage as a selector for enrichment of affinity binders from the pool of LMIPs. After affinity separation, the obtained LMIP was analysed by mass spectrometry. Since the data obtained point to a single product, no further enrichment steps were performed.

Surflex-Dock and Sybyl Leapfrog molecular modelling were applied to predict positions of the LMIP fragments along the PhrA10 sequence^[20] (Figure 1). Acrylic acid trimer showed relatively high and equal affinity for either of two arrangements around the asparagine residue, forming either a four-point interaction (hydrogen-bond donation to the C and N residue carbonyls, and acceptance from the N amine and side-chain amide) or five-point interaction (hydrogen-bond donation to the carbonyls of G and the N side chain, acceptance from the N amide and amines of the G and N backbone), in which the two interactions with the G residue are relatively weaker. The strongest interaction found by Leapfrog was a three-point interaction between the acrylam-

Figure 1. Molecular complex between the LMIP fragments and the C-terminal end of the PhrA peptide (SNGLDVGKAD), as predicted by computational modelling. N-terminal C added for SH coupling onto the solid phase.

ide trimer with the carbonyl and amine of the leucine residue and the carboxylate of the aspartic acid residue. The butyl acrylamide trimer also showed three points of interaction with PhrA10, all hydrogen-bond donations going from the fragment amides to the backbone carbonyls of the V, G, and K residues. The LMIP structure is predicted to be X-AA-AA-AA-X-Ac-Ac-Ac-X-BA-BA-BA-X, where AA = acrylic acid, Ac = acrylamide, BA = butyl acrylamide, and X = either a join between the adjacent subunits or another fragment of the polymer, with the total mass of X being approximately 195 Da (Figure S4). This approach is similar in nature to fragment-based drug discovery, with the added advantage that the sequence of fragments is guided by a self-assembly process that takes place during molecular imprinting.^[21] The affinity of LMIP-PhrA for the PhrA10 peptide was studied by surface plasmon resonance (SPR; Figure 2). The results indicate that the LMIP binds PhrA10 in a dose-dependent and sequence-specific manner. The dissociation constant (K_D) was estimated to be 8.16 μ M (Figure 2 A), which correlates with the bioactive concentration range. Conversely, the K_D of the interaction between LMIP-PhrA and a scrambled peptide with the same amino acids (DAKGVDLGNS) and in similar concentration range was much higher (>0.13 mM) and practically no binding was observed (Figure 2 B).

The effect of LMIP-PhrA/PhrA10 binding on to TprA receptor was assessed by surface plasmon resonance (SPR). For this, TprA was immobilized on a SPR chip and then PhrA10 (ranging from 0.2 to 50 μ M) was injected in order to establish the minimal concentration of PhrA10 necessary for binding to occur to TprA, which was 25 μ M. PhrA10 at 25 μ M (Figure 2 C) was then co-injected onto the TprA receptor with the LMIP at a concentration of 100 nM, as used in the microbiological inhibition assays (see the Supporting Information). LMIP-PhrA significantly altered the binding behaviour of PhrA10 and TprA, with an 84 % reduction in binding response and a reduction in the dissociation rate of the complex PhrA10/LMIP-PhrA/TprA, resulting in irreversible binding. No measurable interaction was observed upon

Figure 2. SPR sensorgrams. A) Injections of PhrA10 onto the immobilized LMIP. B) Injections of scrambled PhrA10 onto the LMIP; no response at any of the concentrations tested. C) PhrA10 at 25 μM injected onto immobilized TprA (curve PhrA10), and PhrA10 (at 25 μM) injected alongside LMIP-PhrA onto immobilized TprA.

injection of 100 nM LMIP-PhrA onto immobilized TprA in the absence of PhrA10. Having demonstrated the specific binding and consequent blocking of the TprA receptor by PhrA10/LMIP-PhrA, LMIP-PhrA was then tested to determine whether it would attenuate the induction of β -galactosidase activity by the *phrA* promoter (*PphrA*), and decrease pneumococcal growth on galactose (for which the TprA/*PhrA* system is required).^[15] The inhibitory role of LMIP on *PphrA*-driven β -galactosidase activity in the presence of PhrA10 was evaluated since PhrA activates the transcriptional expression of PhrA on galactose. Addition of PhrA10 resulted in an approximately 5.7-fold increase in the activity relative to a control that was not induced with the peptide (Figure 3A). In contrast, in the presence of LMIP, the β -galactosidase activity was still higher than the control (no PhrA10) but was significantly lower (by 1.8-fold) compared to induction by PhrA10 alone ($p < 0.001$), thus demonstrating the efficacy of the LMIP in inhibiting promoter activation by PhrA10. The results were also confirmed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR; see Supporting Information).

To demonstrate the growth inhibitory effect of the LMIP, the wild-type strain of *S. pneumoniae* was grown in chemically

Figure 3. Phenotypic evaluation of the LMIP (A) and growth assay (B and C). A) The LMIP inhibits β -galactosidase activity driven from the promoter of *phrA* (*PphrA*) in $\Delta phrA:phrA$, a strain of *S. pneumoniae* deficient in endogenous production of PhrA. *PphrA* was induced with 10 nM synthetic PhrA10 peptide or with PhrA10 plus 100 nM LMIP, and galactosidase activity without PhrA in CDM supplemented with galactose is also shown. In growth assays, 25–100 nM LMIP attenuates *S. pneumoniae* growth in medium supplemented with galactose (B) but not with glucose (C). Control cultures did not receive the LMIP. Each experiment was performed at least in triplicate; ** $p < 0.01$, **** $p < 0.0001$.

defined medium (CDM) supplemented with galactose, in the presence of varying concentrations of the LMIP (25–100 nM; Figure 3B). The growth yield was significantly lower at all concentrations tested, and the growth rate was significantly reduced at 100 nM LMIP-PhrA compared to the control in absence of LMIP-PhrA (Figure 3B and Figure S1). Consistent with the patterns of TprA expression (Table S1), LMIP-PhrA had no influence on growth on glucose (Figure 3C), thus showing a lack of toxicity at these concentrations. TprA was also found to regulate neuraminidase (Figure S5), so neuraminidase activity was assessed in pneumococcal cultures grown on galactose with or in absence of 100 nM LMIP-PhrA. Neuraminidase plays an important role in pneumococcal colonization and invasiveness.^[22–24] There was a significant decrease in neuraminidase activity (26.7 ± 1.5 U) in the presence of LMIP-PhrA compared to the control that did not contain LMIP-PhrA (53.3 ± 3.0 U; $p < 0.001$), thus indicating that by blocking TprA, LMIP-PhrA abrogates neuraminidase activity and hence virulence. The impact of LMIP-PhrA on virulence was further confirmed by in vivo evaluation against lethal microbial challenge in a mouse model of pneumococcal pneumonia (Figure S2). Three groups were infected intranasally, either with 7.5×10^5 pneumococci/mouse in 50 μ L phosphate-buffered saline (PBS; control), or with 1.4×10^6 /mouse suspended in the same volume containing the 100 nM LMIP-PhrA solution in PBS. The third group was infected with 1.4×10^6 /mouse in the presence of 100 nM of a LMIP targeting the scrambled 10 aa peptide (LMIPscramble). After 24 h, the results show that despite receiving a higher dose, the cohort that received the inoculum together with LMIP-PhrA had lower blood counts (Log_{10} 0.61 ± 0.28 CFU mL^{-1} , $n = 15$) than the cohort that received

the dose in PBS ($\text{Log}_{10} 2.96 \pm 0.54 \text{ CFU mL}^{-1}$; $n = 13$, $p < 0.01$). There was no difference between the number of pneumococci in the control and LMIPscramble groups ($\text{Log}_{10} 2.49 \pm 1.04 \text{ CFU mL}^{-1}$, $n = 5$) ($p > 0.05$). This shows that LMIP-PhrA prevents the translocation of pneumococci from lungs to blood. Consistent with reduction in bacterial blood count, the cohort that received LMIP-PhrA survived significantly longer ($65 \text{ h} \pm 15.1$, $n = 13$) than the controls ($37 \text{ h} \pm 8.4$; $n = 15$, $p < 0.0001$), whereas there was no difference between the LMIPscramble group ($44 \text{ h} \pm 4.6$, $n = 5$) and the control ($p > 0.05$).

In conclusion, the LMIP developed herein can interfere with QS signals selectively, thereby curtailing the phenotypic manifestation of this system in vitro and in vivo and so can be developed as an anti-infective against pathogenic Gram-positive bacteria. In the future, we plan to use this technology in a more challenging clinical situation and to determine an optimal dose/dosing regimen. We will also assess the performance of synthetic ligands (such as peptides) against PhrA10 with an equivalent sequence of functional groups along the backbone mimicking that of the LMIP to validate this approach for drug discovery.

Experimental Section

Synthesis of the LMIP by solid-phase imprinting: In 100 mL deionised (DI) and degassed water, the following monomers were dissolved: 24 mg acrylamide ($3.3 \times 10^{-4} \text{ mol}$), 33 mg *N*-tert-butylacrylamide ($2.60 \times 10^{-4} \text{ mol}$) and 2.2 μL acrylic acid ($2.24 \times 10^{-5} \text{ mol}$), 5.5 mg *N*-(3-aminopropyl)methacrylamide hydrochloride ($3.1 \times 10^{-5} \text{ mol}$). Then, 60 g of PhrA10- or scrambled-peptide-derivatised glass beads (see the Supporting Information for the coupling) were placed in a 100 mL glass bottle and then 50 mL of the monomer solution added under N_2 atmosphere. Polymerization was initiated with 0.5 mL ammonium persulfate solution (60 mg mL^{-1}) containing 54 mg mL^{-1} sodium bisulfite at RT for 15 h. Afterwards, beads and solution were decanted onto an SPE cartridge, washed with $5 \times 40 \text{ mL}$ DI water at RT, followed by $4 \times 40 \text{ mL}$ DI water at 70°C to elute the high-affinity LMIP. See the Supporting Information for additional methods.

Conflict of interest

The authors declare no conflict of interest.

Keywords: antibacterial agents · quorum sensing · receptors · molecular imprinting · virulence factors

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- [1] B. D. Brooks, A. E. Brooks, *Adv. Drug Delivery Rev.* **2014**, *78*, 14.

- [2] M. B. Miller, B. L. Bassler, *Annu. Rev. Microbiol.* **2001**, *55*, 165.
[3] S. T. Rutherford, B. L. Bassler, *Cold Spring Harbor Perspect. Med.* **2012**, *2*, a012427.
[4] J. Zhu, M. B. Miller, R. E. Vance, M. Dziejman, B. L. Bassler, J. J. Mekalanos, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 3129.
[5] A. E. Clatworthy, E. Pierson, D. T. Hung, *Nat. Chem. Biol.* **2007**, *3*, 541.
[6] J. J. Huang, J. I. Han, L. H. Zhang, J. R. Leadbetter, *Appl. Environ. Microbiol.* **2003**, *69*, 5941.
[7] J. E. Gonzalez, N. D. Keshavan, *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 859.
[8] Y. H. Dong, L. H. Wang, J. L. Xu, H. B. Zhang, X. F. Zhang, L. H. Zhang, *Nature* **2001**, *411*, 813.
[9] M. Hentzer, H. Wu, J. B. Andersen, K. Riedel, T. B. Rasmussen, N. Bagge, N. Kumar, M. A. Schembri, Z. Song, P. Kristoffersen, M. Manefield, J. W. Costerton, S. Molin, L. Eberl, P. Steinberg, S. Kjelleberg, N. Høiby, M. Givskov, *EMBO J.* **2003**, *22*, 3803.
[10] C. T. O'Loughlin, L. C. Miller, A. Siryaporn, K. Drescher, M. F. Semmelhack, B. L. Bassler, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 17981.
[11] E. V. Piletska, G. Stavroulakis, L. D. Larcombe, M. J. Whitcombe, A. Sharma, S. Primrose, G. K. Robinson, S. A. Piletsky, *Biomacromolecules* **2011**, *12*, 1067.
[12] E. V. Piletska, G. Stavroulakis, K. Karim, M. J. Whitcombe, I. Chianella, A. Sharma, K. E. Eboigbodin, G. K. Robinson, S. A. Piletsky, *Biomacromolecules* **2010**, *11*, 975.
[13] E. Cavaleiro, A. S. Duarte, A. C. Esteves, A. Correia, M. J. Whitcombe, E. V. Piletska, S. A. Piletsky, I. Chianella, *Macromol. Biosci.* **2015**, *15*, 647.
[14] E. Magennis, F. Fernandez-Trillo, C. Sui, S. Spain, D. Bradshaw, D. Churchley, G. Mantovani, K. Winzer, C. Alexander, *Nat. Mater.* **2014**, *13*, 748.
[15] S. E. Hoover, A. J. Perez, H.-C. T. Tsui, D. Sinha, D. L. Smiley, R. D. DiMarchi, M. E. Winkler, B. A. Lazazzera, *Mol. Microbiol.* **2015**, *97*, 229.
[16] see Ref. [15].
[17] M. Robb, J. K. Hobbs, S. A. Woodiga, S. Shapiro-Ward, M. D. L. Suits, N. McGregor, H. Brumer, H. Yesilkaya, S. J. King, A. B. Boraston, *PLoS Pathog.* **2017**, DOI: <https://doi.org/10.1371/journal.ppat.1006090>.
[18] H. F. Kahya, P. W. Andrew, H. Yesilkaya, *PLoS Pathog.* **2017**, DOI: <https://doi.org/10.1371/journal.ppat.1006263>.
[19] A. Poma, A. Guerreiro, S. Caygill, E. Moczek, S. Piletsky, *RSC Adv.* **2014**, *4*, 4203.
[20] A. N. Jain, *J. Med. Chem.* **2003**, *46*, 499.
[21] M. Baker, *Nat. Rev. Drug Discovery* **2013**, *12*, 5.
[22] V. S. Terra, K. A. Homer, S. G. Rao, P. W. Andrew, H. Yesilkaya, *Infect. Immun.* **2010**, *78*, 348.
[23] S. Manco, F. Hernon, H. Yesilkaya, J. C. Paton, P. W. Andrew, A. Kadioglu, *Infect. Immun.* **2006**, *74*, 4014.
[24] H. Yesilkaya, S. Manco, A. Kadioglu, V. S. Terra, P. W. Andrew, *FEMS Microbiol. Lett.* **2008**, *278*, 231.

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