Details of the research work

Next-generation therapeutic and biomaterial strategies to tackle drug-resistance

Antimicrobial resistance (AMR) currently claims around seven hundred thousand lives annually and this is expected to rise to ten million by 2050 if not tackled immediately. India is the highest contributor to this staggering statistic. Over the past few decades, many drug-resistant super bugs have emerged, from both, Gram-positive as well as Gram-negative bacteria. The World Health Organization (WHO) has come up with a list of pathogens which are top priority ones, and present complicated and dangerous clinical repercussions. And the number of such pathogens is continually on the rise, with more and more antibiotics being relegated from the clinic to the shelf. Given the reduced number of new antibiotics being approved, with even less number of new targets being identified, there is a need to develop newer strategies which target pathogenic bacteria non-specifically, are non-toxic towards mammalian cells, against which feeble or no resistance development is seen, and which can act against all sub-populations of bacteria, including metabolically dormant cells and biofilms. Our laboratory seeks to provide a platform to integrate medicinal chemistry and pharmacology with biology to combat infectious diseases. We are involved in creating novel antimicrobial agents for the prevention and treatment of infectious diseases and combating the emergence of antimicrobial resistance. Some of the strategies for tackling infectious diseases and antimicrobial resistance are discussed here –

1. Semi-synthetic glycopeptide antibiotics: Vancomycin, an important antibiotic of the last decade, is increasingly becoming inactive against Gram-positive pathogens, due to the emergence of vancomycin resistance. Very few treatment options are currently available for curing infections caused by vancomycin-resistant *S. aureus* (VRSA), vancomycin-resistant *E. faecium* (VRE), both of which are designated as high priority pathogens. With a view of addressing the complications arising due to these pathogens, a major aspect of our work involves undertaking semi-synthetic modifications to these antibiotics, with the aim of restoring their activity, either by countering the resistance element, or by conferring additional mechanisms of action. Our group has immensely contributed to developing various vancomycin analogues, which display highly enhanced activity against VRSA, VRE, and Gram-negative pathogens. Described briefly here are some of the successful strategies from our group –

(i) Membrane active vancomycin analogues to combat acquired and inherent drug resistance: We have engineered lipophilic cationic vancomycin analogues which exhibit very high antibacterial activity against both multidrug-resistant Gram-positive bacteria (VRSA and VRE) and antibiotic-resistant Gram-negative bacteria (*E. coli, A. baumanii, K. pneumoniae*). These membrane active analogues possess cationic lipophilic substitutions at the C-terminus of vancomycin. Compared to vancomycin, efficacy >1000-fold and >100-fold was demonstrated against VRE and VRSA thus overcoming the acquired bacterial resistance. Additionally, these analogues could overcome the inherent resistance of vancomycin towards Gram-negative bacteria *A. baumannii* exhibiting very high antibacterial activity, which was a first example of its kind. Significantly, unlike vancomycin, these compounds are rapidly bactericidal and do not induce bacterial resistance. An optimized compound in the series, compared to vancomycin and linezolid, showed higher activity in MRSA, VISA and VRE infected mouse model, exhibited improved pharmacological properties with no observed toxicity. The lead compounds, VanQAmC10, which possesses a quaternary ammonium group (cationic charge), and a decyl chain (lipophilic group), separated by an amide spacer displayed

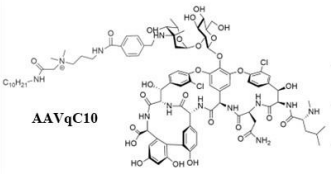
significant activity against vancomycin-resistant Gram-positive bacteria, VRSA, VRE, VISA (MIC = 0.25 – 4 μM), as well as against a broad panel of multidrug resistant *A. baumannii* (MIC = 3.9 – 15.5 μM). These derivatives also displayed activity against intracellular MRSA infections. In another recent study, our lead AAvqC10 derivative of vancomycin (**Figure 1(i)**), possessing an aryl-alkyl group appended to the amino terminal of the vancosamine group, displayed potent activity against vancomycin-resistant pathogens such as VISA, VRSA, VRE strains (MIC = 0.45 – 3.6 μM). This derivative, while showing membrane perturbation, also inhibited bacterial cell division at different stages. The antibacterial activity of this analogue was also validated in an *in-vivo* mice model of MRSA thigh infection, where reduction of ~3 Log CFU was observed in the treated mice. The *in-vivo* toxicity of the molecule was determined and LD_{50} via i.v. and i.p. dosage was found to be >55 mg/kg. The remarkable activity of these analogues can be attributed to the incorporation of a new membrane disruption mechanism into vancomycin and opens up a great opportunity for the development of life saving antibiotics. We have done a detailed study of the structure and activity profile gives alkyl-cationic substitutions an edge over aryl analogs, where the lead molecule $VanQAmC_{10}$ also hampers the distribution of MinD, a protein which has an integral role in cell division regulation machinery.

(ii) Tackling of vancomycin-resistant bacteria through enhanced binding affinity towards cell wall precursor peptide: Vancomycin-resistant bacteria (VRB) sense a glycopeptide antibiotic challenge and remodel their cell wall precursor peptidoglycan terminus from D-Ala-D-Ala to D-Ala-D-Lac, reducing the binding of vancomycin to its target 1000-fold and accounting for the loss in antimicrobial activity. To overcome this reduced binding, we developed various vancomycin-sugar analogues designed to exhibit increased binding affinity to both D-Ala-D-Ala and D-Ala-D-Lac thereby reinstating activity against VRB. Optimized conjugate YV4465 (**Figure 1(ii)**), bearing lactobionic acid conjugated to the C-terminus, with lipophilicity exhibited enhanced binding affinity for VRE with excellent antibacterial activity against all, MRSA, VRE and VISA (MIC = 0.2 – 1 μM). This conjugate is highly bactericidal and showed excellent *in-vivo* antibacterial activity against vancomycin resistant bacterial infection of VISA, where a >2.0 log CFU/g reduction in count was observed upon treatment with the conjugate. The *in-vivo* toxicity of the molecule was determined and LD_{50} via i.v. dosage was found to be >100 mg/kg. The conjugate also displayed improved pharmacological properties over vancomycin. In fact, this derivative was found to be more effective than vancomycin, linezolid. Therefore, our rational approach could potentially lead to the development of new generation of antibiotics to tackle multidrug-resistant bacteria.

(iii) Incorporation of membrane disruption properties and improved binding affinity in vancomycin to tackle vancomycin-resistant bacteria: Given the increasing occurrence of glycopeptide resistance, it is imperative to develop multipronged strategies to tackle vancomycin-resistant bacteria. Towards this goal, incorporating two mechanisms in one molecule is an interesting approach. Having shown that the conjugation of sugar moiety enhances the binding affinity of the drug and incorporation of permanent cationic hydrophobic moiety imparts membrane disruption properties; we aimed at imparting both the properties to the same molecule to effectively tackle drug resistance. With this rationale, we developed a vancomycin analogue which had the lactobionic acid sugar conjugated to the C-terminus, and a permanent cationic lipophilic group conjugated to the vancosamine sugar amino terminal. An optimized compound from this series, LB-VanPyC8 (**Figure 1(iii)**), bearing the octyl chain, showed outstanding activity against MRSA, VISA and VRE, being >8000-fold more effective than vancomycin (MIC = 0.09 – 1.3 μM). This analogue was also active against biofilms of

MRSA, and displayed >5 log CFU/mL reduction in bacterial titre as compared to control. As was envisaged, the analogue displayed significant membrane perturbation, while retaining its

(i) Membrane active vancomycin analogues to combat acquired and inherent drug resistance

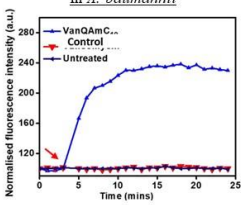


AAVqC10

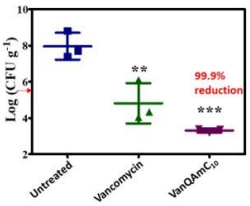
- MIC = 0.45–3.6 µM
- HC_{50} >250 µM
- LD_{50} (i.v., i.p.) >55 mg/kg

- Kills 10^6 CFU/mL planktonic MRSA cells in 6 h
- Eradicates *A. baumannii* biofilms
- Kills bacterial persisters and stationary phase cells
- Inhibits bacterial cell division

Membrane Permeabilisation in *A. baumannii*

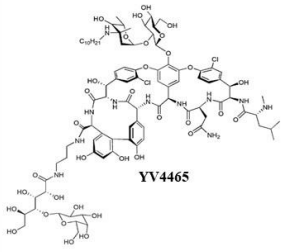


In-vivo activity in MRSA thigh infection



Publications: (i) *J. Med. Chem.* **2021**, *64*, 10185. (ii) *ACS Chem Biol*, **2020**, *15*, 884. (iii) *ACS Infect. Dis.* **2016**, *2*, 132. (iv) *J. Glob. Antimicrob. Resist.* **2016**, *5*, 71. (v) *ACS Infect. Dis.* **2015**, *1*, 469. (vi) *Int. J. Antimicrob. Agents* **2015**, *45*, 627. (vi) *Chem Sci.*, **2023,** *14,* 2386.
Patents: WO2013072838 A1, US10081655 B2, KR101816228 B1, CA2855753 C, EP2780359 B1, US20140308347 A1, AU2012338461 C1.

(ii) Tackling vancomycin-resistant bacteria through enhanced binding affinity to cell wall precursor peptide

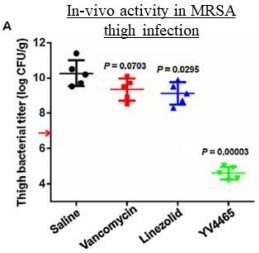


YV4465

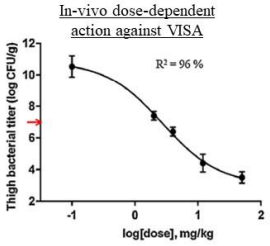
- MIC = 0.2–1 µM
- HC_{50} >100 µM
- LD_{50} (i.v.) >100 mg/kg

- Kills 10^6 CFU/mL planktonic MRSA in 6 h
- Eradicates *A. baumannii* biofilms
- Kills bacterial persisters and stationary phase cells
- Inhibits bacterial cell division

In-vivo activity in MRSA thigh infection

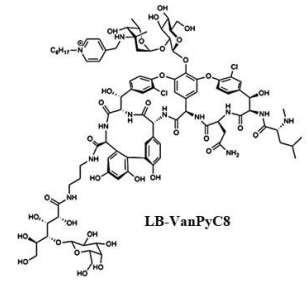


In-vivo dose-dependent action against VISA



Publications: (i) *Int. J. Antimicrob. Agents* **2015**, *46*, 446. (ii) *J. Antibiot.* **2015**, *68*, 302.
Patents: IN2013CH04314A, CA2925005 A1, WO2015040467A1, US20160303184A1, AU2014322817A1, EP3049115A1.

(iii) Incorporation of membrane disruption properties and improved binding affinity in vancomycin to tackle vancomycin-resistant bacteria

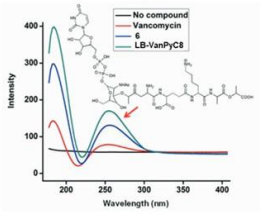


LB-VanPyC8

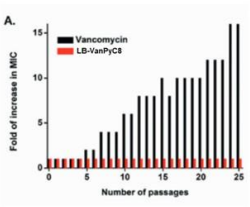
- MIC = 0.9–1.3 µM
- HC_{50} >100 µM
- LD_{50} (i.v.) > 100 mg/kg

- Shows higher binding constant than Van
- Eradicates MRSA biflms
- No resistance development seen even after 25 passages

Accumulation of cell-wall precursor in treated case

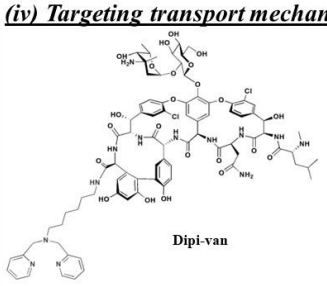


Resistance development in MRSA



Publications: i) *Angew. Chem. Int. Ed.* **2015**, *54*,13644.
Patents: WO2014097178 A1, US9783490 B2, CA2894202 A1, JP6533466 B2, CN104981249 B, AU2013365769 B2, BR112015014391 A2, HK1210437 A1.

(iv) Targeting transport mechanism of cell wall precursor through vancomycin derivatives

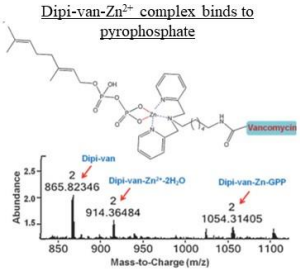


Dipi-van

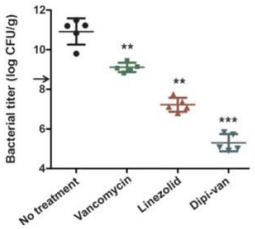
- MIC = 1–2 µM
- HC_{50} >1000 µM
- LD_{50} (i.v.) > 100 mg/kg

- Shows higher binding constant than Van
- Binds to pyrophosphate of C_{55}-lipid
- No resistance development seen even after 20 passages

Dipi-van-Zn^{2+} complex binds to pyrophosphate



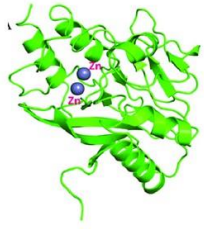
In-vivo activity in VRE kidney infection model



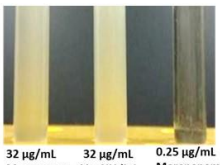
Publications: i) *Angew. Chem. Int. Ed.* **2016**, *55*, 7836.
Patents: WO2016103284A1 CA2972276 A1, EP3240574 A1, US20170342110 A1.

(v) Vancomycin derivatives acting as metallobetalactamase inhibitors to re-sensitise carbapenems against Gram-negative superbugs

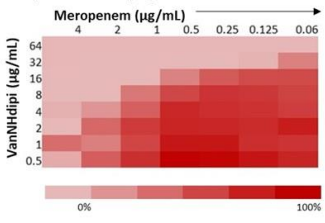
Structure of NDM-1



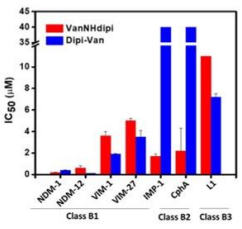
Activity of combination



Chequerboard assay against *A. baumannii*



Inhibition of metallobetalactamases



Publications: (i) *ACS Infect. Dis.* **2018**, *4*, 1093. (ii) *Angew. Chem. Int. Ed.* **2016**, *27*, 7836.
Patents: WO2016103284A1 CA2972276 A1, EP3240574 A1, US20170342110 A1.

Figure 1: Semi-synthetic glycopeptide antibiotics

inherent cell wall biosynthesis inhibition mode of action. In fact, this compound displayed enhanced cell wall inhibition compared to vancomycin and showed excellent *in-vivo* activity against vancomycin-resistant bacterial murine kidney infection (VRE), with a 6 log CFU reduction in bacterial count. The *in-vivo* toxicity of the molecule was determined and LD_{50} via i.v. dosage was found to be >100 mg/kg. Further, negligible resistance development was observed for the compound even after 25 passages. Conversely, the MIC of vancomycin increased by more than 15 times over the same no of passages. Thus, our multipronged approach bears immense potential in the field of antibiotic development for the treatment of vancomycin-resistant bacterial infections.

(iv) Targeting transport mechanism of cell wall precursor through vancomycin derivatives: An interesting strategy to overcome vancomycin resistance in bacteria can be to look at other imperative processes within the cell wall biosynthesis process. We have in one of our works, conjugated dipicolyl-1,6-hexadiamine (a Zn^{2+}-binding ligand), to the C-terminal of vancomycin, to develop Dipi-van (**Figure 1(iv)**). This chelating dipicolyl amine groups chelates Zn^{2+} ions with very high selectivity. Our analogue also forms a complex with the divalent Zn^{2+} ions, through the dipicolyl amine moiety. We hypothesised that this Zn-Dipi-Van complex can bind very strongly with the pyrophosphate group of the Lipid-II transporter, present in the membrane. This molecule exhibited potent activity (MIC = 1-2 μM), and a ~370 fold increase as compared to vancomycin, against vancomycin-resistant bacteria (VISA, VRE). We validated the enhanced cell wall biosynthesis inhibition by this derivative through mass spectrometric analysis of accumulation of cell wall precursor. Apart from that, the derivative did not display development of resistance even after 25 passages against MRSA, while vancomycin's MIC increased by >16 fold. Further, we also validated the activity of this molecule in an *in-vivo* mice model of VRE kidney infection. Dipi-van displayed ~5 Log CFU reduction in bacterial burden, which was far superior than vancomycin, or another conventional antibiotic linezolid. The *in-vivo* toxicity of the molecule was determined and LD_{50} via i.v. dosage was found to be >100 mg/kg. Taken together, this molecule was significantly active against vancomycin-resistant bacteria, displayed potent *in-vivo* activity, and owing to its multimodal mechanisms of action, displayed negligible resistance development propensity.

(v) Vancomycin derivatives acting as bacterial cell-division inhibitor and exhibiting broad spectrum antibacterial activity in-vivo: In our search for next-generation membrane-active glycopeptide antibiotics, we report a new derivative of vancomycin where we explored the antibacterial mechanisms beyond the conventional notion of D-Ala-D-Ala binding. These modified vancomycin derivatives show several alterations in the bacterial cells, which facilitate inactivation of the pathogens (**Figure 1(v)**). A detailed study of the structure and activity profile gives alkyl-cationic substitutions an edge over aryl analogs. The lead candidate, $VanQAmC_{10}$ shows a significant extent of depolarization and permeabilization of bacterial membrane. Interestingly, $VanQAmC_{10}$ shows retardation of growth during cell division, which is the first of its kind observation setting $VanQAmC_{10}$ apart from other glycopeptide antibiotics. The compound also hampers the distribution of MinD, a protein which has an integral role in cell division regulation machinery. Not only *in-vitro,* $VanQAmC_{10}$ showed superior activity in a mice model of thigh infection. VanQAmC10. Overall, this study investigates multiple mechanisms of action of a novel vancomycin derivative which contribute to the negligible resistance induction and superior antibacterial properties as compared to the parent drug.

(vi) Vancomycin derivatives acting as metallobetalactamase inhibitors to re-sensitise carbapenems against Gram-negative superbugs: Betalactams are one of the most populous class of antibiotics, consisting of multiple sub-classes. However, the emergence of hydrolytic

betalactamase enzymes has rendered them ineffective against a variety of pathogens. One such kind of betalactamase is the nearly a decade old New Delhi Metallobetalatamase (NDM). This enzyme is extremely efficient in cleaving even the highest generation of cephalosporins, and hence, no betalactams can work against bacteria possessing it. In a first of its kind study, our group has used our dipicolylamine moiety-conjugated vancomycin derivative (Dipi-van) as a metallobetalactamase inhibitor, in combination with meropenem, to treat infections caused by New Delhi Metallobetalactamase (NDM) positive *E. coli*. Dipi-van acts by inhibiting this NDM enzyme, and preventing the cleavage of meropenem. The chelating moiety of Dipi-van has a high binding affinity for Zinc (II) ions, which are a crucial part of the active site of the NDM enzyme. Dipi-van binds to the active Zinc (II) centre, forming a Dipi-van-Zn^{2+} conjugate, thus preventing it from catalysing the betalactam cleavage. This is a promising and unique strategy to tackle increasing NDM-resistance in Gram-negative bacteria. We demonstrated in this study that at sub-active concentrations, Dipi-van was able to re-sensitise meropenem against NDM-expressing *K. pneumoniae*, and increase its activity by >80 fold (MIC of meropenem in presence of 28.7 μg/mL Dipi-van = 1.5 μg/mL). Similar synergistic effect was observed for all NDM-expressing clinical isolates. Dipi-van displayed outer membrane permeabilization of Gram-negative *K. pneumoniae*. The ability of inhibition of NDM-1 by Dipi-van was assessed on purified recombinant enzyme *in-vitro* were an IC_{50} of ∼6 μM (13.8 μg/mL) was obtained. The study has also attested to the potential mode of NDM-inhibition, as the synergistic efficacy reduced in presence of Zinc (II) ions. The most significant contribution of the study was the proof of *in-vivo* activity of this combination, in a murine model of sepsis. The combination of meropenem and Dipi-van cleared ∼3−4 $\log_{10}$ CFU/g of *K. pneumoniae* from most of the organs of the animal, as compared to control. This study was an important milestone, as it is, to the best of our knowledge, the first report of a glycopeptide analogue being used as an NDM inhibitor, with validated *in-vivo* activity.

2. Small-molecular peptidomimetic antimicrobial agents: Antimicrobial peptides (AMPs) are naturally occurring antibacterial agents which target the membrane. They represent effective therapeutic alternatives to antibiotics, but their limited success is due to high cost of production, low stability *in-vivo* and unwanted toxicity to mammalian cells. In our lab, towards the goal of addressing the problem of antibiotic resistance, and persistent infections by dangerous drug-resistant superbugs, we have developed synthetic mimics of AMPs targeted towards bacterial membrane, biofilm matrix and lipopolysaccharides (LPS). We utilize different chemical strategies to design such molecules. The basic design is based on naturally

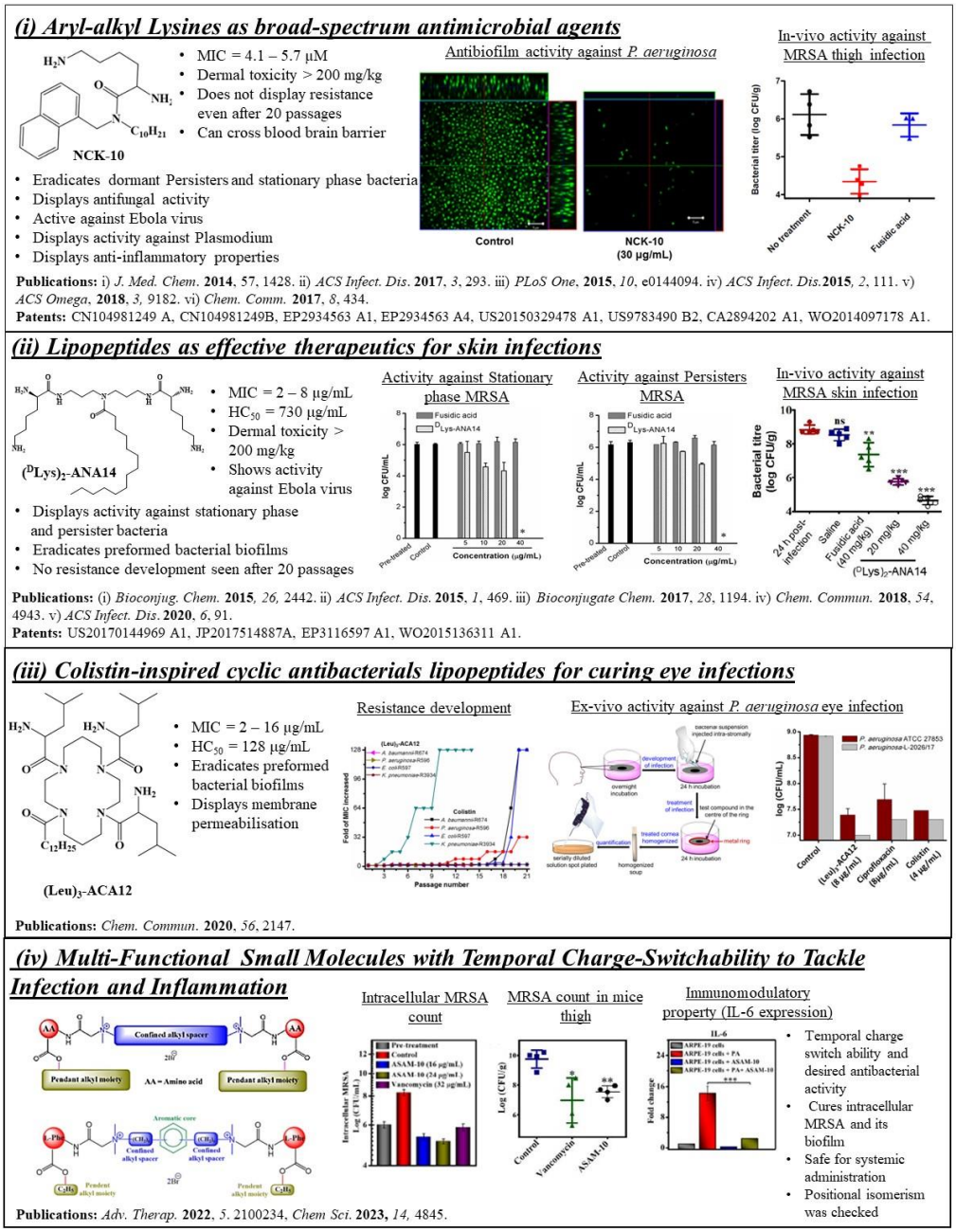


Figure 2: Small-molecular peptidomimetic antimicrobial agents

available amino acids, and involves alkyl and aryl long chains, which confer hydrophobicity. Our designs have various advantages over natural antimicrobial peptides, as they are easy to synthesise, do not undergo enzymatic cleavage, and are less toxic. Various designs have been

developed in our lab for tackling drug-resistant bacteria. Some of our designs have been patented and are being tested for their antifungal and antiviral effects too. Our molecules have shown very good *in-vivo* efficacy in mice infection models (burn wound, superficial skin and thigh infections).

A few of the lead identified from the different designs of small-molecular peptidomimetic antimicrobial agents are discussed in detail here –

(i) Aryl-alkyl Lysines as broad-spectrum antimicrobial agents: In our research we developed a library of small molecular antibacterial peptoids where an aryl group, an alkyl group, and a lysine moiety have been assembled through a tertiary amide linkage. The compounds exhibited excellent broad-spectrum antibacterial potency *in-vitro* against clinical isolates of ESKAPE group of pathogens. These non-toxic rapidly bactericidal compounds permeabilized and depolarized bacterial membrane. The lead molecule isolated from the preliminary screening was highly active against bacteria (MIC ranged from 1.5 to 9 μM), and least toxic towards mammalian cells. It possesses a naphthyl group and a decyl chain appended to a lysine (NCK-10) (**Figure 2(i)**). This drug-candidate is particularly different from conventional antibiotics such as norfloxacin, as it does not lead to resistance development even after more than 20 passages, while norfloxacin displays close to 400-fold increase in its active concentration after the same number of passages. Owing to the membrane active mechanism of the lead, it could kill metabolically dormant stationary phase and persister bacterial populations too, as opposed to conventional antibiotics. Further, good efficacy in clearing of bacterial biofilms was also demonstrated by the lead. The lead displayed excellent efficacy in curing skin infection caused by methicillin-resistant *S. aureus* planktonic cells as well as biofilms, in a murine model (~2.5 Log CFU reduction). While the control antibiotic fusidic acid at similar concentration could not reduce the infection, lead drug-candidate was able to significantly clear planktonic cells and reduce bacterial burden in biofilms. The lead drug-candidate displayed excellent ability to clear infections by drug-resistant superbug *A. baumannii* in a burn-wound infection model in mice (~2.5 Log CFU reduction in bacterial burden). No dermal toxicity in mice was observed for the molecule upto 200 mg/kg. The activity profiles of the compounds were also extended to fungi, and fungal biofilms. Additionally, the lead molecule displayed different effects apart from its antimicrobial activity, such as ability to cross the blood brain barrier and anti-inflammatory properties. Further, another molecule from this series, possessing the hexyl chain (NCK-6) displayed potent activity in murine models of cerebral malaria. Upon treatment with 20 mg/kg of this compound, parasitemia in mice was lowered by more than 60%, increasing mice survival as compared to control case. Thus, this set of molecules yielded a drug-candidates with highly broad-spectrum activity against multiple classes of pathogens, with selective activity in mouse-models, and shows good potential to be developed clinically into multi-purpose antimicrobial agents. One of the other molecules, NCK-8, has been shortlisted as potential drug candidate against Ebola by Public Health England (PHE) from many candidates all over the world. This has displayed *in-vitro* activity against the Ebola virus, with a potent reduction in the viral RNA titre upon treatment. It has also shown very good activity *in-vivo* against Ebola virus in a Guinea pig model (~99% reduction).

(ii) Lipopeptides as effective therapeutics for skin infections: This series of AMP-mimicking molecules is based on two symmetric triamines, norspermidine and bis-hexamethylenediamine, which have different hydrophobicity. To the central secondary amine, an alkyl chain is attached, which can further be used to vary hydrophobicity. In this design, cationic amino acids such as arginine, histidine, diaminobutanoic acid, ornithine and lysine, attached to the terminal primary amines bestow this positive charge. Thus, through different

permutations and combinations, thirty different compounds were synthesized, with varying backbone hydrophobicity (due to differing core triamines), varying cationic charge and side chain hydrophobicity (differing amino acids) and different pendant hydrophobicity (differing alkyl chains). From a preliminary activity-toxicity screening, the lysine bearing norspermidine analogue with a C_{14} chain was found to be the most selective molecule, with potent activity against multidrug-resistant pathogens (MIC = 3.1-6.3 μg/mL for Gram-positive and Gram-negative bacteria) and least toxicity towards mammalian cells. To achieve enhanced stability to enzymatic cleavage, D-lysine was used to synthesize the same molecule. This lead, identified based on its selectivity, possessing the amino acid D-Lysine, at the terminals, and a tetradecanoyl chain in the centre, (^{D}Lys)$_2$-ANA14 (**Figure 2(ii)**), has been studied extensively for its antibacterial properties. This lead is highly active against multidrug-resistant superbugs (MIC values of 5.8 μg/mL, 1.6 μg/mL, 4 μg/mL against MRSA, VRE and VRSA respectively). As opposite to conventional, the lead effectively eradicates bacterial biofilms, displaying close to 80% reduction in biofilm biomass upon treatment with compound and demonstrates ~6 log reduction in the embedded bacteria. The molecule is highly active against metabolically dormant stationary phase bacteria. While fusidic acid is completely inactive against stationary phase MRSA, (^{D}Lys)$_2$-ANA14 displayed a concentration dependent killing efficacy, eradicating ~6 log bacteria at a concentration of 40 μg/mL. Similarly, contrary to fusidic acid, the lead displayed complete eradication of close to 6 log persister cells of MRSA at similar concentrations. Both, the antibiofilm activity and the activity against dormant bacteria, prove the lead molecule's superiority over conventional antibiotics such as vancomycin, fusidic acid, etc. It displays more than 100-fold selectivity for bacterial killing, within an hour for ~6 log bacterial burden. This molecule is membrane active, and stable in physiological fluids such as plasma, liver homogenate, etc. Its efficacy has been assessed in a murine model of skin infection, where it shows potent *in-vivo* anti-MRSA activity (~3 log reduction at 40 mg/kg treatment), and does not show any skin toxicity even at 200 mg/kg of the compound exposure. Additionally, MRSA could not develop resistance against (^{D}Lys)$_2$-ANA14 even after 18 subsequent passages, whereas the topical anti-MRSA antibiotic fusidic acid succumbed to rapid resistance development. All of these characteristics indicate to the highly promising nature of the lead drug-candidate for further assessment.

(iii) Colistin-inspired cyclic antibacterials lipopeptides for curing eye infections: Colistin, a last resort antibiotic for multidrug-resistant Gram-negative bacterial infections, is a naturally available lipopeptide, with a cyclic ring, a connecting peptide, and a hydrophobic alkyl chain. Inspired from the structure of colistin, we have designed small-molecular antibacterial agents, with the cyclic tetraamine Cyclam as the core of the design. To integrate the physicochemical features of colistin, namely, positive charge and hydrophobicity, three amino acid moieties, and a long hydrophobic chain were appended to the four amino groups of cyclam. The amino groups of the amino acid confer positive charge, while the long chain confers hydrophobicity to the design. These molecules, unlike colistin, display good activity against Gram-positive and Gram-negative bacteria, with MIC of the most active compounds ranging between 2-16 μg/mL, due to their membrane depolarization and membrane disrupting activity. They are fast-killing, leading to >6 log reduction of bacteria within 120 minutes. These molecules show least propensity to resistance development. The lead molecule possessing the dodecanoyl long chain (Leu)$_3$-ACA12 (**Figure 2(iii)**) has been identified through preliminary screening as the most selective molecule. It is highly active against drug-resistant bacteria (MIC values of 1-2 μg/mL against Gram-positive bacteria and 2-8 μg/mL against Gram-negative bacteria) and displays least cytotoxicity. It is highly active against stationary phase bacteria as well as pre-formed biofilms. While colistin, the inspiration for this design, has high propensity for resistance development against various Gram-negative pathogens, the lead molecule displays no

resistance even after 21 passages for a large palette of multidrug-resistant Gram-negative pathogens. The active concentration of the lead is very low as compared to its toxic dose. Thus, owing to its easy synthesis, broad-spectrum activity, non-specific mode of action, and no propensity for resistance development, this design is highly superior as compared to colistin. Further, the efficacy of the lead has been assessed in an *ex-vivo* model of eye infections. It demonstrates good activity against bacterial infections of the human cornea, reducing 99% (~2 log) of bacterial burden upon treatment. Taken together, this design shows good potential for development as an antibacterial therapeutic agent.

(iv) Multifunctional small molecules with temporal charge-switchability tackle infection and inflammation: Colistin, a last resort antibiotic for multidrug-resistant Gram-negative bacterial infections, is a naturally available lipopeptide, with a cyclic ring, a connecting peptide, and a hydrophobic alkyl chain. However, it suffers tremendously from an exorbitant toxicity owing to its charged nature which limits its application in the real settings. Recently, we have introduced an innovative category of versatile amino acid-conjugated small antibacterial molecules that effectively address complicated infections and associated inflammation. These molecules demonstrate a wide-ranging bactericidal effect against bacteria that are resistant to multiple drugs. The lead molecule, ASAM-10 incorporating phenylalanine, efficiently targets bacterial dormant subpopulations, biofilms, and intracellular pathogens simultaneously (**Figure 2(iv)**). A noteworthy feature of this work is its ability to mitigate the toxicity concern linked to cationic lipopeptides such as colistin, achieved through a temporal charge shift from cationic to zwitterionic due to the breakdown of labile ester connections after furnishing desired antibacterial action. The significant decrease in the expression of pro-inflammatory cytokines (IL-6, IL-8, TNF-$\boldsymbol{\alpha}$, and IL-1$\boldsymbol{\beta}$) in infected macrophages upon ASAM-10 treatment underscores its potent anti-inflammatory properties. Not only in-vitro, administering ASAM-10 in a mice with thigh infection showed a substantial reduction in bacterial load (2 Log CFU/g). Collectively, this novel class of multifunctional molecules demonstrates its safety and promises advanced therapeutic potential in addressing complex bacterial infections and inflammation. We have also developed another isoamphipathic antibacterial molecules (IAMs: 1–3) where positional isomerism was introduced as one of the guiding factors for molecular design.

3. Macromolecular therapeutics: Naturally occurring AMPs possess various disadvantages, as discussed earlier. To tackle these, AMP-inspired polymeric antimicrobial agents are a promising strategy. We have worked towards developing such macromolecular therapeutics, by functionalising easily available polymeric scaffolds with various groups such as cationic-alkyl moieties, or amino acids. In one of our earlier designs, we designed and developed a series of cationic amphiphilic polymers based on poly(isobutylene-alt-N-alkyl maleimide), with varying amphiphilicity. This was achieved by appending through quaternization, alkyl chains of different lengths (ethyl to decyl). We also introduced amide or ester bonds between the quaternary charge and hydrophobicity, to generate two lead molecules, Q*n*-prAP and Q*n*-

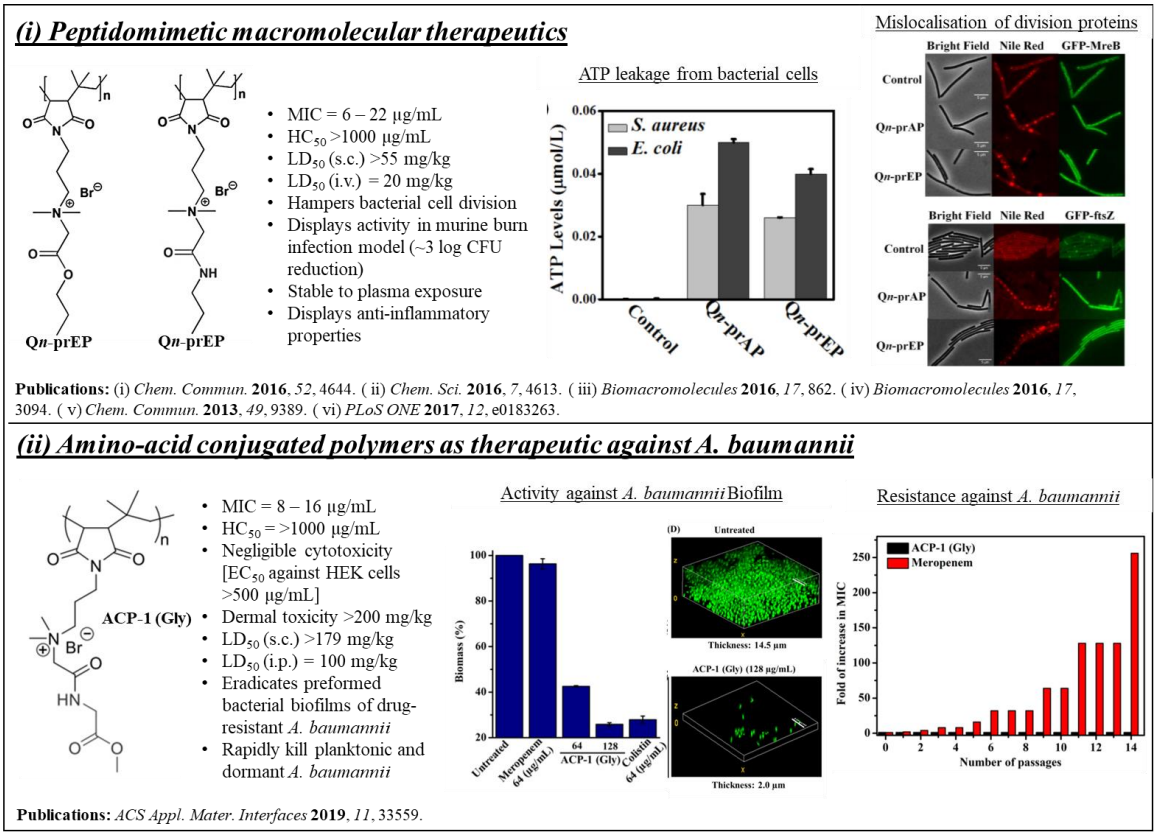


Figure 3: Macromolecular therapeutics

prEP respectively (**Figure 3(i)**). It was observed that the molecule bearing propyl chain (Q*n*-prAP), separated from quaternary charge by amide bond was the most selective one from among the large library. It displayed significant activity against multiple drug-resistant bacterial strains. These molecules were found to peremabilise and depolarise bacterial membranes, owing to their membrane-active mechanism of action. This was also validated through various assays such as Potassium leakage or ATP leakage assays. Apart from this, the macromolecules also disrupted the process of bacterial cell division. This was proven through analysis of cell-division proteins and their distribution upon treatment with the macromolecules. These macromolecules retained activity even upon incubation with human blood plasma, which indicated their stability. We further explored the exact modes of binding and reasons for differential selectivity of ester-containing, amide-containing or ester/amide-devoid macromolecules, through theoretical simulations. We observed through these studies that the additional hydrogen bonding donor/acceptor capabilities contributed by the amide substituents conferred higher selectivity to the amide-containing macromolecules, over the ester-containing, or amide/ester-devoid ones. The efficacy of the lead was also validated in an *in-vivo* murine model of *A. baumanni* skin infection, where it displayed ~3 Log CFU reduction in bacterial count upon treatment. The *in-vivo* toxicity of the molecules was assessed through different dosage routes, and it was found to be safe at the therapeutic concentration (LD_{50} (s.c.) >55 mg/kg and LD_{50} (i.v.) = 20 mg/kg). These macromolecules displayed a range of other important properties, such as the ability to control inflammation due to LPS-exposure in immune cells. We also investigated the reason for these anti-inflammatory effects, and found that our macromolecules form pseudoaggregates with LPS, which hampers the recognition of LPS by our immune system. We attempted to build up on this design and introduced naturally available amino acids in the design of macromolecular therapeutics. Inspired from the molecular architecture of AMPs, we developed a class of side-chain amino acid tuneable

peptidomimetic antibacterial polymers (ACPs). The main highlight of these polymers was their potent activity (MIC = 8-16 µg/mL) against *A. baumannii*, which is considered the worlds' deadliest pathogen. The optimized polymer (ACP-1 (Gly)) (**Figure 3(ii)**) was rapidly bactericidal and killed metabolically inactive stationary phase *A. baumannii* entirely within 2 min. Importantly, negligible toxicity was observed for the lead polymer (HC_{50} >1000 µg/mL, Dermal toxicity >200 mg/kg, LD_{50} (s.c.) >179 mg/kg, LD_{50} (i.p.) = 100 mg/kg). This polymer was also capable of eradicating preformed biofilms of drug-resistant *A. baumannii* clinical isolates. Furthermore, no propensity of resistance development was detected against this polymer after 14 subsequent passages, unlike the carbapenem antibiotic meropenem, or the lipopeptide colistin.

4. Antibiotic Adjuvants for Gram-negative superbugs: In this era of dearth of effective antibiotics, it has become evident to invest in the 'repurposing and rehabilitation' of already existing drugs. This would make use of the existing antibiotic arsenal in tackling multidrug-resistant bacteria, thus obliterating the redundancy of such antibiotics, and reinforcing the efficacy of already approved drugs. Membrane-perturbing adjuvants can help to rehabilitate

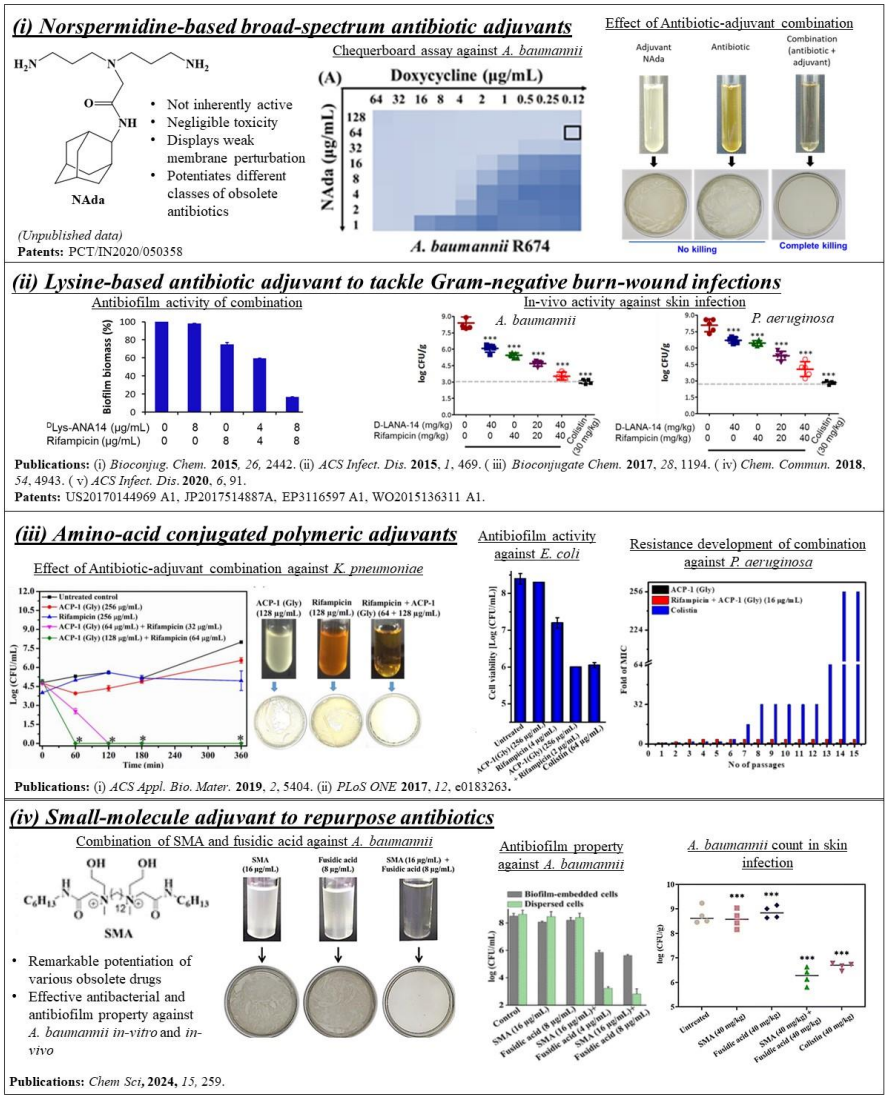


Figure 4: Antibiotic adjuvants for Gram-negative superbugs

various antibiotics excluded due to permeability issues and extrusion due to efflux pumps. Employing this approach, our group has developed different designs of non-toxic small molecular adjuvants to repurpose and rehabilitate obsolete antibiotics against the most critical Gram-negative pathogens including New-Delhi metallo β-lactamase (NDM) producing bacteria. Some of our small molecular adjuvants lead to potentiation of conventional antibiotics fusidic acid, rifampicin, minocycline, tetracycline, and linezolid, when used in combination with them, against these pathogens.
Some of our successful designs have been discussed here –

(i) Norspermidine-based broad-spectrum antibiotic adjuvants: One of our designs was based on the triamine norspermidine, where the cage-like adamantly group is appended to the central amine through an amide spacer. This molecule NAda (**Figure 4(i)**), was completely inactive against different bacteria, with MIC > 500 μg/mL. However, this molecule displayed membrane perturbation, and depolarisation. We exploited this property of the molecule and used it as an adjuvant for resensitising obsolete drugs which have been rendered useless due to resistance development in the form of efflux forms. Very promising results were observed for this molecule, as it was capable of potentiating different classes of antibiotics such as tetracyclines like doxycycline, macrolides like erythromycin, chloramphenicol, glycopeptide vancomycin, etc. Of itself, the antibiotic or the adjuvant displayed little effect in reducing bacterial burden, but the combination (at concentrations lower than individual) displayed excellent antibacterial activity against multidrug-resistant Gram-negative pathogens, including top priority pathogen *A. baumannii*. This adjuvant, was able to potentiate the antibiotics by 8 to >512 times for different pathogens, indicating its non-specific and broad-spectrum effect. As a representative example, as it is seen from the chequerboard assay results, NAda reduces the MIC of Doxycycline against *A. baumannii* from 64 μg/mL to 0.12 μg/mL, i.e. a ~270 times increase in activity when used in combination. Further studies are underway in the experimental as well as theoretical domain, to improve the potentiation, as well as develop a clear understanding of the mechanistic nitty-gritties of the adjuvants.

(ii) Lysine-based antibiotic adjuvant to tackle Gram-negative burn-wound infections: Along with this design, we also observed that at sub-MIC concentrations against Gram-negative bacteria, our cationic lipophile $(^{D}Lys)_2$-ANA14 was able to perturb the bacterial outer membranes of potent Gram-negative pathogens such as *A. baumannii* and *P. aeruginosa* (**Figure 4(ii)**). Due to this effect, while being less active against these pathogens, $(^{D}Lys)_2$-ANA14 at lower concentrations worked as an adjuvant and could resensitize the inactive antibiotics, tetracycline, and rifampicin against these critical superbugs at a low concentration (at 4-8 μg/mL) with the FICI (fractional inhibitory concentration index) value ranged between 0.09-0.38. It displayed an enhancement in the range of 64 to ≥1024 times in the activity of obsolete antibiotics against different pathogens. Additionally, the combination is capable to inactivate the non-dividing stationary phase cells of these clinical isolates too, where the killing efficacy may be majorly attributed to the adjuvant. The combination also exhibits synergistic efficacy in disrupting the biofilms formed by these critical pathogens. In combination (4 μg/mL of adjuvant and 4 μg/mL of antibiotic rifampicin), around 80% reduction was observed in the biomass of preformed biofilms of both *A. baumannii* and *P. aeruginosa*. Most importantly, while poorly effective when used independently, the adjuvant-antibiotic combination retains its synergy in the *in-vivo* setting too, where burn-wound infection has been cured in a murine model, with a drastic reduction of bacterial burden (~4-6 log reduction at combined treatment of 40 mg/kg of adjuvant and rifampicin each).

(iii) Amino-acid conjugated polymeric adjuvants: In today's era, very few treatment options

are left to tackle the Gram-negative superbugs, harbouring New Delhi metallo β-lactamase-1 (NMD-1) and other extended spectrum β-lactamases (ESBL). To tackle such multidrug resistant pathogens, we demonstrated the potent resensitisation capabilities of our amphiphilic macromolecule (ACPs) with obsolete antibiotics (**Figure 4(iii)**). Our amino acid conjugated macromolecules worked as adjuvants capable of resensitising the obsolete antibiotic rifampicin against such type of superbugs, by 4-66 fold. Importantly, the combination was extremely potent against different Gram-negative bacterial biofilms, and reduced 3-5 Log CFU/mL of bacteria upon treatment. We validated the safety and nontoxicity of ACP-1(Gly) in mice. The superiority of the combination was further evident from the negligible propensity of resistance development against different Gram-negative bacteria, even after 15 passages. Conversely, the activity of conventional antibiotic colistin increased by 256-fold in the same number of passages. All of these results indicate the potent applicability of the combination in tackling Gram-negative multidrug resistant pathogens.

(iv) Small-molecule adjuvant to repurpose antibiotics: Gram-negative bacterial infections are challenging due to the impermeability of their outer membrane and the overexpression of efflux pumps, both contributing to antibiotic resistance. Additionally, multispecies biofilms, involving superbugs, complicate treatment as they are resistant to most antibiotics. Combining obsolete antibiotics with non-antibiotic adjuvants that target bacterial membranes has shown promise but is hindered by the toxicity of these adjuvants, due to limited understanding of their structure and action. To address this, we designed a small molecular adjuvant by optimizing structural parameters like the balance between hydrophilic and hydrophobic groups, and hydrogen bonding interactions ((**Figure 4(iv)**). This induces moderate membrane perturbation in bacterial cells, enhancing antibiotic internalization and increasing intracellular drug concentration by inhibiting efflux mechanisms, without mammalian cell toxicity. This significantly boosts antibiotic efficacy by 32–512 fold. The leading combination shows potent activity against *A. baumannii* biofilms and disrupts mature biofilms of *A. baumannii* and MRSA. It also demonstrates good biocompatibility and excellent in vivo efficacy (>99% reduction) in an A. baumannii skin infection model. Importantly, *A. baumannii* shows reduced resistance development against this combination, highlighting its potential for treating multi-drug-resistant infections.

5. Local delivery of antibacterial agents: Tackling infections in relatively less vascular tissues such bone infections, cartilage infections or surgical site infections is limited by low penetration of antimicrobials into the target sites. We have engineered different polymeric systems which act as drug-depots and can locally deliver antimicrobials achieving high local therapeutic concentrations of antibacterial agents at the infection site. In one design, a small molecular cationic biocide was encapsulated in dextran methacrylate, a bio-compatible and photopolymerizable polymer (**Figure 5(i)**). The hydrogel was highly active against drug-sensitive and drug resistant bacteria (*S. aureus* and *E. coli*) and their biofilms. The biocide showed sustained release and prolonged activity. Hydrogel displayed >99.99% reduction in the bacterial viability *in-vivo* MRSA skin infection mode in mice. It demonstrated excellent results in rat model of acute dermal toxicity, guinea pig model of skin sensitization, and rabbit model of skin irritation. We also prepared an injectable dual active hydrogel with contact active as well as release active property (**Figure 5(ii)**). The hydrogel was prepared by covalent conjugation of a bioadhesive polymer, polydextran aldehyde (PDA) and inherently antibacterial polymer HTCC. Vancomycin was also loaded in the hydrogel matrix through imine chemistry leading to its sustained pH dependant release. The hydrogel displayed excellent antibacterial efficacy *in-vivo* MRSA infection model (>99.999%) upon direct contact as well as at distal site from the gel implantation. Similary, we have prepared an ocular hydrogel

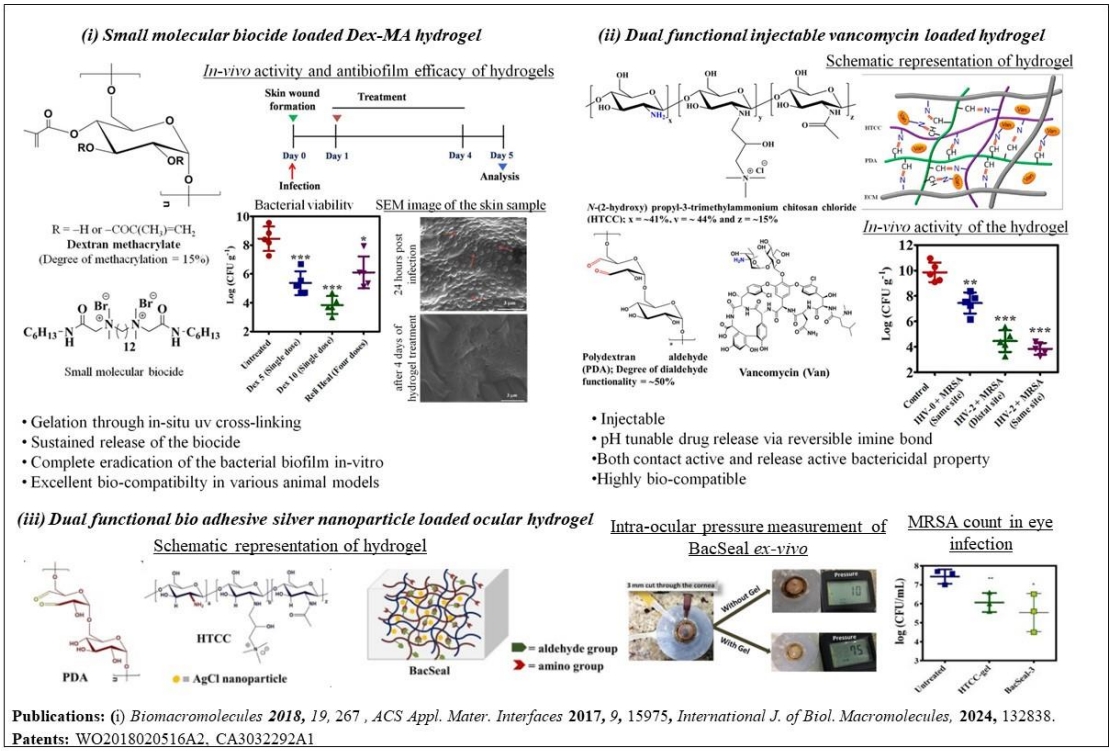


Figure 5: Local delivery of antibacterial agents

by conjugating polydextran aldehyde (PDA) and inherently antibacterial polymer HTCC loaded with silver nanoparticles for tackling eye infections (BacSeal). BacSeal effectively reduced biofilm-embedded MRSA and Pseudomonas aeruginosa by approximately 99.99%. In an ex-vivo human cornea infection model, BacSeal demonstrated around 99% reduction in ocular infection. Additionally, the hydrogel showed excellent sealing properties, maintaining ocular pressure up to 75 mm-Hg when applied to human corneal trauma (**Figure 5(iii)**).

6. Engineering of polymeric antimicrobial biomaterials and surface coatings: In the current times of the dangerously spreading and infectious COVID-19, the need for preventive strategies is being reiterated extensively. We have contributed on that front through the development of antibacterial and antifungal paints which can be coated on various surfaces to prevent the easy spread of infections. Recently we have reported a covalent antimicrobial coating with multivariate activity against bacteria, fungi, influenza and SARS-CoV-2 viruses. We have also engineered antimicrobial coatings for biomedical devices (i.e., catheters). Our biomaterials research spectrum also encompasses development of polymeric hydrogels which have shown the potential of treating skin tissue infections *in-vivo*. Such approach can be useful to prevent surgical site infections, one of the major hospital-acquired infections claiming a high number of lives worldwide.

Described briefly here are some of the successful strategies from our group –

Antimicrobial coating to tackle surface associated infections: Microbial attachment and subsequent colonization onto surfaces lead to community- and hospital-acquired infections. Concomitant emergence of drug-resistant pathogens resulted in a fatal havoc with rise in cases of surface associated infections. As a preventive approach, we developed organo-soluble and water insoluble quaternary antimicrobial polymers based on polyethyleneimine. They were coated on glass surfaces through easy techniques such as painting, dipping or brush-coating.

Coated surfaces showed activity against various bacteria including drug-resistant MRSA and VRE. The coatings also completely killed pathogenic fungi *Candida* spp. and *Cryptococcus* spp. This paint was coated on common surfaces showing complete killing of influenza viruses. In a recent report, we have developed a covalent coating QBAm (**Figure 6(i)**) which showed antiviral activity against the notorious influenza virus with 100% killing within 30 minutes upon contact. The coated surfaces killed SARS-CoV-2 cells within half an hour (>99.9% reduction). The coated surfaces also completely killed different drug-resistant bacteria and fungi pathogens such as methicillin resistant *S. aureus* (MRSA) and fluconazole resistant *C. albicans* spp with 99.99% of reduction in microbial count. >99.99% reduction in MRSA burden in mice subcutaneous model. Our group also developed a chitin-based antimicrobial biocompatible polymeric paint. Apart from showing activity against both drug-sensitive and drug-resistant bacteria, the coatings exerted minimal toxicity against mammalian cells. Catheters coated with the polymer inhibited bacterial biofilm formation in mice. Polymer discs showed substantial temporal degradation in *in-vivo* conditions. In implant-associated infections, a high bacterial load prevails in tissue environment. We engineered a dual active antimicrobial coating from previously described contact active quaternary chitin-based polymers through in-situ reduction of silver nitrate to silver nanoparticles (**Figure 6(ii)**). The polymer-nanocomposite coatings were found to inactivate both drug-sensitive and drug-resistant bacteria and fungi more rapidly than polymer alone. The composites released silver ions displaying long-lasting activity and inhibited both bacterial and fungal biofilm formation. Catheters coated with nanocomposites, upon implantation in mice body, showed >99.99% reduction on catheter and >99.999% reduction in surrounding tissues.

Intrinsically antibacterial hydrogels: In the hydrogel front, we reported an antibacterial hydrogel developed by exploiting imine-bond chemistry between modified chitosan (HTCC) and polydextran aldehyde (PDA) (**Figure 6(iii)**). The engineered formulation killed drug-resistant bacteria (MRSA, VRE, and β-lactam-resistant *K. pneumoniae*). Easy synthesis of its precursors and injectability widened the spectrum of its application. This injectable hydrogel also exhibited hemostatic, bio-adhesive and wound-healing properties. In a cecal ligation and puncture model in mice, hydrogel-treated mice showed higher survival than control mice. We also developed quaternary lipophilic chitosan derivatives which exhibited improved water solubility and enhanced antibacterial activity against multidrug-resistant Gram-positive and Gram-negative bacteria including clinical isolates. These chitosan derivatives were further

developed into antibacterial hydrogels by cross-linking the free amino groups with biocompatible gelatin. The hydrogel showed ~5–7 log reduction of various multidrug-resistant bacteria including the stationary-phase cells within 6 h.

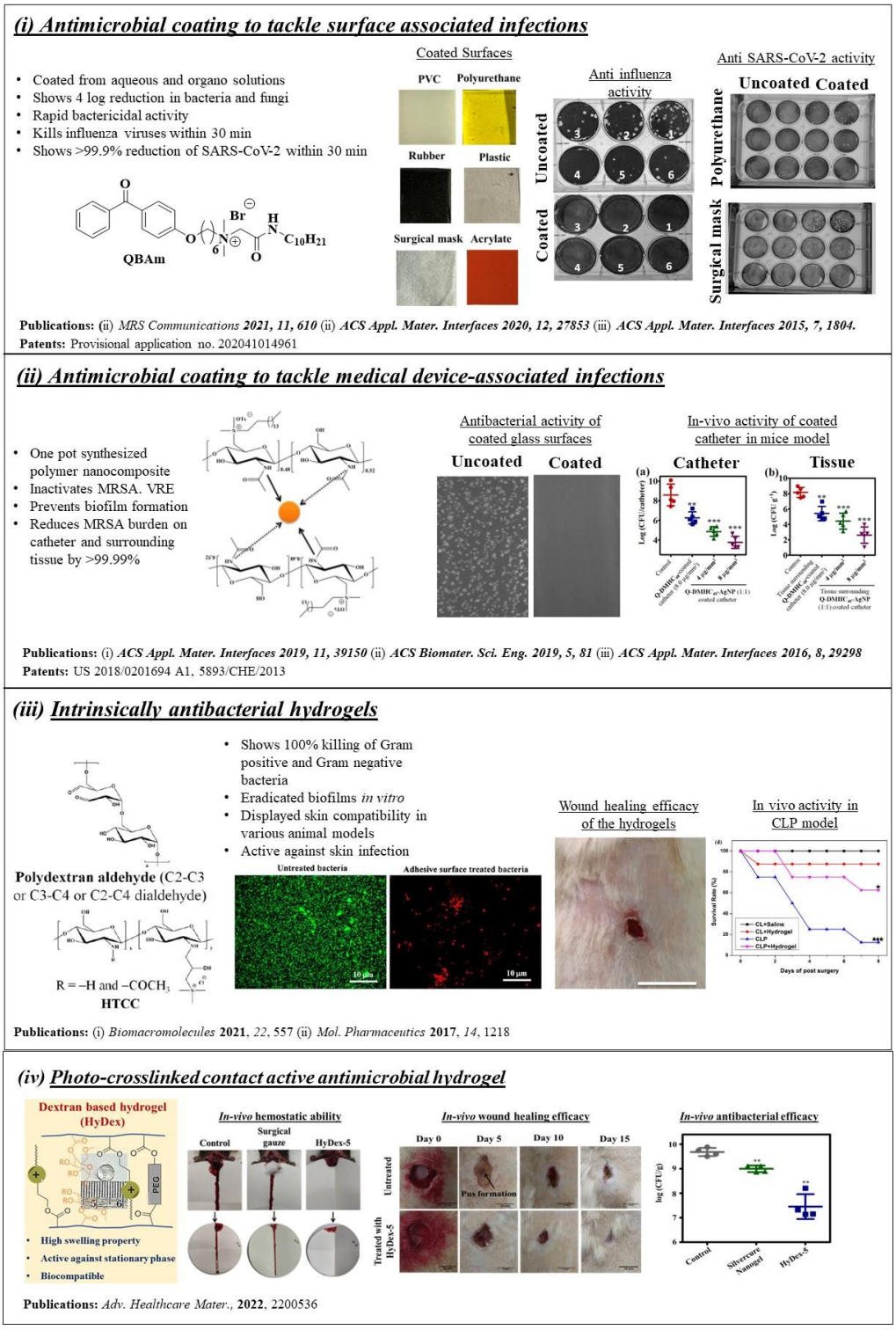


Figure 6: Engineering of polymeric antimicrobial biomaterials and surface coatings

Photo-crosslinked contact active antimicrobial hydrogel: Further in our biomaterial pursuit, we have exploited an UV-irradiation mediated crosslinking polymerization to obtain a highly intrinsically active, wound healing hydrogel (**Figure 6(iv)**). In a recent report, we have engineered an intrinsically antimicrobial hydrogel (HyDex) in a one-pot UV crosslinking technique, employing dextran methacrylate, polyethylene glycol diacrylate, and cationic lipophilic methacrylate with varied hydrophobic chain. The optimized hydrogel exhibits potent antimicrobial efficacy against multidrug-resistant Gram-positive and Gram-negative bacteria as well as against pathogenic fungus *Candida albicans*. A major problem, traumatic or wound-associated haemorrhage is addressed rapidly using this hydrogel in a mice liver puncture model. The hydrogel kills carbapenem-resistant *Acinetobacter baumannii* in a murine model of burn wound infection with >99% reduction in bacterial burden, with accelerated wound healing in rat deep wound model.

Ongoing and future research activities

Our lab has been working to mitigate the problem of infectious diseases, antimicrobial resistance, and chronic infections, through a multifaceted and interdisciplinary approach. An important aspect of our upcoming research is focused on the development of new generation of betalactam antibiotics, and extended spectrum betalactamase inhibitors against Gram-negative superbugs. We are attempting to understand the complexities of microbial infections and target the underlying reasons for virulence. Understanding of bacterial virulence pathways will facilitate better targeting of bacteria, and thereby, effective treatment of chronic infections. Towards this goal, we intend to develop various quorum sensing inhibitors, which can act as therapeutics, and prevent bacterial virulence also. An important strategy involving the development of siderophore-based small molecular peptidomimetics, which can target multidrug resistant Gram-negative bacteria, is being explored too. Concomitantly, we wish to explore in greater detail, the mechanisms of action of our developed therapeutics, through advanced omics techniques, such as metabolomics, proteomics, as well as microscopy. We are exploring, and will continue to explore in greater detail, the exact mechanisms of action and targets of our molecules through simulations and in silico studies.

We are expanding the spectrum of our biomaterials for treatment of polymicrobial infections, consisting of pathogenic fungi and bacteria also. We plan to further our research on hydrogels and materials which possess wound-healing properties, along with their antibacterial nature. The most important applications of such materials will be in treating deep tissue infections, or diabetic and gangrenous wounds, where the antibacterial nature will lead to bacterial load reduction, while wound healing properties will help in fast recuperation. In our recent research endeavors, we have extended our biomaterials research spectrum towards generation of hemostatic materials incorporating anti-infective properties. While a major focus of our earlier work has been on broad spectrum antibacterial agents, we now wish to move ahead to polymicrobial therapeutics, which can work even in case of co-infections by multiple microorganisms, which is a major clinical occurrence. Additionally, renewed focus is being devoted towards addressing specific infections, such as eye infections, respiratory tract infections, etc. at the organ level, along with development of delivery platforms for the same. The therapeutic inventions developed by our lab have shown a broad spectrum of activity, against different classes of multidrug-resistant Gram-positive, Gram-negative bacteria and pathogenic fungi. With our initial foray in the field of antiviral materials, we are attempting to develop hemagglutinin inhibiting, membrane active peptidomimetic anti-influenza therapeutics, which can tackle resistant strains of the virus.

Jayanta Haldar

Applicant: Prof. Jayanta Haldar