Development of L-Lysine-based Small Molecules as Broad-spectrum Antimicrobial Agents

A Thesis Submitted for the Degree of

Doctor of Philosophy

By

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March - 2017

Dedicated to late Fr. Boris D'Santos and my parents

Declaration

I hereby declare that the matter embodied in the thesis entitled "**Development of L-Lysine-based Small Molecules as Broad-spectrum Antimicrobial Agents**" is the result of the research carried out by me at the New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India under the supervision of **Dr. Jayanta Haldar** and that it has not been submitted elsewhere for the award of any degree or diploma.

In keeping with the general practice in reporting the scientific observations, due acknowledgement has been made whenever the work described is based on the findings of other fellow researchers. Any omission that might have occurred due to oversight or error is regretted.

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Chandradhish Ghosh

Certificate

I hereby certify that the matter embodied in the thesis entitled "**Development of L-Lysine-based Small Molecules as Broad-spectrum Antimicrobial Agents**" has been carried out **by Chandradhish Ghosh** at the New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India under my supervision and that it has not been submitted elsewhere for the award of any degree or diploma.

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Dr. Jayanta Haldar

(Research Supervisor)

Acknowledgments

Firstly, I would like to thank my research supervisor Prof. Jayanta Haldar for his guidance, encouragement and freedom that he had offered me to explore new things. Without this freedom, none of this would have been possible. I would like to thank him for inculcating in me a confidence to take up any new challenge in life.

I owe everything to my parents, my sister and my brother-in-law for encouraging me in all my endeavours. They have believed in me more than I can ever believe in myself. It is for their constant support that I could achieve whatever I have, so far in life.

I have been extremely fortunate to have been in a department chaired by Prof. C.N.R. Rao. His zeal for research and his knowledge of science has been a constant source of inspiration. I am also thankful to all the faculty members of New Chemistry Unit for their support.

My course instructors Prof. Subi George (NCU), Prof. Jayanta Haldar (NCU), Prof. T. Govindaraju (NCU), Prof. P. Balaram (IISc) and Prof. Sidharth Sharma (IISc) have been instrumental in teaching me to approach a problem scientifically. I will be grateful to them for exposing me to the wonderful world of research.

I sincerely acknowledge all collaborators Dr. Krishnamoorthy and Dr. Shome (NICEDI, Bangalore), Vikas Yadav and Prof. Kaustuv Sanyal (MBGU, JNCASR), Shweta Chaubey and Prof. Utpal Tatu (Biochemistry, IISC), Haroun Mohammad and Prof. Mohamed N. Seleem (Purdue University), Nicole Harmouche and Prof. Burkhard Bechinger (CNRS, Strasbourg), Ashvini Ray and Prof. Swapan K. Pati (TSU, JNCASR) and colleagues at Public Health England for their help in preparing this thesis.

I am indebted to my labmates for their support, encouragement and constructive criticism. I extend my deep sense of gratitude to Venky, Jiaul, Divakara, Padma, Goutham, Mohini, Paramita, Sandip, Swagatam, Pakruddin, Vijay, Kathakali, Brinta, Sreyan, Ashly, Sridhar and Pinky for maintaining a healthy and competitive environment in the laboratory. I also thank my short-term students Ahanjit, Neha, Deepti and Souradeep for their help.

I acknowledge technical staff Dr. R.G. Prakash (Animal house in-charge), Vasu (UV/ PL/ IR), Mahesh (NMR) and Sivakumar (HR-MS). I am especially thankful to Dr. R.G. Prakash for helping with animal studies.

I thank my friends Syamantak, Dipanwita, Soumyabrata, Sohini, Ananya, Payel, Abishek, Somananda, Arpan, Sarada for making my stay in JNCASR pleasant and enjoyable. It is because of them that I have been able to cope with the pressure of PhD life. I thank the bunch of guys I played football with at JNCASR. Football was my constant source of rejuvenation.

I would like to express my sincere thanks to all the academic, administrative, security, library, comp-lab and health centre staff for making our campus life smooth and easy.

Lastly, I would thank my teachers at St. Xavier's Collegiate School, Kolkata for instilling a sense of integrity and discipline, which has and will continue to take me forward. Especially, Late Father Boris D' Santos without whom I would not have studied chemistry this far.

Preface

The thesis, divided into seven chapters, aims to document the design principles, synthetic strategies underlying the development of simple small molecules that act as broad-spectrum antimicrobial agents by targeting the membrane of pathogenic microorganisms.

Chapter 1 provides an introduction to the predicament of antimicrobial resistance (AMR) and endorses the significance of antimicrobial peptides (AMPs) as templates for next generation of drugs. In an attempt to emulate the properties of AMPs, in **Chapter 2,** the design and synthesis of simple antibacterial agents involving only one amino acid (L-Lysine) and two lipid tails are reported. The use of two short lipid tails over a single long one is shown to have increased selective activity against bacteria over mammalian cells significantly. Bacteria found it difficult to develop resistance against the best compound in multiple passages. Fluorescence spectroscopic studies corroborated the membrane-active mechanism of action of a representative compound. Topical application of the representative compound reduced bacterial burden by 2.1 logs in mice inflicted with burn-infection caused by *Acinetobacter baumannii*.

In **Chapter 3** another series of small molecules have been described wherein aromatic moieties, alkyl groups and L-lysine moieties were assembled together through a tertiary amide. The aromatic moieties were varied from anthracene naphthalene to benzene. These molecules, termed as aryl-alkyl-lysines, exhibited high *in vitro* potency against a variety of multidrug-resistant Gram-positive and Gram-negative bacteria including the anaerobic bacteria, *Clostridium difficile* (MIC for the best compound was less than 3 μg mL^{-1}) Nontoxic toward mammalian cells, these rapidly bactericidal compounds primarily acted by permeabilization and depolarization of bacterial membrane and withstood resistance development. In a methicillin-resistant *Staphylococcus aureus* model of skin infection and *A. baumannii* model of burn-infection in mice, bacterial burden was reduced by 2.1 logs and 2.4 logs respectively.

In **Chapter 4**, lipidated-biphenyl-lysines which contain four cationic charges two lipid tails and a biphenyl core has been described. Increase in positive charge residues led to an increase in selectivity and it was observed that this class of compounds was able to inhibit cell wall biosynthesis of bacteria in addition to infiltrating membranes. Against this class of compounds too, bacteria could not develop resistance in multiple passages. Additionally, the compound was able to inhibit growth of bacteria within mammalian cells. In MRSA model of skin infection and *A. baumannii* model of burn infection in mice, bacterial burden was reduced by 2.7 logs and >5 logs respectively.

Chronic or persistent infections belligerent to antibiotic treatment are often caused by stable communities of bacteria, termed biofilms, which grow on surfaces. In **Chapter 5**, the ability of the best two compounds (identified from the previous chapters) to act against dormant cells and biofilms of Gram-positive and Gram-negative bacteria have been studied. The efficacy of the compounds were also validated in a murine models of biofilm infection caused by MRSA and *P. aeruginosa*. Further, the ability of one of the compounds to prevent endotoxin stimulated inflammatory response (sepsis) have been described.

In **Chapter 6** the antifungal properties of aryl-alkyl-lysines has been documented. The activity of the best compound (MIC <2 μg mL-1) was performed against different clinical isolates of pathogenic fungi and also against biofilms of *Candida albicans*. It was observed that polarization and permeability of fungal cell membrane was compromised upon addition of the compound. Additionally, the compound was found to possess the ability to cross the blood-brain-barrier in an *in vitro* model. Overall, aryl-alkyl-lysines were found to be excellent antifungal compounds with scope for further development to clinical antifungals.

In **Chapter 7**, the potential of aryl-alkyl-lysines to as antimalarial agents was tested. The optimal compound (IC_{50}=1 μM) acted by arresting the development of *Plasmodium falciparum* at the ring stage inside the erythrocytes. The compound could perturb the plasma membrane potential and the digestive vacuole of parasites. The compound was also able to increase the survival of mice infected by *Plasmodium bergheii* by at least 5 days when administered via intra-peritoneal route, but showed no apparent toxicity to mice at the concentration tested. Thus, this class of compounds bear potential to be developed as novel antimalarial drugs.

Contents

Chapter 1

An Introduction to Antimicrobial Resistance and the potential of synthetic mimics of antimicrobial peptides as potential drugs

1.1 Infectious diseases and Antimicrobial resistance

Infectious diseases continue to wreak havoc in hospitals and health care settings. The World Health Organization has published a Global Report on Surveillance which summarizes the dire situation created by increasing antimicrobial resistance to drugs. The situation is worst in case of bacteria but resistance is rampant in other pathogenic microorganisms such as parasites, fungi and viruses.[1] According to the report of the O'Neill commission, AMR already causes around 700,000 deaths annually which is estimated to increase to 10 million deaths annually by 2050. Moreover, it is expected to cost global healthcare around $100 trillion by 2050. This has also prompted the governments of USA and UK to set up strategic plans to combat antimicrobial resistance.

Several other problems with respect to infectious diseases needs attention. A major problem is that of infection due to biofilms and metabolically inactive bacteria. Although around 80% of all infections are related to biofilms, no dedicated treatment towards such infections exist. Sepsis, is another problem which is responsible for significant mortality, especially for children below the age of five years. Sepsis is overreaction of the immune system of the host in response to endotoxins released by bacteria. The condition of sepsis is also often overlooked and no dedicated treatment exists. Despite several unmet needs, several other factors have aggravated the problem.

Major pharmaceutical companies such as Roche, Bristol-Myers, Squib, Lilly, Abbott, Bayer and Pfizer had shut down their antibiotic research due to increasing rate of bacterial resistance and slow approval of drugs. This is reflected in the data given in Figure 1.1. In a period of twenty years from 1995 to 2014, till 2009, there was a steady decrease in the number of drugs approved by the Food and Drug Administration (FDA) of United States of America (USA) for infections caused by bacteria; only one, Vibativ® (Telavancin), was approved between 2005 and 2009. However, the threat of antimicrobial resistance has compelled companies, health agencies and governments to implement new programs such as the GAIN (Generating Antibiotics Incentives Now) act, (effective in USA since 2012) "the '10' × '20' initiative" and ReAct (Action on Antibiotic Resistance).[2] This in turn has prompted drug approval agencies such as the FDA to respond. In 2014 itself, as many as 9 drugs have been approved for infectious diseases, out of which 4 are for antibacterial purposes. In the period of 2009-2014, as many as 11 drugs were approved for treating bacterial infections.

As can be seen from Figure 1.1, the number of drugs approved for fungi is few and those approved for parasites are even fewer. However, both parasites and fungi are major contributors to human morbidity. In case of immunocompromised patients, fungal infections claim a lot of lives. Malaria is still rampant in low-income tropical countries and reports of resistance against artemisinin, the most active drug, has already emerged. As long as the elusive malarial vaccine is not available, it is important to invest research in developing new drugs. Against both the class of pathogens, drugs with novel mechanism of action are needed

Historically, nature has been a constant supplier of new antimicrobial agents.[3] Ever since penicillin was discovered by Alexander Fleming, significant amount of research had been dedicated towards isolation of novel antimicrobial agents from nature. Indeed, all the major classes of antibiotics, such as aminoglycosides, β-lactams, cephalosporins, macrolides, tetracyclines, glycopeptides and lipopeptides have all been isolated from nature. Although the sulphonamides, quinolones and oxazolidinones have been derived synthetically, significantly higher number of products have come from nature. Improved innovations has slowly made it possible to culture the previously uncultured microorganisms as well.[4-7] These might possess a range of undiscovered antibiotics which might have clinical importance. The recently reported lassomycin and teixobactin are already being considered as future antibiotics.[8, 9] The incorporation of newer technologies into drug discovery, such as computer aided drug discovery,[10, 11] high-throughput

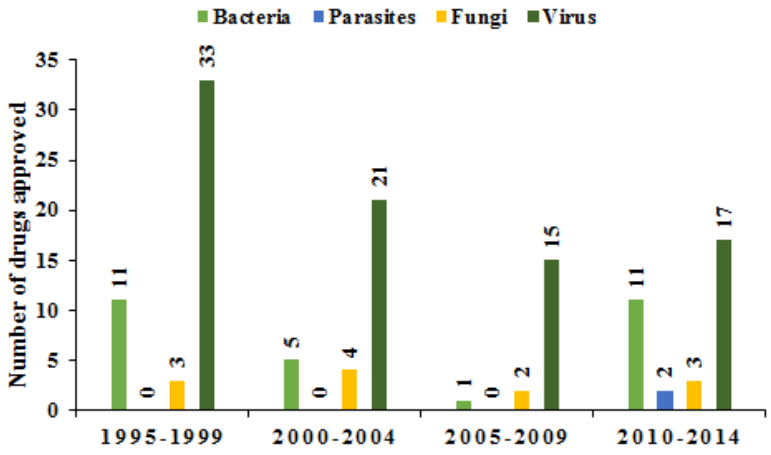

Figure 1.1: The number of drugs approved by FDA in the category of infectious diseases from 1995 to 2014.

screening (HTS),[12] Quantitative Structure Activity Relationship (QSAR) studies[13] have made it possible to screen a large number of molecules for potential antibacterial activity. Synthetic modification of existing natural products is also another approach that is being successfully exploited to enrich antibiotic arsenal.[14] Semi-synthetic vancomycin derivatives with improved biological activity have also been designed.[15, 16] Another design principle that could be employed is to mimic the properties of natural compounds. This approach would basically be a combination of all the approaches mentioned before. Such an approach could be used to design more effective drugs with properties that could be controlled. The synthetic designs would involve novel challenges for an organic chemist making the approach intellectually gratifying. Even against fungi and parasites, similar strategy can be followed.

In this chapter, one such approach of producing novel antimicrobial agents has been described, that is making small molecular mimics of antimicrobial peptides. First, a small introduction to natural antimicrobial peptides have been given; their properties, merits and demerits have been dealt with. Subsequently, synthetic attempts towards mimicking the properties of antimicrobial peptides have been classified. After briefly touching upon the macromolecular and oligomeric mimics of antimicrobial peptides, details of the design principles involved towards making small molecular mimics of antimicrobial peptides have been provided. Further, some of the unmet problems in the field have been discussed in details and how this field can tackle such problems have been described. Finally, the scope of the thesis has been furnished.

1.2 Natural antimicrobial peptides

Antimicrobial peptides (AMPs) and host-defense-peptides (HDPs), the first line of defense in many multicellular organisms, possess a broad range of biological activities including antibacterial, antifungal, antiviral, anticancer, antiplasmodial, antiprotistal, insecticidal, spermicidal and immunomodulatory activities, have been touted as future drugs.[17, 18] Membrane active nature of such molecules are considered to be largely responsible for such a broad range of activity of these compounds. Targeting the cell membrane of microorganisms is an effective and selective antimicrobial approach as they bear subtle differences with the mammalian cell membrane.[19, 20]

Facial amphiphilicity, common to all cationic AMPs, helps in interacting with microbial membranes.[17, 20] For example, it is widely accepted that the positive charges on the peptides

interact with the negatively charged bacterial cell envelope which leads to attraction and attachment.[21, 22] This is followed by interaction of the hydrophobic residues of the peptide with the negatively charged lipid rich bacterial membrane, which allows the disintegration of cell membrane, thereby causing cell death. The selectivity of AMPs towards bacterial cells (negatively charged) over mammalian cells (zwitterionic) is mainly due to the differences of the lipid components of the respective cell membrane. For example, the lipid composition of *S. aureus*, a typical Gram-positive bacteria, is 58% phosphatidylglycerol (PG) and 42% cardiolipin (CL) while that of *E. coli* is 80% phosphatidylethanolamine (PE), 15% (PG) and 5% cardiolipin. The mammalian cell has two leaflets; for a RBC the outer leaflet is composed of 33% phosphatidylcholine (PC), 9% PE, 18% sphingomyelin (SM) and 25% cholesterol (CH). The inner leaflet is composed of 10% PC, 25% PE, 10% phosphatidylserine and 5% SM [20, 23, 24]

AMPs and HDPs offer more advantages as therapeutic agents over conventional antibiotics, which mostly target biosynthesis of bacterial cell wall, biosynthesis of proteins,

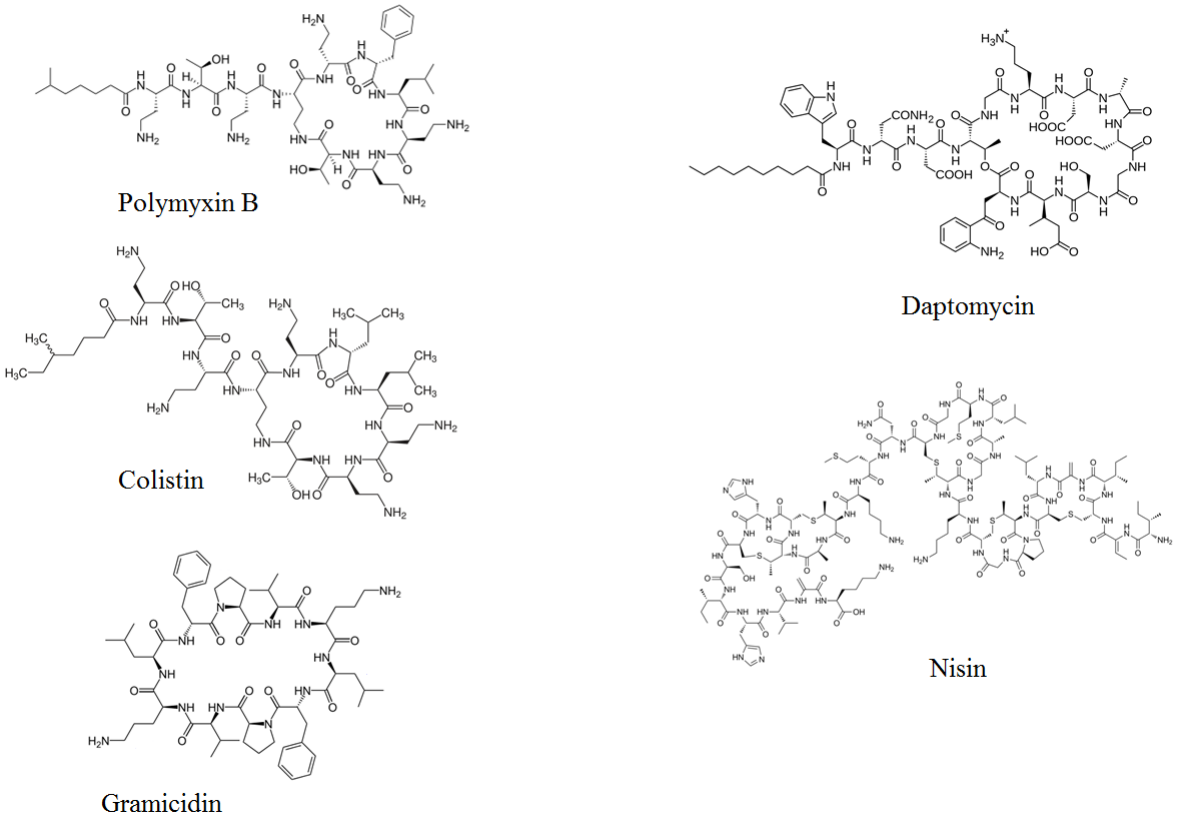

Figure 1.2: The peptide antibiotics that have been approved for clinical use.

replication and folic acid synthesis. However, bacteria quickly develop resistance against such antibiotics.[25-27] However, because AMPs target the cell membrane of the microorganisms for direct antimicrobial action bacteria find it difficult to develop resistance. Although there are reports of resistance even against antimicrobial peptides, the frequency for resistance development is much less.[23, 28] Another advantage offered by AMPs is their ability to modulate the immune system to fight infections.[17, 20, 29] Towards this, AMPs can alter host gene expression, induce chemokine production, inhibit production of pro-inflammatory cytokines, promote wound healing and so on.[30]

Despite so many advantages, few peptide antibiotics have had little clinical success largely due to their *in vivo* toxicity, limited bioavailability and large cost of production. The therapeutic potential of AMPs range from antibacterial to antiviral to antifungal to antiplasmodial. Very few peptide antibiotics have been used in the market, although several of them are undergoing clinical trials.[14] The only peptide antibiotics that have been used in clinics if given in Figure 1.2 and include Polymyxin B (a lipopeptide obtained from *Bacillus polymyxa*), Colistin (Polymyxin E, also from *Bacillus polymyxa*), Gramicidin (a linear polypeptide derived from *Bacillus brevis*), daptomycin (a cyclic anionic lipopeptide) and nisin (a food preservative obtained from *Lactococcus lactis*). The successes and failures of other antimicrobial peptides are well documented in some excellent reviews.[31, 32] In Table 1.1, an update on the current status of various synthetic and natural AMPs that are undergoing clinical trials has been provided. The search has been conducted by visiting clinicaltrials.gov and the individual websites of the companies.

1.3. Synthetic mimics of antimicrobial peptides

The field of medicinal chemistry is flooded with examples where synthetic chemistry has been used to mimic medicinally important molecules obtained from nature. Structure-activity-relationship studies on various successful compounds have also yielded drugs with enhanced properties. In the field of antimicrobial peptides, chemists have developed sound synthetic strategies to overcome the problems faced by natural antimicrobial peptides. The different strategies can be broadly classified into three categories: 1. Macromolecular approaches 2. Oligomeric approaches and 3. Small molecular approaches. In this chapter, for the sake of simplicity macromolecular compounds have been defined as those which are high in molecular weight and are polydisperse. The oligomers are smaller in size and have a well-defined molecular weight. Small molecules are compounds which are less than 1000Da in weight.

1.3.1 Macromolecular approach

Macromolecular approaches have also been successfully used to mimic the properties antimicrobial peptides. Again, amphiphilicity and overall cationic nature were important structural parameters which needed to be integrated into the designs of the polymers. Most of these designs could be categorized into one of the three following approaches: segregated monomer approach,

Table 1.1: Antimicrobial Peptides in clinical trials.

Compounds	Indication	Status	Company
Locilex (Pexiganan)	Diabetic foot ulcers	Phase III	Dipexium Pharma, USA
Omiganan	Rosacea	Phase II	Cutanea life sciences, USA
LL-37	Chronic Leg Ulcers Melanoma	Phase II Phase II	Pergamum, Sweden M. D. Anderson Cancer Centre, USA
OP-145	Middle ear infection	Phase II	OctoPlus, Netherlands
DPK-060	Atopic Dermatitis Acute external otitis	Phase II Phase I	Pergamum, Sweden
NP123	Onychomycosis	Phase II	Novabiotics, UK
Hlf1-11	Bacterimia and fungal infections	Phase II	AM-Pharma, Netherlands
XOMA-629	Impetigo	Phase II	XOMA, Netherlands
PAC-113	Oral candidiasis	Phase II	Pacgen Biopharmaceuticals, USA
CZEN-002	Vulvivaginal candidiasis	Phase II	Zengen Inc., USA
SGX 942	Oral Mucositis	Phase II	Soligenix Inc., USA
AP 138	MRSA implant infections	Phase I	Adenium Biotech, Denmark
Avidocin and purocin	Narrow spectrum antibiotic	Preclinical	Avid Biotics, USA
HB 1345	Acne	Preclinical	Helix Biopharma, Canada
HB 1275	Trychophyton infections	Preclinical	Helix BioPharma, Canada
Plectasin	Gram-positive infections	Preclinical	Novozymes, Denmark

same-centered approach and facially-amphiphilic approach.[33] Same-centered approach has also been used by us to develop selective antibacterial polymers.[34] Several excellent designs have been effectively used to obtain a plethora of antimicrobial polymers.[35-43] The field of polymer antimicrobials are now being explored for other interesting activities like antifungal activity, siRNA delivery, anti-biofilm properties or action in combination with other drugs.[44-47] The macromolecular approach towards the development of antimicrobial peptide mimics have been very well documented in literature and would provide excellent platform for future designs.[33, 48-50]

1.3.2 Oligomeric approach

Several groups around the world have tried to develop strategies to counter the problems faced by AMPs. Most of the initial approaches focused on using peptidomimetic approaches. The peptidomimetic approaches were focused on modification of the peptide backbone, either by extending α-peptides[51, 52] to β-peptides [53, 54] or by replacing the amide bonds by urea bond,[55, 56] a peptoid linkage[57] and so on. Other important contributions in the field include those of oligoacyl lysines[58] and α-AA peptides.[59] Although most of the designs centered on mimicking the peptide bonds, they kept the cationic and amphiphilic nature of antimicrobial peptides constant in their designs. These compounds were oligomeric and were thus able to form secondary structures necessary for potent antimicrobial activity. Due to the presence of abiotic moieties these peptides were usually resistant to degradation. Some excellent reviews have covered the advent of the field of antimicrobial peptidomimetics.[49, 60-62]

1.3.3 Small molecular approach

Small molecular membrane active agents are interesting antimicrobial agents which can be potentially explored for activity against a plethora of pathogens. Around 90% of the drugs that are used in the market today are small molecules.[14] There are many reasons as to why still small molecules rule pharmaceutical industry: they are relatively simple to synthesize, they offer a broad diversity of biological activities, and they are easily cleared from the body. Small molecules can be processed easily in large scale industrial production. They can be incorporated easily into tablets and capsules which when digested will pass easily into the blood stream via the intestinal wall. Through the blood stream they can reach almost anywhere in the body. Moreover, their small size and chemical structures allow them to easily permeate through the walls of cell membranes.

This section deals with the various design strategies employed in the field of small molecular membrane active agents so far. The different compounds have been classified into four categories. 1. Structures that employ internal H-bonding for facial amphiphilicity. 2. Structures that use central aromatic cores. 3. Structures that use amino acids/peptides. 4. Other interesting designs.

1.3.3.1 Structures that employ internal H-bonding

Facial segregation of hydrophobic and hydrophilic residues is a conserved characteristic of most natural antimicrobial peptides. It was important to incorporate this property into any mimic of antimicrobial peptides. DeGrado and co-workers used hydrogen bonding as a tool to incorporate facial amphiphilicity into a polymer.[63] In this design they have carried out polymerization of a diamine [2, 6-Diamino-4-t-butyl-1-(2-t-butoxycarbonylaminoethyl) sulfanylbenzene] and isophthaloyl chloride (Figure 1.3 Structure 1). It was expected that the methylene group bonded to the thioether would lie away from a plane of the aromatic ring and allow weak hydrogen bond interactions with the amide protons.

In an improved design, the primary compound which served as a template for further functionalization contained a central aromatic ring was flanked on both sides (through amide bonds) by the diamine (2,6-Diamino-4-t-butyl-1-(2-t-

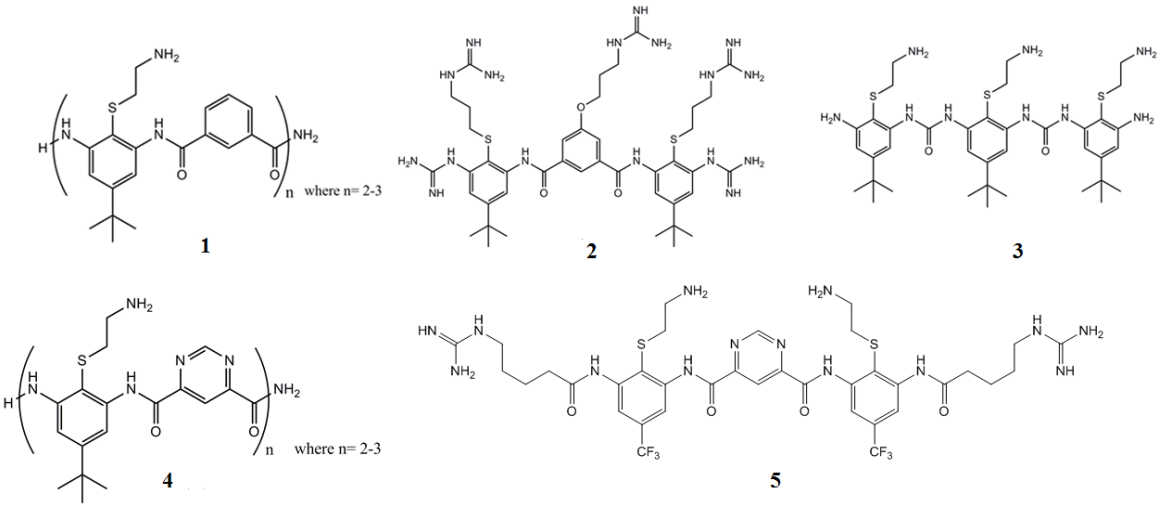

Figure 1.3: The structures of compounds which incorporated H-bonding in their designs.

butoxycarbonylaminoethyl)sulfanylbenzene) as shown in Figure 1.3 Structure 2.[64] The terminal amine groups of the diamines allowed further functionalization. Similarly, on increasing the positive charge residues, a different set of SAR was performed. It was observed that greater selectivity could be obtained by increasing the number of positive charges without compromising on activity significantly. The compound which was considered to be the most selective had 4 positive charges, which were contributed by two arginine residues.

In a separate study, Tew and co-workers sought to improve the design by replacing the amide moieties by urea and also by varying the aromatic rings from one to four.[65] The aromatic ring containing the thioether moiety was kept constant in the design. The newly incorporated urea analogues allowed H-bonding interactions between S and NH on every side. This allowed for greater conformational rigidity in the molecule due to hydrogen bonding. The compound containing three aromatic rings was the most active compound (Figure 1.3 Structure 3). If the central ring was replaced by one in which the positions ortho to the carbonyl atoms contain a hydrogen bonding acceptor like Nitrogen, then the degree of the stiffness in the molecule can be further increased leading to better facial amphiphilicity. This design was envisioned and executed by Tew and co-workers in a further improvement of the aryl amide foldamers (Figure 1.3 Structure 4). [66]

DeGrado and co-workers on the other hand wanted to increase rigidity of the molecules by replacing the central isophthalic acid moiety by 4, 6-dialkoxy-substituted isophthalic acid 4, 6-dialkoxy-substituted isophthalic acid. This allowed the formation of extra intra-molecular O…H—N hydrogen bonds which restricted the rotation around the aryl—CO bond. The effect of greater positive charge density was also studied by the introduction of guanidine groups (Figure 1.3 Structure 5).[67]

In another design, three aromatic rings formed the backbone but the central ring was para substituted (Figure 1.4 Structure 6).[68] An important feature was the systematic use of hydrogen bonding via the optional use of nitrogen atoms in the central ring. Furthermore the effect of hydrophobicity was studied by variation of polar and non-polar side chains. The NH_2 providing functionality was varied from β-alanine to aminovaleric acid. The polar groups were varied from tertiary butyl to CF_3.

1.3.3.2 Structures that use central aromatic cores

Tew and co-workers had introduced abiotic designs devoid of amide bonds in the form of polymers of phenylene ethylenes.[69-71] In these designs the phenyl rings were joined by ethylene bonds. The hydrophobicity was brought about aromatic rings which are optionally substituted with an alkoxy group while hydrophilicity was brought about amine groups joined to the ring via ethyl groups. In the very first report the polymers length as varied from n = 6 to n = 20. A further advancement of the work, they had made a shorter derivative consisting of three aromatic rings.[72] Upon variation of the number of methylene groups connecting the amine groups to the ring, a novel SAR was done (Figure 1.4 Structure 7).

A new series of small molecular membrane active agents were designed by Tew *et al.* wherein four to six charges were decorated on another three aromatic moieties.[73] This time the central ring was varied from benzene, naphthalene and phenyl benzene. The positive charges were contributed by ethanolamine moieties conjugated to the adjacent aromatic rings via ether bonds.

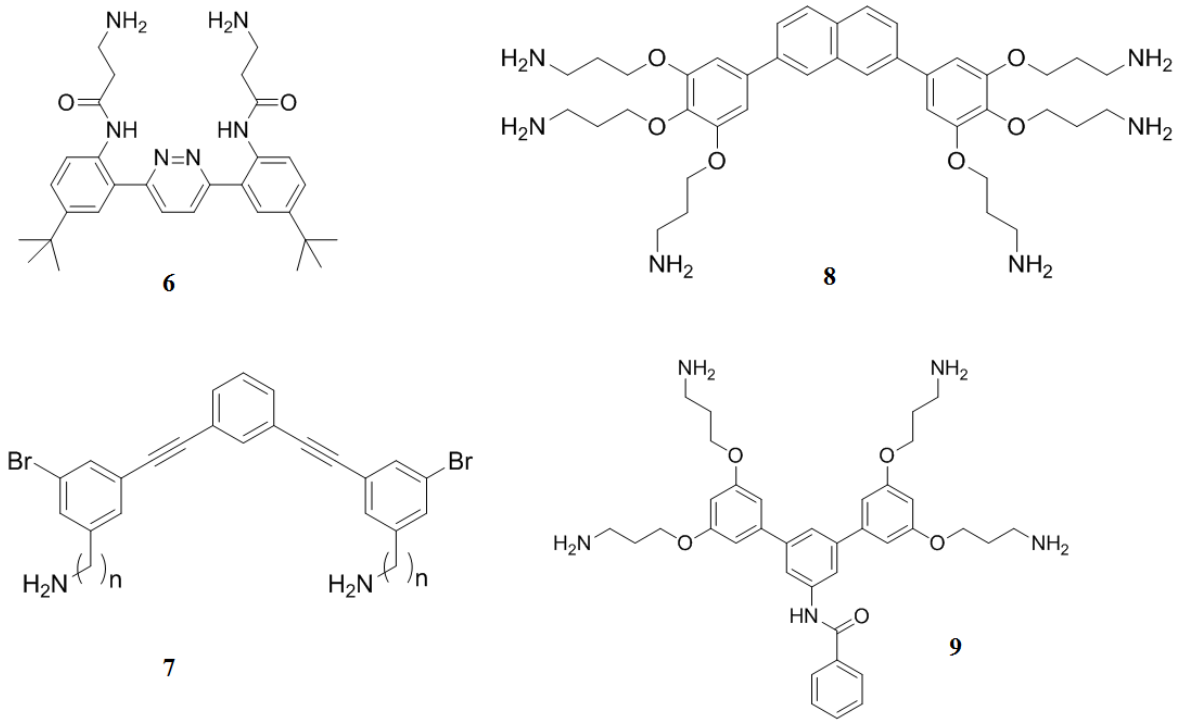

Figure 1.4: Structures of compounds based on aromatic cores.

(Figure 1.4 Structure 8). Using a similar design they had further shown that if the facial amphiphilicity of such molecules are disrupted, then the compounds lose their broad spectrum activity (Figure 1.4 Structure 9).[74] Some more molecules based on the same design principles were created but the central aromatic core was connected to the adjacent aromatic moieties via triazole moieties generated by click chemistry.[75]

1.3.3.3 Structures that use amino acids/peptides

Svendsen et al. had been interested in finding the pharmacophore of bovine lactoferrcin derivatives. In miniaturization attempt, they had made hexapeptides to dipeptides. Their systematic truncation of the original peptide and structure activity study with different derivatives allowed them to come to the conclusion that depending on the strain of bacteria a minimum of 4 amino acid residues were required for substantial activity.[76] Two of the residues required to be cationic and two of them required them to be bulky hydrophobic amino acid residues. Further, they considered the activity of molecules which only two amino acids but a C-terminal appended to hydrophobic bulky groups. Of the two amino acid, arginine formed the C-terminal amino acid while several hydrophobic unnatural amino acid were the N-terminal ones.[77] The free N-terminus and the guanidine moiety of the arginine moiety provided the two cationic charges. The best molecule identified from the study comprised of 2,5,7-tri-tert-butyltryptophan connected to an arginine moiety whose C-terminus was capped by benzyl amine. They had further designed tripeptides wherein, a central unnatural amino acid (with provision for adding aromatic moieties to it for increasing hydrophobicity) flanked on two sides by arginine residues.[78] But studies conducted later showed that the compounds generated from tripeptides with the C-terminus capped with benzyl group, and highly hydrophobic central amino acid is the most effective. If the C-terminal of this molecule is capped by an ethylphenyl group, LTX-109 is obtained, which is undergoing clinical trials (Figure 1.5 Structure 10).[79]

Over several years Bremner et al. had been trying to mimic vancomycin action by design of small cationic cyclic peptoids.[80-84] They had explored a variety of structures and designs towards this aim. In their most successful approach they had developed binaphthyl based dicationic peptoids (Figure 1.5 Structure 11).[85] In this elegant design, the amino acids lysine and arginine contributed the positive charges. The hydrophobicity was provided by the binaphthyl core and a leucine moiety which was capped on the C-terminus.

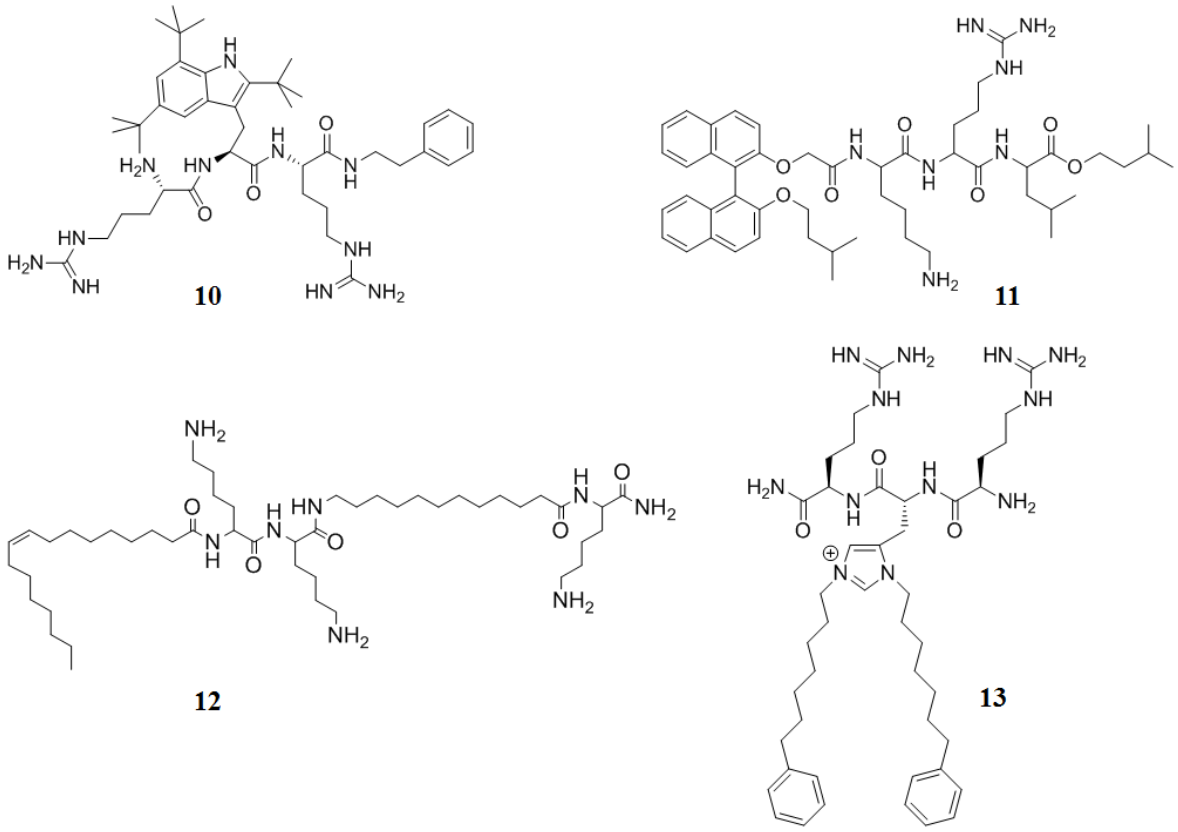

Figure 1.5: Structures based on amino acids/peptides.

Oligoacyllysines, developed by Amram Mor and co-workers were excellent mimics of host defense peptides with promising antimicrobial properties.[58] But most of the compounds generated were oligomers with M.W. >1000 Da. Thus discussion regarding their evolution and the principles behind their designs has been reviewed excellently by them.[86] Subsequently however, short oligoacyllysines had been designed. These molecules were an assembly of 3 lysines and 2 fatty acids.[87, 88] The compound can be described as aminolauryl-[lysyl-aminolauryl]-lysyl. The design of the molecules is shown below (Figure 1.5 Structures 12). These molecules tend to aggregate in solution which limits their antibacterial efficacy and enhances their hemolytic activity. In order to disrupt their aggregation properties, the second molecule was designed which contained an unsaturated long chain. Their hypothesis was confirmed as the second molecule showed reduced aggregation and toxicity maintaining a similar antibacterial activity.

In another novel design, several Histidine derived ultrashort peptides were created. The N(π)- and N(τ)- positions of histidine were appended to lipophilic groups. The lipophilic groups were typically propyl-cyclohexanes. Histidine (His) and Arginines (Arg) were repeated to form dimers and trimers. Cationic nature of the compounds were varied by varying the arginine groups (Figure 1.5 Structure 13).[89]

α-Mangostin is a natural compound which acts against bacteria like MRSA by disrupting the cytoplasmic membrane. In order to improve the selectivity of α-Mangostin, several novel membrane active agents based on the xanthone core were designed. In this design the Xanthone core was functionalized on two sides with amine moieties with different pKa values to fine tune the amphiphilic nature of the molecules.[90] In a further advancement to this work, cationic amino acids were coupled to the xanthone core to provide more potent and selective compounds.[91] In a representative structure (Figure 1.6 Structure 14), the four positive charges were contributed by the guanidine groups of arginine.

In another design a norspermidine derivative was functionalized in a way to have at least two positive charges and a pendant aliphatic group (Figure 1.6 Structure 15). Phenylalanine was conjugated to the primary amines of norspermidine while an aliphatic chain was appended to the secondary amine to create a library of compounds. A structure-activity relationship was delineated by varying the length of the long chain, varying the stereoisomerism and type of hydrophobicity. To understand the role of phenylalanine conjugation and appended long chains, control compounds without the phenylalanine conjugation as well as those without long chains were also synthesized. These compounds too were effective designs of small molecular membrane active agents.[92]

1.3.3.4 Other interesting designs

Savage and co-workers had decorated cholic acid derivatives with amines and guanidines to mimic the various derivatives of polymyxins B. It was observed that upon introduction of a lipophilic alkyl chain to the C17 position of the cholic acid derivatives made the compounds active. Furthermore, the activity of compounds wherein, the C-24 of the cholic acid was functionalized by various polyamines were also prepared. Significant improvement in activity was not observed, however. The work has been very well reviewed.[93] The best compound of the study turned out to be CSA-13 the structure of which is represented in Figure 8 (Structure 16).

Benzophenone based membrane active antibiotics were initially designed as DNA targeting antibiotics (Figure 1.6 Structure 17). In their design they had segregated the molecules into three distinct regions: tail, heterocyclic moiety and linker. The symmetric benzophenone moiety was the linker, connected to it, via an amide bond, was *N*-methylimidazole, which served as the heterocycle. The tail region, which provided cationicity to the molecules were varied from methylpiperazine, dimethylaniline, morpholine, piperidine, or pyrrolidine.[94] Subsequent studies on these compounds elucidated their mechanism of action which has been discussed later.[95]

Another new class of antimicrobial agents were based on L-arginine coupled to an achiral lipophilic 3-amino-2,2-disubstituted propionic acid on the C-terminal. The lipophilic moieties had been varied from alkyl to cyclohexyl to aromatic derivatives. [96] As a further improvement to the design the L-arginine moiety was replaced with various functionalities containing different amine groups (Figure 1.6 Structure 18). Various lipophilic groups were also varied; mostly based on

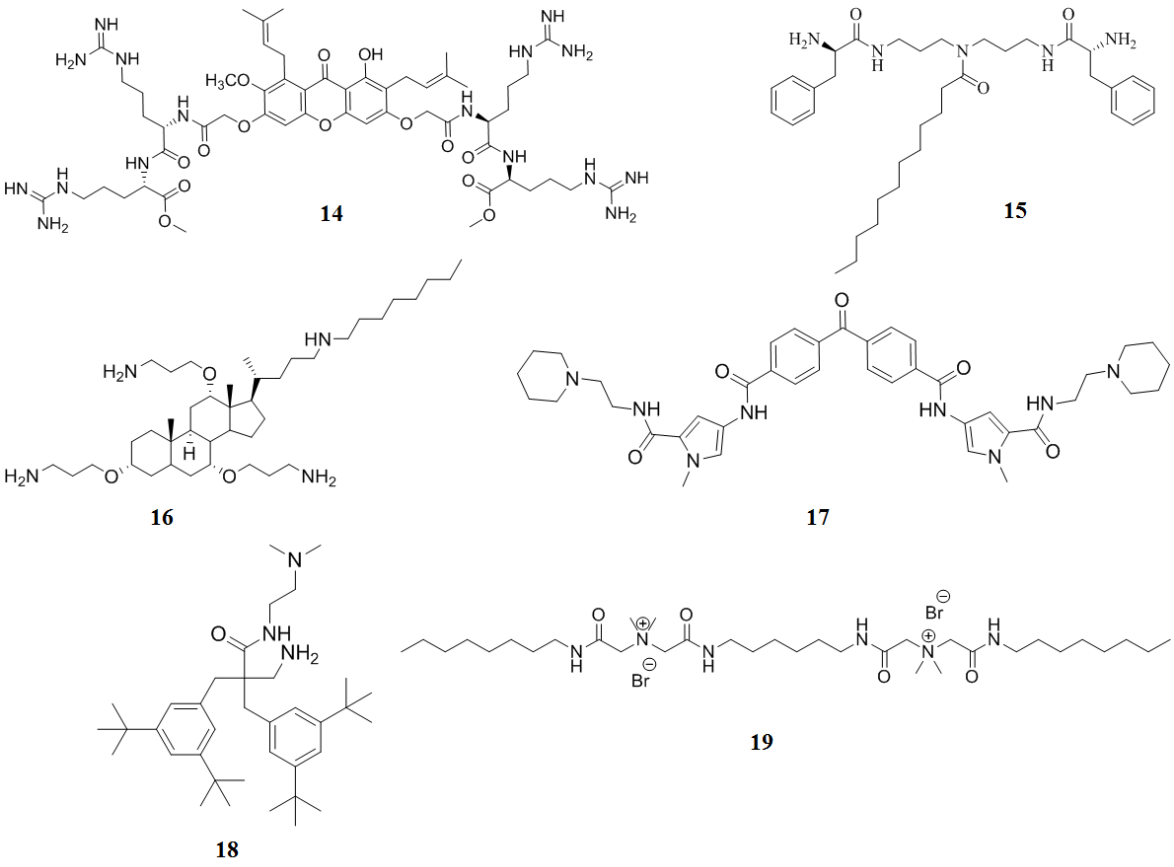

Figure 1.6: Other interesting designs of membran-active small molecules.

aromatic moieties. The most potent compounds were those in which the lipophilic moiety was based on tertiary butyl substituted benzenes.[97]

A set of novel cationic amphiphilic small molecular biocides was also reported from Haldar and co-workers (Figure 1.6 Structure 19). Three important features of these compounds are- variable hydrophobic character not only from pendent lipophilic alkyl chains but also from the lipophilic spacer chains in their design; inclusion of non-peptidic amide bonds in lipophilic alkyl chain; and permanent positive charges from quaternary ammonium groups instead of soft charges provided by natural amino acids. In subsequent studies, non-peptidic amide bonds were introduced into the lipophilic spacer chains. In another design both the lipophilic spacer and the pendant alkyl chains bore non-peptidic amide bonds.[98]

1.3.4 Therapeutic implications of small molecular mimics of antimicrobial peptides

Small molecular membrane active agents have been shown to have a variety of antimicrobial properties against several microorganisms including bacteria, fungi and parasites. Some of these compounds also show antifungal properties. The problems caused by these pathogens and activities towards these pathogens have been further subdivided into sections for better understanding.

1.3.4.1 Selective activity against bacteria

The field of antimicrobial peptidomimetics progressed largely due to the evolution of designs with respect to selective antibacterial activity. In other words, compounds should be non-toxic to eukaryotic cells at the concentration at which they are toxic to bacterial cells. The activity of the best compounds in every series has been presented in Table 1.2. The activity against *S. aureus* and *E. coli* have been presented to give an idea of the broad spectrum activity of the compounds.

Generally, synthetic mimics of AMPs, such as the polymers and oligomers, as described above, possess potent activity against Gram-positive bacteria over Gram-negative bacteria. Development of drugs against Gram-negative bacteria has become absolutely crucial due to the rampant increase of resistance. The prevalence of ESBL (Extended spectrum β-lactamase) producing Gram-negative species such as *K. pneumonia* and *E. coli* strains has further aggravated the problem. Carbapenems, once effective against such ESBL producing strains have now been

rendered ineffective due to the expression of carbapenem hydrolyzing enzymes.[99] It has been particularly observed that certain bacteria belonging to the class of Gram-negative pathogens such as *P. aeruginosa* and *K. pneumoniae* are less susceptible to small molecular mimics. It is worth understanding why some of these highly potent membrane active agents fail to exhibit significant activity against these two bacteria. In fact structure activity relationship studies dedicated to understand the necessary parameters required solely for achieving Gram-negative activity is lacking. This needs to be addressed fast, primarily because of the growing number of drug resistant Gram-negative pathogens.

Most of the compounds in this class show potent activity against Drug-resistant Gram-positive bacteria such as MRSA and some of the compounds also show activity against VRE. This in itself shows the therapeutic efficacy and importance of these molecules. However, the field lacks study against several other important pathogens such as *A. baumannii* and *C. difficile*. Considering the mechanism of action of these class of compounds, they are expected to show activity against all classes of bacteria, albeit to different extent. The important point that needs to

Table 1.2: Antibacterial activity of synthetic mimics of AMPs.

Compounds	Minimum Inhibitory Concentration (μg mL^{-1})	
	S. aureus	*E. coli*
Arylamide foldamers	0.05	0.4
Aromatic ring based	3.3	3.3
Short CAPs	12	12
Ceragenins	0.4	3
Short OAKs	3.5	28
β-scaffold based compounds	3.8	7.4
Benzophenone based membrane targeted antibiotics	0.5	16
Binaphthyl-based Dicationic peptoids	4	16
Xanthone derivatives	0.78	-
HDAMPs	2	4
PANA	3.5	10
Cationic amphiphilic biocides	0.5	1

be highlighted here is that every compound does not need to be active against all bacteria, what is needed is a long-lasting antibiotic against each of these. Since membrane active agents bear much promise as antibiotics which are less prone to trigger bacterial resistance, each of them can be deemed long lasting.

All the compounds that have already been designed or are going to be designed to target an individual type of bacteria have a greater chance of being developed as a future antibiotic. Locilex®, for example is being developed for diabetic foot ulcers and omiganan for rosacea. While it is important to have a broad-spectrum candidate, it is necessary to solve individual bacterial infections. An important parameter to be considered while talking about antibacterial activity is selectivity. The ultimate aim in the field is to develop novel compounds with broad-spectrum selective antibacterial profiles. The basic toxicity profile of the compounds is usually provided by hemolytic activity. Toxicity against other mammalian cells are also evaluated to get an idea of the *in vitro* selectivity of the compounds. The selectivity of the compounds is an important factor directing the possible applications of these compounds. As long as the compound does not show any toxicity at its therapeutic concentration, it is generally considered safe. However, higher selectivity is always preferable. Selective antibacterial activity has been achieved by almost all the compounds of concern in laboratory settings. They vary substantially in their *in vitro* selectivity though. However, *in vitro* toxicity is not directly translated to *in vivo* toxicity. Toxicity *in vivo* is thus a more accurate reflection of the potential of the compounds in clinical level.

Extensive studies on the *in vivo* profiles of the arylamide foldamers have been reported. The maximum tolerated doses (MTD) of these compounds were 20 mg kg^{-1}. In a thigh-infection model of mice, these compounds were able to bring bacterial load down by 4 logs at two dosages of 20 mg kg^{-1} (separated by 6 hours). The authors have also reported multiple dosage patterns, which enhanced the activity of the compounds. These studies indicate the potential of these compounds as possible antibiotics for the treatment of systemic infections.[67] Arylamide foldamers are undergoing Phase II clinical trials as antibiotic against Acute Bacterial Skin and Skin Structure Infections (ABSSSI). Lytix Biopharma, licensed to perform the preclinical and clinical studies of LTX-109 (short cationic peptidomimetic) studied the potential of the compound as a topical antibacterial agent.[79] From the data furnished in the company website, it is apparent that these short CAPs were able to achieve antimicrobial activity against MRSA in a mouse-skin infection

model at efficacies better than the approved drugs Albatax and Fucidin.[100] In fact, very recently they have shown the efficacy of LTX-109 as a novel agent for nasal decolonization of methicillin-resistant and -sensitive *S. aureus* in humans.[101] The short OAKs, at concentrations of 2 and 5 mg kg^{-1} were able to increase the survival of mice, infected in the thigh with a clinical isolate of *S. aureus* by 30% to 90%. The maximum tolerated dose of these compounds varied from 5 mg kg^{-1} to 20 mg kg^{-1} depending upon the route of administration.[88] In a novel study, the Xanthone based antimicrobial compounds were tested for their ability to cause wound healing in a rabbit cornea.[90] Subsequently, the authors had also studied the ability of xanthone derivatives to cure bacterial infection in the cornea of mice.[91] This new model of study is another eye-opener to the immense possibilities these classes of compounds hold as next generation antibiotics.

1.3.4.2 Mechanism of action

Natural antimicrobial peptides have been shown to act primarily by attacking the bacterial cell membrane. The peptides, upon interaction with the cell membranes could bring about bacterial cell death by i) depolarization of the bacterial cell membrane ii) permeabilization of the bacterial cell membrane iii) leakage of K^+ iv) action on intracellular targets. Extensive biophysical studies have been done to explain the possible way of interacting with the bacterial cell membranes. Most of these studies were based on solid state NMR spectroscopy,[102-104] circular dichroism, [105-107] neutron scattering, [108, 109] microscopy and X-ray diffraction studies.[52, 105] Based on information from these studies, the mechanism by which antimicrobial peptides act can be explained by the following models: Barrel stave model,[110-112] toroidal pore model,[113-115] carpet model,[116, 117] Shai-Matsuzaki-Huang model,[17, 118] lipid-clustering model[119, 120] and interfacial activity model.[121, 122] Several peptides also exert their activity by targeting internal components of bacterial cells.

The initial studies conducted to establish the mechanism of action of these compounds dealt with the ability of the compounds to induce leakage of fluorescent dyes from lipid vesicles mimicking bacterial and mammalian cell membranes, which proved their selective membrane active nature.[64, 78]

Most of these compounds respond positively to the membrane depolarization experiments conducted with the membrane potential sensitive dye $DiSC_3(5)$.[123] The ability to permeabilize the membrane of Gram-positive bacteria and the inner membrane of Gram-negative bacteria are

studied using Propidium Iodide (PI). Only membrane compromised cells allow the entry of the dye and upon binding to DNA there is an increase in fluorescence of the dye.[124] The ability to cause outer membrane permeabilization of Gram-negative bacteria is studied by using the dye *N*-phenylnaphthylamine (NPN).[125] Most compounds respond positively to either or all of these experiments. The membrane active benzophenone derivatives were shown to bind to Lipopolysaccharides (LPS) and Lipoteichoic acids (LTA) of Gram-negative and Gram-positive bacteria respectively in addition to their membrane depolarization properties. Moreover these compounds also showed the ability to release K^+ from the bacterial cells.[95] Some of the compounds are expected to have action after internalization into the cells as well. Unfortunately, not a great deal of study has been reported about the internalization of the small molecular membrane active agents.

Significant amount of study has been done with the mechanism of action of arylamide foldamers especially with their lead compound Brilacidin.[67] Brilacidin was able to cause depolarization and permeabilization of bacterial cell membranes and gain entry inside the cytoplasm. Transcriptional profiling showed that treatment with Brilacidin led to upregulation of genes belonging to the *rcs* regulon (which plays a role in the transcriptional regulation of the genes for capsular polysaccharide in *E. coli*) and *cpxAR* regulon (which plays a role in membrane reorganization).[126] In a further work, the efficacy of brilacidin, was compared with that of daptomycin and LL-37. Transcriptional profiling showed that treatment with all three drugs resulted in induction of the *NsaSR* (responsible for cell-wall synthesis, membrane transport, redox stress, DNA remodeling and general cell metabolism), *VraSR* (responds to cell wall stress and up regulates genes involved in LTA and peptidoglycan synthesis), *WalKR* (cell-wall and membrane turnover and maintenance) and *GraSR* (involved in reduction of overall negative charge of bacterial membrane) two-component system regulons. The effect of brilacidin on all of these systems was comparatively more in comparison to daptomycin and LL-37.[127]

Perturbation of membrane potential also results in development of defective cell division. Proper localization of proteins such as MinD, FtsA and MreB is extremely important for effective cell division to take place. Brilacidin treated cells showed a down regulation of genes involved in such processes. The short RW rich hexapeptide, which was established to have membrane active antibacterial action was also found to delocalize the proteins MinD and MurG.[128] This

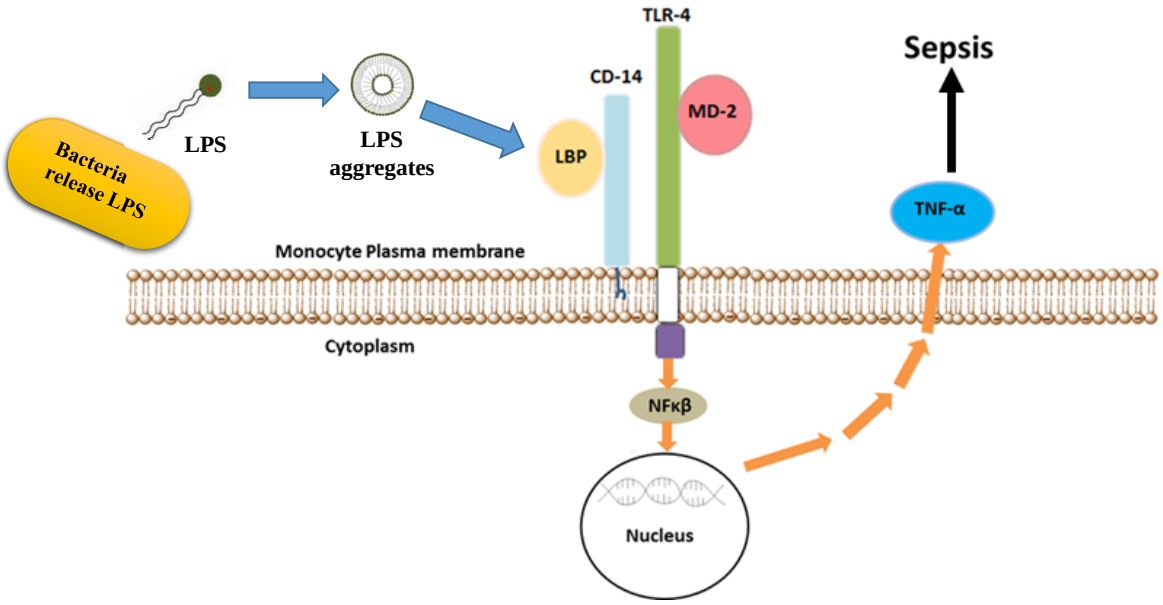

Figure 1.7: The mechanism of LPS induced sepsis.

delocalization would result in improper cell division and lipid II biosynthesis. Furthermore, the hexapeptide was shown to reduce precursor incorporation, activate the promoter of cell wall stress-responsive genes, up-regulate proteins indicative of cell-wall biosynthesis stress and diminish the integrity of the cell wall.

1.3.4.3 Development of bacterial resistance

It has been established already that bacteria find it difficult to develop resistance against AMPs. This offers an additional advantage to AMPs for development as long lasting antibacterial agents. However, there have been several reports wherein, resistance to AMPs have been described. So far resistance to AMPs have been developed through 1. Modification of bacterial surface, 2. External trapping of AMPs, 3. Active efflux pumps, 4. Proteolysis degradation, 5. Bacterial gene regulation, 6. Bacterial regulation of host AMP production and 7. Complete loss of LPS production. The various mechanisms of resistance to AMPs have been documented by others.[19, 23, 28, 129]

It was observed for the arylamide foldamers, ceragenins, oligo-acyl-lysines, and phenylalanine conjugated norspermidine derivatives that even in multiple passages bacteria do not

develop resistance against these compounds. In comparison, cationic lipopeptides like colistin were found to develop resistance in as little as five passages. This result is intriguing because colistin belongs to lipopeptides class of antibiotics, which are also membrane active agents. This does open up an entire field of research as to why bacteria find it difficult to develop resistance against some of such membrane active agents and not against others.

1.3.4.4 Activity against Stationary phase bacteria, persister cells and biofilms

Chronic bacterial infections are often associated with slowly dividing bacteria, metabolically inactive cells and biofilms. Biofilms, are stable communities of bacteria, embedded within a matrix a polysaccharides, which are recalcitrant to antibiotic treatment.[130-132] They are an eclectic mixture of extracellular polymeric substances (EPS) including polysaccharides, proteins, extracellular DNA, bacteria and other components of bacterial membrane.[133, 134] It has been estimated that around 80% of all infections are biofilm related. The EPS prevents antibiotics from entering the biofilms and that is one of the reasons why antibiotics are ineffective. Further there are diffusion barriers and genetic mutations which also contribute to the belligerence of biofilms to antibiotics.[135, 136]

Often, within bacteria lie bacteria which are non-dividing or slowly dividing cells. In the bacterial growth curve, such cells are encountered in stationary phase.[137] Since antibiotics usually target a physiological processes within bacteria, they are inactive against such stationary phase bacteria. Apart from that upon antibiotic treatment, around 1% cells survive by down-regulating their metabolic activity. These cells, known as persister cells, can get back to normalcy upon removal of antibiotic pressure.[138, 139] These cells are also encountered in biofilms. Biofilms are responsible for a variety of chronic diseases such as cystic fibrosis, urinary tract infections, catheter infections, middle-ear infections, formation of dental plaques, gingivitis and so on. Overall, biofilms are extremely tolerant to antibiotics and is one of the impending dangers facing the world today. It is imperative to develop new strategies to counter this growing threat.

Several strategies have been used to solve the problem of bacterial biofilms which include use of natural products, enzymes, small molecules, quorum sensing inhibitors, siRNA, etc. In worst cases surgical removal of the biofilms is needed.[140]

Ceragenins, particularly CSA-13 has been shown to have activity against established bio-films of *P. aeruginosa*, *K. pneumonia*, *S. epidermis* and *S. aureus*. The effect of CSA-13 toward

the biofilms formed by these pathogens were comparable to that of the clinically used antibiotic ciprofloxacin.[141] In a further advancement to the study, the same group showed that CSA-13, when incorporated into a polydimethylsiloxane polymer for development of release based antimicrobial coatings, it showed excellent activity both *in vitro* and *in vivo*. In fact this coating was able to prevent bio-film related infection development in a sheep model of simulated Type IIIB open fracture.[142]

The short cationic tripeptides developed also showed potent activity against staphylococcal bio-films. In their study they considered the effect of the compounds/antibiotics toward the metabolic activity of the bio-films formed by various species of Staphylococci. It was shown that while some of the frontline antibiotics were not able to suppress the metabolic activity of the bio-films, the synthetic antimicrobial peptidomimetics were able to completely eradicate the metabolic activity of the biofilms.

1.3.4.5 Immunomodulation

An exciting approach towards the treatment of infections is inducing the immune system to act against the invading pathogens. Innate immunity is responsible for preventing the body from succumbing to infections, considering the constant bombardment of pathogens our body faces. Stimulating the innate immunity by inducing the production of chemokines, suppressing the production of pro-inflammatory cytokines, promotion of wound healing are some of the ways that infections can be tackled. Since this process will have no direct impact on the pathogens, chances of resistance are considered to be low. Natural AMPs and some synthetic peptides have demonstrated excellent ability to not only activate the immune system but also have endotoxin neutralizing abilities. This is a novel way of treating infections which has several advantages. The immunomodulatory activities of natural and synthetic peptides have been covered in some excellent reviews.[143-147]

During infection, bacteria release several endotoxins which are recognized by the immune system of the host. These pathogen associated molecular patterns target different receptors on the host immune cells.[148] Upon recognition, it leads to a cascade of reactions which results in the production pro-inflammatory cytokines such as TNF-α and IL-6. These, in turn causes excess inflammation and in worst cases sepsis (Figure 1.7). Sepsis is a cause of severe morbidity amongst humans but there is no dedicated treatment against sepsis. Lipopolysachharide, a component of

Gram-negative cell wall is released during proliferation of bacteria and sometimes also due to the action of antibiotics. Aggregates of LPS is recognized by the host immune system and often leads to sepsis. Similarly, Gram-positive bacteria also release another endotoxin known as lipoteichoic acid, albeit, LPS causes more damage. In order, to prevent sepsis, it is necessary to develop molecules that will prevent the excessive interaction of the endotoxins with the host immune system.[149, 150]

The aromatic ring based compounds described by Tew and co-workers was the first report wherein small molecular membrane active agents showed potent immunomodulatory activities along with potent antibacterial activity. One of the compounds SMAMP-4 induced the production of TNF-α on its own and, did not down regulate the activity of LPS. This activity is different from the immunomodulatory activities of natural peptides and the synthetic IDR peptide.[20] Moreover, SMAMP-4 could induce the production of neutrophil chemo attractant, murine KC, in mouse primary cells. This was the first report of a non-peptidic membrane active agent which displayed both potent antimicrobial activity and immunomodulatory activity.[73]

Some aryl amide foldamers were able to bind to LTA of *S. aureus* and prevented the induction of TNF, IL-6 and IL-10. They synergistically exhibited potent antibacterial activity as well as inhibited LTA induced sepsis *in vivo*.[151]

The HDAMPs also showed potent anti-inflammatory activity. One of the compounds, which had two arginines flanking a central histidine, which in turn was functionalized with a lipophilic group, was able to suppress LPS induced NO production and TNF-α production at efficiency similar to LL-37.[89]

1.3.4.7 Activity against parasites

Parasites such as *Plasmodium sp.*, *Leishmania sp.* and *Trypanosoma sp.* contribute majorly to mortality in the tropics.[152-154] The malarial parasite *Plasmodium sp.* are notorious because of their ability to develop resistance against drugs rapidly. Development of resistance is known even in the other parasites. Moreover some of the frontline drugs which are approved for treatment against Chagas disease are quite toxic. Thus there is a constant need for drugs against such parasites. Slowly, due to their ability to act against malarial parasites, several groups around the world have started to develop synthetic peptides as potential antimalarial drug candidates.[155-158]

Ceragenins such as CSA-8, CSA-13 and CSA-54 showed antitrypanostomatid activity and antileshmanial activity *in vitro*. All though the compounds showed substantial toxicity they bore promise as antiparasitic compounds. However, the authors believed that the activity of the compounds could be enhanced further if a suitable drug-delivery agent is employed.[159]

Arylamide foldamers also showed potent antimalarial activity. These compounds were active at nanomolar ranges even against chloroquine resistant *Plasmodium* strains. Fluorescence dye based experiments revealed that these compounds acted mostly on the digestive vacuole of the parasites, and activity on the parasite membrane was minimal, if at all. In a murine model too these compounds were able to decrease the burden of parasites and increased mice survival significantly.[158] To the best of our knowledge no other published data exists with respect to activity of membrane active small molecules against parasites.

1.3.4.8 Activity against fungi

Pathogenic fungi are notorious for causing both superficial and life-threatening infections both in healthy and immunocompromised individuals.[160] More than 300 million individuals worldwide are afflicted with a serious fungal infection. Although infections caused by bacteria, viruses and parasites are often highlighted, infections caused by pathogenic fungi have largely remained out of limelight. The emergence of resistance to front-line antifungal drugs (including fluconazole) has further exacerbated the issue. This necessitates the identification and development of new antifungal agents to address this significant public health challenge. Few small molecules described earlier have been investigated in details for their potential antifungal properties. Only LTX-109 has been studied in details for their antifungal properties.[161] As topical agents, these membrane active small molecules can be exploited as drugs for the treatment of fungal infections of the skin, nails and so on. Future studies must consider antifungal aspects of membrane active agents.

1.4 Scope of the thesis

The thesis, divided into seven chapters, aims to document the design principles, synthetic strategies underlying the development of simple small molecules that act as broad-spectrum antimicrobial agents by targeting the membrane of pathogenic microorganisms.

Chapter 1 provides an introduction to the predicament of antimicrobial resistance (AMR) and endorses the significance of antimicrobial peptides (AMPs) as templates for next generation of drugs. In an attempt to emulate the properties of AMPs, in **Chapter 2,** the design and synthesis of simple antibacterial agents involving only one amino acid (L-Lysine) and two lipid tails are reported. The use of two short lipid tails over a single long one is shown to have increased selective activity against bacteria over mammalian cells significantly. Bacteria found it difficult to develop resistance against the best compound in multiple passages. Fluorescence spectroscopic studies corroborated the membrane-active mechanism of action of a representative compound. Topical application of the representative compound reduced bacterial burden by 2.1 logs in mice inflicted with burn-infection caused by *Acinetobacter baumannii*.

In **Chapter 3** another series of small molecules have been described wherein aromatic moieties, alkyl groups and L-lysine moieties were assembled together through a tertiary amide. The aromatic moieties were varied from anthracene naphthalene to benzene. These molecules, termed as aryl-alkyl-lysines, exhibited high *in vitro* potency against a variety of multidrug-resistant Gram-positive and Gram-negative bacteria including the anaerobic bacteria, *Clostridium difficile* (MIC for the best compound was less than 3 $\mu g\ mL^{-1}$) Nontoxic toward mammalian cells, these rapidly bactericidal compounds primarily acted by permeabilization and depolarization of bacterial membrane and withstood resistance development. In a methicillin-resistant *Staphylococcus aureus* model of skin infection and *A. baumannii* model of burn-infection in mice, bacterial burden was reduced by 2.1 logs and 2.4 logs respectively.

In **Chapter 4**, lipidated-biphenyl-lysines which contain four cationic charges two lipid tails and a biphenyl core has been described. Increase in positive charge residues led to an increase in selectivity and it was observed that this class of compounds was able to inhibit cell wall biosynthesis of bacteria in addition to infiltrating membranes. Against this class of compounds too, bacteria could not develop resistance in multiple passages. Additionally, the compound was able to inhibit growth of bacteria within mammalian cells. In MRSA model of skin infection and *A. baumannii* model of burn infection in mice, bacterial burden was reduced by 2.7 logs and >5 logs respectively.

Chronic or persistent infections belligerent to antibiotic treatment are often caused by stable communities of bacteria, termed biofilms, which grow on surfaces. In **Chapter 5**, the ability of the best two compounds (identified from the previous chapters) to act against dormant cells and biofilms of Gram-positive and Gram-negative bacteria have been studied. The efficacy of the compounds were also validated in a murine models of biofilm infection caused by MRSA and *P. aeruginosa*. Further, the ability of one of the compounds to prevent endotoxin stimulated inflammatory response (sepsis) have been described.

In **Chapter 6** the antifungal properties of aryl-alkyl-lysines has been documented. The activity of the best compound (MIC <2 μg mL-1) was performed against different clinical isolates of pathogenic fungi and also against biofilms of *Candida albicans*. It was observed that polarization and permeability of fungal cell membrane was compromised upon addition of the compound. Additionally, the compound was found to possess the ability to cross the blood-brain-barrier in an *in vitro* model. Overall, aryl-alkyl-lysines were found to be excellent antifungal compounds with scope for further development to clinical antifungals.

In **Chapter 7**, the potential of aryl-alkyl-lysines to as antimalarial agents was tested. The optimal compound (IC_{50}=1 μM) acted by arresting the development of *Plasmodium falciparum* at the ring stage inside the erythrocytes. The compound could perturb the plasma membrane potential and the digestive vacuole of parasites. The compound was also able to increase the survival of mice infected by *Plasmodium bergheii* by at least 5 days when administered via intra-peritoneal route, but showed no apparent toxicity to mice at the concentration tested. Thus, this class of compounds bear potential to be developed as novel antimalarial drugs.

In summary, this thesis describes the design and development of small molecular antimicrobial agents that selectively target microbial cell membrane with low toxicity to mammalian cells. This approach has been exploited for development of potential drug candidates against acute and chronic infections caused by bacteria, fungi and parasites.

Chapter 2

Lipidated lysines: Broad-spectrum antibacterial compounds that target the bacterial membrane

Abstract

In the global effort to thwart antimicrobial resistance lipopeptides represent an important class of antimicrobial agents, especially against Gram-negative infections. In an attempt to circumvent their synthetic complexities, the design of simple membrane-active agents involving only one amino acid and two lipid tails has been reported here. It has been shown that use of two short lipid tails over a single long one increases selective antibacterial activity significantly. The study yielded several selective antibacterial compounds and investigation of the properties of the class of compounds were conducted with the most active compound. Fluorescence spectroscopic studies revealed the ability of the compound to cause depolarization and permeabilization of bacterial cell membrane. Topical application of the compound reduced bacterial burden in mice inflicted with burn-infection caused by Acinetobacter baumannii. The design principles described in this chapter has laid the foundation for the development of better antimicrobial agents.

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(1) Ghosh, C. *et al.* "Designing simple lipidated lysines: Bifurcation imparts selective antibacterial activity". *ChemMedChem* **2016** (DOI: 10.1002/cmdc.201600400)

2.1 Introduction

The current predicament of antimicrobial resistance has not only put the world health at risk but also expected to severely affect global economy.[162, 163] As mentioned in Chapter 1, natural antimicrobial peptides are an inspiration for designing of next generation of drugs. The advent of synthetic membrane active agents have bolstered the antimicrobial pipeline with a new class of drugs.[17, 20, 38, 73, 79, 86, 164, 165]Although much remains to be achieved in terms of clinical success, their importance as future antibiotics cannot be undermined. Peptidomimetics and development of synthetic mimics of antimicrobial peptides (SMAMPs) have gained popularity among scientists as a successful method of producing broad-spectrum antibacterial agents. Lipidation of currently used antibiotics have also been reported in the literature.[15, 58, 92, 166-173] Cellceutix's brilacidin and Lytix biopharma's LTX 109 are successful examples of small molecular mimics of antimicrobial peptides.[79, 165] Some other examples include the binaphthyl based dicationic peptoids,[85] $\beta^{2,2}$-amino acid derivatives[96, 97] and those based on aryl scaffolds.[73, 74] Although there is no doubt about the efficacy of such designs, there was a scope to make a simpler and more cost-effective compounds.

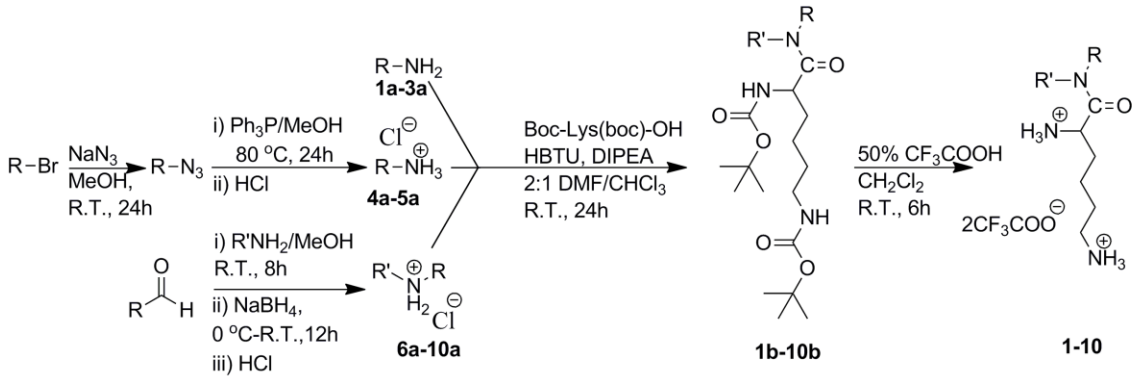
Here we describe the development of simple lipidated lysine based membrane-active molecules and one possible approach of achieving selective antibacterial activity. Further, the mechanism of antibacterial action, ability to stall resistance development in bacteria has been studies. Finally, the activity of the compound was also validated in a murine model of *Acinetobacter baumannii* (*A. baumannii*) burn infection.

2.2 Results and discussion

2.2.1 Design and Synthesis

In our attempt to design simplest membrane-active agents, we first identified the necessary parameters required. After surveying the literature, we concluded, that for an agent to be membrane-active and antibacterial, minimum two positive charges were necessary. Hydrophobicity was also important, but the nature of the moiety varied from design to design. We then looked for a simple molecule that will not only provide the necessary positive charges, but also allow for further functionalization with hydrophobic groups. Due to the presence of two amine groups, L-lysine seemed to be the perfect molecule to base our design on. Further, the COOH

group allowed for further functionalization. We envisioned that the structure of a simple lysine based membrane-active compound would be an alkyl chain conjugated to it. In order to achieve any significant activity, the alkyl chain has to be sufficiently long, which will facilitate better interaction with the bacterial membranes. In the very first example, we chose to couple dodecylamine to lysine using simple HBTU coupling chemistry to obtain $C_{12}K$ (**1**) (Scheme 2.1). Gradual increase of alkyl chains yielded compounds with tetradecyl chain ($C_{14}K$, **2**), hexadecyl chain ($C_{16}K$, **3**), octadecyl chain ($C_{18}K$, **4**) and eicosyl chain ($C_{20}K$, **5**).



where, **1a:** R=$C_{12}H_{25}$,**1b** and**1**: R=H, R'=$C_{12}H_{25}$; **2a:** R=$C_{14}H_{29}$; **2b** and **2**: R=H, R'=$C_{14}H_{29}$; **3a:**R'=$C_{16}H_{33}$; **3b** and **3**: R=H, R'=$C_{16}H_{33}$; **4a:** R=$C_{18}H_{37}$, **4b** and **4**: R=H, R'=$C_{18}H_{37}$; **5a:** R=$C_{20}H_{41}$, **5b** and **5**: R=H, R'=$C_{20}H_{41}$; **6a, 6b** and **6**: R=C_6H_{13}, R'=C_6H_{13}; **7a, 7b** and **7**: R=C_6H_{13}, R'=C_8H_{17}; **8a, 8b** and **8**: R=C_8H_{17}, R'=C_8H_{17}; **9a, 9b** and **9**: R=$C_{10}H_{21}$, R'=C_8H_{17}; **10a, 10b** and **10**: R=$C_{10}H_{21}$, R'=$C_{10}H_{21}$

Scheme 2.1: General scheme for the synthesis of the compounds

To understand, how different properties can be obtained by keeping the same amphiphilic properties, we bifurcated the alkyl chain e.g. splitting the hexadecyl long chain to two octyl chains. Based on this, we designed a set of new molecules containing two short alkyl chains instead of one long chain. We symmetrically and asymmetrically varied the length of the two chains from hexyl to decyl. We deliberately avoided using chains of odd number of carbons to obtain asymmetry in the designs. The synthetic strategy is outlined in Scheme 2.1. In the first step of synthesis, alkanals were first reacted with alkylamines in dry methanol and then reduced by sodium borohydride to obtain dialkyl amines. These dialkyl amines were then coupled to Boc-Lys(Boc)-OH in $CHCl_3$/DMF mixture using HBTU chemistry. In compounds **1** to **5**, single long chain amines were directly coupled to Boc-Lys(Boc)-OH). After purifying the compounds using column chromatography the Boc groups were subsequently deprotected using 50% trifluoroacetic acid in

DCM to yield the final compounds. They were purified by HPLC to more than 95% purity and were subsequently characterized using ^{1}H NMR, ^{13}C NMR, IR and High Resolution Mass Spectrometry (HRMS).

2.2.2 Antibacterial activity and selectivity

The activity of the compounds were evaluated against *Staphylococcus aureus* (*S. aureus*), *Enterococcus faecium* (*E. faecium*), *Escherichia coli* (*E. coli*) and *A. baumannii* (Table 2.1). It was observed that the first three compounds possessed good antibacterial activity. However, as we moved to $C_{18}K$ and $C_{20}K$, the activity of the compounds was lost. Evaluation of their toxicity against erythrocytes, as indicated by their HC_{50} values (concentration at which 50% erythrocytes are lysed), revealed that all the active compounds possessed substantial toxicity. The activity of bifurcated compounds (**6**-**10**) were then tested against *S. aureus*, *E. faecium*, *E. coli*, *A. baumannii* and human erythrocytes. For Gram-negative bacteria, colistin was used as a comparator and for Gram-positive bacteria, vancomycin was used as a comparator drug (Table 2.1). Compound C_6-K-C_6, which is the bifurcated analogue of C_{12}-K, retained the activity against *S. aureus* (MIC of 23 μM), *E. faecium* (MIC of 46 μM) and *E. coli* (MIC of 23 μM), but lost the activity against *A. baumannii* (MIC > 50 μM). However, with a HC_{50} value of 390 μM, it was significantly less toxic to erythrocytes in comparison to C_{12}-K (HC_{50} of 120 μM). The asymmetric bifurcated analogue of C_{14}-K, consisted of an octyl chain and a hexyl chain. Other than *E. faecium*, the parent compound C_{14}-K, was active at 11 μM. The bifurcated compound C_6-K-C_8 displayed two fold less activity (MIC of 22 μM against all of those three bacteria) but it was more active against *E. faecium*. The HC_{50} value of compound C_6-K-C_8 was 172 μM while that of compound C_{14}-K was 105 μM. Like its single chain counterpart, C_{16}-K, C_8-K-C_8 displayed good activity against all bacteria (MIC ranged from 5 μM to 10.5 μM) but was more active against *E. faecium* and relatively less toxic. C_{18}-K, was inactive against all the bacteria tested till 50 μM. In comparison, C_{10}-K-C_8 turned out to be a very active compound in the series. It displayed MICs of 5 μM against both *S. aureus* and *E. coli* while against *E. faecium* and *A. baumannii*, the MIC was 10.5 μM. C_{10}-K-C_{10} was as active as C_{10}-K-C_8 (MIC of 6 μM) against *S. aureus*, *E. faecium* and *E. coli* but slightly more active against *A. baumannii*. Although the single chain analogue 5 was less toxic (HC_{50} of 185 μM), it was inactive against all bacteria. Since, selective antibacterial activity is the most desired condition for membrane active agents, it could be envisioned from this study that bifurcation of long chains

in any membrane active antibiotics containing such feature should substantially increase the selectivity of the resultant compound. Since C_{10}-K-C_8 turned out to be the most selective compound, further studies were conducted with that.

Table 2.1: Antibacterial and haemolytic activity of the compounds.

Compounds	Minimum Inhibitory Concentration (μM)				HC_{50} (μM)
	S. aureus	*E. faecium*	*E. coli*	*A. baumannii*	
C_{12}-K (**1**)	23	46	23	23	120
C_{14}-K (**2**)	11	44	11	11	105
C_{16}-K (**3**)	5	21	5	10.5	73
C_{18}-K (**4**)	>50	>50	>50	>50	114
C_{20}-K (**5**)	>50	>50	>50	>50	185
C_6-K-C_6 (**6**)	23	46	23	>50	390
C_8-K-C_6 (**7**)	22	22	22	22	172
C_8-K-C_8 (**8**)	5	10.5	5	10.5	110
C_{10}-K-C_8 (**9**)	5	10	5	10	108
C_{10}-K-C_{10} (**10**)	6	6	6	6	88
Colisin	25	N.D.	0.4	0.4	N.D.
Vancomycin	0.5	0.5	0.5	>100	N.D.

Values are the averages of at least two experiments, each done in triplicate (error <5%)

The microorganisms which are often associated with such rapid resistance development are commonly termed as ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), because of their ability to "escape" frontline antibiotic treatment. These pathogens are involved in a significantly high percentage of infections in intensive care units (ICUs). Although the rate of resistance varies, some of them are strong enough to completely wipe out the use of front line antimicrobials. In order to test the overall properties of the molecule, the antibacterial activity of C_{10}-K-C_8 toward several ESKAPE pathogens are given in Table 2.2. Most of these strains were isolated from patients at National Institute of Mental Health and Neurosciences (NIMHANS, Bangalore). Against a vancomycin resistant strain of *E. faecium* the compound was active at 5 μM. There were a total of three strains of *Staphylococcus aureus* against which the compound was tested. One of them was a laboratory strain obtained from ATCC while the other two were MDR clinical isolates. All the strains were susceptible to 0.7 μM of vancomycin. Against methicillin-susceptible and methicillin-resistant strains of *S. aureus*, C_{10}-K-C_8 exhibited an MIC of 5 μM as well. This emphasized on the strong activity of the compound against Gram-positive strains. Subsequently, we tested the activity of the compound against the Gram-negative strains of ESKAPE groups of pathogens which included *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. coli*. The compound displayed good activity against all the Gram-negative pathogens. Against the ATCC strain of *K. pneumoniae* (resistant to β-lactam antibiotics) the compound was active at 10 μM, while against the clinical isolate R3421, an MIC of 5 μM was observed. The MIC of C_{10}-K-C_8 against both the strains of *A. baumannii* (R674 and R676) was also 5 μM. The activity of the compound against the *P. aeruginosa* clinical isolates were moderate. It was active at 10 μM against *P. aeruginosa* R590, but inactive against the other strains. The last representative of the ESKAPE group of pathogens was *E. coli* which belong to the family of Enterobacteriaceae. Against the clinical isolate *E. coli* R250, the compound displayed an activity of 5 μM. Further, activity of the compound against New Delhi-metallo-β-Lactamase producing Gram-negative bacteria was also conducted.

The emergence of New Delhi-metallo-β-lactamase carrying bacteria, which remain susceptible only to polymyxins and tigecycline, have set alarm bells ringing in every health agency. As a further dent to the problem, there are reports of bacteria developing resistance to the

drugs of last resort, colistin and tigecycline. Carbapenem-resistant New Delhi-metallo-β lactamase were isolated from patients of National Institute of Mental Health and Neurosciences, Bangalore.

The bacteria against which the study was conducted included *E. coli* 3336, *K. pneumoniae* R3934, *P. aeruginosa* R596 and *E. clocae* R2928. Against *E. coli* R3336 and *P. aeruginosa* R596,

Table 2.2: Activity against drug-resistant strains of ESKAPE pathogens

Bacterial Strains		MIC of C_{10}-K-C_8 (μM)
E	Vancomycin-resistant *Enterococcus faecium*	5
S	MRSA ATCC 33591	5
	MRSA R3889	5
	MRSA R3890	5
K	*K. pneumoniae* ATCC 700603	10
	K. pneumoniae R3421	5
A	*A. baumannii* R674	5
	A. baumannii R676	5
P	*P. aeruginosa* R590	10
	P. aeruginosa R3324	>50
E	*E. coli* R250	5
NDM-1	*E. coli* R3336	10
(+ve)	*K. pneumoniae* R3934	5
	P. aeruginosa R596	10
	Enterobacter cloacae R2928	5

Values are the averages of at least two experiments, each done in triplicate (error <5%)

C_{10}-K-C_8 was active at 10 µM and both while against *K. pneumoniae* R3934 and *E. clocae*, a MIC of 5 µM was displayed by the compound. The activity of the compound against selected strains of ESKAPE have been compared with vancomycin and tetracycline and presented in Figure 2.1A.

2.2.3 Kinetics of antibacterial activity

Kinetics of bactericidal action of C_{10}-K-C_8 was then studied. Within minutes of treatment at concentrations only thrice its MIC, C_{10}-K-C_8 was able to lyse cells of both Gram-negative and Gram-positive bacteria. In Figure 2.1B, time kill kinetics against *E. coli* has been furnished. The ability of the compound to lyse bacterial cells could be attributed to their membrane active nature.

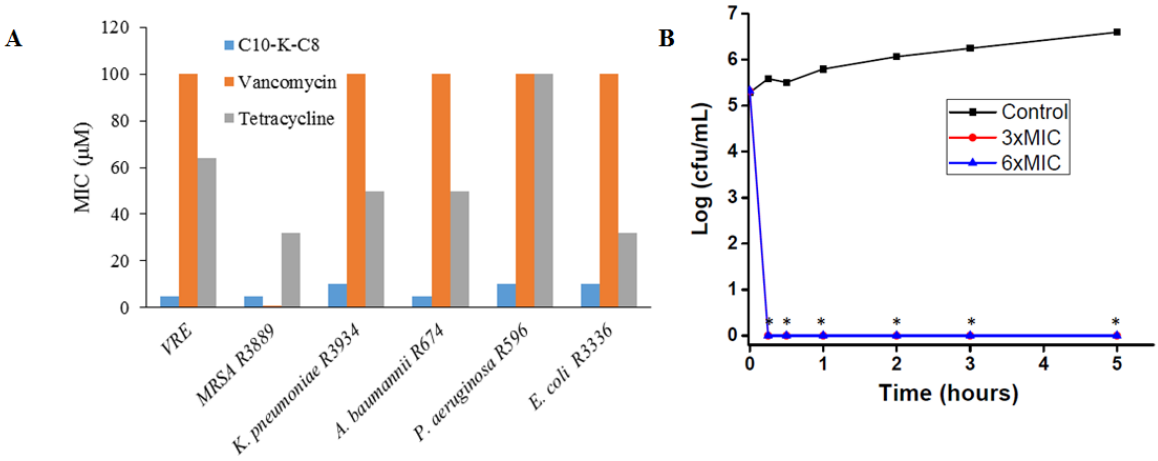


Figure 2.1: A) Activity of the compound C_{10}-K-C_8 in comparison to that of vancomycin and tetracycline against representative strains of ESKAPE pathogens. B) Time kill-kinetics of C_{10}-K-C_8 against *E. coli*. * indicate no bacteria observed. Detection limit is 50 CFU mL^{-1}. The MIC considered is 5 µM.

2.2.4 Activity in different physiological conditions

Another important point that has to be considered in detail in development of new antimicrobial agents is their activity in different physiological conditions such as pH, salinity and in whole blood. Membrane active agents can have a variety of applications as antibacterial agents: as topical antibiotics for skin-infections, antibiotics for systemic infections, as food preservatives and so on.

Although all of the applications mentioned above are equally important, the compound's performance in different physiological conditions determines its ultimate use.

The pH of the site of infection varies from place to place inside the body. Infections of the vagina are also regulated by changes in pH. In cystic fibrosis too it is believed that low pH reduces the antimicrobial activity of airway surface liquid. Thus, it is important to understand the antimicrobial activity of the test compound at different pH conditions. The balance of NaCl is critical in different aspects of antimicrobial treatment. Osmotic stress is known to increase capsule formation in *E. coli* thereby inducing antibiotic resistance in the infecting strain. Many natural membrane active agents such as the Antimicrobial peptide Human-β-defensin is inactivated by high concentration of NaCl. Moreover, use of hypertonic saline (concentration of NaCl ≥3%) is common in the treatment of cystic fibrosis for improved mucociliary clearance and sputum expectoration. Several food-borne pathogens are known to have decreased antibiotic susceptibility on exposure to NaCl.

To gain insight into the potential areas of application of the compound, we chose to study its antibacterial efficacy at different pH (varied from 5.5 to 8.5) and salinity (varied from 1% to 3%). The compound was found to retain its activity against both *S. aureus* and *E. coli* in different physiological conditions. In different percentage of salinity the compound maintained its MIC of 5 μM against both *S. aureus* and *E. coli*. At pH 5.5, the compound was active at 10 μM against *S. aureus* and 5 μM against *E. coli*. No variation in MIC was observed against either bacteria at any other pH. We also tested the activity of the compound in presence of 4% bovine serum albumin (BSA). Compound C_{10}-K-C_8 was active at 10 μM against both *S. aureus* and *E. coli* in presence of BSA. The stability of the compound at such broad ranges of conditions suggested that they could be used for treating bacterial infections at various regions in the body.

2.2.5 Propensity to induce resistance development

Since it is widely known that bacteria find it difficult to develop resistance against membrane active agents, the belligerence of the compound to induce resistance development was studied (Figure 2.2). Serial passage of bacterial culture (both *S. aureus* and *E. coli*) in sub-MIC concentrations of C_{10}-K-C_8 yielded no resistant mutants. In comparison norfloxacin (comparator drug for *S. aureus*) and colistin (comparator drug for *E. coli*), yielded resistant mutants within 6

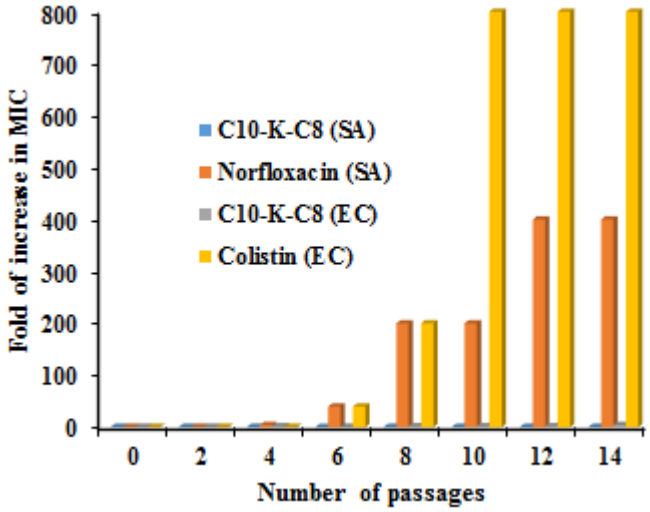


Figure 2.2: Ability of the compound C_{10}-K-C_8 to halt resistance development in bacteria in comparison to norfloxacin and colistin. SA stands for *S. aureus* and EC stands for *E. coli*.

passages and in fourteen passages a 400 fold increase in MIC was observed for norfloxacin and an 800 fold increase was observed for colistin.

2.2.6 Mechanism of antibacterial action

AMPs and their synthetic mimics have different modes of action such as cytoplasmic membrane depolarization, membrane permeabilization, potassium efflux etc. Since these compounds were designed to mimic the biological properties of natural AMPs, we hypothesized that these compounds would also act via infiltration of the membrane.

2.2.6.1 Cytoplasmic membrane depolarization

Altering the membrane potential across the cytoplasmic cell membrane of the bacteria has been shown to be one of the main mechanisms of action of natural AMPs. Ability of the compound to depolarize bacterial plasma membrane was measured using a membrane potential sensitive dye, $DiSC_3$ (5) ((3, 3'-dipropylthiadicarbocyanine iodide). $DiSC_3$ (5) is known to intercalate into the cytoplasmic membrane of energized cells resulting in quenching of fluorescence. Upon

perturbation of the membrane potential the dye fluoresces in aqueous environment. Experiments with this dye showed that upon addition of C_{10}-K-C_8 (15 µM) potential of *E. coli* membrane was dissipated (Figure 2.3 A).

2.2.6.2 Outer membrane permeabilization

The outer membrane of Gram-negative bacteria prevents the entry of several antibiotics rendering them ineffective. Natural AMPs and their synthetic mimics are known to permeate the outer membrane of Gram-negative bacteria. Ability of the C_{10}-K-C_8 to cause outer membrane permeabilization was studied by measuring the uptake of a fluorescent probe *N*-phenylnaphthylamine (NPN). The fluorescence of this dye is quenched in the lipophilic region of the outer cell membrane and fluoresces in aqueous solution. Studies with this dye showed that the outer membrane of *E. coli* was compromised within minutes of treatment with 15 µM of C_{10}-K-C_8 (Figure 2.3 B).

2.2.6.3 Cytoplasmic inner membrane permeabilization

Natural AMPs and cationic and amphiphilic small molecules can also cause membrane permeabilization and disruption causing loss of membrane integrity of the bacteria. Kinetics of membrane permeabilization was studied by measuring the uptake of a fluorescent probe propidium iodide (PI). This dye enters only membrane compromised cells and fluoresces upon binding to nucleic acids. Treatment with the compound (15 µM) also allowed the entry of propidium iodide

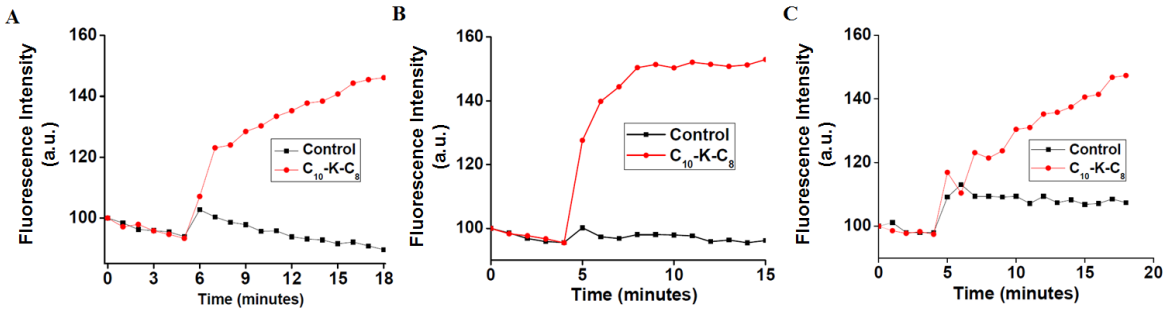


Figure 2.3: Ability of the compound C_{10}-K-C_8 at 15 µM to act on *E. coli* membrane. A) Depolarization of membrane B) Outer membrane permeabilization C) Inner membrane permeabilization.

(PI) inside *E. coli* cells as was evident from increase in fluorescence intensity of the dye upon compound treatment (Figure 2.3 C)

2.2.7 *In vivo* activity

2.2.7.1 Acute Dermal toxicity in mice

The acute dermal toxicity was performed in accordance with the OECD guidelines. Mice skin were shaved and the shaved regions were treated with 200 mg kg^{-1} of C_{10}-K-C_8. The mice were observed for irritation, tremors, convulsions, salivation or diarrhoea for 14 days. Careful visual observation of the animals showed that upon application of the compound the mice showed no sign of irritation or unnatural behaviour. However, the skin showed development of corrosion on the first and second day but appeared to heal subsequently. Normal generation of fur was observed henceforth (within a week) proving that the compound possessed acute dermal toxicity but not chronic.

2.2.7.2 *In vivo* murine model of *Acinetobacter baumannii* burn-infection

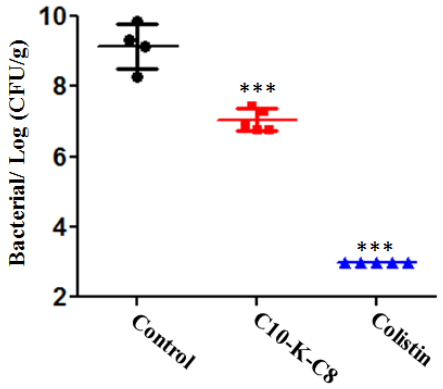


Figure 2.4: *In vivo* activity against *A. baumannii* burn infection. Concentration of C_{10}-K-C_8 was 40 mg kg^{-1} and that of colistin was 5 mg kg^{-1}. Statistical analysis was performed using Student's t-test. Differences are considered statistically significant from the untreated group with a value of $P<0.05$ with 95% confidence intervals (*** indicate $P<0.001$).

Infections caused by *A. baumannii* is a growing problem with growing reports of resistance against even drugs of last resort, such as colistin. A serious problem associated with burns is infection caused by *A. baumannii*. We, thus chose to address this serious problem by checking the activity of our compounds against a murine model of burn-infection caused by *A. baumannii*. In order to do the experiments, a burn infection was induced on the skin at the back of the mice. Subsequently, the burn wounds of mice inflicted with *A. baumannii* were continuously treated for six days with C_{10}-K-C_8 at concentrations of 40 mg kg^{-1} (no toxicity was observed at this concentration). It can be seen from Figure 2.4 that serial plating of homogenates of the severed part of the mice showed that treatment with compound brought down the bacterial burden by 2 log in comparison to the untreated case in six days (P value 0.0003). Colistin, which was used as a positive control completed cleared the bacterial burden even at 5 mg kg^{-1}. Although, the efficacy of the compounds falls short of colistin, it should be kept in mind that colistin is prone to triggering resistance development in bacteria unlike C_{10}-K-C_8 (Figure 2.2B). The rapid development of resistance against colistin could be attributed to its lipopolysaccharide-mediated mechanism of action.

2.3 Conclusions

In summary, this chapter describes the development of simple membrane active agents using two long chains and one amino acid (L-lysine). An important conclusion of the study is that using two short chains instead of one single long chain can effectively increase the selective antibacterial activity of the resultant compounds. Through the study, one potent membrane antimicrobial agent was discovered which acted on both Gram-positive and Gram-negative bacteria. Although slightly toxic in mice, the compound was active in treating burn infection caused *A. baumanii*. The compound retained activity at different physiological conditions and did not select resistant mutants. Although, a lot more improvement is warranted, initial data reveals the potential of this class of compounds for treatment of topical Gram-negative infections.

2.4 Experimental section

2.4.1 Materials and instrumentation: All the solvents were of reagent grade and were distilled and dried prior to use wherever required. Chloroform and methanol were supplied by Merck-India. Dimethylformamide, Dicholoromethane, Diethyl ether and other solvents were supplied either by SDFCL (India) or Spectrochem (India). L-Lysine, Di-tert-butyl carbonate, Diisopropylethylamine,

HBTU, Hexylamine, Octylamine, Trifluoroacetic acid were purchased from Spectrochem (India). Hexanal, Octanal, Decanal and Decylamine were purchased from Sigma-Aldrich. All the chemicals were used as supplied. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates pre-coated with silica gel 60 F254 (250 μm thickness). Visualization was accomplished using UV light and Iodine. Column chromatography was performed on silica gel (60-120 Å pore size). HPLC analysis was performed on a Shimadzu-LC 8A Liquid Chromatograph instrument (C18 column, 10 mm diameter, 250 mm length) with UV detector monitoring at 254 nm. Nuclear magnetic resonance spectra were recorded on Bruker (AV-400) 400 MHz spectrometer in deuterated solvents. High-resolution Mass Spectrometry was recorded on Agilent 6538 Q-TOF LC-MS system and Shimadzu LC-MS 2020 spectrometer. Optical density was measured by TecanInfinitePro series M200 Microplate Reader. Bacterial strains, *S. aureus* (MTCC 737), *E. coli* (MTCC 443), *A. baumannii* (MTCC 1425) were obtained from MTCC (Chandigarh, India). *E. faecium* was obtained from ATCC (19634). *A. baumannii* R674 was a clinical isolate obtained from the Department of Neuromicrobiology, National Institute of Mental Health and Neurosciences, Hosur Road, Bangalore 560029, India. *S. aureus* and *A. baumannii* was cultured in nutrient media (peptone broth), *E. coli* was grown in Luria-Bertani broth, *E. faecium* was cultured in brain-heart infusion broth.

Animals: 6-8-week old Balb/c female mice weighing 20 to 25 g were used for all studies. The animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) and carried out as per the guidelines of Committee for the purpose of Supervision and Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi.

2.4.2 Synthetic procedure and characterization of compounds

2.4.2.1 Synthesis of azidoalkanes: Alkyl bromides (2.3 mmols) were dissolved in dry methanol in a pressure tube and to it sodium azide (3.6 mmols) was added. The mixture was stirred at R.T. for 24h. At the end of the reaction, the organic layer was filtered and evaporated under reduced pressure. The contents were dissolved in chloroform and filtered again. Finally, the organic layer was evaporated under reduced pressure to obtain the alkyl azides in quantitative yields.

Azidooctane ($C_{18}H_{37}N_3$): Yield: 100%. ^{1}H-NMR: (400 MHz, $CDCl_3$) δ/ppm: 3.25 (t, N_3-CH_2-$C_{17}H_{35}$, 2H), 1.6 (m, N_3-CH_2-C*H*$_2$-$C_{16}H_{33}$ 2H), 1.26 (s, N_3-CH_2-CH_2-C_{15}*H*$_{30}$$CH_3$, 30H). 0.8 (t, terminal CH_3 groups, 3H). FT-IR (cm^{-1}): 2928-2843 (sp^3 C-H str.), 2100 (N_3 str.)

Azidodecane ($C_{20}H_{41}N_3$): Yield: 100%. ^{1}H-NMR(400 MHz, $CDCl_3$) δ/ppm: 3.25 (t, N_3-CH_2-$C_{19}H_{39}$, 2H), 1.6 (m, N_3-CH_2-C*H*$_2$-$C_{18}H_{37}$ 2H), 1.26 (s, N_3-CH_2-CH_2-C_{17}*H*$_{34}$$CH_3$, 34H). 0.8 (t, terminal CH_3 groups, 3H).. FT-IR (cm^{-1}): 2928-2843 (sp^3 C-H str.), 2100 (N_3 str.)

2.4.2.2 Synthesis of alkylamines: The azidoalkanes (2.5 mmols) were dissolved in dry methanol in a pressure tube and to it triphenyl phosphine (3 mmols) was added. The mixture was heated at 80 °C for 24 h. At the end of the reaction, methanol was removed under reduced pressure to a minimum volume. To the mixture HCl (4N) was added and thick white precipitates of the salts of alkylamines were obtained. This was filtered, washed several times with hexanes and the residue was characterized and used for further synthesis. The compounds were obtained in more than 90% yields.

Octadecylaminium chloride (4a): Yield: 90%. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 2.65 (t, NH_2-CH_2-$C_{17}H_{35}$, 2H), 1.56 (m, NH_2-CH_2-C*H*$_2$-$C_{16}H_{33}$, 2H), 1.26 (s, NH_2-CH_2-CH_2-C_{15}*H*$_{30}$$CH_3$, 30H). 0.8 (t, terminal CH_3 groups, 3H). FT-IR (cm^{-1}): 3059 (NH_2 str.) 2928-2843 (sp^3 C-H str.).

Eicosanylaminium chloride (5a): Yield-92%. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 2.65 (t, NH_2-C*H*$_2$-$C_{19}H_{39}$, 2H), 1.56 (m, NH_2-CH_2-C*H*$_2$-$C_{18}H_{37}$, 2H), 1.26 (s, NH_2-CH_2CH_2-$C_{17}H_{34}$-CH_3, 34H). 0.8 (t, terminal CH_3 groups, 3H). FT-IR (cm^{-1}): 3059 (NH_2 str.) 2928-2843 (sp^3 C-H str.).

2.4.2.3 Synthesis of *N*-dialkylaminium chlorides (6a-10a): In a typical reaction amines (3.87 mmols) and alkanal (4.65 mmols) were dissolved in dry methanol (20 mL) and stirred at room-temperature (under Nitrogen atmosphere) for 8 hrs. The resulting clear solution was then cooled to 0°C, and to it sodium borohydride (7.74 mmol) was added. This was allowed to come to room temperature and stirred for 12 h. Then the solvents were evaporated under reduced pressure (not to dryness) and diluted with diethyl ether. To this 2N NaOH (20 mL) was added and stirred for 15 minutes. After separation from the NaOH layer, the organic layer was subsequently washed

with water (twice), brine and dried over $MgSO_4$. The organic layer was then evaporated under reduced pressure and the residue was dissolved minimum volume of methanol. To this of 4N HCl (3 mL) was added and instantaneous formation of precipitate was observed. The solvents were completely removed and the precipitate was dissolved in minimum volume of ethyl acetate (a few drops of methanol was added to dissolve completely). To this hexanes were added to obtain pure crystals of the target compound (Yield: 65-80%). These crystals were filtered, dried and subsequently characterized using ^{1}H NMR, HRMS.

Dihexylammonium chloride (6a): Yield: 70%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 9.5 (s, C_6H_{13}-*NH_2*-C_6H_{13}, 2H), 3.0 (m, RC*H_2*-NH_2-*CH_2*-R, 4H), 1.84 (m, R-C*H_2*-CH_2-NH_2-CH_2- C*H_2*-R, 4H), 1.4-1.1 (s, *R*-CH_2-CH_2-NH_2-CH_2- CH_2-*R*, 12H), 0.84 (t, *CH_3*-R‘--NH_2-R-*CH_3*, 6H). $[M+H]^+$ obsd. = 186.2209 (calc. = 186.2216)

***N*-hexyloctan-1-aminium chloride (7a):** Yield: 72%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 9.5 (s, C_8H_{17}-*NH_2*-C_6H_{13}, 2H), 3.0 (m, RC*H_2*-NH_2-*CH_2*-R‘, 4H), 1.89 (m, R-C*H_2*-CH_2-NH_2-CH_2- C*H_2*-R‘, 4H), 1.4-1.1 (m, *R*-CH_2-CH_2-NH_2-CH_2- CH_2-*R‘*, 16H), 0.84 (t, *CH_3*-R‘—NH_2-R-*CH_3*, 6H). $[M+H]^+$ obsd. = 214.2535 (calc. = 214.2529)

Dioctylammonium chloride (8a): Yield: 68%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 9.5 (s, C_8H_{17}-*NH_2*-C_8H_{17}, 2H), 3.0 (m, RC*H_2*-NH_2-*CH_2*-R, 4H), 1.84 (m, R-C*H_2*-CH_2-NH_2-CH_2- C*H_2*-R, 4H), 1.4-1.1 (s, *R*-CH_2-CH_2-NH_2-CH_2-CH_2-*R*, 20H), 0.84 (t, *CH_3*-R‘--NH_2-R-*CH_3*, 6H). $[M+H]^+$ obsd. = 242.2842 (calc. = 242.2848)

***N*-octyldecan-1-aminium chloride (9a):** Yield: 67%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 9.5 (s, C_8H_{17}-*NH_2*-$C_{10}H_{21}$, 2H), 3.0 (m, R’C*H_2*-NH_2-*CH_2*-R, 4H), 1.84 (m, R‘-C*H_2*-CH_2-NH_2-CH_2- C*H_2*-R, 4H), 1.4-1.1 (s, *R‘*-CH_2-CH_2-NH_2-CH_2- CH_2-*R*, 24H), 0.84 (t, *CH_3*-R‘--NH_2-R-*CH_3*, 6H). $[M+H]^+$ obsd. = 270.3175 (calc. = 270.3155)

Didecylammonium chloride (10a): Yield: 75%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 9.5 (s, $C_{10}H_{21}$-*NH_2*-$C_{10}H_{21}$, 2H), 3.1-2.5 (RC*H_2*-NH_2-*CH_2*-R, 4H), 1.95-1.1 (*R*-CH_2-NH_2-CH_2-*R‘*, 28H), 0.84 (t, *CH_3*-R-NH_2-R-*CH_3*, 6H). $[M+H]^+$ obsd. = 242.2841 (calc. = 242.2842)

2.4.2.4 General synthetic procedure for amide coupling reactions of dialkylamines or alkylamines with Boc-Lys(Boc)-OH: In a typical reaction, to a stirred solution of Boc-Lys(Boc)-OH (1.97 mmol) in 2:1 DMF/$CHCl_3$(9 mL), *N*,*N*-Diisopropylethylamine (DIPEA) (4.92 mmol) was added at 0°C. To this solution was added HBTU (1.97 mmol). This reaction mixture was stirred for 5 minutes at 0 °C and subsequently the dialkylamines or alkylamines (1.64 mmol) was added to it. The mixture was stirred at 0 °C for 30 minutes and subsequently at RT for 24 hrs typically. At the end, $CHCl_3$was evaporated under reduced pressure and the resulting solution was diluted to 2 times its original volume by addition of ethyl acetate. This mixture was subsequently washed with 0.5 M $KHSO_4$, H_2O (thrice) and brine. After passage through anhydrous Na_2SO_4, the organic layer was evaporated under reduced pressure and the residue was purified using column chromatography (only $CHCl_3$) to obtain the products in 70-90% yield. The purified compound was subsequently characterized using ^{1}H NMR, IR and HRMS.

Boc-Lys(Boc)-*N*-aminododecane (1b): Yield: 86%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.4 (m, Lys (ε-NH-Boc)-α-N*H*-boc, 1H), 4.6 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.0 (t, α-C*H* of Lys(boc)$_2$, 1H), 3.3-3 (δ-C*H*$_2$ of Lys(boc)$_2$ and R'-C*H*$_2$-NH-Lys(boc)$_2$, 4H), 1.7-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH$_2$)$_{10}$*-CH$_3$ of R group, 44H), 0.8 (the terminal CH_3 of alkyl chains, 3H). FT-IR (cm^{-1}): 3437 (carbamate N-H str.), 2984-2858 (sp^3 C-H str.), 1706 (C=O str. of carbamate), 1669 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 514.4208 (calc. = 514.4220).

Boc-Lys(Boc)-*N*-aminotetradecane (2b): Yield: 90%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.4 (m, Lys (ε-NH-Boc)-α-N*H*-boc, 1H), 4.6 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.0 (t, α-C*H* of Lys(boc)$_2$, 1H), 3.3-3 (δ-C*H*$_2$ of Lys(boc)$_2$ and R'-C*H*$_2$-NH-Lys(boc)$_2$, 4H), 1.7-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH$_2$)$_{12}$*-CH$_3$ of R group, 48H), 0.8 (the terminal CH_3 of alkyl chains, 3H). FT-IR (cm^{-1}): 3437 (carbamate N-H str.), 2984-2858 (sp^3 C-H str.), 1706 (C=O str. of carbamate), 1669 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 542.45218 (calc. = 542.4533).

Boc-Lys(Boc)-*N*-aminohexadecane (3b): Yield: 84%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.4 (m, Lys (ε-NH-Boc)-α-N*H*-boc, 1H), 4.6 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.0 (t, α-C*H* of Lys(boc)$_2$, 1H), 3.3-3 (δ-CH_2 of Lys(boc)$_2$ and R'-CH_2-NH-Lys(boc)$_2$, 4H), 1.7-1.2 (-CO-[CH-CH_2-CH_2-CH_2-CH_2-NH-COO-C(CH_3)$_3$]-NH-COO-C(CH_3)$_3$ of Lys(boc)$_2$ and –CH_2-$(CH_2)_{14}$-CH_3 of R group, 52H), 0.8 (the terminal CH_3 of alkyl chains, 3H). FT-IR (cm^{-1}): 3437 (carbamate N-H str.), 2984-2858 (sp^3 C-H str.), 1706 (C=O str. of carbamate), 1669 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 570.47813 (calc. = 570.4846).

Boc-Lys(Boc)-*N*-aminooctadecane (4b): Yield: 89%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.4 (m, Lys (ε-NH-Boc)-α-N*H*-boc, 1H), 4.6 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.0 (t, α-C*H* of Lys(boc)$_2$, 1H), 3.3-3 (δ-CH_2 of Lys(boc)$_2$ and R'-CH_2-NH-Lys(boc)$_2$, 4H), 1.7-1.2 (-CO-[CH-CH_2-CH_2-CH_2-CH_2-NH-COO-C(CH_3)$_3$]-NH-COO-C(CH_3)$_3$ of Lys(boc)$_2$ and –CH_2-$(CH_2)_{16}$-CH_3 of R group, 56H), 0.8 (the terminal CH_3 of alkyl chains, 3H). FT-IR (cm^{-1}): 3437 (carbamate N-H str.), 2984-2858 (sp^3 C-H str.), 1706 (C=O str. of carbamate), 1669 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 598.51529 (calc. = 598.5159)

Boc-Lys(Boc)-*N*-aminoicosane (5b): Yield: 90%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.4 (m, Lys (ε-NH-Boc)-α-N*H*-boc, 1H), 4.6 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.0 (t, α-C*H* of Lys(boc)$_2$, 1H), 3.3-3 (δ-CH_2 of Lys(boc)$_2$ and R'-CH_2-NH-Lys(boc)$_2$, 4H), 1.7-1.2 (-CO-[CH-CH_2-CH_2-CH_2-CH_2-NH-COO-C(CH_3)$_3$]-NH-COO-C(CH_3)$_3$ of Lys(boc)$_2$ and –CH_2-$(CH_2)_{18}$-CH_3 of R group, 60H), 0.8 (the terminal CH_3 of alkyl chains, 3H). FT-IR (cm^{-1}): 3437 (carbamate N-H str.), 2984-2858 (sp^3 C-H str.), 1706 (C=O str. of carbamate), 1669 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 626.5462 (calc. = 626.5472)

Boc-Lys(Boc)-*N,N*-aminodihexane (6b): Yield: 82%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.4 (m, Lys (ε-NH-Boc)-α-N*H*-boc, 1H), 4.7-4.5 (Lys (ε-N*H*-Boc)-α-NH-boc, and α-C*H* of Lys(boc)$_2$, 2H), 3.5-3 (δ-CH_2 of Lys(boc)$_2$ and R'-CH_2-N(-CH_2-R)Lys(boc)$_2$, 6H), 1.7-1.2 (-CO-[CH-CH_2-CH_2-CH_2-NH-COO-C(CH_3)$_3$]-NH-COO-C(CH_3)$_3$ of Lys(boc)$_2$ and –CH_2-$(CH_2)_4$-CH_3 of R group and –CH_2-$(CH_2)_4$-CH_3, of R' group, 40H), 0.8 (the terminal CH_3 of alkyl chains, 6H). FT-IR (cm^{-1}): 3437 (carbamate N-H str.), 2928-2863 (sp^3 C-H str.), 1706 (C=O str. of carbamate), 1638 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 542.4524 (calc. = 542.4533)

Boc-Lys(Boc)-*N,N*-octylaminohexane (7b): Yield: 85%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.4 (m, Lys (ε-NH-Boc)-α-N*H*-boc, 1H), 4.7-4.5 (Lys (ε-N*H*-Boc)-α-NH-boc, and α-C*H* of Lys(boc)$_2$, 2H), 3.5-3 (δ-C*H*$_2$ of Lys(boc)$_2$ and R'-C*H*$_2$-N(-C*H*$_2$-R)Lys(boc)$_2$, 6H), 1.7-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH*$_2$)$_6$-CH$_3$ of R group and –CH$_2$-*(CH*$_2$)$_4$-CH$_3$, of R' group, 44H), 0.8 (the terminal CH_3 of alkyl chains, 6H). FT-IR (cm^{-1}): 3437 (carbamate N-H str.), 2928-2863 (sp^3 C-H str.), 1710 (C=O str. of carbamate), 1634 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 542.4524 (calc. = 542.4533)

Boc-Lys(Boc)-*N,N*-aminodioctane (8b): Yield: 78%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.4 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.7-4.5 (Lys (ε-NH-Boc)-α-N*H*-boc, and α-C*H* of Lys(boc)$_2$, 2H), 3.5-3 (δ-C*H*$_2$ of Lys(boc)$_2$ and R-C*H*$_2$-N(-C*H*$_2$-R)Lys(boc)$_2$, 6H), 1.7-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH*$_2$)$_6$-CH$_3$ of R group and –CH$_2$-*(CH*$_2$)$_6$-CH$_3$, of the other R group, 48H), 0.8 (the terminal CH_3 of alkyl chains, 6H). FT-IR (cm^{-1}): 3437 (carbamate N-H str.), 2984-2858 (sp^3 C-H str.), 1706 (C=O str. of carbamate), 1669 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 570.4780 (calc. = 570.4846)

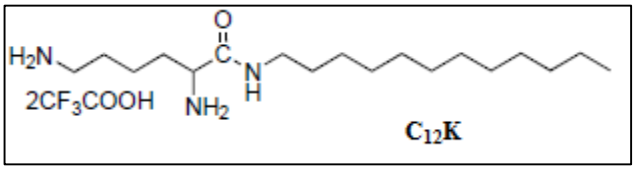
Boc-Lys(Boc)-*N,N*-octylaminodecane (9b): Yield: 79%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.4 (m, Lys (ε-NH-Boc)-α-N*H*-boc, 1H), 4.7-4.5 (Lys (ε-NH-Boc)-α-N*H*-boc, and α-C*H* of Lys(boc)$_2$, 2H), 3.5-3 (δ-C*H*$_2$ of Lys(boc)$_2$ and R'-C*H*$_2$-N(-C*H*$_2$-R)Lys(boc)$_2$, 6H), 1.7-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH*$_2$)$_6$-CH$_3$ of R group and –CH$_2$-*(CH*$_2$)$_8$-CH$_3$, of R' group, 52H), 0.8 (the terminal CH_3 of alkyl chains, 6H). FT-IR (cm^{-1}): 3437 (carbamate N-H str.), 2928-2863 (sp^3 C-H str.), 1706 (C=O str. of carbamate), 1670 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 598.51518 (calc. = 598.5159)

Boc-Lys(Boc)-*N,N*-aminodidecane (10b): Yield: 78%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.4 (m, Lys (ε-NH-Boc)-α-N*H*-boc, 1H), 4.7-4.5 (Lys (ε-NH-Boc)-α-N*H*-boc, and α-C*H* of Lys(boc)$_2$, 2H), 3.5-3 (δ-C*H*$_2$ of Lys(boc)$_2$ and R'-C*H*$_2$-N(-C*H*$_2$-R)Lys(boc)$_2$, 6H), 1.7-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH*$_2$)$_8$-CH$_3$ of R group and –CH$_2$-*(CH*$_2$)$_8$-CH$_3$, of R' group, 52H), 0.8 (the terminal CH_3 of alkyl chains, 6H). FT-

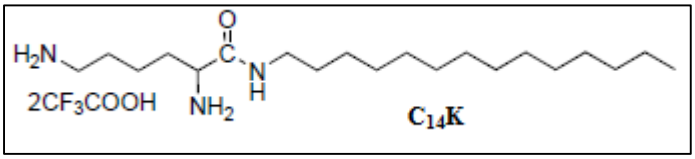
IR (cm^{-1}): 3437 (carbamate N-H str.), 2928-2863 (sp^3 C-H str.), 1706 (C=O str. of carbamate), 1638 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 626.54392 (calc. = 626.5472)

2.4.2.5. Deprotection of Boc groups: Boc-Lys(Boc)-*N*,*N*-alkylaminoalkanes or Boc-Lys(Boc)-*N*,*N*-aminoalkanes (1.2 mmol) were dissolved in DCM and subsequently CF_3COOH (50% by volume) was added and stirred at RT for 6h. The reactions were monitored by TLC until complete removal of starting material was observed. All the volatile components were removed and the compound was dried overnight in a high vacuum oven. Then the compounds were purified to more than 95% purity using reverse phase HPLC and subsequently characterized by ^{1}H NMR, ^{13}C NMR, IR and HRMS.

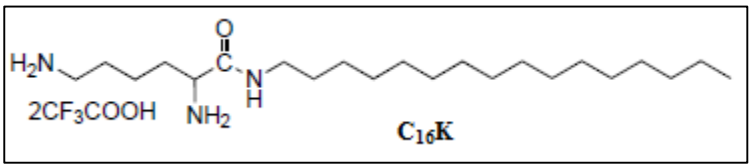
Lys-*N*-aminododecane (C_{12}-K, 1): ^{1}H NMR (400 MHz, D_2O) δ/ppm: 4.0 (t, α-C*H* of Lys, 1H), 3.3 (residual solvent peak for MeOH) 3.2-2.8 (R'-CH_2-NH-Lys and ε-CH_2 of Lys, 4H), 1.8-1.2 (β- CH_2 γ- CH_2 δ- CH_2 of Lys and –CH_2-$(CH_2)_{10}$-CH_3 of R group, 26H), 0.8 (the terminal CH_3 of alkyl chains, 3H). ^{13}C-NMR (100 MHz, D_2O with drops of DMSO-d_6):168, 53.12, 48.94, 39.03, 38.35, 38.18, 37.96, 37.75, 31.61, 30.55, 29.32, 28.83, 28.45, 26.92, 26.39, 22.37, 21.42, 13.64 FT-IR (cm^{-1}): 2912-2847 (sp^3 C-H str.), 1675 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 314.3152 (calc. = 314.3171)



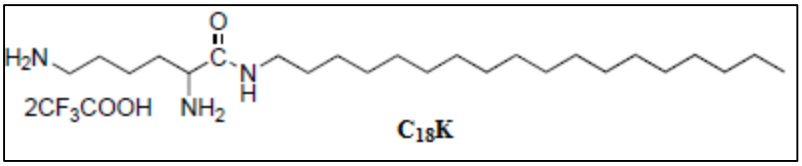
Lys-*N*-aminotetradecane (C_{14}-K, 2): ^{1}H NMR (400 MHz, D_2O) δ/ppm: 4.79 (residual peak for D_2O) 4.0 (t, α-C*H* of Lys, 1H), 3.3 (residual solvent peak for MeOH) 3.2-2.8 (R'-CH_2-NH-Lys and ε-CH_2 of Lys, 4H), 1.8-1.2 (β- CH_2 γ- CH_2 δ- CH_2 of Lys and –CH_2-$(CH_2)_{12}$-CH_3 of R group, 30H), 0.8 (the terminal CH_3 of alkyl chains, 3H). ^{13}C-NMR (100 MHz, D_2O with drops of DMSO-d_6): 168.8, 53.00, 48.93, 38.92, 38.34, 38.14, 37.92, 37.71, 37.50, 31.90, 30.59, 29.83, 29.41, 29.27, 28.66, 26.87, 26.28, 22.56, 21.42, 13.71. FT-IR (cm^{-1}): 2912-2847 (sp^3 C-H str.), 1675 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 342.3521 (calc. = 342.3484).



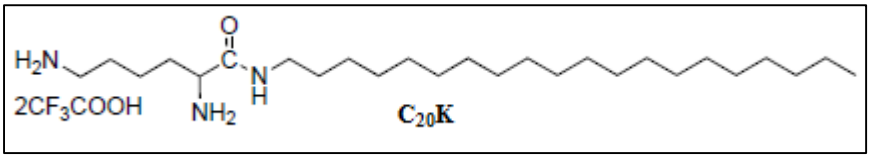
Lys-*N*-aminohexadecane (C_{16}-K, 3): ^{1}H NMR (400 MHz, D_2O) δ/ppm: 4.0 (t, α-C*H* of Lys, 1H), 3.3 (residual solvent peak for MeOH) 3.2-2.8 (R'-C*H*$_2$-NH-Lys and ε-C*H*$_2$ of Lys, 4H), 1.8-1.2 (β-C*H*$_2$ γ- C*H*$_2$ δ- C*H*$_2$ of Lys and –CH$_2$-*(CH*$_2$*)*$_{14}$-CH$_3$ of R group, 34H), 0.8 (the terminal CH$_3$ of alkyl chains, 3H). ^{13}C-NMR (100 MHz, D_2O with drops of DMSO-d_6): 168.9, 53.04, 48.92, 38.98, 38.37, 38.16, 37.96, 37.74, 37.54, 31.97, 30.65, 29.93, 29.81, 29.48, 29.34, 28.70, 26.93, 26.34, 22.62, 21.46, 13.79. FT-IR (cm^{-1}): 2912-2847 (sp^3 C-H str.), 1675 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 370.37887 (calc. = 370.3797)



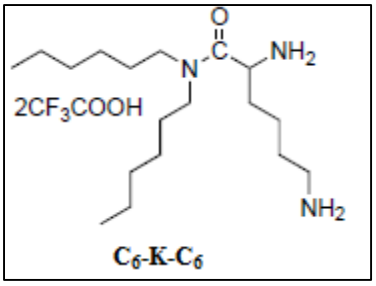
Lys-*N*-aminooctadecane (C_{18}-K, 4): ^{1}H NMR (400 MHz, D_2O) δ/ppm: 4.0 (t, α-C*H* of Lys, 1H), 3.3 (residual solvent peak for MeOH) 3.2-2.8 (R'-C*H*$_2$-NH-Lys and ε-C*H*$_2$ of Lys, 4H), 1.8-1.2 (β- C*H*$_2$ γ- C*H*$_2$ δ- C*H*$_2$ of Lys and –CH$_2$-*(CH*$_2$*)*$_{16}$-CH$_3$ of R group, 38H), 0.8 (the terminal CH$_3$ of alkyl chains, 3H). ^{13}C-NMR (100 MHz, D_2O with drops of DMSO-d_6): 168.9, 53.12, 48.94, 38.92, 38.32, 38.11, 37.90, 37.69, 37.47, 31.99, 30.60, 30.00, 29.93, 29.81, 29.52, 28.83, 28.45, 26.92, 26.29, 22.63, 21.44, 13.79. HR-MS (m/z): $[M+H]^+$ obsd. = 398.4139 (calc. = 398.4110)



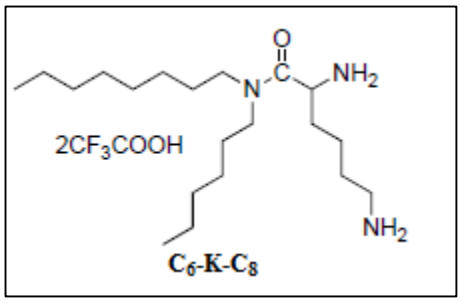
Lys-*N*-aminoicosane (C_{20}-K, 5): ^{1}H NMR (400 MHz, D_2O) δ/ppm: 4.0 (t, α-C*H* of Lys, 1H), 3.3 (residual solvent peak for MeOH) 3.2-2.8 (R'-C*H*$_2$-NH-Lys and ε-C*H*$_2$ of Lys, 4H), 1.8-1.2 (β- C*H*$_2$ γ- C*H*$_2$ δ- C*H*$_2$ of Lys and –CH$_2$-*(CH*$_2$*)*$_{18}$-CH$_3$ of R group, 42H), 0.8 (the terminal CH$_3$ of alkyl chains, 3H). ^{13}C-NMR (100 MHz, D_2O with drops of DMSO-d_6): 53.12, 48.94, 38.92, 38.34, 38.14, 37.93, 37.72, 37.51, 31.99, 30.60, 30.10, 30.00, 29.93, 29.81, 29.52, 28.83, 28.45, 26.92, 26.29, 22.63, 21.44, 13.79. HR-MS (m/z): $[M+H]^+$ obsd. = 426.44359 (calc. = 426.4423)



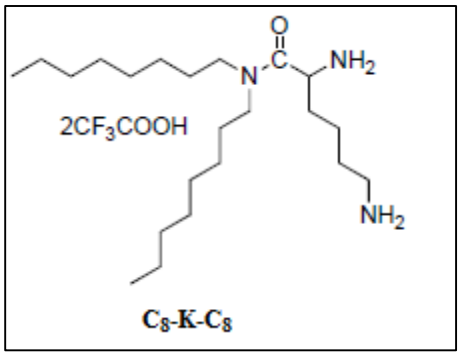
Lys-*N*,*N*-aminodihexane (C_6-K-C_6, 6): ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 4.3 (α-C*H*$_2$ of Lys, 1H), 3.7-2.8 (δ-C*H*$_2$ of Lys and R'-C*H*$_2$-N(-C*H*$_2$-R)Lys, 6H), 1.9-1.2 β-C*H*$_2$ of Lys, γ-C*H*$_2$ of Lys, δ-C*H*$_2$ of Lys and –CH_2-*(CH$_2$)$_4$*-CH_3 of R group and –CH_2-*(CH$_2$)$_4$*-CH_3, of R' group, 22H), 0.8 (the terminal CH_3 of alkyl chains, 6H). FT-IR (cm^{-1}): 2912-2847 (sp^3 C-H str.), 1675 (C=O str. of tertiary amide). ^{13}C-NMR (100 MHz, CD_3OD): 168.58, 163, 162.7, 53.58, 52.63, 50.46, 47.87, 46.36, 39, 30.85, 30.22, 28.09, 26.61, 26.5 25.93, 25.64, 22.0, 21.9 21.15 13.37. HR-MS (m/z): $[M+H]^+$ obsd. = 314.3190 (calc. = 314.3171)



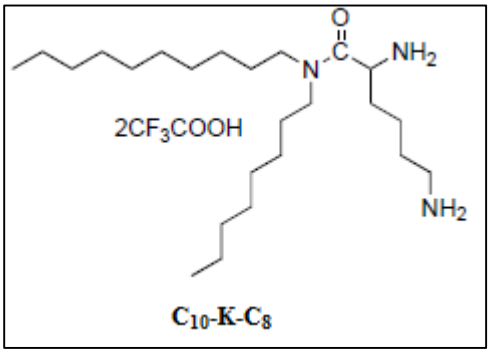
Lys-*N*,*N*-octylaminohexane (C_6-K-C_8, 7): ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 4.3 (α-C*H*$_2$ of Lys, 1H), 3.7-2.8 (δ-C*H*$_2$ of Lys and R'-C*H*$_2$-N(-C*H*$_2$-R)Lys, 6H), 1.9-1.2 β-C*H*$_2$ of Lys, γ-C*H*$_2$ of Lys, δ-C*H*$_2$ of Lys and –CH_2-*(CH$_2$)$_6$*-CH_3 of R group and –CH_2-*(CH$_2$)$_4$*-CH_3, of R' group, 26H), 0.8 (the terminal CH_3 of alkyl chains, 6H). ^{13}C-NMR (100 MHz, D_2O with drops of DMSO-d_6): 50.48, 48.94, 47.88, 38.15, 37.95, 37.73, 30.97, 30.26, 28.72, 28.24, 26.52, 26.03, 22.28, 22.04, 21.23, 13.60. FT-IR (cm^{-1}): 2912-2847 (sp^3 C-H str.), 1676 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 342.34797 (calc. = 342.3484)



Lys-*N*,*N*-aminodioctane (C_8-K-C_8, 8): ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 4.3 (α-C*H*$_2$ of Lys, 1H), 3.7-2.8 (δ-C*H*$_2$ of Lys and R'-C*H*$_2$-N(-C*H*$_2$-R)Lys, 6H), 1.9-1.2 β-C*H*$_2$ of Lys, γ-C*H*$_2$ of Lys, δ-C*H*$_2$ of Lys and –CH_2-*(CH$_2$)$_6$*-CH_3 of R group and –CH_2-*(CH$_2$)$_6$*-CH_3, of R' group, 30H), 0.8 (the terminal CH_3 of alkyl chains, 6H). ^{13}C-NMR (100 MHz, D_2O with drops of DMSO-d_6): 48.93, 47.77, 37.93, 37.71, 31.73, 31.68, 30.31, 29.03, 28.86, 28.76, 28.23, 26.83, 26.67, 26.49, 26.13, 22.48, 22.43, 21.22, 13.68. FT-IR (cm^{-1}): 2912-2847 (sp^3 C-H str.), 1675 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 370.3787 (calc. = 370.3797)

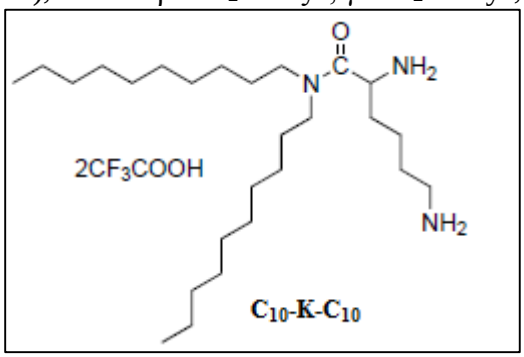


Lys-*N*,*N*-octylaminodecane (C_8-K-C_{10}, 9): ^{1}H-NMR (400 MHz, CD_3OD) δ/ppm: 4.3 (α-C*H*$_2$ of Lys, 1H), 3.7-2.8 (δ-C*H*$_2$ of Lys and R'-C*H*$_2$-N(-C*H*$_2$-R)Lys, 6H), 1.9-1.2 β-C*H*$_2$ of Lys, γ-C*H*$_2$ of Lys, δ-C*H*$_2$ of Lys and –CH_2-*(CH$_2$)$_6$*-CH_3 of R group and –CH_2-*(CH$_2$)$_8$*-CH_3, of R' group, 34H), 0.8 (the terminal CH_3 of alkyl chains, 6H). FT-IR (cm^{-1}): 3350 (carbamate N-H str.), 2912-2847 (sp^3 C-H str.), 1675 (C=O str. of tertiary amide). ^{13}C-NMR (100 MHz, CD_3OD): 169.51, 163, 162.7, 53.65, 47.57, 40.25, 33.05, 32.96, 31.91, 30.99, 30.674, 30.50, 30.43, 30.35, 30.16, 28.47, 28.23, 27.99, 27.75, 23.74, 23.07, 22.68, 13.79. HR-MS (m/z): $[M+H]^+$ obsd. = 398.4089 (calc. = 398.4110)



C_{10}-K-C_8

Lys-*N*,*N*-aminodidecane (C_{10}-K-C_{10}, 10): ^{1}H-NMR: (400 MHz, D_2O) δ/ppm: 4.3 (α-C*H*$_2$ of Lys, 1H), 3.7-2.8 (δ-C*H*$_2$ of Lys and R'-C*H*$_2$-N(-C*H*$_2$-R)Lys, 6H), 1.9-1.2 β-C*H*$_2$ of Lys, γ-C*H*$_2$ of Lys, δ-C*H*$_2$ of Lys and –CH_2-*(CH$_2$)$_8$*-CH_3 of R group and –CH_2-*(CH$_2$)$_8$*-CH_3, of R' group, 38H), 0.8 (the terminal CH_3 of alkyl chains, 6H). FT-IR (cm^{-1}): 3350 (carbamate N-H str.), 2912-2847 (sp^3 C-H str.), 1675 (C=O str. of tertiary amide). ^{13}C-NMR (100 MHz, CD_3OD): 169.51, 163, 162.7, 51.65, 47.57, 40.27, 33.03, 31.91, 30.66, 30.64, 30.46, 30.41, 30.38, 30.11, 28.45, 28.23, 27.97, 27.75, 22.63, 21.44, 13.79. HR-MS (m/z): $[M+H]^+$ obsd. = 426.44036 (calc. = 426.4423).



C_{10}-K-C_{10}

2.4.3 *In vitro* biological assays

2.4.3.1 *In vitro* susceptibility studies: The MIC of the compounds against different bacterial strains were determined using a standardized protocol.[15] Briefly, 50 µL of serially diluted compounds (starting concentrations were 50 µg mL^{-1}) were added to wells of 96 well plates containing 150 µL of media containing bacteria (10^5 CFU mL^{-1}). The plate was then incubated at 37 °C for 24 h and then the O. D. value was measured at 600 nm using TECAN (Infinite series, M200 pro) Plate Reader. Each concentration had triplicate values and the whole experiment was done at least twice and the MIC value was determined by taking the average of triplicate O. D.

values for each concentration and plotting it against concentration. The minimum concentration at which no bacterial growth was observed was considered to be the MIC of the compound. The value was converted to μM and presented in Table 2.1.

2.4.3.2 Hemolytic Activity: Hemolytic experiments were performed using a standardized protocol.[174] Erythrocytes were isolated from freshly drawn, heparinized blood and re-suspended to 5 vol% in PBS (pH 7.4). In a 96-well microtiter plate, 150 μL of erythrocyte suspension was added to 50 μL of serially diluted compound. 50 μL of 1 vol% solution of Triton X-100 served as positive control while in negative control 50 μL of water was used. After incubation for 1 h at 37°C, the plate was centrifuged at 3,500 rpm for 5 min. Subsequently, 100 μL of the supernatant from each well was carefully transferred to a fresh microtiter plate, and absorbance at 540 nm was measured. Percentage of hemolysis was determined as $(A - A_0)/(A_{total} - A_0) \times 100$, where A is the absorbance of the test well, A_0 the absorbance of the negative controls (without compound) and A_{total} the absorbance of wells with 100% hemolysis (with Triton X-100) all at 540 nm. The HC_{50} values are reported as averages of at least two independent experiments (each experiment was performed in triplicates) respectively. The error of the experiments is less than 10%.

2.4.3.3 Bactericidal time-kill kinetics: Kinetics of bactericidal activity of **9** was performed according to a standardized protocol.[92] The experiment was performed against both *S. aureus* and *E. coli* cells using the same protocol. The procedure for conducting the study against *E. coli* is described. Briefly, *E. coli* (MTCC) was grown in nutrient broth at 37 °C for 6 h. Test compounds were inoculated with the aliquots of bacteria resuspended in fresh media at ~10^5 CFU mL^{-1}. After specified time intervals (0 min, 15 min, 30 min, 60 min, 120 min, 180 min and 360 min), 20 μL aliquots were serially diluted 10 fold in 0.9 % saline, plated on sterile MacConkey's agar plates and incubated at 37 °C overnight. The viable colonies were counted the next day and represented as $\log_{10}$ (CFU mL^{-1}). Experiment against *S. aureus* was also done using the same protocol.

2.4.3.4 Propensity to induce resistance development in bacteria: This was performed following a standardized protocol.[92] For this experiment, we had chosen norfloxacin as a comparator drug for *S. aureus* and colistin as a comparator drug for *E. coli*. MIC values of the compounds (**9** and antibiotics) were determined against *S. aureus* (MTCC 737) and *E. coli* (MTCC 443) as described

above. Subsequently, bacterial cells growing at the highest concentration of the compound were harvested and inoculated into fresh media (Luria Bertani broth in case of *E. coli* and nutrient broth in case of *S. aureus*) to a concentration of 10^5 CFU mL^{-1}. This inoculum was subjected to another MIC assay. After 24 h incubation period, cells growing in the highest concentration of the compound from the previous passage were once again harvested and assayed for the MIC. The process was repeated for 14 passages. The MIC value of the compound was plotted against the number of passages, and the fold increase in MIC was determined. The results indicate the fold of increase in MIC every two days.

2.4.3.5 Antibacterial activity in different physiological conditions: The experiment was performed exactly as reported earlier. First the pH and salinity of the culture medium was brought to the desired pH by adding NaOH (1N) or HCl (1N) or to the desired salt concentration by adding NaCl to the medium. The different pH conditions considered were 5.5, 6.5, 7.4, and 8.5. The different percentages of NaCl considered were 1%, 2% and 3%. Then the antibacterial activity of the compounds was determined in this medium by the same assay as described above.

2.4.4 Mechanism of action: The assays for determining the mechanism of action of the compounds were performed according to a standardized protocol.[92] Brief description of the assays are provided below.

2.4.4.1 Cytoplasmic membrane depolarization assay: The assay was performed in a solution containing 5 mM glucose, 5 mM HEPES buffer and 100 mM KCl solution in 1:1:1 ratio. Mid-log phase *E. coli* cells were harvested, washed with and resuspended in the solution at concentrations of 10^8 CFU mL^{-1}. $DiSC_3$ (5) dye was added to this solution to a final concentration of 2 µM. After preincubation for 40 mins in a well of a black 96-well plate with transparent bottom for dye uptake, and resultant self-quenching, the fluorescence of the bacterial suspension was measured (excitation wavelength: 622 nm; emission wavelength: 670 nm) and allowed to stabilize for 5 min at room temperature before the addition of 2 µL of compound **9** (3×MIC). After addition fluorescence intensity was measured every minute or two for 20 min. The resultant plot was obtained by joining the data points.

2.4.4.2 Outer membrane permeabilization assay: Mid-log phase *E. coli* cells were harvested, washed with 5 mM HEPES and 5 mM glucose (10^8 CFU mL^{-1}) and resuspended in a 1:1 solution of the same. To this solution *N*-phenylnaphthylamine dye was added to the bacterial suspension containing the dye (200 µL) to give a final concentration of 10 µM in the bacterial suspension. The suspension containing the dye (200 µL) was then added to the well of a 96-well plate (black plate, clear bottom with lid) and stabilized for 5 minutes. Then 2 µL of compound **9** was added to the solution at 10 µg mL^{-1}. After addition fluorescence intensity (excitation wavelength: 350 nm; emission wavelength: 420 nm) was measured every minute or two for 20 min. The resultant plot was obtained by joining the data points.

2.4.4.3 Inner membrane permeabilization assay: Mid-log phase *E. coli* cells were harvested washed, and resuspended in 5 mM HEPES and 5 mM glucose pH 7.2. To this solution propidium iodide (PI) dye was added to a final concentration of 15 µM. The suspension containing the dye (200 µL) was then added to the well of a 96-well plate (black plate, clear bottom with lid) and then 2 µL of compound **9** was added to the solution to a final at concentrations of 10 µg mL^{-1}. Fluorescence intensity was measured at excitation wavelength of 535 nm (slit width: 10 nm) and emission wavelength of 617 nm (slit width: 5 nm). The uptake of PI was measured by the increase in fluorescence of PI for 10 min as a measure of inner membrane permeabilization.
Persister cells were isolated as described above and diluted to 10^8 cells in respective media. Mechanistic studies were performed with them using the same protocol the only difference being freshly isolated persister cells were used instead of mid-log phase cells.

2.4.5 *In vivo* studies

The mice were housed in individually ventilated cages (IVC) maintained with controlled environment as per the standards. They are housing—pathogen free conventional caging system, bedding material (Corn Cob). The husbandry conditions:-Light: dark cycle- 12:12 hours, Animal Room Temp: 22 ± 2°C, Relative humidity: 30–40%, Access to feed and water: ad libitum and Water: RO Water. Animals were randomly selected, marked to permit individual identification and kept in their cages for at least 5 days before the experiment to allow for acclimatization to the experimental conditions. Animal handling and experimentation protocols were followed according

to OECD Guidelines for the Testing of Chemicals (OECD 425). All care was taken to cause no pain to the animals. Humane endpoints were used to avoid unnecessary distress and suffering in animals following an experimental intervention that would lead to death. The animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) and carried out as per the guidelines of Committee for the purpose of Supervision and Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi.

2.4.5.1 Acute dermal toxicity: This study was performed following a standardized protocol. The acute dermal toxicity was performed on five 6 to 8 weeks-old male BALB/c mice (18-22 g) following the OECD guidelines. Approximately 24h before the experiment, fur was removed from the back of the mice, first by clipping and then shaving. Care was taken to avoid abrasion of the skin. The area of the shaved portion was roughly 2 cm^2. Compound **9** was dissolved in saline at concentrations of 100 mg mL^{-1} to make working stocks. From this stock, 40 µL was added to the shaved area of the skin to give a concentration of 200 mg kg^{-1} per animal. Following the application of the compound, the animals were observed carefully and then observed carefully once every day for 14 days. Particular attention was paid to the changes in fur, eyes and mucous membranes, and observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

2.4.5.2 *A. baumannii* burn infection: This experiment was performed using a standardized protocol. Female Balb/c mice (6-8 weeks, 22-25 g) were anesthetized with ketamine-xylazine cocktail, their dorsal surface shaved and cleansed. Around 6 mm burn wounds were created by applying a 120 s heated brass bar for 10s. Immediately after injury, burn wounds were infected with a mid-log phase bacterial inoculum of 10^7 CFU of *Acinetobacter baumannii* (MTCC 1425) prepared in PBS. Treatment was started 4 h post infection. Burn wounds were treated every 24 h for 7 days. Compound **9** and colistin were dissolved in saline. Burn wounds were treated with 40 µL of solutions containing compound **9** (40 mg kg^{-1}) or colistin (5 mg kg^{-1}) whereas only saline was used as untreated control. Mice were euthanized 7 days post-injury; the wounded muscle tissue was excised, weighed, and homogenized in 10 mL of PBS. Serial homogenate dilutions were plated on MacConkey agar (Himedia, India) and the results were stated as log (CFU g^{-1}) of tissue. P value was calculated using unpaired Student's t test (2 tailed 2 samples assuming equal

variances) between the control group and the treatment group and a value of $P < 0.05$ was considered significant.

Chapter 3

Aryl-alkyl-lysines: Broad-spectrum antibacterial agents

Abstract

The emergence of multi-drug resistant bacteria compounded by depleting arsenal of antibiotics has accelerated efforts towards development of antibiotics with novel mechanism of action. In chapter 2, simple lipidated lysines were designed which possessed broad-spectrum antibacterial activity. Due to the preponderance of aromatic moieties in several successful membrane-active agents, in this chapter a new series of small molecules is presented, wherein, an aromatic moiety is introduced into the design. These compounds exhibit high in vitro potency against a variety of Gram-positive and Gram-negative bacteria, including drug-resistant species. Highlight of this class of compounds is their superior activity against the major nosocomial pathogen Pseudomonas aeruginosa and anaerobic bacteria Clostridium difficile. Non-toxic towards mammalian cells, these rapidly bactericidal compounds primarily act by permeabilization and depolarization of bacterial membrane. Unlike the previous class of compounds (Chapter 2), the optimal compound of this series was non-toxic to mice skin at till 200 mg kg^{-1}. In addition to having activity against A. baumannii burn infection, the compound was effective in bringing bacterial burden down in a MRSA model of skin infection. Synthetically simple, selectively antibacterial, these compounds can be developed into a newer class of therapeutic agents against multi-drug resistant bacterial species.

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(1) Ghosh, C. *et al.* "Small Molecular Antibacterial Peptoid Mimics: the simpler the better!" *J. Med. Chem*, **2014**, *57*, 1428–1436.

(2) Ghosh, C. *et al.* "Aryl-alkyl-lysines: Membrane active small molecules active against murine model of burn infection." *ACS Infect. Dis.*, **2016**, *2*, 111-122.

(3) Ghosh, C. *et al.* "Aryl-alkyl-lysines: Agents that kill planktonic cells, persister cells, biofilms of MRSA and protect mice from skin-infection." *PLoS One*, **2015**, *10*, e0144094.

3.1 Introduction

In an effort towards solving the problem of antimicrobial resistance, simple small molecules based on L-lysine had been introduced in the previous chapter. These compounds were designed on the premise that they would be mimicking the properties of natural antimicrobial peptides in terms of activity and not retain the demerits of AMPs. Although the compounds were found to possess broad-spectrum bactericidal activity, they were toxic to mice skin at 200 mg kg^{-1} and were not active against methicillin-resistant *S. aureus* in a murine model of skin infection. Thus there was scope for improvement in both of these aspects.

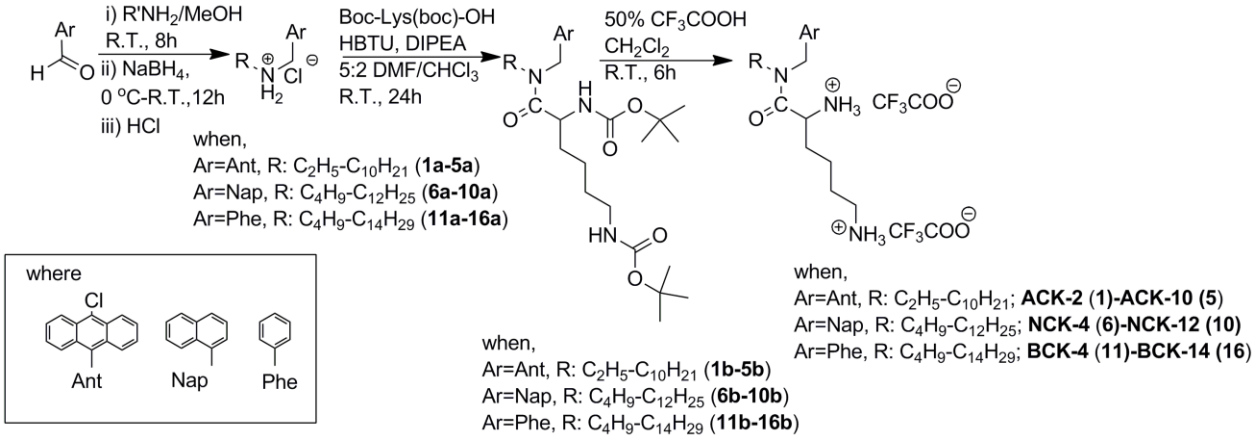
Several synthetic membrane-active agents reported in the literature have used aromatic moieties in their designs. The cationic antibacterial tripeptides reported by Svendsen et al. have aromatic moieties in their designs.[76, 78, 79] All of the antibacterial small molecules reported from the groups of DeGrado and Tew are based on aryl scaffolds.[64-68, 72-74, 126, 158, 165, 175] Aromatic moieties have also been used by Barron *et al.* in their design of antibacterial peptoids.[57, 167] In Chapter 1 several other designs have been presented which have used aromatic moieties in their designs. Thus, we surmised that introduction of aromatic moieties might lead to compounds with different properties.

In this chapter, the design and development of aryl-alkyl-lysines have been reported in which, the two positive charges were again contributed by an L-lysine moiety; hydrophobicity was brought about by an aromatic core (methyl anthracene, methyl naphthalene or methyl benzene) and an alkyl chain. The hydrophobicity of the lipophilic alkyl chain and the bulky aromatic core was varied systematically to understand the role of these parameters towards selective antibacterial activity. The efficacy of the compounds were conducted against different clinical isolates of pathogenic bacteria including the notorious anaerobic bacteria *Clostridium difficile*. Their mechanism of action was then studied using fluorescence spectroscopy, fluorescence microscopy as well as FESEM. Additionally, their efficacy against MRSA model of skin-infection as well as against that of *A. baumannii* model of burn infection have been presented.

3.2 Results and discussion

3.2.1 Design and Synthesis

As mentioned earlier, in this chapter an aromatic moiety was introduced in place of one of the lipophilic tails of the compounds reported in the previous chapter. Herein, we thought an effective way to introduce hydrophobicity was to attach an aromatic moiety with a lipid chain first. To synthesize the compounds, first aromatic aldehydes (10-Chloro-9-Anthracenaldehyde, naphthyldehyde and benzaldehyde) were reacted with alkylamines to form Schiff's bases, followed by reduction with sodium borohydride, to give secondary amines (Scheme 3.1). Then secondary amines were coupled to Boc-Lys(Boc)-OH using HBTU coupling and finally the Boc groups were deprotected using trifluoroacetic acid to obtain the required compounds. The final compounds were purified by HPLC to more than 95% purity and characterized by ^{1}H-NMR, ^{13}C-NMR, IR and HR-MS. Two significant features of these compounds are that there is no imposed structural rigidity in their design and that they include an *N*-disubstituted or tertiary amide bond.



Scheme 3.1: General scheme for the synthesis of the compounds

3.2.2 Antibacterial activity and selectivity

The antibacterial efficacy (Table 3.1) of these compounds was evaluated against different Gram-positive (*S. aureus* and *E. faecium*) and Gram-negative (*E. coli, K. pneumoniae* and *P. aeruginosa*)

bacteria including MRSA and VRE by determination of their minimum inhibitory concentrations (MICs; the concentration of compounds required to completely inhibit the growth of bacteria).

The shortest alkyl chain derivative ACK-2 was found to be moderately active against both Gram-positive and Gram-negative bacteria with MICs of 18 μM and 40 μM against *S. aureus* and *E. coli* respectively. Increasing the long chain to butyl improves antibacterial activity as was observed in ACK-4. Further increase in alkyl chain yielded ACK-6 (hexyl) which displayed improved MICs of 3.5 μM and 5.1 μM against *S. aureus* and *E. coli* respectively. The antibacterial activity was found to increase even further on increasing the long chain to octyl chain; ACK-8 displayed MICs of 3 μM and 4.1 μM against *S. aureus* and *E. coli* respectively. However, further increase in alkyl chain compromised the activity of the compounds as was observed for ACK-10 (decyl). ACK-8 emerged as the most potent compound in the series. In comparison, vancomycin displayed MIC of 0.6 μM against *S. aureus* while colistin exhibited MIC of 0.4 μM against *E. coli*. The Structure-activity-relationship study indicates that if the anthracene core was kept constant, the optimum chain length lied between butyl to octyl.

All the compounds were active against *E. faecium*, in fact ACK-2, the least effective compound of the series also displayed moderate activity (MIC of 22 μM) against the bacterium. ACK-8 with MIC of 3.5 μM was again the most potent compound while vancomycin had MIC of 0.6 μM.

The highlight of these set of compounds, however, is their superior activity towards opportunistic human pathogen *P. aeruginosa* which, is a leading cause of hospital-acquired infections and is known to show resistance to almost all clinically approved antibiotics. All the compounds of ACK series are extremely active against *P. aeruginosa*. Against this bacterium, the minimum MIC value was displayed by ACK-6 (MIC = 2.3 μM). ACK-4 also showed excellent activity towards *P. aeruginosa* with MIC of 3 μM.

In order to elucidate the role of the hydrophobic core towards antibacterial activity we synthesized methyl naphthalene (NCK series) analogues of the molecules with varying chain lengths. It was hypothesized that decreasing the bulkiness from anthracene to naphthalene might yield a more selective antibacterial agent. Unlike in the case of ACK series, the small chain analogues of NCK series, e.g. NCK-4, were devoid of any activity. NCK-6, the hexyl analogue, was moderately active and showed potency against all the drug sensitive bacteria, especially *P. aeruginosa* (MIC = 18 μM). NCK-8 exhibited potent activity against all pathogens comparable to

its anthracenyl counterpart (ACK-8), for example MICs against *S. aureus* and *E. coli* were 10 μM and 8 μM respectively. Its activity towards *P. aeruginosa* was also commendable (MIC = 8.6 μM). NCK-10 and NCK-12 were the most active compounds in the series (Table 3.1) with superior activity against all the drug sensitive bacteria (MIC ranged from 2.3 to 9 μM). The compound NCK-10 was one of the most active compounds in the entire study.

In order to see the effect of a further decrease of aromatic core, methyl benzene series of compounds (BCK series) was prepared. In the BCK series of compounds, however, no significant activity was observed till BCK-8. The decyl analogue, BCK-10 showed potent activity against all the pathogens. With MICs of 9.4 μM and 11 μM against *S. aureus* and *E. coli*, its activity was comparable to NCK-8. BCK-12 and BCK-14 had comparable antibacterial activity against all the tested pathogens (Table 3.1). Against *S. aureus*, BCK-12 was the most potent compound in the series with MIC of 4.3 μM. The activity of BCK-14 was slightly better against all other pathogens.

Toxicity of these compounds towards mammalian cells was evaluated by their ability to lyse human erythrocytes and represented as their HC_{50} values (i.e. the concentration at which 50% of the red blood cells are lysed). HC_{50} of the compounds ranged from 72 μM to >1000 μM. Hemolytic toxicity studies reveal that these compounds are selectively toxic towards bacterial cells. Although some of the compounds reported in this chapter were more selective than the optimum compound reported in the previous chapter, the difference was not very significant. *In vitro* selectivity does not always correlate to *in vivo* toxicity. Thus, it was imperative to check the *in vivo* toxicity of a representative compound, which has been furnished below.

It was observed that most of the ACK series compounds had a tendency to aggregate in water and media at very high concentrations, which deemed them unsuitable for clinical use. As a proof of concept, we did use ACK-6 in some of the further studies, but for *in vivo* applications NCK series of compounds were chosen. Activity against clinical isolates of pathogenic bacteria were tested with both NCK-series and BCK-series of compounds. But it is known in the field, that use of very long chains are also toxic to erythrocytes, which limited further use of BCK series of compounds. This is evident in the HC_{50} values given in Table 3.1. Albeit none of the compounds were toxic at their minimum inhibitory concentrations.

Table 3.1: Antibacterial and haemolytic activity of the compounds.

Compounds	Minimum Inhibitory Concentration (μM)							HC_{50} (μM)
	Drug sensitive bacteria				Drug resistant bacteria			
	S. aureus	*E. faecium*	*E. coli*	*P. aeruginosa*	MRSA	VRE	*K. pneumoniae*	
ACK-2 (**1**)	18	22	40	6.4	34	11.5	50	188
ACK-4 (**2**)	8	7	7.3	3	10	8.1	26	139
ACK-6 (**3**)	3.5	4.8	5.1	2.3	4.1	7.6	24	120
ACK-8 (**4**)	3	3.5	4.1	5.4	3.2	4.2	6.1	90
ACK-10 (**5**)	9.6	6.6	35.3	15	6.2	7.6	10.3	96
NCK-4 (**6**)	>50	>50	>50	>50	>50	>50	>50	>1000
NCK-6 (**7**)	34	>50	42	18	>50	>50	>50	850
NCK-8 (**8**)	10	8.8	8	8.6	7	11	21	96
NCK-10 (**9**)	4	5.4	6	4.6	4	2.4	9	85
NCK-12 (**10**)	4.4	2.3	4.5	4.7	4	5	6	83
BCK-4 (**11**)	>50	>50	>50	>50	>50	N.D.	>50	>1000
BCK-6 (**12**)	>50	>50	>50	>50	>50	N.D.	>50	>1000
BCK-8 (**13**)	>50	>50	>50	>50	>50	>50	>50	565
BCK-10 (**14**)	9.4	11	11	6.6	26	9.6	>50	160
BCK-12 (**15**)	4.3	4	8	6.3	4.6	5.2	4.4	72
BCK-14 (**16**)	4.7	3	4.7	4.2	3.8	3.8	6.1	76
Vancomycin	0.6	0.6	N.D.	N.D.	0.6	>100	N.D.	N.D.
Colistin	20.0	>100	0.4	0.4	>50	>100	1	N.D.

Values are the averages of at least two experiments, each done in triplicate (error <5%)

To emphasize on the efficacy of the active compounds, their activity was tested against drug resistant superbugs such as VRE, MRSA and β-lactam resistant *K. pneumoniae*. Most of these compounds possessed remarkable activity against VRE (Table 3.1). ACK-8, NCK-10 and BCK-14 were the most potent compounds with MIC values of 4.2 μM, 2.4 μM and 3.8 μM respectively while vancomycin remained inactive till a concentration of 100 μM. ACK-8, NCK-10 and BCK-14 were also potent against MRSA with MICs around 3-4 μM for all. Other compounds of ACK series were also active against MRSA but to a lower extent. ACK-8 was also potent against β-lactam resistant *K. pneumoniae* (MIC = 6.1 μM). NCK-12 and BCK-14 were the most active compounds in their respective series with MICs of 6 μM each whereas colistin showed MIC of 1 μM.

In Table 3.2 we have listed the activity of four of the compounds (NCK-8, NCK-10, BCK-10 and BCK-12) against some clinical isolates of pathogenic bacteria. Against MRSA R3889 and MRSA R3890, which were isolated from two different patients at NIMHANS Bangalore. NCK-

Table 3.2: Activity of the compounds against clinical isolates of drug-resistant bacteria.

Bacterial Strains	Minimum Inhibitory Concentration (μM)			
	NCK-8	NCK-10	BCK-10	BCK-12
MRSA R3889	10.8	5.7	10.9	5.5
MRSA R3890	7.9	4.8	10.2	4.3
K. pneumoniae ATCC 700603	21	9	9.6	9
K. pneumoniae R3421	24	4.5	48	4.5
A. baumannii R674	11	1.5	23	1.5
A. baumannii R676	10	2.3	10.7	1.5
P. aeruginosa MTCC 424	8.5	4.5	6.6	6.3
P. aeruginosa R590	48	10.5	25	10.7
P. aeruginosa R3324	24	10.5	23	10.7
E. coli MTCC 443	8	5	10.7	10.7
E. coli R250	4.8	1.5	10	7.6
E. coli colistin resistant	10	4.7	4.6	1.6

Values are the averages of at least two experiments, each done in triplicate (error <5%)

10 displayed MICs of 5.7 μM and 4.8 μM respectively. BCK-12 was as active as NCK-10 against these strains with MICs of 5.5 μM and 4.3 μM respectively. Vancomycin was active at 0.6 μM and 0.7 μM respectively. NCK-8 and BCK-10 too were equally active against both the strains, but their MICs were around two-fold higher than their long chain homologues. The compounds displayed potency against clinical isolates of Gram-negative pathogens as well (Table 3.2). Against the clinical isolate of *K. pneumoniae* R3421, both NCK-10 and BCK-12 were equally potent with MIC of 4.5 μM. NCK-8 and BCK-10 were less active against *K. pneumoniae* R3421. The activity of NCK-10 and BCK-12 against *A. baumannii* was a highlight of these compounds. Against, a clinical isolate R674, both the compounds displayed a superior MIC of 1.5 μM. Again, both NCK-8 and BCK-10 was much less active than their higher long chain homologues. Since

the activity of these set of compounds toward a laboratory strain of *P. aeruginosa* (MTCC 424) was very good (MIC ranged from 4.5 μM to 9 μM), two clinical isolates were selected for the study. Both the strains of *P. aeruginosa* were MDR strains and the compounds displayed very good activity against them. Again, NCK-10 and BCK-12 turned out to be the most potent compounds. *P. aeruginosa* R590, was a little less susceptible to the compounds; NCK-10 and BCK-12 were active at 10.5 μM and 6 μM. Although BCK-10 displayed MIC of 25 μM against R590, NCK-8 was active only at 48 μM. The activity of the compounds varied from 6 μM to 11 μM against the laboratory strain of *E. coli* (MTCC). However against the clinical isolate *E. coli* R250, both NCK-10 and BCK-12 displayed a superior activity of 1.5 μM. The compounds displayed good potency against a colistin-resistant strain of *E. coli* as well (MIC ranged from 1.6 μM to 10 μM). Due to the excellent efficiency displayed by these compounds toward inhibiting the growth MDR clinical isolates, we were motivated to study their activity against New Delhi-metallo-β-lactamase 1 producing Gram-negative bacteria.

The activity of these compounds was evaluated against carbapenem-resistant *bla*$_{\text{NDM-1}}$ containing Gram-negative pathogens (Table 3.3). All the compounds studied were active against this group of bacteria at low concentrations. Against *E. coli* R3336, NCK-8 and BCK-10 were active at 20 μM and both NCK-10 and BCK-12 were four-fold more active with MICs of 4.5 μM each. Similar activity profile was observed against *K. pneumoniae*, NCK-10 and BCK-12 were around four-fold more active than NCK-8 and BCK-10. The compounds were found to be very

Table 3.3: Activity of the compounds against NDM-1 producing Gram-negative bacteria.

Compounds	Minimum Inhibitory Concentration (μM)			
	E.coli R3336	*K. pneumoniae* R3934	*P. aeruginosa* R596	*E. cloacae* R2928
NCK-8	20	20	20	9.5
NCK-10	4.5	4.5	4.5	4.5
BCK-10	20	20	10	10
BCK-12	4.5	4.5	4.5	4.5
Meropenem	>50	>50	N.D.	>50
Tetracycline	>50	>50	N.D.	>50

Values are the averages of at least two experiments, each done in triplicate (error <5%)

active against *P. aeruginosa* R596 with NCK-10 and BCK-12 displaying comparable activity (MIC 4.5 µM). Against *E. cloacae* too NCK-10 and BCK-12 were equally active (MICs of around 4.5 µM), whereas the activity of the other two compounds were two-fold less (MIC of 10 µM).

3.2.3 Kinetics of antibacterial activity

In order to obtain a deeper understanding of the nature of the activity of these compounds against bla_{NDM-1} containing pathogens, the kinetics of bactericidal activity of NCK-10, one of the most active compounds, was studied (Figure 3.1). In order to do the experiment, we chose to treat the bacteria with three different concentration of the compounds: at MIC/2, MIC and at 2 times its

MIC. As expected at MIC/2, no activity was observed. However, more than 3 log reduction in CFU was observed within the first thirty minutes upon treatment of compound even at its MIC. Even ACK-6 was found to be rapidly bactericidal against MRSA.

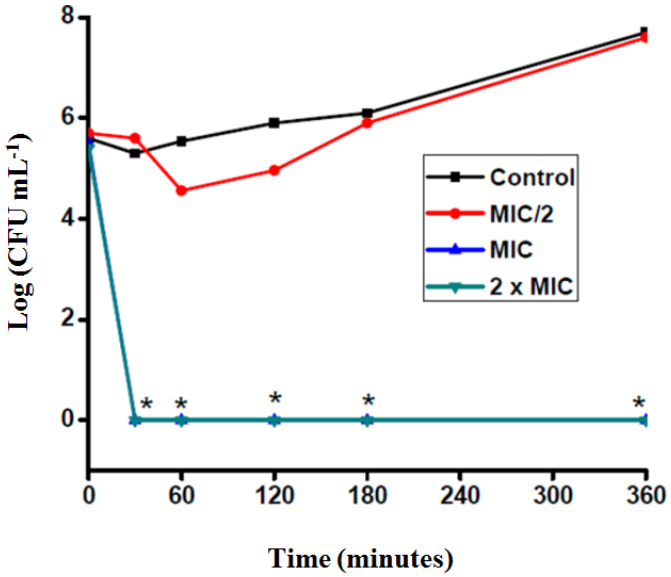


Figure 3.1: Time kill-kinetics of NCK-10 against *E. coli*. * indicate no bacteria observed. Detection limit is 50 CFU mL^{-1}. MIC considered is 5 µM.

3.2.4 Activity in different physiological conditions

The antibacterial activity of NCK-10 towards *S. aureus* was then studied in varying physiological conditions. This study was expected to provide an idea of the efficacy of the compound for different clinical applications. Pathogens such as *Mycobacterium tuberculosis, Helicobacter pylori* and even *S. aureus* can survive at very low pH after colonizing macrophages and gastric lining of the stomach.[176] Moreover, change in vaginal pH also leads to infection.[177] In cystic fibrosis too it is believed that low pH reduces the antimicrobial activity of airway surface liquid.[178] More importantly, the pH of the human skin is acidic (around pH 5.5).[179, 180]The study was carried out in varying pH and varying salinity. It is well known that different regions of the body have different pH. Thus, in order to gauge the potential of the compounds to treat infections in different regions of the body, it was important to understand its antimicrobial activity in different pH conditions. The antibacterial effect of NCK-10 was first determined in pH ranging from 3.5 to 8.5. At pH 3.5 and 4.5, almost no growth of *S. aureus* was observed. At pH 5.5 NCK-10 showed an activity of 9.4 µM against *S. aureus*. At pH 6.5, 7.4 and 8.5, the MIC remained the same at around 5 µM. Likewise, NCK-8, BCK-10 and BCK-12 displayed MICs of 10.8 µM, 10.9 µM and 5.5 µM against *S. aureus* at all pH tested (5.5 to 8.5). No bacterial growth was observed at pH 3.5, thus the activity of the compound at that pH could not be determined. However, at pH 4.5 and 5.5 the MIC of the compound against *E. coli* was 10 µM, while, at pH 6.5 two-fold more potency was observed against *E. coli*. At alkaline pH of 8.5, the compound retained its activity against *E. coli* (MIC of 5 µM).

Many natural membrane active agents have been reported to lose activity in physiological NaCl concentration of 140 mM.[181, 182] Thus, the efficacy of the compound in presence of varying salinity (percentage of NaCl) was subsequently studied. The concentrations of NaCl (85 mM, 170 mM, 260 mM and 1030 mM) in the growth media was varied first and the efficacy of the compound in the modified growth media was studied. Against *S. aureus* the compound retained its antibacterial activity at all concentrations. Not only NCK-10, the other compounds also retained their activity in these concentrations of NaCl. Against *E. coli* the activity of the compound was reduced by two-fold at the highest concentration of 3% NaCl.

On varying the concentration of $MgCl_2$ from 20 mM to 80 mM, it was observed that at higher concentrations of the metal, the compound loses activity significantly but no loss of antibacterial activity was observed at 20 mM. Since, the serum concentration of divalent cations like Mg^{2+} are

around 3 mM, the compound is expected to work in presence of serum. Antimicrobial peptides are known to lose their activity in presence of divalent cations such as Ca^{2+} and Mg^{2+}. Thus we studied the antibacterial effect of the NCK-10 in different concentrations of $MgCl_2$. The concentration of $MgCl_2$ was varied from 20 mM to 80 mM. As expected the effect against *E. coli* was much more pronounced. Even at 20 mM, the activity of the compound dropped to 19 µM in case of *E. coli* while at higher concentrations the compound was not active till 50 µM.

3.2.5 Propensity to induce resistance development

As mentioned earlier, the current crisis in antibiotic drug development is the rapid rate at which bacteria develop resistance toward drugs. Since in this report we have explored the possibility of these compounds as antibacterial agents towards treatment against MDR clinical isolates of pathogens, it was imperative to study if the compounds themselves possess any propensity to induce bacterial resistance. Thus, in order to evaluate the potential of these compounds as long-lasting antibacterial agents, the ability of *E. coli* to develop resistance against these compounds were studied (Figure 3.2). NCK-10 was chosen as a model compound for this study. As a positive control for *E. coli* colistin was used. The MIC of colistin increased on the fourth day of the experiment. The MIC of NCK-10 toward *E. coli* did not change even after 20 passages respectively, whereas the MIC of colistin increased by 250 fold. As a positive control for *S. aureus*,

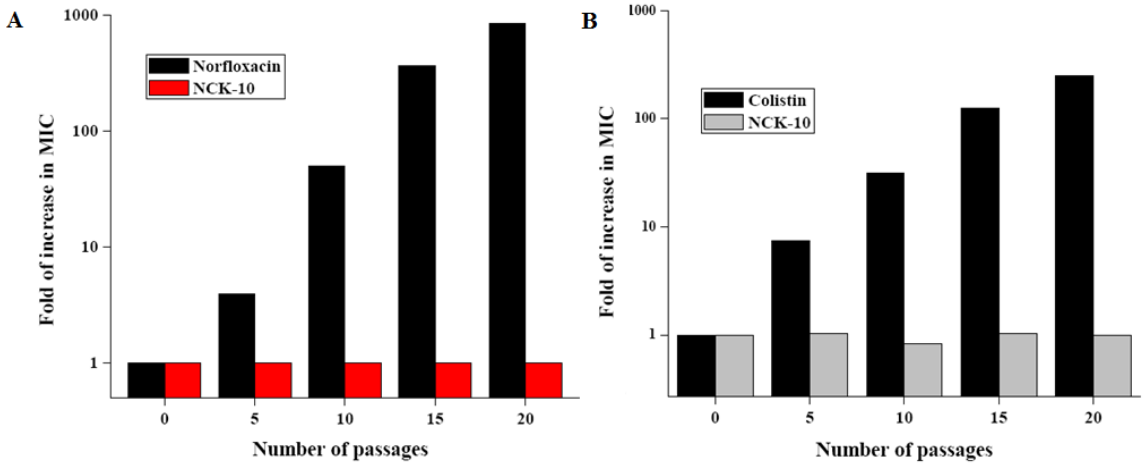


Figure 3.2: Ability of the compound to halt resistance development in bacteria. A) *S. aureus* (norfloxacin is used as a comparator) and B) *E. coli* (colistin is used as a comparator).

norfloxacin was used. The MIC of NCK-10 toward *S. aureus* (MTCC strain) remained unchanged even after 20 passages, whereas the MIC of norfloxacin increased by 800 fold. This proved that bacteria found it more difficult to develop resistance against NCK-10 in comparison to a clinically used antibiotic norfloxacin

3.2.6 Mechanism of antibacterial action

Investigation into the mechanism of action of the most selective compounds ACK-6 and NCK-10 (model compounds) using various spectroscopic techniques revealed that these compounds primarily act by targeting the bacterial cell membrane.

3.2.6.1 Cytoplasmic membrane depolarization

Experiments with the membrane-potential sensitive dye $DiSC_3(5)$ showed that ACK-6 (Figure 3.3A) rapidly depolarize the membrane of *S. aureus* cells. In fact, the membranes of Gram-negative species *P. aeruginosa* and *E. coli* were also depolarized by ACK-6.

3.2.6.2 Inner membrane permeabilization

Bacterial cytoplasmic membrane permeabilization was studied using the fluorescent probe propidium iodide. Although both ACK-6 and NCK-10 were able to permeabilize the *S. aureus*

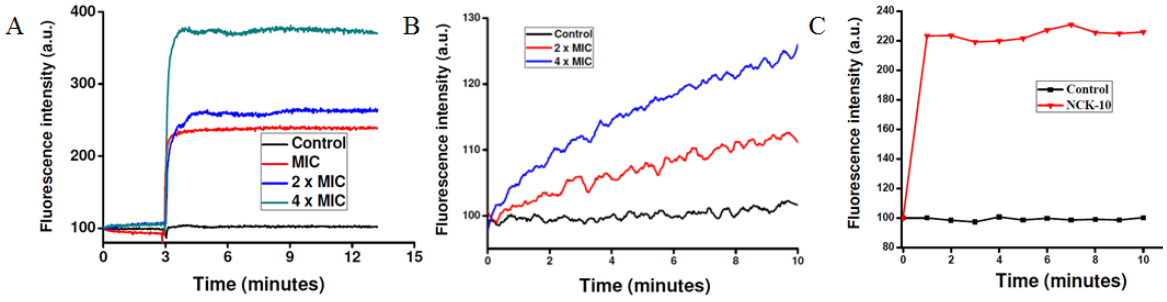


Figure 3.3: Ability of the compounds to act on bacterial membrane. A) Depolarization of *S. aureus* membrane by ACK-6. B) Inner membrane permeabilization of *S. aureus* by ACK-6. MIC of ACK-6 considered is 3.5 µM. C) Outer membrane permeabilization of *E. coli* by NCK-10 at 15 µM.

membrane, similar effect was not observed against Gram-negative cells. In Figure 3.3B the effect of ACK-6 on cytoplasmic membrane of *S. aureus* has been furnished.

3.2.6.3 Outer membrane permeabilization

Experiments using fluorescent probe *N*-phenylnaphthylamine, with NCK-10 showed that it could cause permeabilization of Gram-negative outer membrane at 3×MIC (Figure 3.3C). The same experiment could not be performed with ACK-6 as it absorbs at the same wavelength as the dye.

3.2.6.4 Live/dead assay

To shed further light on the mechanism of action of the compounds, fluorescence microscopic studies were carried out using the so called "live-dead stain". To carry out microscopic studies, the bacteria were incubated with a mixture of two dyes, SYTO-9 (green fluorescence) and Propidium iodide (red fluorescence). Both the dyes act on the nucleic acids of the cells. However, while SYTO-9 can pass through the membranes of both live and dead cells, PI can pass through only the membrane-compromised cells and compete with SYTO-9. As a result when viewed under different filters, the live cells appear green while the dead cells appear red. It was observed that in the control (untreated bacteria), the colour of the overlapped image was green, proving that the

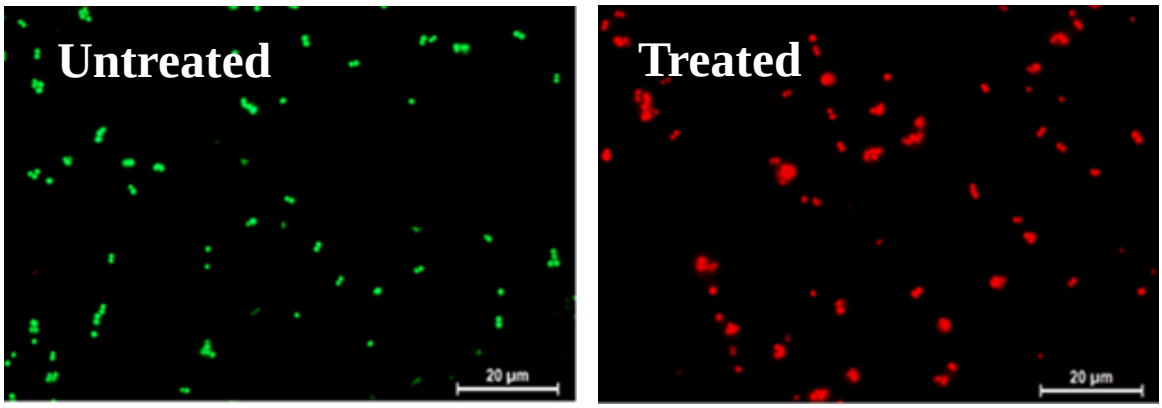


Figure 3.4: Ability of the ACK-6 (10×MIC) to permeabilize the membrane of *S. aureus* to allow the influx of Propidium iodide (red) in comparison to untreated control wherein the cells are stained by SYTO-9 (green) instead of PI. MIC considered is 3.5 µM.

membranes were intact (Figure 3.4). Whereas, in case of ACK-6 treated bacteria, the observed colour was red, proving that bacterial cell membranes were indeed compromised (Figure 3.4).

3.2.6.5 Visualization of the effect on membranes of bacteria

To visualize the effect of the compounds on bacterial cells, *E. coli* and *S. aureus* cells were incubated with ACK-6 (10 × MIC) and observed under Scanning electron microscope (Figure 3.5). As can be observed from Figure 3.5A, in the control the *E. coli* cells were intact, however *E. coli* cells treated with ACK-6 were deformed. Their membranes were disrupted as a result more than one bacteria merge with each other and cannot be distinguished. Similarly, intact *S. aureus* cells

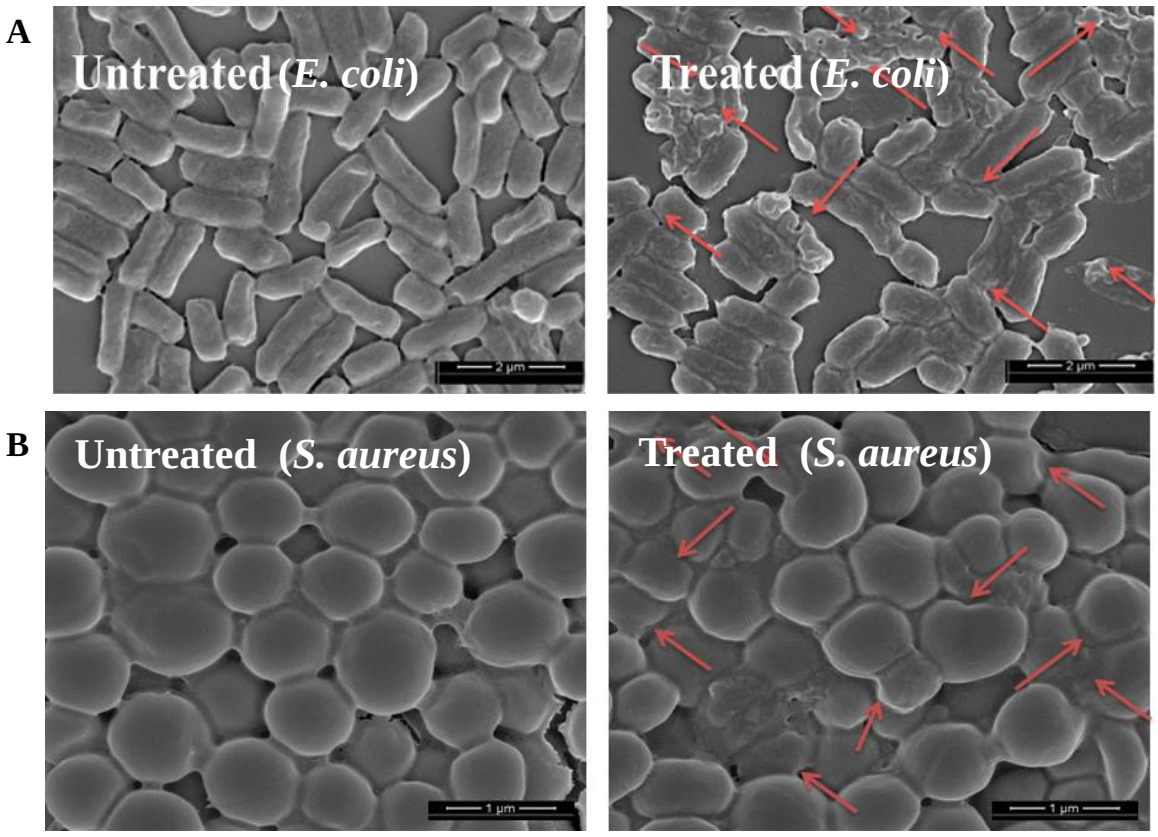


Figure 3.5: FESEM studies to visualize the effect of the compounds on bacteria A) Membranes of *E. coli* cells are damaged upon treatment with ACK-6 in comparison to untreated control. B) *S. aureus* are intact in untreated control but holes are observed when treated with ACK-6 at 10×MIC. MIC considered is 3.5 µM.

were observed in the control and in the treated sample, formation of holes on the surface of *S. aureus* was observed (Figure 3.5 B). These images are a visual proof of the membrane damaging properties of the compounds.

3.2.7 Activity against anaerobic bacteria

3.2.7.1 Activity against different strains of *Clostridium difficile*

Colitis or inflammatory bowel disease caused by *Clostridium difficile* is a growing health problem in all parts of the world. CDC reports 29,000 deaths in USA alone for *C. difficile* infections. It is also responsible for a burden of around $4.8 billion to the economy of US health care.[183] The major problem associated with *C. difficile* is relapse of disease condition once antibiotics are taken off. Moreover, spores of *C. difficile* are recalcitrant to heat and sanitizers, which make them ideal transmissible agents. In this respect developing new antibacterial agents to treat *C. difficile* infections represents an urgent need.[184] Given, the ability of these compounds to infiltrate the membrane of bacteria and the fact that they retain activity in acidic pH, we tested the antibacterial activity of the NCK series of compounds against different strains of *C. difficile*. Vancomycin and metronidazole, which are clinically used to treat *C. difficile* infections were used as comparator drugs. NCK-6 was not active against these strains till 50 µM, while the MIC of NCK-8 ranged from 3 µM to 13 µM. Both NCK-10 and NCK-12 were quite active against these strains with MIC ranging from 1 µM to 6 µM. The strains were susceptible to vancomycin and metronidazole on an average at MIC of 0.5 µM and 1.2 µM respectively. This study showed that the activity of NCK-10 was comparable to that of vancomycin and metronidazole. However, as mentioned earlier, one of the major problems associated with these infections is their recurrence after antibiotic treatment is taken off. Spores of these bacteria were responsible for recurrence of the infection. Hence, we proceeded to check the activity of the compounds against *C. difficile* spores. Only NCK-10 were active against the spores at MIC of 6 µM.

3.2.7.2 Activity against human gut normal flora

We next checked whether the commensal bacteria were susceptible to NCK-10 at the same concentrations. We carried out the activity of the compounds against commensals such as *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium bifidium* and *Bifidobacterium*

breve. Again, vancomycin and metronidazole were used as comparator antibiotics. Metronidazole was inactive against *Lactobacillus sp.* even till 46 µM. Vancomycin was also inactive till 5.5 µM against *L. casei* but active against *L. acidophilus* at 0.7 µM. Although NCK-8 and NCK-12 were active only at 12 µM and 23 µM, NCK-10 was active at 2 µM itself. In case of the bacteria as well, a similar trend was observed. *Bifidobacterium sp.* was susceptible to NCK-10 at 1.5 µM. It could thus be concluded that NCK series of compounds, especially NCK-10 was not selectively active against pathogenic cells, leaving a scope for further improvement.

Table 3.2: Activity against *C. difficile* strains

C. difficile (strain)	Minimum inhibitory concentration (µM)					
	NCK-6	NCK-8	NCK-10	NCK-12	Vancomycin	Metronidazole
P8 (32888)	107	13	1.5	6	0.25	0.29
P13 (32891)	107	13	3.1	3	0.25	0.56
Isolate 1 (13427)	107	13	3.1	6	2.00	0.56
Isolate 10 (13436)	107	13	1.5	3	0.25	2.94
Isolate 4 (13430)	107	13	3.1	3	0.13	0.13
Isolate 5 (13431)	54	3	<0.7	1	0.25	0.56
Isolate 9 (13435)	54	6	1.5	3	0.34	0.20
P 21 (32897)	54	6	1.5	3	0.17	0.41
P 29 (32903)	54	13	1.5	3	0.17	0.20
P 20 (32896)	107	13	1.5	3	>0.7	0.82
P 2 (32883)	107	6	1.5	1	0.17	0.20
P 15 (32892)	107	13	1.5	3	0.34	0.20
Isolate 6 (13432)	107	13	1.5	3	0.34	0.41
P 15 (32892)	54	6	<0.7	1	0.17	0.10
Isolate 2 (13428)	107	13	1.5	3	0.67	0.20
P 4 (32889)	107	13	3.1	6	1.35	0.41
Spores	>200	51	6.1	47	<0.7	<3

Values are the averages of at least two experiments, each done in triplicate (error <5%)

3.2.8 *In vivo* activity

3.2.8.1 Acute Dermal toxicity in mice

The acute dermal toxicity was performed in accordance with the OECD guidelines. To do the study we used 200 mg kg^{-1} of NCK-10 (5 times the concentration used in the subsequent infection study). Careful visual observation of the animals showed that application of the compound caused no irritation, tremors, convulsions, salivation or diarrhea. In fact, steady growth of fur was observed on the mice even after the application of the compound. By the end of seven days, almost complete regeneration fur was observed in all the test animals. No morbidity was observed among the test animals and it was concluded that the compound caused no toxicity to the skin even at concentrations as high as 200 mg kg^{-1}.

3.2.8.2 *In vivo* murine model of skin-infection caused by MRSA

In vitro activity of the compounds against MRSA was translated into mice model of skin infection. To do the experiment, a wound was first induced while shaving the back of each mouse until skin tissue was red and glistening. The wounds were then inoculated with approximately 10^7 CFU of multi drug-resistant methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33592). In this model treatment of the compounds was initiated four hours after infection was set. NCK-10 or fusidic acid, was dosed at 40 mg kg^{-1} (once a day) for seven days by adding a 40 µL droplet (concentration 20 mg mL^{-1}) on the infected site. The droplet was gently spread on the entire surface of the wound to avoid any drop from rolling down the sides. The compound was able to bring down bacterial burden significantly (p value 0.00014) in a course of seven days (Figure 3.6A). In fact the effect of the compound was better than that of the approved drug fusidic acid at the same concentration.

3.2.8.3 *In vivo* murine model of *Acinetobacter baumannii* burn-infection

This experiment was performed as reported in Chapter 2. The wound was treated with NCK-10 at a concentration of 40 mg kg^{-1} and colistin was used as a positive control (concentration used was 5 mg kg^{-1}). After treating with the compound for 7 days (once daily), the bacterial burden was brought down significantly in comparison to control (Figure 3.6B). Colistin, on the other hand, was extremely effective; no colony was observed within the detection limit. However, colistin, as mentioned previously, is prone to triggering resistance development unlike NCK-10.

Overall, it is quite clear that in this series of molecules, more selective compounds were obtained which could act against both MRSA model of skin-infection as well *A. baumannii* model of burn-infection.

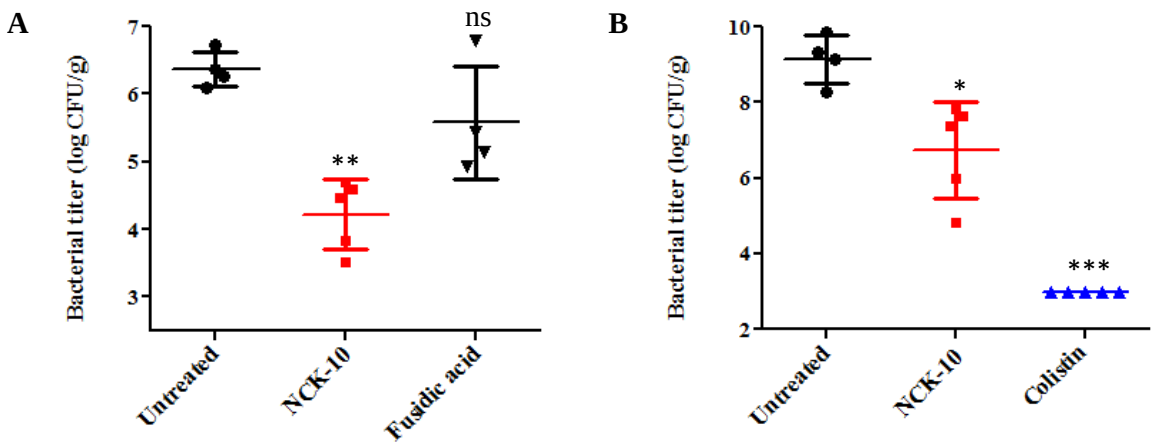


Figure 3.6: *In vivo* activity A) Against MRSA models of skin-infection, NCK-10 (at 40 mg kg^{-1}) is more active in comparison to fusidic acid (40 mg kg^{-1}). B) Against *A. baumannii* burn infection. Concentration of NCK-10 was 40 mg kg^{-1} and that of colistin was 5 mg kg^{-1}. Statistical analysis was performed using Student's t-test. Differences are considered statistically significant from the untreated group with a value of $P<0.05$ with 95% confidence intervals (*** indicate $P<0.001$).

3.3 Conclusions

A significant improvement in broad-spectrum activity was noticed upon introduction of the aromatic moiety. This is best reflected in the *in vivo* activity studies. Unlike C10-K-C8, NCK-10 was not at all toxic to the skin of mice emphasizing on the selective efficacy of this set of compounds. Membrane-activity remained the primary mechanism of action and thus no resistance development was triggered in either *S. aureus* or *E. coli*. Excellent activity was obtained against *C. difficile* related infections although the compound was also toxic to commensal bacteria.

Although NCK-10 was quite effective in murine models of infection, there is still scope for improvement.

3.4 Experimental Section

3.4.1 Materials and Instrumentation:

All the solvents were of reagent grade and were distilled and dried prior to use wherever required. Chloroform and methanol were supplied by Merck-India. Dimethylformamide, Dicholoromethane, Diethyl ether and other solvents were supplied either by SDFCL (India) or Spectrochem (India). L-Lysine, Di-*tert*-butyl carbonate, Diisopropylethylamine, HBTU, Butylamine, Hexylamine, Octylamine, Benzaldehyde, Trifluoroacetic acid were purchased from Spectrochem (India). 10-choloro-9-anthraldehyde, 1-Naphthaldehyde, Ethylamine, Decylamine, Dodecylamine and Tetradecylamine were purchased from Sigma-Aldrich. All the chemicals were used as supplied. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates pre-coated with silica gel 60 F_{254} (250 μm thickness). Visualization was accomplished using UV light and Iodine. Column chromatography was performed on silica gel (60-120 Å pore size). HPLC analysis was performed on a Shimadzu-LC 8A Liquid Chromatograph instrument (C18 column, 10 mm diameter, 250 mm length) with UV detector monitoring at 254 nm. Nuclear magnetic resonance spectra were recorded on Bruker (AV-400) 400 MHz spectrometer in deuterated solvents. Infrared (IR) spectra of the solid compounds were recorded on Bruker IFS66 V/s spectrometer using KBr pellets. IR spectra of the compounds soluble in low-boiling solvents were recorded with the same instrument using NaCl crystal. High-resolution Mass Spectrometry was recorded on Agilent 6538 Q-TOF LC-MS system and Shimadzu LC-MS 2020 spectrometer. Optical density was measured by TecanInfinitePro series M200 Microplate Reader. Bacterial strains, *Staphylococcus aureus* (MTCC 737), *Pseudomonas aeruginosa* (MTCC 424) and *Escherichia coli* (MTCC 443) were obtained from MTCC (Chandigarh, India). *Enterococcus faecium* (ATCC 19634), methicillin resistant *S. aureus* (ATCC 33591), β-lactamase producing and drug-resistant *Klebsiella pneumonia* (ATCC700603) and vancomycin resistant *Enterococcus faecium* (ATCC 51559) were obtained from ATCC.

Animals: 6-8-week old Balb/c mice weighing 20 to 25 g were used for all studies. The animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) and carried out

as per the guidelines of Committee for the purpose of Supervision and Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi.

3.4.2 Synthetic procedure and characterization of compounds

3.4.2.1 General procedure for synthesis of *N*-alkylaminomethylarene hydrochlorides (1a-16a): In a typical reaction aromatic aldehydes (2.08 mmol) and alkyl amines (2.08 mmol) were dissolved in a 1:1 mixture of dry chloroform and methanol (20 ml) and stirred at room-temperature (under Nitrogen atmosphere) for 6 h. The resulting clear solution was then cooled to 0°C, and to it Sodium borohydride (3.75 mmol) was added. This was allowed to come to room temperature and stirred overnight. Then the solvents were evaporated under reduced pressure (not to dryness) and diluted with diethyl ether. To this 2N NaOH (20 ml) was added and stirred for 15 minutes. After separation from the NaOH layer, the organic layer was subsequently washed with water twice, brine and dried over $MgSO_4$. The organic layer was then evaporated under reduced pressure and the residue was dissolved minimum volume of methanol. To this 4N HCl (3 ml) was added and instantaneous formation of precipitate was observed. The solvent was completely removed and the precipitate was dissolved in minimum volume of ethyl acetate (a few drops of methanol was added to dissolve completely). To this hexane was added to obtain pure crystals of the target compound (Yield: 65-90%). These crystals were filtered, dried and subsequently characterized using ^{1}H NMR, and Mass spectrometry.

***N*-ethyl-10-aminomethyl-9-chloroanthracene hydrochloride (1a):** Yield-80%. ^{1}H-NMR ($CDCl_3$) δ/ppm: 10.0 (s, Ar-CH_2-*NH_2*-C_2H_5, 2H), 8.52 (d, Ar*H*, 2H), 8.35 (d, Ar*H*, 2H), 7.70 (t, Ar*H*, 2H), 7.61 (t, Ar*H*, 2H), 4.9 (s, Ar-*CH_2*-NH_2-, 2H), 2.75 (d,-NH_2-*CH_2*-CH_3, 2H), 1.24 (t, -NH_2-CH_2-*CH_3*, 3H); HRMS (m/z): $[M+H]^+$ obsd. = 270.1052 (calc. = 270.1050).

***N*-butyl-10-aminomethyl-9-chloroanthracene hydrochloride (2a):** Yield-100%. ^{1}H-NMR ($CDCl_3$) δ/ppm: 9.9 (s, Ar-CH_2-*NH_2*-C_4H_9, 2H), 8.52 (d, Ar*H*, 2H), 8.38 (d, Ar*H*, 2H), 7.72 (t, Ar*H* 2H), 7.62 (t, Ar*H*, 2H), 4.97 (s, Ar-*CH_2*-NH_2-, 2H), 2.65 (d, -NH_2-*CH_2*-C_3H_7, 2H), 1.73 (m, -NH_2-CH_2-*CH_2*-C_2H_5, 2H), 1.18 (q, -NH_2-C_2H_4-*CH_2*-CH_3, 2H), 0.74 (t, -NH_2-C_3H_6-*CH_3*, 3H); HR-MS (m/z): $[M+H]^+$ obsd. = 298.1337 (calc. = 298.1357).

***N*-hexyl-10-aminomethyl-9-chloroanthracene hydrochloride (3a):** Yield- 75%. ^{1}H-NMR ($CDCl_3$) δ/ppm: 9.9 (s, Ar-CH_2-*NH_2*-C_6H_{13}, 2H), 8.52 (d, Ar*H*, 2H), 8.38 (d, Ar*H*, 2H), 7.72 (t, Ar*H*, 2H), 7.62 (t, Ar*H*, 2H), 4.97 (s, Ar-*CH_2*-NH_2-, 2H), 2.64 (d, -NH_2-*CH_2*-C_5H_{11}, 2H), 1.74 (t,-NH_2-CH_2-*CH_2*-C_4H_9, 2H), 1.18-1.0 (m, -NH_2-C_2H_5-*$(CH_2)_3$*-CH_3, 6H), 0.74 (t, -NH_2-C_5H_{10}-*CH_3*, 3H); HR-MS (m/z): $[M+H]^+$ obsd. = 326.1670 (calc. = 326.1676).

***N*-octyl-10-aminomethyl-9-chloroanthracene hydrochloride (4a):** Yield-75%. ^{1}H-NMR ($CDCl_3$) δ/ppm: 9.9 (s, Ar-CH_2-*NH_2*-C_8H_{17}, 2H), 8.52 (d, Ar*H*, 2H), 8.38 (d, Ar*H*, 2H), 7.72 (t, Ar*H*, 2H), 7.62 (t, Ar*H*, 2H), 4.97 (s, Ar-*CH_2*-NH_2-, 2H), 2.64 (d,-NH_2-*CH_2*-C_7H_{14}, 2H), 1.74 (t,-NH_2-CH_2-*CH_2*-C_6H_{13}, 2H), 1.2-1.0 (m, -NH_2-C_2H_5-*$(CH_2)_5$*-CH_3, 10H), 0.79 (t, -NH_2-C_7H_{14}-*CH_3*, 3H); HR-MS (m/z): $[M+H]^+$ obsd. = 354.1960 (calc. = 354.1983).

***N*-decyl-10-aminomethyl-9-chloroanthracene hydrochloride (5a):** Yield-80%. ^{1}H-NMR ($CDCl_3$) δ/ppm: 9.9 (s, Ar-CH_2-*NH_2*-$C_{10}H_{21}$, 2H), 8.52 (d, Ar*H*, 2H), 8.38 (d, Ar*H*, 2H), 7.72 (t, Ar*H*, 2H), 7.62 (t, Ar*H*, 2H), 4.97 (s, Ar-*CH_2*-NH_2-, 2H), 2.64 (d, -NH_2-*CH_2*-C_9H_{19}, 2H), 1.74 (t, -NH_2-CH_2-*CH_2*-C_8H_{17}, 2H), 1.18-1.0 (m, -NH_2-C_2H_5-*$(CH_2)_7$*-CH_3, 14H), 0.82 (t, -NH_2-C_9H_{18}-*CH_3*, 3H); HR-MS (m/z): $[M+H]^+$ obsd. = 382.2273 (calc. = 382.2296).

***N*-butyl-1-aminomethylnaphthalene hydrochloride (6a):** Yield-78%. ^{1}H NMR ($CDCl_3$) δ/ppm: 9.9 (s, Ar-CH_2-*NH_2*-C_4H_9, 2H), 8.12 (d, Ar*H*, 1H), 7.85 (m, Ar*H*, 3H), 7.64 (t, Ar*H*, 1H), 7.52 (q, Ar*H*, 2H), 4.5 (s, Ar-*CH_2*-NH_2-, 2H), 2.77 (m, -NH_2-*CH_2*-C_3H_7, 2H), 1.83 (m, -NH_2-CH_2-*CH_2*-C_2H_5, 2H), 1.29 (m, -NH_2-C_2H_4-*CH_2*-CH_3, 2H), 0.82 (t, -NH_2-C_3H_6-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 214.1567 (calc. = 214.159).

***N*-hexyl-1-aminomethylnaphthalene hydrochloride (7a):** Yield-80%. ^{1}H NMR ($CDCl_3$) δ/ppm: 9.9 (s, Ar-CH_2-*NH_2*-C_6H_{13}, 2H), 8.12 (d, Ar*H*, 1H), 7.85 (m, Ar*H*, 3H), 7.64 (t, Ar*H*, 1H), 7.51 (q, Ar*H*, 2H), 4.5 (s, Ar-*CH_2*-NH_2-, 2H), 2.75 (t, -NH_2-*CH_2*-C_5H_{11}, 2H), 1.85 (q, -NH_2-CH_2-*CH_2*-C_4H_9, 2H), 1.2 (m, -NH_2-C_2H_5-*$(CH_2)_3$*-CH_3, 6H), 0.82 (t, -NH_2-C_5H_{12}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 242.1889 (calc. = 242.1903).

***N*-octyl-1-aminomethylnaphthalene hydrochloride (8a):** Yield-78%. ^{1}H NMR ($CDCl_3$) δ/ppm: 9.9 (s, Ar-CH_2-*NH_2*-C_8H_{17}, 2H), 8.12 (d, Ar*H*, 1H), 7.85 (m, Ar*H*, 3H), 7.64 (t, Ar*H*, 1H), 7.51 (q, Ar*H*, 2H), 4.5 (s, Ar-*CH_2*-NH_2-, 2H), 2.75 (t, -NH_2-*CH_2*-C_5H_{11}, 2H), 1.85 (q, -NH_2-CH_2-*CH_2*-C_6H_{13}, 2H), 1.3-1.1 (-NH_2-C_2H_5-(*CH_2*)$_5$-CH_3,10H), 0.82 (t, -NH_2-C_7H_{14}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 270.252 (calc. = 270.2216).

***N*-decyl-1-aminomethylnaphthalene hydrochloride (9a):** Yield-65%. ^{1}H NMR ($CDCl_3$) δ/ppm: 10 (s, Ar-CH_2-*NH_2*-C_8H_{17}, 2H), 8.12 (d, Ar*H*, 1H), 7.85 (m, Ar*H*, 3H), 7.64 (t, Ar*H*, 1H), 7.51 (q, Ar*H*, 2H), 4.5 (s, Ar-*CH_2*-NH_2-, 2H), 2.75 (t, -NH_2-*CH_2*-C_5H_{11}, 2H), 1.85 (q, -NH_2-CH_2-*CH_2*-C_6H_{13}, 2H), 1.3-1.1 (-NH_2-C_2H_5-(*CH_2*)$_5$-CH_3,14H), 0.82 (t, -NH_2-C_7H_{14}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 298.2541 (calc. = 298.2535).

***N*-dodecyl-1-aminomethylnaphthalene hydrochloride (10a):** Yield-65%.^{1}H NMR ($CDCl_3$) δ/ppm: 10 (s, Ar-CH_2-*NH_2*-C_8H_{17}, 2H), 8.12 (d, Ar*H*, 1H), 7.85 (m, Ar*H*, 3H), 7.64 (t, Ar*H*, 1H), 7.51 (q, Ar*H*, 2H), 4.5 (s, Ar-*CH_2*-NH_2-, 2H), 2.75 (t, -NH_2-*CH_2*-C_5H_{11}, 2H), 1.85 (q, -NH_2-CH_2-*CH_2*-C_6H_{13}, 2H), 1.3-1.1 (-NH_2-C_2H_5-(*CH_2*)$_5$-CH_3,18H), 0.82 (t, -NH_2-C_7H_{14}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 326.2839 (calc. = 326.2848).

***N*-butyl-1-aminomethylbenzene hydrochloride (11a)**: Yield-78%. ^{1}H NMR ($CDCl_3$) δ/ppm: 9.87 (s, Ar-CH_2-*NH_2*-C_4H_9, 2H), 7.6 (d, Ar*H*, 2H), 7.39 (m, Ar*H*, 3H), 4.02 (s, Ar-*CH_2*-NH_2-, 2H), 2.79 (t, -NH_2-*CH_2*-C_3H_7, 2H), 1.82 (m, -NH_2-CH_2-*CH_2*-C_2H_5, 2H), 1.4 (-NH_2-C_2H_4-*CH_2*-CH_3, 2H), 0.82 (t, -NH_2-C_3H_6-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 164.1430 (calc. = 164.1439).

***N*-hexyl-1-aminomethylbenzene hydrochloride (12a)**: Yield-80%. ^{1}H NMR ($CDCl_3$) δ/ppm: 9.85 (s, Ar-CH_2-*NH_2*-C_6H_{13}, 2H), 7.6 (d, Ar*H*, 2H), 7.39 (m, Ar*H*, 3H), 4.02 (s, Ar-*CH_2*-NH_2-, 2H), 2.77 (t,-NH_2-*CH_2*-C_5H_{11}, 2H), 1.84 (m, -NH_2-CH_2-*CH_2*-C_4H_9, 2H), 1.25 (m, -NH_2-C_2H_4-*$(CH_2)_3$*-CH_3, 6H), 0.84 (t, -NH_2-C_5H_{10}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 192.1777 (calc. = 192.1747).

***N*-octyl-1-aminomethylbenzene hydrochloride (13a)**: Yield-78%. ^{1}H NMR ($CDCl_3$) δ/ppm: 9.87 (s, Ar-CH_2-*NH_2*-C_8H_{13}, 2H), 7.6 (d, Ar*H*, 2H), 7.39 (m, Ar*H*, 3H), 4.02 (s, Ar-*CH_2*-NH_2-,

2H), 2.77 (m, -NH_2-*CH_2*-C_7H_{15}, 2H), 1.84 (m, -NH_2-CH_2-*CH_2*-C_6H_{13}, 2H), 1.25 (m, -NH_2-C_2H_5-*$(CH_2)_5$*-CH_3, 10H), 0.84 (t, -NH_2-C_7H_{14}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 220.2122 (calc. = 220.206).

***N*-decyl-1-aminomethylbenzene hydrochloride (14a)**: Yield-65%. ^{1}H NMR ($CDCl_3$) δ/ppm: 9.87 (s, Ar-CH_2-*NH_2*-C_8H_{13}, 2H), 7.6 (d, Ar*H*, 2H), 7.39 (m, Ar*H*, 3H), 4.02 (s, Ar-*CH_2*-NH_2-, 2H), 2.77 (m, -NH_2-*CH_2*-C_7H_{15}, 2H), 1.84 (m, -NH_2-CH_2-*CH_2*-C_6H_{13}, 2H), 1.25 (m, -NH_2-C_2H_5-*$(CH_2)_5$*-CH_3, 14H), 0.84 (t, -NH_2-C_7H_{14}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 247.2122 (calc. = 247.23).

***N*-dodecyl-1-aminomethylbenzene hydrochloride (15a)**: Yield-65%.^{1}H NMR ($CDCl_3$) δ/ppm: 9.87 (s, Ar-CH_2-*NH_2*-C_8H_{13}, 2H), 7.6 (d, Ar*H*, 2H), 7.39 (m, Ar*H*, 3H), 4.02 (s, Ar-*CH_2*-NH_2-, 2H), 2.77 (m, -NH_2-*CH_2*-C_7H_{15}, 2H), 1.84 (m, -NH_2-CH_2-*CH_2*-C_6H_{13}, 2H), 1.25 (m, -NH_2-C_2H_5-*$(CH_2)_5$*-CH_3, 18H), 0.84 (t, -NH_2-C_7H_{14}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 276.2693 (calc. = 276.2691).

***N*-tetradecyl-1-aminomethylbenzene hydrochloride (16a)**: Yield-65%. ^{1}H NMR ($CDCl_3$) δ/ppm: 9.87 (s, Ar-CH_2-*NH_2*-C_8H_{13}, 2H), 7.6 (d, Ar*H*, 2H), 7.39 (m, Ar*H*, 3H), 4.02 (s, Ar-*CH_2*-NH_2-, 2H), 2.77 (m, -NH_2-*CH_2*-C_7H_{15}, 2H), 1.84 (m, -NH_2-CH_2-*CH_2*-C_6H_{13}, 2H), 1.25 (m, -NH_2-C_2H_5-*$(CH_2)_5$*-CH_3, 22H), 0.84 (t, -NH_2-C_7H_{14}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 304.3006 (calc. = 304.3004).

3.4.2.2 General synthetic procedure for amide coupling reactions: In a typical amide coupling reaction, to a stirred solution of Boc-Lys(Boc)-OH (0.2 g, 0.58 mmol) in 5:2 DMF/$CHCl_3$(7 ml), was added *N*,*N*-Diisopropylethylamine (DIPEA, 250 µL, 1.44 mmol) at 0°C. To this solution was then added HBTU (0.22 g, 0.58 mmol). This mixture was stirred for 5 minutes at 0°C and subsequently the secondary amines (**1a-16a,** 0.48 mmol) were added to it. The mixture was stirred at 0°C for 30 minutes and subsequently at RT for 24 h typically. At the end, $CHCl_3$wasevaporated under reduced pressure and the resulting solution was diluted to 2 times its original volume by addition of ethyl acetate. This mixture was subsequently washed with 0.5 M $KHSO_4$, H_2O (thrice) and brine. After passage through anhydrous Na_2SO_4, the organic layer was evaporated under reduced pressure and the residue was purified using column chromatography (only $CHCl_3$) to

obtain the product in 65%-90% yield. The purified compound was subsequently characterized using ^{1}H NMR, IR and Mass spectrometry.

Boc-Lys(Boc)-*N*-ethyl-10-Aminomethyl-9-chloroanthracene (1b): Yield-81%. ^{1}H-NMR ($CDCl_3$) δ/ppm: 8.61 (d, Ar*H*, 2H), 8.29 (d, Ar*H*, 2H), 7.60 (m, Ar*H*, 4H), 6.05 (d, Ar-C*H*^{1}H^{2}-N(R)Lys(boc)$_2$, 1H), 5.42 (d, α-N*H*-Boc of Lys(boc)$_2$, 1H), 5.34 (d, Ar-CH^{1}*H*^{2}-N(R)Lys(boc)$_2$, 1H), 4.56 (m, Lys (ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 2H) 3.1-2.81 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-CH$_3$)Lys(boc)$_2$, 4H), 1.5-1.3 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$Lys(boc)$_2$, 24H), 1.03 (t,-CH$_2$-C*H*$_3$ of R group, 3H). FT-IR (cm^{-1}): 3354 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1704 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HRMS (m/z): [M+H]$^+$ obsd. = 598.3043 (calc. = 598.2969).

Boc-Lys(Boc)-*N*-butyl-10-Aminomethyl-9-chloroanthracene (2b): Yield-83%. ^{1}H-NMR ($CDCl_3$) δ/ppm: 8.61 (d, Ar*H*, 2H), 8.29 (d, Ar*H*, 2H), 7.60 (m, Ar*H*, 4H), 6.08 (d, Ar-C*H*^{1}H^{2}-N(R)Lys(boc)$_2$, 1H), 5.42 (d, α-N*H*-Boc of Lys(boc)$_2$, 1H), 5.29 (d, Ar-CH^{1}*H*^{2}-N(R)Lys(boc)$_2$, 1H), 4.56 (m, Lys(ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 2H) 3.1-2.81 (δ-CH$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-CH$_3$)Lys(boc)$_2$, 4H), 1.62-1.3 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$Lys(boc)$_2$ and –CH$_2$-*CH*$_2$-C$_2$H$_5$ of R group, 26H), 1.0 (m,–C$_2$H$_4$-*CH*$_2$-CH$_3$ of R group 2H), 0.63 (t,-C$_3$H$_6$-C*H*$_3$ of R group, 3H). FT-IR (cm^{-1}): 3354 (carbamate N-H str.), 3083 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1711 (C=O str. of carbamate), 1631 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): [M+K]$^+$ obsd. = 664.4545 (calc. = 664.292).

Boc-Lys(Boc)-*N*-hexyl-10-Aminomethyl-9-chloroanthracene (3b): Yield-90%. ^{1}H-NMR ($CDCl_3$) δ/ppm: 8.61 (d, Ar*H*, 2H), 8.29 (d, Ar*H*, 2H), 7.60 (m, Ar*H*, 4H), 6.08 (d, Ar-C*H*^{1}H^{2}-N(R)Lys(boc)$_2$, 1H), 5.42 (d, α-N*H*-Boc of Lys(boc)$_2$, 1H), 5.29 (d, Ar-CH^{1}*H*^{2}-N(R)Lys(boc)$_2$, 1H), 4.56 (m, Lys(ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 2H) 3.1-2.81 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-CH$_3$)Lys(boc)$_2$, 4H), 1.63-1.3 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*CH*$_2$-C$_4$H$_9$ of R group, 26H), 1.05 (m,–C$_2$H$_4$-*CH*$_2$-C$_3$H$_7$ of R group 2H), 0.94 (m,–C$_2$H$_4$-*(CH*$_2$*)*$_3$-CH$_3$ of R group, 4H) 0.73 (t,-C$_5$H$_{10}$-

C*H*$_3$ of R group, 3H). FT-IR (cm^{-1}): 3354 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1705 (C=O str. of carbamate), 1634 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 654.3619 (calc. = 654.3668).

Boc-Lys(Boc)-*N*-octyl-10-Aminomethyl-9-chloroanthracene (4b): Yield-75%. ^{1}H-NMR ($CDCl_3$) δ/ppm: 8.61 (d, Ar*H*, 2H), 8.29 (d, Ar*H*, 2H), 7.60 (m, Ar*H*, 4H), 6.08 (d, Ar-C*H*^{1}H^2-N(R) Lys(boc)$_2$, 1H), 5.42 (d, α-N*H*-Boc of Lys(boc)$_2$, 1H), 5.29 (d, Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, 1H), 4.56 (m, Lys(ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 2H) 3.1-2.81 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-CH$_3$)Lys(boc)$_2$, 4H), 1.63-1.28 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*CH*$_2$-C$_6$H$_{13}$ of R group, 26H), 1.18 (m, –C$_2$H$_4$-*CH*$_2$-C$_5$H$_{11}$ of R group, 2H), 1.12-0.87 (–C$_3$H$_7$-*(CH*$_2$*)*$_4$-CH$_3$ of R group, 8H) 0.73 (t, -C$_7$H$_{14}$-C*H*$_3$of R group, 3H). FT-IR (cm^{-1}): 3354 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1696 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HRMS (m/z): $[M+H]^+$ obsd. = 682.3897 (calc. = 682.3981).

Boc-Lys(Boc)-*N*-decyl-10-Aminomethyl-9-chloroanthracene (5b): Yield-80%. ^{1}H-NMR ($CDCl_3$) δ/ppm: 8.61 (d, Ar*H*, 2H), 8.29 (d, Ar*H*, 2H), 7.60 (m, Ar*H*, 4H), 6.08 (d, Ar-C*H*^{1}H^2-N(R)Lys(boc)$_2$, 1H), 5.42 (d, α-N*H*-Boc of Lys(boc)$_2$, 1H), 5.29 (d, Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, 1H), 4.56 (m, Lys (ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 2H) 3.1-2.81 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-CH$_3$)Lys(boc)$_2$, 4H), 1.62-1.28 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*CH*$_2$-C$_8$H$_{17}$ of R group, 26H), 1.31-0.8 (–CH$_2$-CH$_2$-*C*$_8$*H*$_{17}$ of R group, 17H). FT-IR (cm^{-1}): 3334 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1704 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HRMS (m/z): $[M+H]^+$ obsd. = 710.4220 (calc. = 710.4294).

Boc-Lys(Boc)-*N*-butyl-1-Aminomethylnaphthalene (6b): Yield-87%. ^{1}H NMR ($CDCl_3$) δ/ppm: 8.0-7.74 (Ar*H*, 3H), 7.60-7.34 (Ar*H*, 3H), 7.31-7.14 (Ar*H*, 1H), 5.5-5.0 (Ar-C*H*^{1}H^2-N(R)Lys(boc)$_2$ and α-N*H*-Boc of Lys(boc)$_2$, 2H), 4.75-4.40 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, Lys (ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 3H), 3.40-3.0 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-C$_3$H$_7$)Lys(boc)$_2$, 4H), 1.72-1.12 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-

COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH$_2$)$_2$*-CH$_3$ of R group, 28H), 0.84 (m, Ar-CH$_2$-N(-C$_3$H$_6$-C*H*$_3$)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3331 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2975-2865 (sp^3 C-H str.), 1709 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): [M+H]$^+$ obsd. = 542.3641 (calc. = 542.3594).

Boc-Lys(Boc)-*N*-hexyl-1-Aminomethylnaphthalene (7b): Yield-81%. ^{1}H NMR (CDCl$_3$) δ/ppm: 8.0-7.74 (Ar*H*, 3H), 7.60-7.34 (Ar*H*, 3H), 7.31-7.14 (Ar*H*, 1H), 5.5-5.0 (Ar-C*H*^{1}H^2-N(R)Lys(boc)$_2$ and α-N*H*-Boc of Lys(boc)$_2$, 2H), 4.75-4.40 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$,Lys (ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 3H), 3.40-3.0 (δ-*CH$_2$* of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H$_2$*-C$_5$H$_{11}$)Lys(boc)$_2$, 3H), 1.72-1.12 (-CO-[CH-*CH$_2$*-*CH$_2$*-*CH$_2$*-CH$_2$-NH-COO-C(C*H$_3$*)$_3$]-NH-COO-C(C*H$_3$*)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH$_2$)$_4$*-CH$_3$ of R group, 32H), 0.84 (m, Ar-CH$_2$-N(-C$_5$H$_{10}$-C*H$_3$*)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3331 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1709 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): [M+H]$^+$ obsd. = 570.3954 (calc. = 570.3907).

Boc-Lys(Boc)-*N*-octyl-1-Aminomethylnaphthalene (8b): Yield-85%. ^{1}H NMR (CDCl$_3$) δ/ppm: 8.0-7.74 (Ar*H*, 3H), 7.60-7.34 (Ar*H*, 3H), 7.31-7.14 (Ar*H*, 1H), 5.5-5.0 (Ar-C*H*^{1}H^2-N(R)Lys(boc)$_2$ and α-N*H*-Boc of Lys(boc)$_2$, 2H), 4.75-4.40 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, Lys (ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 3H), 3.40-3.0 (δ-CH$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H$_2$*-C$_5$H$_{11}$)Lys(boc)$_2$, 4H), 1.72-1.12 (-CO-[CH-*CH$_2$*-*CH$_2$*-*CH$_2$*-CH$_2$-NH-COO-C(C*H$_3$*)$_3$]-NH-COO-C(C*H$_3$*)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH$_2$)$_6$*-CH$_3$ of R group, 36H), 0.84 (m, Ar-CH$_2$-N(-C$_7$H$_{14}$-C*H$_3$*)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3331 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1709 (C=O str. of carbamate), 1640 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): [M+H]$^+$ obsd. = 598.4246 (calc. = 598.422).

Boc-Lys(Boc)-*N*-decyl-1-Aminomethylnaphthalene (9b): Yield-98%. ^{1}H NMR (CDCl$_3$) δ/ppm: 8.0-7.74 (Ar*H*, 3H), 7.60-7.34 (Ar*H*, 3H), 7.31-7.14 (Ar*H*, 1H), 5.5-5.0 (Ar-C*H*^{1}H^2-N(R)Lys(boc)$_2$ and α-N*H*-Boc of Lys(boc)$_2$, 2H), 4.75-4.40 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, Lys (ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 3H), 3.40-3.0 (δ-CH$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H$_2$*-C$_5$H$_{11}$)Lys(boc)$_2$, 4H), 1.72-1.12 (-CO-[CH-*CH$_2$*-*CH$_2$*-*CH$_2$*-CH$_2$-NH-COO-C(C*H$_3$*)$_3$]-NH-COO-C(C*H$_3$*)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH$_2$)$_6$*-CH$_3$ of R group, 40H), 0.84 (m, Ar-CH$_2$-N(-C$_7$H$_{14}$-

C*H*$_3$)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3331 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1709 (C=O str. of carbamate), 1640 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 625.445 (calc. = 625.4455).

Boc-Lys(Boc)-*N*-dodecyl-1-Aminomethylnaphthalene (10b): Yield-80%. ^{1}H NMR ($CDCl_3$) δ/ppm: 8.0-7.74 (Ar*H*, 3H), 7.60-7.34 (Ar*H*, 3H), 7.31-7.14 (Ar*H*, 1H), 5.5-5.0 (Ar-C*H*^{1}H^2-N(R)Lys(boc)$_2$ and α-N*H*-Boc of Lys(boc)$_2$, 2H), 4.75-4.40 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, Lys (ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 3H), 3.40-3.0 (δ-CH$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-C$_5$H$_{11}$)Lys(boc)$_2$, 4H), 1.72-1.12 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH$_2$)$_6$*-CH$_3$ of R group, 44H), 0.84 (m, Ar-CH$_2$-N(-C$_7$H$_{14}$-C*H*$_3$)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3331 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1709 (C=O str. of carbamate), 1640 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 654.4842 (calc. = 654.4846).

Boc-Lys(Boc)-*N*-butyl-1-Aminomethylbenzene (11b): Yield-94%. ^{1}H NMR ($CDCl_3$) δ/ppm: δ 7.2 (Ar*H*, 3H), 7.15 (d, Ar*H*, 2H), 5.5 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.97-4.56 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, and α-C*H* of Lys(boc)$_2$, 3H), 4.44 (d, Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, 1H) 3.4-2.9 (δ-CH$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-C$_5$H$_{11}$)Lys(boc)$_2$, 4H), 1.8-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH$_2$)$_2$*-CH$_3$ of R group, 28H), 0.8 (m, Ar-CH$_2$-N(-C$_3$H$_6$-*CH*$_3$)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3354 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1704 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 492.3677 (calc. = 492.3437).

Boc-Lys(Boc)-*N*-hexyl-1-Aminomethylbenzene (12b): Yield-97%. ^{1}H NMR ($CDCl_3$) δ/ppm: 7.2 (Ar*H*, 3H), 7.15 (d, Ar*H*, 2H), 5.5 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.97-4.56 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$ and α-C*H* of Lys(boc)$_2$, 3H), 4.44 (d, Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$,1H) 3.4-2.9 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-C$_5$H$_{11}$)Lys(boc)$_2$, 4H), 1.8-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH$_2$)$_4$*-CH$_3$ of R group, 32H), 0.8 (m, Ar-CH$_2$-N(-C$_5$H$_{10}$-*CH*$_3$)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3354 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1711 (C=O str. of carbamate), 1643 (C=O str. of

tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 520.387 (calc. = 520.375).

Boc-Lys(Boc)-*N*-octyl-1-Aminomethylbenzene (13b): Yield-85%. ^{1}H NMR ($CDCl_3$) δ/ppm: 7.2 (Ar*H*, 3H), 7.15 (d, Ar*H*, 2H), 5.5 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.97-4.56 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$ and α-C*H* of Lys(boc)$_2$, 3H), 4.44 (d, Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, 1H) 3.4-2.9 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-C$_5$H$_{11}$)Lys(boc)$_2$, 4H), 1.8-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH*$_2$*)*$_6$-CH$_3$ of R group, 36H), 0.8 (m, Ar-CH$_2$-N(-C$_7$H$_{14}$-C*H*$_3$)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3354 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1704 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 548.3842 (calc. = 548.4063).

Boc-Lys(Boc)-*N*-decyl-1-Aminomethylbenzene (14b): Yield-71%. ^{1}H NMR ($CDCl_3$) δ/ppm: 7.2 (Ar*H*, 3H), 7.15 (d, Ar*H*, 2H), 5.5 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.97-4.56 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$ and α-C*H* of Lys(boc)$_2$, 3H), 4.44 (d, Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, 1H) 3.4-2.9 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-C$_5$H$_{11}$)Lys(boc)$_2$, 4H), 1.8-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH*$_2$*)*$_6$-CH$_3$ of R group, 40H), 0.8 (m, Ar-CH$_2$-N(-C$_7$H$_{14}$-C*H*$_3$)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3354 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1704 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 576.4376 (calc. = 576.4376).

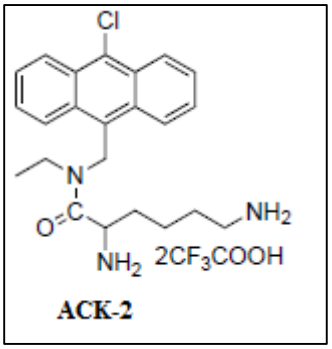
Boc-Lys(Boc)-*N*-dodecyl-1-Aminomethylbenzene (15b): Yield-68%. ^{1}H NMR ($CDCl_3$) δ/ppm: 7.2 (Ar*H*, 3H), 7.15 (d, Ar*H*, 2H), 5.5 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.97-4.56 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$ and α-C*H* of Lys(boc)$_2$, 3H), 4.44 (d, Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, 1H) 3.4-2.9 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-C$_5$H$_{11}$)Lys(boc)$_2$, 4H), 1.8-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH*$_2$*)*$_6$-CH$_3$ of R group, 44H), 0.8 (m, Ar-CH$_2$-N(-C$_7$H$_{14}$-C*H*$_3$)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3354 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1704 (C=O str. of carbamate), 1643 (C=O str. of

tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 604.4678 (calc. = 604.4689).

Boc-Lys(Boc)-*N*-tetradecyl-1-Aminomethylbenzene (16b): Yield-67%. ^{1}H NMR ($CDCl_3$) δ/ppm: 7.2 (Ar*H*, 3H), 7.15 (d, Ar*H*, 2H), 5.5 (m, Lys (ε-N*H*-Boc)-α-NH-boc, 1H), 4.97-4.56 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$ and α-C*H* of Lys(boc)$_2$, 3H), 4.44 (d, Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$, 1H) 3.4-2.9 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-C$_5$H$_{11}$)Lys(boc)$_2$, 4H), 1.8-1.2 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH*$_2$*)*$_6$-CH$_3$ of R group, 48H), 0.8 (m, Ar-CH$_2$-N(-C$_7$H$_{14}$-C*H*$_3$)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3354 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1704 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 632.5005 (calc. = 632.5002).

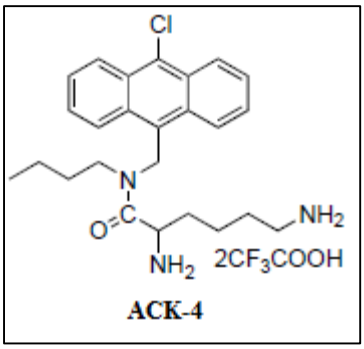
3.4.2.3. Deprotection of Boc groups (1-16): Typically, the Boc-Lys(Boc)-*N*-alkyl-aromatic compounds (**1b-16b**, 0.35 mmol) were dissolved in DCM and subsequently CF_3COOH (50% by volume) was added and stirred at RT. The reactions were monitored by TLC until complete removal of starting material was observed. All the volatile components were removed, and the product is purified by reverse phase HPLC using 0.1% Trifluoroacetic acid (TFA) in water/acetonitrile (0-100%) as mobile phase to more than 95% purity. C_{18} column (10mm diameter, 250 mm length) and UV detector (at 270 nm wavelength) were used. After drying the compounds in freeze drier, the compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR and mass spectrometry. Peptoids of naphthalene have been reported to show presence of rotamers in solution. These compounds (Series I and Series II) too show existence of rotamers, as is evident from their NMR spectra.

Lys-*N*-ethyl-10-Aminomethyl-9-chloroanthracene trifluoroacetate (ACK-2, 1): ^{1}H-NMR (D_2O) δ/ppm: 8.48 (d, Ar*H*, 2H), 8.13 (d, Ar*H*, 2H), 7.71 (m, Ar*H*, 4H), 5.82 (d, Ar-C*H*^{1}H^{2}-N(R)Lys, 1H), 5.16 (d, Ar-CH^{1}*H*^{2}-N(R)Lys, 1H), 4.4 (t, α-C*H* of Lys, 1H), 3.0 (m, ε-*CH*$_2$ of Lys, 2H), 2.67 (m, Ar-CH$_2$-N(*CH*$_2$CH$_3$)Lys, 2H), 1.81 (m, γ-CH$_2$ of Lys, 2H), 1.50 (m, *δ*-*CH*$_2$ of Lys, 2H), 1.28 (m, β-C*H*^{1}H^{2} of Lys, 1H), 1.16 (m, β-CH^{1}*H*^{2} of Lys, 1H), 0.92 (t, *N*-CH$_2$*CH*$_3$ of R, 1H). ^{13}C-NMR (CD_3OD): 168.55, 134.54, 131.22, 129, 128.54, 127.82, 127.45, 126.92, 126.73, 124.92, 124.76, 40.41, 40.13, 39.92, 39.71, 39.5, 39.29, 39.08, 38.87, 38.68, 38.37, 38.24, 26.46, 26.37, 20.76, 19.44, 19.11, 13.16 . FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 398.1976 (calc. = 398.1999).



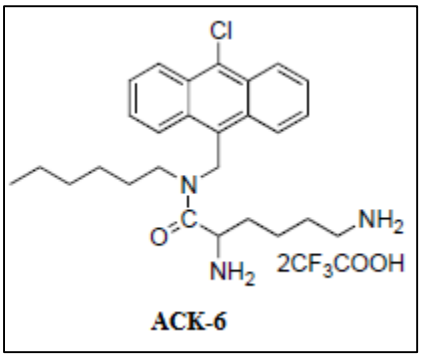
ACK-2

Lys-*N*-butyl-10-Aminomethyl-9-chloroanthracene trifluoroacetate (ACK-4, 2): ^{1}H-NMR (D_2O) δ/ppm: 8.58 (d, Ar*H*, 2H), 8.24 (d, Ar*H*, 2H), 7.71 (m, Ar*H*, 4H), 5.92 (d, Ar-C*H*^{1}H^{2}-N(R)Lys, 1H), 5.28 (d, Ar-CH^{1}*H*^{2}-N(R)Lys, 1H), 4.4 (t, α-C*H* of Lys, 1H), 3.0 (m, ε-*CH*$_2$ of Lys, 2H), 2.67 (m, Ar-CH$_2$-N(*CH*$_2$(CH$_2$)$_2$H$_3$)Lys, 2H), 1.85 (m, γ-*CH*$_2$ of Lys, 2H), 1.54 (m, *δ*-*CH*$_2$ of Lys, 2H), 1.47-1.11 (β-*CH*$_2$ of Lys, 2H) 1-0.7 (m, Ar-CH$_2$-N(CH$_2$*CH*$_2$C$_2$H$_5$)Lys, 2H), 0.2 (m, Ar-CH$_2$-N(C$_2$H$_4$*CH*$_2$CH$_3$)Lys, 2H), 0.1(t, Ar-CH$_2$-N(C$_3$H$_6$*CH*$_3$)Lys, 3H). ^{13}C-NMR (CD_3OD): 169.78, 135.42, 133.04, 129.83, 128.19, 128.09, 126.65, 125.65, 51.92, 46.12, 40.64, 40.14, 32.38, 32.02, 28.18, 22.69, 21.09, 20.73, 13.73. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2962-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 426.2283 (calc. = 426.2312).



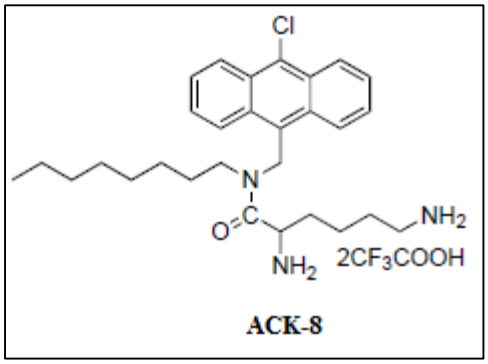
ACK-4

Lys-*N*-hexyl-10-Aminomethyl-9-chloroanthracene trifluoroacetate (ACK-6, 3): ^{1}H NMR (D_2O) δ/ppm: 8.23 (d, Ar*H*, 2H), 7.94 (d, Ar*H*, 2H), 7.49 (m, Ar*H*, 4H), 5.60 (d, Ar-C*H*^{1}H^{2}-N(R)Lys, 1H), 5.03 (d, Ar-CH^{1}*H*^{2}-N(R)Lys, 1H),4.23 (t, α-C*H* of Lys, 1H), 2.78 (m, ε-*CH*$_2$ of Lys, 2H), 2.7-2.23 (m, Ar-CH$_2$-N(*CH*$_2$C$_5$H$_{11}$)Lys, 2H), 1.72 (m, γ-*CH*$_2$ of Lys, 2H), 1.52 (m, δ-*CH*$_2$ of Lys, 2H), 1.41-1.19 (β-*CH*$_2$ of Lys, 2H) 1.0-0.7 (Ar-CH$_2$-N(CH$_2$*CH*$_2$C$_4$H$_9$)Lys, 2H), 0.67-0.11 (Ar-CH$_2$-N(C$_2$H$_4$-*CH*$_2$-*CH*$_2$-*CH*$_2$-*CH*$_3$)Lys, 9H). ^{13}C-NMR (CD_3OD): 169.75, 133.08, 131.51, 129.88, 128.81, 128.22, 128.08, 126.7, 125.63, 51.93, 46.5, 40.85, 40.16, 32.39, 32.13, 29.96, 28.23, 27.11, 23.28, 22.7, 14.19. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): [M+H]$^+$ obsd. = 454.2595 (calc. = 454.2625).



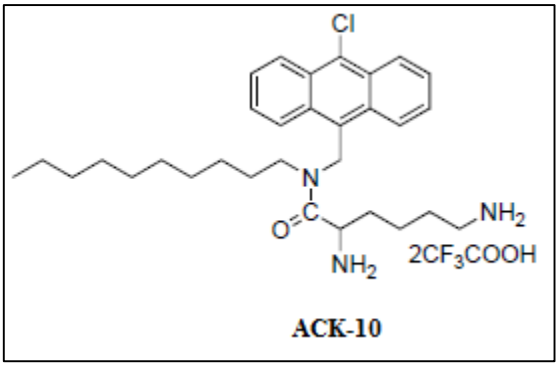
ACK-6

Lys-*N*-octyl-10-Aminomethyl-9-chloroanthracene trifluoroacetate (ACK-8, 4): ^{1}H NMR (D_2O) δ/ppm: 8.04 (d, Ar*H*, 2H), 7.94 (d, Ar*H*, 2H), 7.35 (m, Ar*H*, 4H), 5.46 (d, Ar-C*H*^{1}H^{2}-N(R)Lys, 1H), 5.09 (d, Ar-CH^{1}*H*^{2}-N(R)Lys, 1H), 4.17 (t, α-C*H* of Lys, 1H), 2.82 (m, ε-*CH*$_2$ of Lys, 2H), 2.57-2.29 (m, Ar-CH$_2$-N(*CH*$_2$C$_5$H$_{11}$)Lys, 2H), 1.73 (m, γ-*CH*$_2$ of Lys, 2H), 1.52 (m, δ-*CH*$_2$ of Lys, 2H), 1.40-1.15 (β-*CH*$_2$ of Lys, 2H), 1.0-0.1 (Ar-CH$_2$-N(CH$_3$-*(CH*$_2$*)*$_6$-*CH*$_3$)Lys, 15H). ^{13}C-NMR (CD_3OD): 169.8, 133.1, 131.47, 129.9, 128.81, 128.22, 128.08, 126.7, 125.63, 115.32, 114.2, 53.31, 47, 40.85, 40.2, 32.45, 32.13, 30, 28.3, 27.23, 22.28, 21.7, 14.19. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): [M+H]$^+$ obsd. = 482.290 (calc. = 482.293).



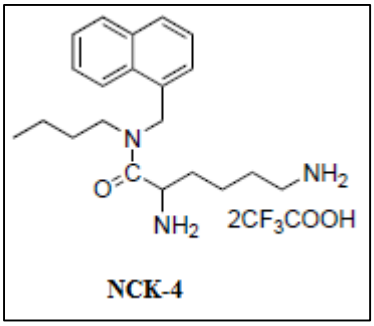
ACK-8

Lys-*N*-decyl-10-Aminomethyl-9-chloroanthracene trifluoroacetate (ACK-10, 5): ^{1}H NMR (CD_3OD) δ/ppm: 8.52 (d, Ar*H*, 2H), 8.48 (d, Ar*H*, 2H), 7.8-7.5 (Ar*H* and Lys-ε-NH_2, 6H), 6.04 (d, Ar-CH^1H^2-N(R)Lys, 1H), 5.54 (d, Ar-CH$^1H^2$-N(R)Lys, 1H), 4.3 (t, α-C*H* of Lys, 1H), 3.0-2.8 (ε-CH_2 of Lys and Ar-CH_2-N($CH_2C_9H_{19}$)Lys, 4H), 1.9-1.7 (γ-CH_2 of Lys, 2H), 1.6-0.8 (β-CH_2 of Lys, δ-CH_2 of Lys and Ar-CH_2-N(CH_3-$(CH_2)_8$-CH_3)Lys, 23H). 169.79, 133.07, 131.5, 129.86, 128.78, 128.16, 126.68, 125.67, 46.53, 40.14, 32.38, 32.02, 28.18, 22.69, 21.09, 20.73, 13.73. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 510.3259 (calc. = 510.3251).



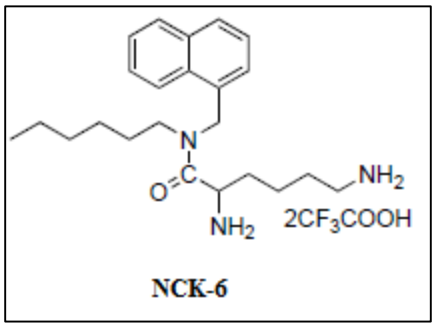
ACK-10

Lys-*N*-butyl-1-Aminomethylnaphthalene trifluoroacetate (NCK-4, 6): ^{1}H NMR (D_2O) δ/ppm: 8.14-7.76 (Ar*H*, 3H) 7.75-7.19 (Ar*H*, 4H), 5.59-5.0 (Ar-CH^1H^2-N(R)Lys, 2H), 4.5 (m, α-C*H* of Lys, 1H) 3.67-3.0 (Ar-CH_2-N($CH_2(CH_2)_2CH_3$)Lys, 2H), 2.84 (d, ε-CH_2 of Lys, 2H), 2.04-1.10 (β-CH_2 of Lys, γ-CH_2 of Lys, δ-CH_2 of Lys and Ar-CH_2-N($CH_2(CH_2)_2CH_3$)Lys, 10H), 0.85 (m, Ar-CH_2-N($C_3H_6CH_3$)Lys, 3H). ^{13}C-NMR (CD_3OD): 170.49, 169.72, 162.94, 135.51, 135.37, 133.19, 132.88, 132.68, 132.15, 130.03, 129.95, 129.64, 128.59, 127.89, 127.51, 127.42, 127.18, 126.47, 126.37, 124.65, 124.28, 123.49, 52.13, 51.92, 49.66, 49.45, 49.23, 49.02, 48.81, 48.59, 48.38, 47.18, 47, 40.19, 32.13, 31.6, 31.45, 30.55, 28.21, 28.09, 22.66, 22.58, 21.15, 20.88, 14.12, 14.05 FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 342.26`29 (calc. = 342.2545).



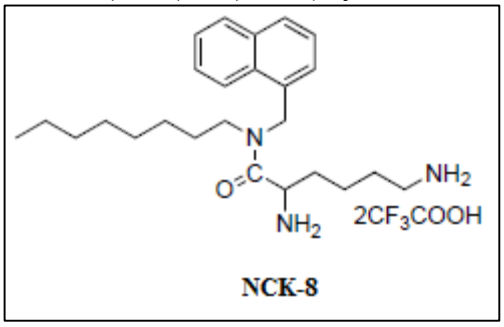
NCK-4

Lys-*N*-hexyl-1-Aminomethylnaphthalene trifluoroacetate (NCK-6, 7): ^{1}H NMR (D_2O) δ/ppm: 7.87-7.5 (Ar*H*, 2H) 7.5-7.0 (Ar*H*, 5H), 5.14 (d, Ar-C*H*^{1}H^{2}-N(R)Lys, 1H), 4.48 (d, Ar-CH^{1}*H*^{2}-N(R)Lys, 1H) 4.37 (m, α-C*H* of Lys, 1H), 3.21-2.7 (Ar-CH_2-N(*CH_2*$(CH_2)_2CH_3$)Lys and ε-C*H_2* of Lys, 4H), 1.94-1.19 (β-C*H_2* of Lys, γ-C*H_2* of Lys, δ-C*H_2* of Lys and Ar-CH_2-N(CH_2*CH_2*C_3H_7)Lys, 8H), 0.85 (m, Ar-CH_2-N(C_2H_4*C_3H_6*CH_3)Lys, 6H), 0.65 (m, Ar-CH_2-N(C_5H_{10}*CH_3*)Lys, 3H). ^{13}C-NMR (CD_3OD): 169.94, 163.28, 135.51, 135.37, 133.22, 132.9, 132.69, 132.18, 130.03, 129.96, 129.65, 128.63, 127.89, 127.52, 127.41, 127.18, 126.47, 126.38, 124.66, 124.38, 123.5, 52.12, 51.93, 47.27, 40.2, 32.64, 32.56, 32.25, 31.73, 29.39, 28.37, 28.24, 28.11, 27.65, 27.37, 23.58, 23.54, 22.71, 22.62, 14.34, 14.31 FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 370.2849 (calc. = 370.2858).



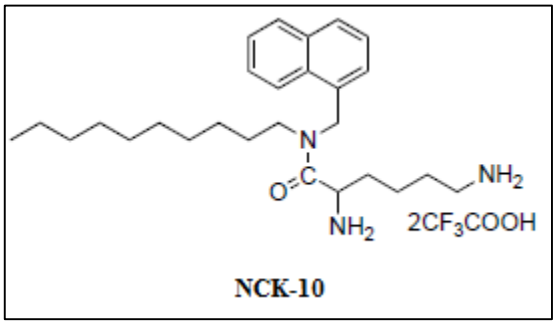
NCK-6

Lys-*N*-octyl-1-Aminomethylnaphthalene trifluoroacetate (NCK-8, 8): ^{1}H NMR (D_2O) δ/ppm: 7.71-7.5 (Ar*H*, 2H) 7.44-6.91 (Ar*H*, 5H), 5.0 (d, Ar-C*H*^{1}H^{2}-N(R)Lys, 1H), 4.4 (d, Ar-CH^{1}*H*^{2}-N(R)Lys, 1H) 4.29 (m, α-C*H* of Lys, 1H), 3.18-2.59 (Ar-CH_2-N(*CH_2*$(CH_2)_2CH_3$)Lys and ε-C*H_2* of Lys, 4H), 1.87-1.60 (d, γ-C*H_2* of Lys, 2H), 1.60-1.02 (β-C*H_2* of Lys, δ-C*H_2* of Lys and Ar-CH_2-N(CH_2*CH_2*C_6H_{13})Lys, 6H), 0.85 (m, Ar-CH_2-N(C_2H_5*$(CH_2)_5$*CH_3)Lys, 10H), 0.56 (m, Ar-CH_2-N(C_7H_{14}*CH_3*)Lys, 3H). ^{13}C-NMR (CD_3OD): 169.94, 163.28, 135.51, 135.37, 133.22, 132.9, 132.69, 132.18, 130.03, 129.96, 129.65, 128.63, 127.89, 127.52, 127.41, 127.18, 126.47, 126.38, 124.66, 124.38, 123.5, 52.12, 51.93, 47.27, 40.2, 32.64, 32.56, 32.25, 31.73, 29.39, 28.37, 28.24, 28.11, 27.65, 27.37, 23.58, 23.54, 22.71, 22.62, 14.34, 14.31. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 398.3228 (calc. = 398.3171).



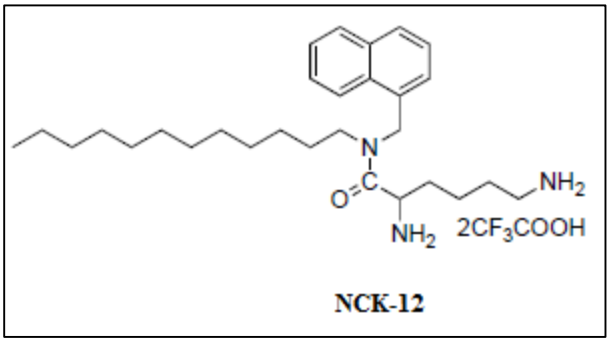
NCK-8

Lys-*N*-decyl-1-Aminomethylnaphthalene trifluoroacetate (NCK-10, 9): ^{1}H NMR (CD_3OD) δ/ppm: 8.2-7.8 (Ar*H*, 3H) 7.7-7.3 (Ar*H*, 4H), 5.6 (d, Ar-C*H*^{1}H^{2}-N(R)Lys, 1H), 4.7 (d, Ar-CH^{1}*H*^{2}-N(R)Lys, 1H) 4.4 (m, α-C*H* of Lys, 1H), 3.6-2.59 (Ar-CH_2-N(*CH_2*$(CH_2)_2CH_3$)Lys and ε-C*H_2* of Lys, 4H), 2-1.8 (d, γ-C*H_2* of Lys, 2H), 1.70-1.4 (β-C*H_2* of Lys, δ-C*H_2* of Lys and Ar-CH_2-N(CH_2*$CH_2C_6H_{13}$*)Lys, 6H), 1.4-1.1 (m, Ar-CH_2-N(C_2H_5*$(CH_2)_5CH_3$*)Lys, 14H), 0.8 (m, Ar-CH_2-N(C_7H_{14}*CH_3*)Lys, 3H). ^{13}C-NMR (CD_3OD): 170.46, 169.72, 163.07, 162.72, 35.51, 135.37, 133.22, 132.92, 132.65, 132.2, 130.04, 129.99, 129.68, 128.67, 127.91, 127.53, 127.42, 127.19, 126.47, 126.37, 124.65, 124.46, 123.5, 119.58, 116.67, 52.15, 51.94, 49.66, 49.45, 49.31, 49.24, 49.02, 48.81, 48.6, 48.39, 47.31, 40.2, 33.03, 32.13, 31.6, 30.62, 30.58, 30.55, 30.39, 30.33, 29.42, 28.36, 28.22, 28.09, 27.94, 27.67, 23.72, 22.67, 22.59, 14.43. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 426.3458 (calc. = 426.3484).



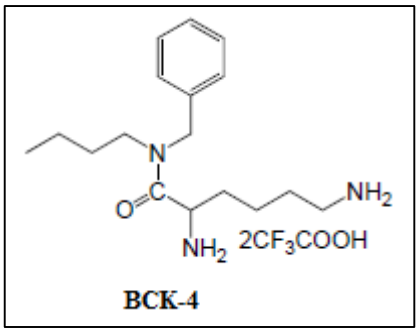
NCK-10

Lys-*N*-dodecyl-1-Aminomethylnaphthalene trifluoroacetate (NCK-12, 10): ^{1}H NMR (CD_3OD) δ/ppm: 8.2-7.8 (Ar*H*, 3H) 7.7-7.3 (Ar*H*, 4H), 5.6 (d, Ar-C*H*^{1}H^{2}-N(R)Lys, 1H), 4.7 (d, Ar-CH^{1}*H*^{2}-N(R)Lys, 1H) 4.4 (m, α-C*H* of Lys, 1H), 3.6-2.59 (Ar-CH_2-N(*CH_2*$(CH_2)_2CH_3$)Lys and ε-C*H_2* of Lys, 4H), 2-1.8 (d, γ-C*H_2* of Lys, 2H), 1.70-1.4 (β-C*H_2* of Lys, δ-C*H_2* of Lys and Ar-CH_2-N(CH_2*$CH_2C_6H_{13}$*)Lys, 6H), 1.4-1.1 (m, Ar-CH_2-N(C_2H_5*$(CH_2)_5CH_3$*)Lys, 18H), 0.8 (m, Ar-CH_2-N(C_7H_{14}*CH_3*)Lys, 3H). ^{13}C-NMR (CD_3OD): 133.23, 132.93, 130.05, 130, 128.71, 127.52, 127.19, 126.37, 124.66, 51.94, 49.65, 49.44, 49.23, 49.01, 48.8, 48.59, 48.37, 47.29, 40.21, 33.06, 32.18, 30.72, 30.58, 30.54, 30.46, 30.34, 29.43, 28.27, 27.68, 23.73, 22.68, 14.43. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 454.3793 (calc. = 454.3797).



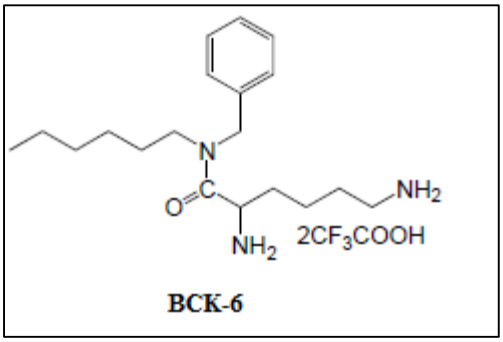
NCK-12

Lys-*N*-butyl-1-Aminomethylbenzene trifluoroacetate (BCK-4, 11): ^{1}H-NMR (D_2O) δ/ppm: 7.36-7.09 (Ar*H*, 5H) 4.63 (t, 1H), 4.53-4.29 (2H), 3.36-3.08 (Ar-CH_2-N(*CH_2*(CH_2)$_2$$CH_3$)Lys, 2H), 2.94-2.87 ((m, ε-C*H_2* of Lys, 2H), 1.90-1.3 (β-C*H_2* of Lys, γ-C*H_2* of Lys, δ-C*H_2* of Lys and Ar-CH_2-N(CH_2*CH_2*C_2H_5)Lys, 8H), 1.10 (m, Ar-CH_2-N(C_2H_4*CH_2*CH_3)Lys, 2H), 0.72 (t, Ar-CH_2-N(C_3H_6*CH_3*)Lys, 3H). ^{13}C-NMR (CD_3OD): 170.14, 170.11, 163.6, 163.26, 162.92, 162.57, 138.38, 137.55, 130.13, 129.76, 129.16, 129.1, 128.75, 128.03, 122.59, 119.68, 116.77, 113.86, 52.02, 51.87, 51.73, 49.85, 49.69, 49.48, 49.27, 49.05, 48.84, 48.63, 48.42, 47.93, 40.27, 40.2, 32.62, 31.89, 31.68, 29.65, 28.17, 28.1, 28.06, 27.62, 27.42, 23.57, 22.65, 22.61, 14.35. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 292.2369 (calc. = 292.2389).



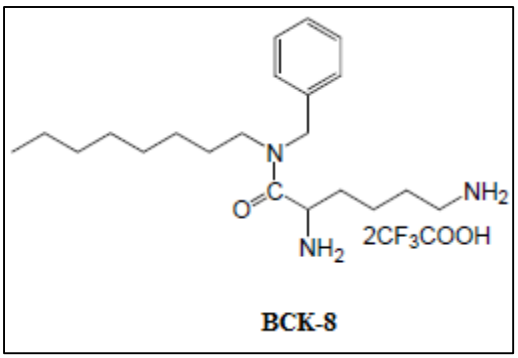
BCK-4

Lys-*N*-hexyl-1-Aminomethylbenzene trifluoroacetate (BCK-6, 12): ^{1}H-NMR (D_2O) δ/ppm: 7.36-7.09 (Ar*H*, 5H) 4.63 (t, 1H), 4.53-4.29 (2H), 3.36-3.08 (Ar-CH_2-N(*CH_2*(CH_2)$_2$$CH_3$)Lys, 2H), 2.94-2.87 (m, ε-C*H_2* of Lys, 2H), 1.90-1.3 (β-C*H_2* of Lys, γ-C*H_2* of Lys, δ-C*H_2* of Lys and Ar-CH_2-N(CH_2*CH_2*C_4H_9)Lys, 8H), 1.10 (d, Ar-CH_2-N(C_2H_4*(CH_2)$_3$*CH_3)Lys, 6H), 0.72 (t, Ar-CH_2-N(C_5H_{10}*CH_3*)Lys, 3H). ^{13}C-NMR (CD_3OD): 170.08, 170, 138.38, 137.57, 130.15, 129.75, 129.18, 129.11, 128.77, 127.93, 52.01, 51.79, 51.69, 49.75, 49.65, 49.43, 49.29, 49.22, 49.01, 48.8, 48.58, 48.37, 48.09, 47.67, 40.27, 40.21, 31.93, 31.77, 31.71, 30.26, 28.24, 28.18, 22.64, 21.11, 20.92, 14.08. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 320.2732 (calc. = 320.2702).



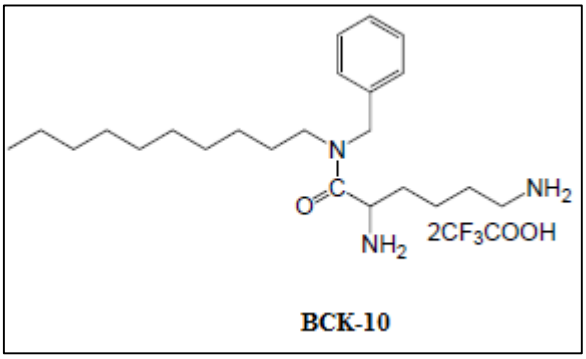
BCK-6

Lys-*N*-octyl-1-Aminomethylbenzene trifluoroacetate (BCK-8, 13): ^{1}H-NMR (D_2O) δ/ppm: 7.36-7.09 (Ar*H*, 5H) 4.8 (d, 1H), 4.6 (d, 1H) 4.53-4.3 (2H), 3.36-3.08 (Ar-CH_2-N(CH_2(CH_2)$_2$$CH_3$)Lys, 2H), 2.94-2.87 ((m, ε-CH_2 of Lys, 2H), 2.1-1.3 (β-CH_2 of Lys, γ-CH_2 of Lys, δ-CH_2 of Lys and Ar-CH_2-N($CH_2$$CH_2$$C_6H_{13}$)Lys, 8H), 1.36-0.97 (Ar-CH_2-N(C_2H_4(CH_2)$_5$$CH_3$)Lys, 10H), 0.84 (t, Ar-$CH_2$-N($C_7H_{14}$$CH_3$)Lys, 3H). ^{13}C-NMR ($CD_3OD$): 170.11, 170.07, 163.63, 163.29, 162.94, 162.6, 138.4, 137.56, 130.13, 129.75, 129.18, 129.11, 128.76, 128.01, 122.6, 119.69, 116.78, 113.87, 52.01, 51.86, 51.71, 49.84, 49.67, 49.46, 49.24, 49.03, 48.82, 48.61, 48.39, 47.94, 40.26, 40.2, 32.95, 31.9, 31.69, 30.39, 30.34, 30.29, 29.71, 28.21, 28.13, 28.09, 27.94, 27.74, 23.69, 22.64, 22.62, 14.43. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 348.3016 (calc. = 348.3015).



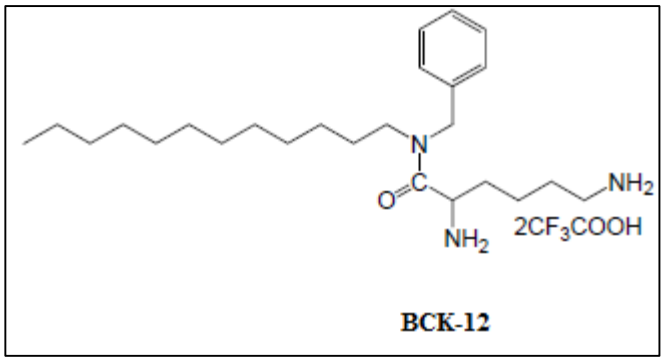
BCK-8

Lys-*N*-decyl-1-Aminomethylbenzene trifluoroacetate (BCK-10, 14): ^{1}H-NMR (CD_3OD) δ/ppm: 7.5-7.2 (Ar*H*, 5H) 4.8 (t, Ar-C$H^1$$H^2$-N(R)Lys, 1H), 4.6 (d, Ar-C$H^1$$H^2$-N(R)Lys, 1H) 4.4 (m, α-C*H* of Lys, 1H) 1H), 3.6-3.2 (Ar-CH_2-N(CH_2(CH_2)$_2$R)Lys, 2H), 2.94-2.87 ((m, ε-CH_2 of Lys, 2H), 2.0-1.4 (β-CH_2 of Lys, γ-CH_2 of Lys, δ-CH_2 of Lys and Ar-CH_2-N($CH_2$$CH_2$$C_6H_{13}$)Lys, 8H), 1.4-1.2 (Ar-$CH_2$-N($C_2H_4$($CH_2$)$_5$$CH_3$)Lys, 14H), 0.84 (t, Ar-$CH_2$-N($C_7H_{14}$$CH_3$)Lys, 3H). ^{13}C-NMR ($CD_3OD$): 170.06, 170.02, 162.97, 162.62, 138.4, 137.55, 130.14, 129.75, 129.2, 129.11, 128.77, 128, 119.53, 116.63, 52.02, 51.88, 51.72, 49.86, 49.66, 49.45, 49.31, 49.24, 49.02, 48.81, 48.6, 48.38, 47.99, 40.26, 40.2, 33.03, 31.9, 31.69, 30.65, 30.61, 30.41, 30.36, 29.72, 28.21, 28.14, 28.09, 27.93, 27.75, 23.72, 22.63, 14.43. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 376.3317 (calc. = 376.3328).



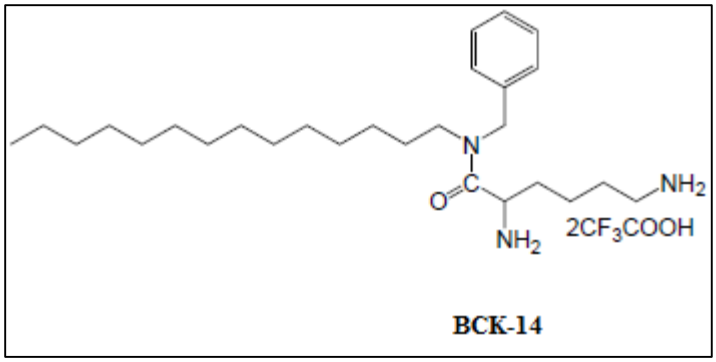
BCK-10

Lys-*N*-dodecyl-1-Aminomethylbenzene trifluoroacetate (BCK-12, 15): ^{1}H-NMR (CD_3OD) δ/ppm: 7.5-7.2 (Ar*H*, 5H) 4.8 (t, Ar-C*H*^{1}H^{2}-N(R)Lys, 1H), 4.6 (d, Ar-CH^{1}*H*^{2}-N(R)Lys, 1H) 4.4 (m, α-C*H* of Lys, 1H) 1H), 3.6-3.2 (Ar-CH_2-N(*CH_2*(CH_2)$_2$R)Lys, 2H), 2.94-2.87 ((m, ε-C*H_2* of Lys, 2H), 2.0-1.4 (β-C*H_2* of Lys, γ-C*H_2* of Lys, δ-C*H_2* of Lys and Ar-CH_2-N(CH_2*CH_2*C_6H_{13})Lys, 8H), 1.4-1.2 (Ar-CH_2-N(C_2H_4*(CH_2)*$_5CH_3$)Lys, 18H), 0.84 (t, Ar-CH_2-N(C_7H_{14}*CH_3*)Lys, 3H). ^{13}C-NMR (CD_3OD): 170.08, 170.04, 163.28, 162.93, 162.58, 138.39, 137.55, 130.14, 129.75, 129.19, 129.11, 128.76, 128.01, 119.5, 116.59, 52.02, 51.88, 51.72, 49.86, 49.67, 49.46, 49.25, 49.04, 48.82, 48.61, 48.4, 47.99, 40.26, 40.2, 33.07, 31.89, 31.68, 30.74, 30.69, 30.61, 30.47, 30.43, 30.37, 29.71, 28.2, 28.09, 27.94, 27.75, 23.74, 22.63, 14.45. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 404.3628 (calc. = 404.3641).



BCK-12

Lys-*N*-tetradecyl-1-Aminomethylbenzene trifluoroacetate (BCK-14, 16): ^{1}H-NMR (CD_3OD) δ/ppm: 7.36-7.09 (Ar*H*, 5H) 4.6 (t, 1H), 4.53-4.3 (2H), 3.36-3.08 (Ar-CH_2-N(*CH_2*(CH_2)$_2CH_3$)Lys, 2H), 2.94-2.87 ((m, ε-C*H_2* of Lys, 2H), 2.1-1.3 (β-C*H_2* of Lys, γ-C*H_2* of Lys, δ-C*H_2* of Lys and Ar-CH_2-N(CH_2*CH_2*C_6H_{13})Lys, 8H), 1.36-0.97 (Ar-CH_2-N(C_2H_4*(CH_2)*$_5CH_3$)Lys, 22H), 0.84 (t, Ar-CH_2-N(C_7H_{14}*CH_3*)Lys, 3H). ^{13}C-NMR (CD_3OD): 170.04, 169.99, 138.4, 137.55, 130.14, 129.74, 129.21, 129.12, 128.77, 128, 52.03, 51.89, 51.72, 49.44, 49.23, 49.01, 48.8, 48.59, 40.27, 40.21, 33.06, 31.9, 31.69, 30.77, 30.74, 30.68, 30.59, 30.45, 30.36, 29.72, 28.23, 28.15, 28.1, 27.93, 27.75, 23.72, 22.62, 14.41. FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 432.3954 (calc. = 432.3954).



BCK-14

3.4.3 *In vitro* biological assays

3.4.3.1 *In vitro* susceptibility studies: The MIC of the compounds against different bacterial strains were determined using a standardized protocol as described in chapter 2 (Section 2.4.3.1).

3.4.3.2 Hemolytic Activity: Hemolytic experiments were performed using a standardized protocol as described in chapter 2 (Section 2.4.3.2).

3.4.3.3 Bactericidal time-kill kinetics: Kinetics of bactericidal activity was performed according to the protocol described in chapter 2 (Section 2.4.3.3).

3.4.3.4 Propensity to induce resistance development in bacteria: This was performed following the protocol described in chapter 2 (Section 2.4.3.4).

3.4.3.5 Antibacterial activity in different physiological conditions: The experiment was performed using the protocol described in Chapter 2 (2.4.3.5)

3.4.3.6 Mechanism of action: The assays for determining the mechanism of action of the compounds were performed according to a standardized protocol as described in chapter 2 (Section 2.4.4).

3.4.3.6.4 Live dead assay: To 1 mL of bacterial suspension containing 10^9 CFU/mL, 10 times MIC of ACK-6 was added to make a final volume of 1 mL of suspension (final concentration 1×10^9 cells/mL), and another 1 mL was left untreated as a control. The mixture was incubated for 1.5 h, centrifuged (12,000 rpm for 1 min), and resuspended in 50 μL of PBS. 5μL of the bacterial suspension was combined with 20 μL of a fluorescent probe mixture containing 3.0 μM green fluorescent nucleic acid stain SYTO 9 (Invitrogen, USA) and 15.0 μM red fluorescent nucleic acid stain PI (Sigma-Aldrich, USA). The mixture was incubated in the dark for 15 min, and a 5 μL aliquot was placed on a glass slide, which was then covered with a coverslip, sealed, and examined under a fluorescence microscope. Excitation was carried out for SYTO 9 at 450−490 nm and for

PI at 515−560 nm. Emission was collected using a band-pass filter for SYTO 9 at 500−550 nm and a long-pass filter for PI at 590− 800 nm. In all cases, a 100× objective was used with immersion oil, giving a total magnification of 1000×. Images were captured with a Leica DM 2500 fluorescence microscope.

3.4.3.6.5 Visualization of the effect on membranes of bacteria: The bacterial cells were cultured for 6 h in suitable media (LB broth for *E. coli* and yeast dextrose broth for *S. aureus*) at 37 °C. The cells were centrifuged and resuspended in nutrient media at pH 7.4 (10^6 cells/mL). The suspension was divided into two portions. To one portion (1 mL) was added 10 × MIC of compounds **3** and **7**. The other portion was left untreated as a control. The suspension was then incubated at 37°C for 2 h (at 250 rpm shaking speed), and the cells from both tubes were harvested by centrifugation at 12,000 rpm. After treatment, the cells were dehydrated sequentially with 30, 50, 70, 80, 90, and 100% ethanol for 15 min. Later, 5 μL of dehydrated cells was dropped on a small piece of silicon wafer and dried at room temperature. Before being imaged, the silicon wafer containing *S. aureus* was sputter coated, and *E. coli* was used directly for imaging without sputtering. Images were recorded by using Quanta 3D FEG FEI field-emission scanning electron microscopy at 20 kV for *S. aureus* and 10 kV for *E. coli*.

3.4.3.7 MICs of the compounds against several strains of *Clostridium difficile* and its spores: The *Clostridium difficile* strains are deposited at Purdue University and their corresponding strain numbers have been mentioned. They were obtained from Purdue University. The strains were grown on Anaerobic blood agar plate (Becton Dickinson, BD) and 0.5 McFarland standard solution was prepared and diluted 1:300 in brain heart infusion supplemented medium (Brain heart infusion medium, BD, supplemented with yeast extract, L-cysteine, Vitamin K1 and Hemin, Sigma). Drugs were added at the required concentration in the first row of a 96-well plates, 100 μL of the bacterial suspension was added to all the wells of the plates and two-fold serial dilution was done in each plate. Plates were then incubated at 37° C anaerobically for 48 hours. MICs reported in Figure 3.6 are the Minimum concentrations of the drugs that could inhibit the visual growth of the bacteria.

Spores were prepared by streaking *C. difficile* HM-88 on BHIS agar plate for 4 days followed by scrapping the colonies and suspending them in sterile water and incubation at 4˚C

overnight. The suspension is then heat shocked at 70˚ C for 20 minutes and stored at 4˚C till use. Before use the spore preparation was serially diluted and counted using BHIS agar plate containing 0.1% taurocholic acid sodium salt as a germinant. Spore preparation was diluted to about 10^6 spore mL^{-1} in BHIS broth containing 0.1% taurocholic acid and incubated with drugs at the required concentration for 24 hours at 37 ˚C before the outgrowth inhibitory concentration (OIC) was recorded for each drug. The OIC is the minimum concentration required to inhibit the visual growth of the spores after 24-hour incubation in a medium containing germinant.

3.4.3.8 Effect of the compounds on human gut normal flora: Two types of bacterial species were used in this experiment, namely *Bifidobacterium* and *Lactobacillus*. Both bacteria were first grown for 48 hours at 37˚ C, anaerobically using anaerobic blood agar for Bifidobacterium and in 5% CO2 using MRS agar plate for Lactobacillus. Each bacterium was suspended in phosphate buffered saline (PBS) to form a solution with optical density equal to 0.5 McFarland standard and diluted in brain heart infusion supplemented medium for *Bifidobacterium* or in MRS broth for *Lactobacillus* to achieve bacterial concentration of approximately 1 x 10^5 CFU mL^{-1}. Bacteria were then added to 96-well plates containing serial dilutions of the compounds and incubated as mentioned for each species for 48 hours. MICs reported are the Minimum concentrations of the drugs that could inhibit the visual growth of the bacteria.

3.4.4 *In vivo* studies

3.4.4.1 Acute dermal toxicity: Acute dermal toxicity of NCK-10 was determined as mentioned in Chapter 2 (Section 2.4.5.1)

3.4.4.2 MRSA skin infection: The experiment was performed following several previously published protocols with modification. [185-188] 6 to 8 weeks-old male BALB/c mice (18-22g) were used for the experiments. The mice were anesthetized by intraperitoneal injection of xylazine-ketamine cocktail. The fur on the back of the mice were then shaved using a sterile razor. The fur was shaved in a manner to induce a wound, that is, reddening and glistening of the exposed skin was observed without bleeding. To this visibly damaged area (~2 cm^2) of the skin, a bacterial infection was initiated by placing on the skin a 20 µL droplet containing 10^7 cells of MRSA. This

was allowed to dry for 20 minutes and it was ensured that the droplet stayed within the shaved area. In the first experiment one group of mice (n=5) were treated after 4h with 40 µL of NCK-10 (concentration of 20 mg mL^{-1}) at the site of infection (on the shaved area of the skin where bacteria was added). The droplet was gently spread on the entire surface of the wound to avoid any drop from rolling down the sides. Another group of mice (n=4) were dosed with Fusidic acid, a comparator drug, at exactly the same concentration. The dosage for both the compounds were continued for seven days. One group of mice (n=) were left untreated and served as a control. 18h after the last dose (to prevent carryover effects) the mice were sacrificed using isofluorane and the infected skin (on the back of the mice) was severed aseptically. The severed part was weighed and placed into about 10 mL of sterile saline and homogenized. The dilutions of the homogenate were plated onto agar plates, which were incubated overnight at about 37°C. The bacterial titer was expressed as $\log_{10}$ CFU g^{-1} of weight of the tissue collected and expressed as mean ± S.E.M (standard error of mean).

3.4.4.3 *A. baumannii* burn infection: Activity of NCK-10 against burn infections caused by *A. baumannii* was determined as mentioned in Chapter 2 (Section 2.4.5.2).

Chapter 4

Biphenyl-alkyl-lysines: Selective antibacterial agents that also kill intracellular bacteria

Abstract

In Chapters 2 and 3, several novel small molecules which possessed potent antibacterial activity against aerobic and anaerobic bacteria were introduced. Although, the compound of study in Chapter 2 (C_{10}-K-C_8) was toxic to mice skin, that of Chapter 3 (NCK-10) was not. However, their in vitro selectivity warranted much improvement. In this Chapter, biphenyl based novel cationic antibacterial agents that inactivate various Gram-positive and Gram-negative bacteria are described. An optimized compound was shown to inhibit cell-wall bio-synthesis of bacteria and also perturbed the polarization of cell membranes. Although, the activity against almost all microorganisms were retained, there was significant increase in selectivity. An interesting property of this class of compounds was their ability to clear intracellular infections of methicillin resistant S. aureus (MRSA). This compound too was non-toxic to mice skin till 200 mg kg^{-1}. In murine models of infection, this compound was found to outperform NCK-10 against both MRSA model of skin infection and A. baumannii model of burn infection.

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(1) Ghosh, C. *et al.* "L-lysine based lipidated biphenyls as agents with anti-biofilm and anti-inflammatory properties that also inhibit intracellular bacteria" (Manuscript under revision)

4.1 Introduction

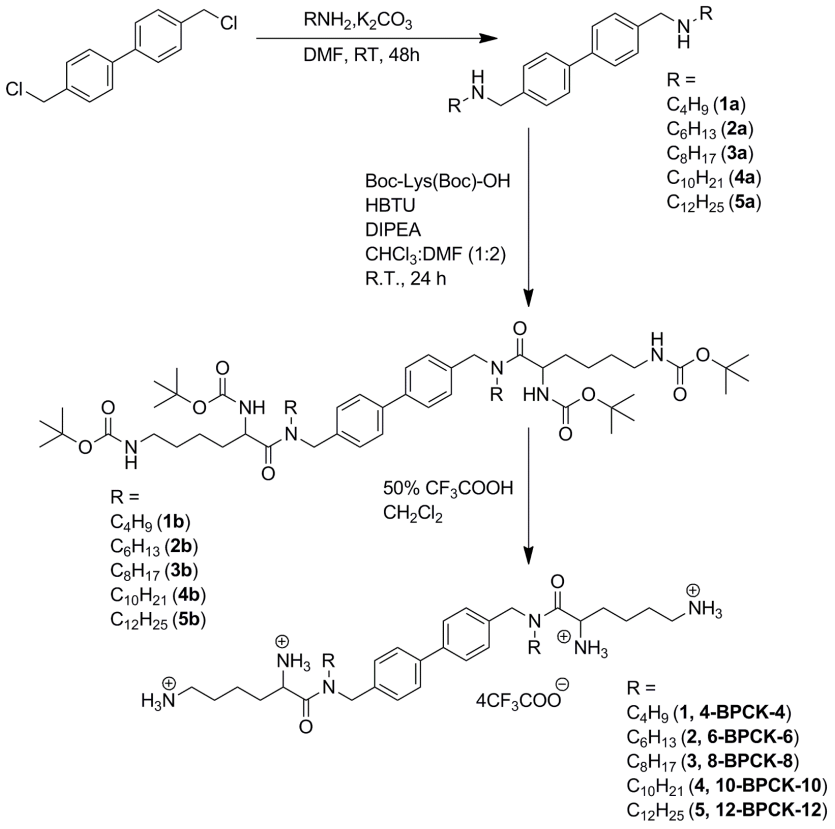
So far, in Chapters 2 and 3 we had introduced several membrane-active small molecules that, although extremely effective, warranted improvement in selectivity. Apart from achieving selective antibacterial activity several other manifestations of bacteria need to be taken care of. Persistent infections caused by bacteria, especially *S. aureus,* continue to pose a severe hurdle for health care personnel all over the world.[189] Other than causing mild skin infections and wound infections, they are known to cause serious diseases such as endocarditis, pneumonia, osteomyelitis and sepsis.[190] Further, *S. aureus* can also evade phagocytosis and cause intracellular infections.[191] Of more serious concern is the fact that *S. aureus* has developed resistance against frontline antibiotics such as vancomycin, linezolid, daptomycin which has left the antibiotic coffers almost empty.[192] A bigger cause of worry is that MRSA is not confined to the clinics anymore and causes significant problem in community. Moreover, no single drug exists that can treat all the manifestations of *S. aureus* infections. In this regard, there is a continuous need for novel antistaphylococcal agents, preferably ones with a novel mechanism of action. Although antimicrobial peptides and their mimics represent promising therapeutic candidates, they are yet to achieve clinical translation.[54, 58, 73, 79, 85, 166, 167, 170, 172, 193-196]

4.2 Results and discussion

4.2.1 Design and Synthesis

Herein, we report lipidated-biphenyl-lysines which contain four cationic charges, two lipid tails and a biphenyl core. It was envisioned that increase in positive charge residues might lead to an increase in selectivity, in addition to imparting new properties to the molecule. The compounds were prepared in three simple synthetic steps which is depicted in Scheme 4.1. The first step involved reaction of 4,4´-Bis(chloromethyl)-1,1´-biphenyl with alkylamines (which were butylamine, hexylamine, octylamine, decylamine and dodecylamine). The resultant secondary amines were reacted with Boc-Lys(boc)-OH using HBTU coupling chemistry and removal of the Boc groups yielded the final compounds:4-BPCK-4 (**1**), 6-BPCK-6 (**2**), 8-BPCK-8 (**3**), 10-BPCK-10 (**4**), 12-BPCK-12 (**5**). The final compounds were then purified (to >95%) using reverse phase HPLC and characterized using NMR, IR and HR-MS. The details of synthesis and characterization

has been furnished in the experimental section. The final compounds were purified by HPLC to more than 95% purity and characterized by ^{1}H-NMR, ^{13}C-NMR, IR and HR-MS. Two significant features of these compounds are that there is no imposed structural rigidity in their design and that they include an *N*-disubstituted or tertiary amide bond.



Scheme 4.1: General scheme for the synthesis of the compounds

4.2.2 Antibacterial activity and selectivity

The antibacterial efficacy of these compounds was evaluated against various Gram-positive (*S. aureus* and *E. faecium*) and Gram-negative (*E. coli, K. pneumoniae* and *P. aeruginosa*) bacteria and the results are presented in Table 4.1. The hemolytic activity has also been represented in Table 4.1. 4-BPCK-4 (**1**), which contains a butyl chain was not active against most of the bacteria tested. Activity of 6-BPCK-6 (**2**), which contains hexyl chains, against all the strains of bacteria

tested is commendable. It shows MICs of 2.8 µM against *S. aureus* and 5.7 µM against *E. faecium*. Against the Gram-negative bacteria, *E. coli*, *P. aeruginosa* and *K. pneumoniae*, it displayed MICs of 4.1 µM, 4.6 µM and 5.7 µM respectively. 8-BPCK-8, the octyl analogue was quite active against most of the bacteria tested. Against *S. aureus* it was active at 2.7 µM while against both *E. coli* and *P. aeruginosa*, the activity was 2 µM. Only against *K. pneumoniae*, there was little loss in activity (MIC of 4.6 µM). The decyl analogue, 10-BPCK-10, was not very active against *P. aeruginosa* and *K. pneumoniae*. But against *S. aureus* and *E. coli*, activity ranged from 2.6 µM to 4.2 µM. On moving to the dodecyl chain analogue, the activity was totally compromised (MIC >50 µM).

None of the compounds were haemolytic at their MICs which was determined by evaluation of the HC_{50} values (the concentration at which 50% of human erythrocytes were lysed). As can be seen in Table 4.1, compound **4** (10-BPCK-10) was the most haemolytic compound (HC_{50} of 65 µM) while **1** (4-BPCK-4) was the least toxic compound (HC_{50} of 390 µM). However, among the

Table 4.1: Activity against Gram-positive and Gram-negative pathogens

Compounds	Minimum inhibitory concentration (µM)					HC_{50} (µM)
	S. aureus	*E. faecium*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	
4-BPCK-4	24	50	50	50	>50	390
6-BPCK-6	2.8	5.7	4.1	4.6	5.7	210
8-BPCK-8	2.7	2.7	2.0	2.0	4.6	115
10-BPCK-10	2.6	2.6	4.2	>50	30	65
12-BPCK-12	>50	>50	>50	>50	>50	80

Values are the averages of at least two experiments, each done in triplicate (error <5%)

active compounds, compound **2** (6-BPCK-6) emerged to be the most selective compound (HC_{50} of 210 μM). Due to its superior selectivity, further studies were carried out with this compound.

Against a vancomycin resistant strain of *E. faecium* the compound (6-BPCK-6) was active at 5 μM. There were a total of three strains of *Staphylococcus aureus* against which the compound was tested. One of them was a laboratory strain obtained from ATCC while the other two were MDR clinical isolates. All the strains were susceptible to 0.7 μM of vancomycin. Against methicillin-susceptible and methicillin-resistant strains of *S. aureus*, 6-BPCK-6 exhibited an MIC of 3 μM as well. This emphasized on the strong activity of the compound against Gram-positive strains. Subsequently, we tested the activity of the compound against the Gram-negative strains of ESKAPE groups of pathogens which included *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and

Table 4.2: Activity against drug-resistant strains of ESKAPE pathogens

Bacterial Strains		MIC of 6-BPCK-6 (μM)
E	Vancomycin-resistant *Enterococcus faecium*	3
S	MRSA ATCC 33591	3
	MRSA R3889	3
	MRSA R3890	3
K	*K. pneumoniae* R3421	>50
	*K. pneumoniae*R2146	>50
A	*A. baumannii* R674	5.7
	A. baumannii R676	5.7
P	*P. aeruginosa* R590	5.7
E	*E. coli* R250	10

Values are the averages of at least two experiments, each done in triplicate (error <5%)

E. coli. Although the compound was active against the ATCC strain of *K. pneumoniae* (Table 4.1), it was not active against the clinical isolates. The MIC of the compound against both the strains of *A. baumannii* (R674 and R676) was also 5.7 μM. The activity of the compound against *P. aeruginosa* R590 was 5.7 μM. The last representative of the ESKAPE group of pathogens was *E. coli* which belong to the family of Enterobacteriaceae. Against the clinical isolate *E. coli* R250, the compound displayed an activity of 10 μM. Further, activity of the compound against some New Delhi-metallo-β-Lactamase producing Gram-negative bacteria was also conducted, however, the compound was not very active against most of these strains.

4.2.3 Kinetics of antibacterial activity

First we checked, the time kill kinetics of 6-BPCK-6 against *S. aureus* (Figure 1A). The compound showed static effect at MIC. Only at 5×MIC, did it show bactericidal activity in 6h. Aryl-alkyl-lysines were earlier shown to be rapidly bactericidal; this property was attributed to their membrane active nature. The fact that this compounds were not rapidly bactericidal, pointed towards a different mechanism of action, other than infiltration of bacterial membrane.

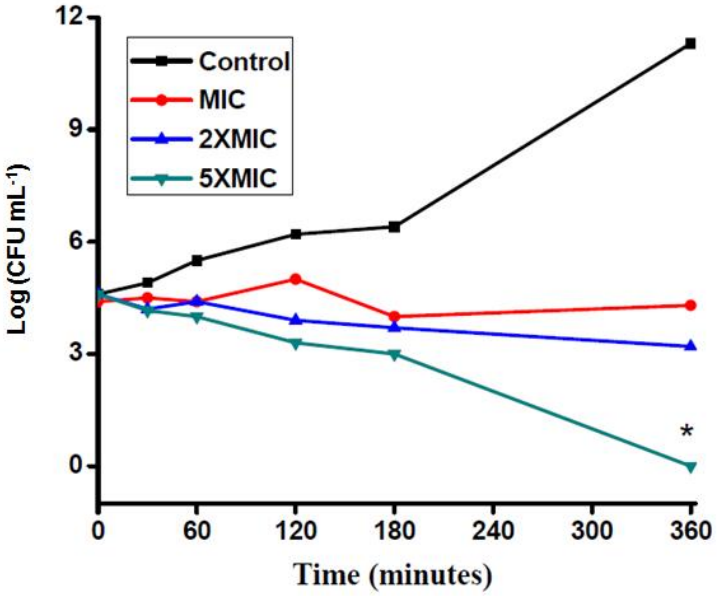


Figure 4.1: Time kill-kinetics of 6-BPCK-6 against *S. aureus*. * indicates no bacteria observed. Detection limit is 50 CFU mL^{-1}. MIC considered is 3 μM.

4.2.4 Propensity to induce resistance development

We then checked the ability of 6-BPCK-6 to stall resistance development in *S. aureus* and *E. coli*. Serial passage of bacterial culture (both *S. aureus* and *E. coli*) in sub-MIC concentrations of 6-BPCK-6 did not select resistant mutants. In comparison, resistance against norfloxacin (comparator drug for *S. aureus*) and colistin (comparator drug for *E. coli*) was developed within six passages and in fourteen passages respectively.

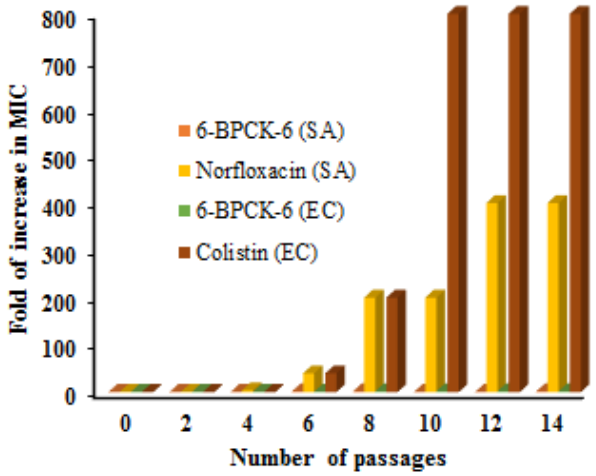


Figure 4.2: Ability of the compound to halt resistance development in *S. aureus* (norfloxacin is used as a comparator) and *E. coli* (colistin is used as a comparator).

4.2.5 Mechanism of antibacterial action

Next, investigation into the mechanism of action of the most selective compounds 6-BPCK-6 against *S. aureus* was performed.

4.2.5.1 Cytoplasmic membrane depolarization

Experiments with the membrane-potential sensitive dye $DiSC_3(5)$ showed that Compound **2**, at its bactericidal concentration (10 µM) was able to depolarize the membranes of *S. aureus* rapidly (Figure 4.3 A), but was not able to permeabilize the cell membranes effectively (data not shown). This was plausibly the reason for slow antibacterial kinetics of the compound. This also proves that other mechanisms of action exist.

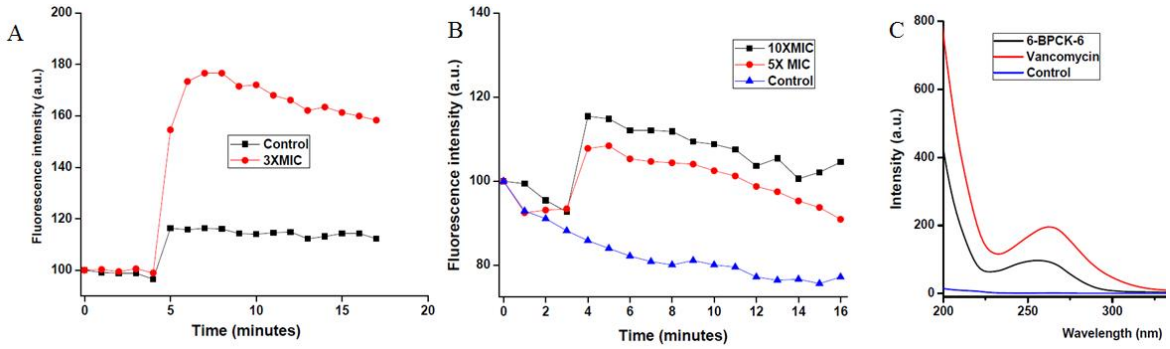


Figure 4.3: Mechanism of action.A) Ability of 6-BPCK-6 to depolarize the cell membrane of *S. aureus* B) Ability of 6-BPCK-6 to cause leakage of intracellular K^+. C) Intracellular accumulation of cell wall biosynthesis precursor UDPMurNAc-pp upon treatment with 5×MIC of 6-BPCK-6 (MIC is 3 µM) and vancomycin (5 µM) in comparison to control.

4.2.5.2 Leakage of intracellular K^+

We further investigated the release of potassium ion caused by 6-BPCK-6 using potassium ion sensitive fluorophore, PBFI-AM against *S. aureus*. There is an increase in the fluorescence of PBFI upon binding to released K^+. As can be seen from Figure 4.3B, significant release of K^+ was observed upon addition of 6-BPCK-6.

4.2.5.3 Inhibition of cell-wall biosynthesis

We then looked into its ability to inhibit biosynthesis of cell wall. The accretion of soluble cell wall precursor undecaprenyl-*N*-acetylmuramic acid-pentapeptide (UDP-MurNAc-pentapeptide) indicates that one of the membrane-associated steps in the biosynthesis of peptidoglycan is blocked.[9, 197]Treatment with the compound at 15 µM lead to the accumulation of UDP-MurNAc-pentapeptide which leads to blockage of biosynthesis of *S. aureus* cells (Figure 4.3C). On treatment with 6-BPCK-6, an intense peak corresponding to UDP-MurNAc-pp was observed at 260 nm. The accumulation was also confirmed by mass spectrometry studies. The observed m/z was 1150.357 (for $[M+H]^+$) whereas the calculated value was 1150.94 [UDPMurNAc-pp ($C_{40}H_{64}N_8O_{27}P_2$): $[M+H]^+$]. In fact, the compound was as efficient as vancomycin (6 µM) in inhibiting the biosynthesis of cell wall of *S. aureus* cells.

4.2.6 Activity against anaerobic bacteria

4.2.6.1 Activity against different strains of *Clostridium difficile*

The activity of the most active compounds against clinical isolates of *Clostridium difficile* was then tested. The information about the strains have already been provided in the experimental section of Chapter 3. Vancomycin and metronidazole, which are currently used for treating *C. difficile* infections, were used as comparative antibiotics. The most active compounds 6-BPC6-6 (2), 8-BPCK-8 (3) and 10-BPCK-10 (4) were tested against fourteen clinical isolates of *C. difficile*. Against the toxigenic strain P8, the activity of the compounds ranged from 1.7 µM to 3.7 µM while vancomycin and metronidazole were active at 0.2 µM and 0.5 µM respectively. Against strain P13, 6-BPCK-6 lost two-fold activity while 8-BPCK-8 and 10-CK-10 was active at 3.5 µM. Isolate 1, was resistant to vancomycin (MIC of 2 µM) but susceptible to metronidazole (MIC 1 µM). Against this strain, 6-BPCK-6 was active at 7.4 µM while compounds 8-PCK-8 and 10-BPCK-10 were active at 1.7 µM respectively. Isolate 10 was susceptible to metronidazole at 5.3 µM, to 6-BPCK-6 at 7.3 µM, 8-PCK-8 and 10-BPCK-10 at 0.9 µM respectively. Against isolates 4, 5, 6 and 9, all the compounds were extremely active with MIC values ranging from 0.8 µM to 1.8 µM. The susceptibility of these strains to vancomycin varied from 0.1 µM to 0.3 µM. The compounds displayed similar efficacy against all the other clinical isolates as well. The MIC of the compounds varied from 0.8 µM to 3.7 µM. Particular attention needs to be paid to strain P 20 against which vancomycin was inactive till 1 µM and metronidazole at 1.5 µM.

4.2.6.2 Activity against human gut normal flora

We next checked whether the commensal bacteria were susceptible to the compounds at the same concentrations. We carried out the activity of the compounds against commensals such as *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium bifidium* and *Bifidobacterium breve*. Again, vancomycin and metronidazole were used as comparator antibiotics. The compounds (2-4), were active against *L. acidophilus* at very low concentrations (highest MIC was 3.7 µM). Even against *L. casei* the activity varied from 1 µM to 7.3 µM. A similar trend was observed in case of *B. bifidum*. The MIC of the compounds ranged from 1.7 µM to 7.3 µM. Among the compounds, the highest MIC observed against *B. breve* was 3.5 µM (6-BPCK-6). Vancomycin and metronidazole were selectively active towards C. difficile strains over commensal bacteria.

4.2.7 Activity against intracellular bacteria

S. aureus, as mentioned earlier, have been reported to infiltrate mammalian cells and survive within. Treatment with current antibiotics does not clear intracellular *S. aureus* infections effectively. The surviving cells might lead to recurrent infections and also spread of the infection to other parts of the body. Thus, apart from having extracellular activity, a good clinical candidate must also possess activity against intracellular infection. Several peptides have been shown to possess intracellular activity.[198, 199]

4.2.7.1 Toxicity against RAW macrophages

First, we evaluated the toxicity of compound 6-BPCK-6 against RAW 264.7 cells using MTT assay. Our results showed that the EC_{50} of the compound against RAW cells was 35 μM. At concentrations of 5×MIC, no toxicity was observed.

4.2.7.2 Toxicity against intracellular MRSA within RAW macrophages

Then, we tested the ability of 6-BPCK-6 to act against MRSA cells surviving within RAW 264.7 macrophages (Figure 2D). RAW 264.7 macrophages were initially allowed to phagocytize the MRSA cells (~10^5 CFU mL^{-1}); compound treatment was initiated after removal of extracellular bacteria by gentamicin. The bacterial titre prior to initiation of infection was 4.8 log and in 24 h, the infection increased to 8 log in the untreated control. Vancomycin at 100×MIC was used as a control. At such a high concentration only could it bring down the bacterial infection to 4.2 log in 6h and 3.9 log in 24 h. Treatment with 6-BPCK-6 at only 5×MIC was effective in inhibiting the growth of bacteria within the mammalian cells. Bacterial population in 6h and 24h were 4.2 log and 4.9 log respectively. It must be mentioned that treatment with 6-BPCK-6 caused no harm to the RAW macrophages when treated at this con-centration for 24h. This result opens up the potential use of the compound in chronic diseases especially osteomyelitis.

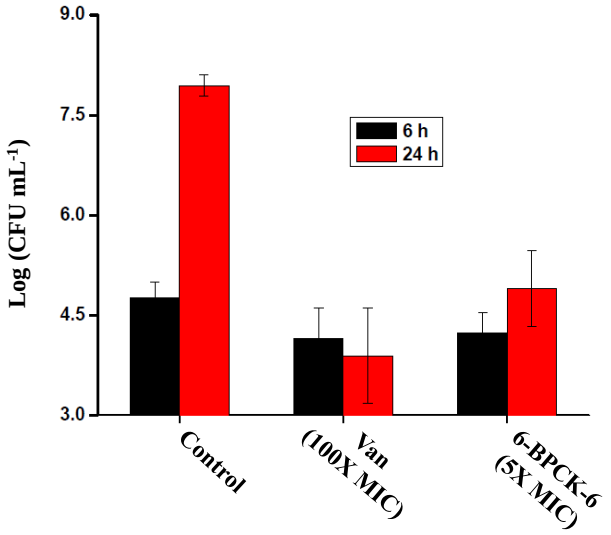


Figure 4.4: Intracellular activity of 6-BPCK-6 (**2**) against MRSA in presence of RAW macrophages. MIC of vancomycin is 0.6 μM and that of 6-BPCK-6 is 3 μM.

4.2.8 *In vivo* activity

4.2.8.1 Acute Dermal toxicity in mice

The acute dermal toxicity was performed as described in the previous chapters. Application of 200 mg kg^{-1} of 6-BPCK-6 (5 times the concentration used in the subsequent infection study) on mice skin caused no irritation, tremors, convulsions, salivation or diarrhea in the animals. Steady growth of fur was observed and by the end of seven days, almost complete regeneration fur was observed in all the test animals. No morbidity was observed among the test animals and it was concluded that the compound caused no toxicity to the skin even at concentrations as high as 200 mg kg^{-1}.

4.2.8.2 *In vivo* murine model of skin-infection caused by MRSA

Activity of the 6-BPCK-6 was determined in a murine model of MRSA skin infection as described in Chapter 3. 6-BPCK-6 or fusidic acid, was dosed at 40 mg kg^{-1} (once a day) for seven days and 6-BPCK-6 was able to bring down bacterial burden significantly in comparison to fusidic acid. In fact 6-BPCK-6 was better than NCK-10 at the same concentration.

4.2.8.3 *In vivo* murine model of *Acinetobacter baumannii* burn-infection

This experiment was performed as reported in Chapter 2 and 3 but the strain of *A. baumannii* was a multi-drug resistant clinical isolate R674. Upon dosage of the compound, 6-BPCK-6 (40 mg kg^{-1}), it was found that it was as effective as colistin. No colony was observed within the detection limit (data not shown) for either of the compounds at the end of the experimental time point.

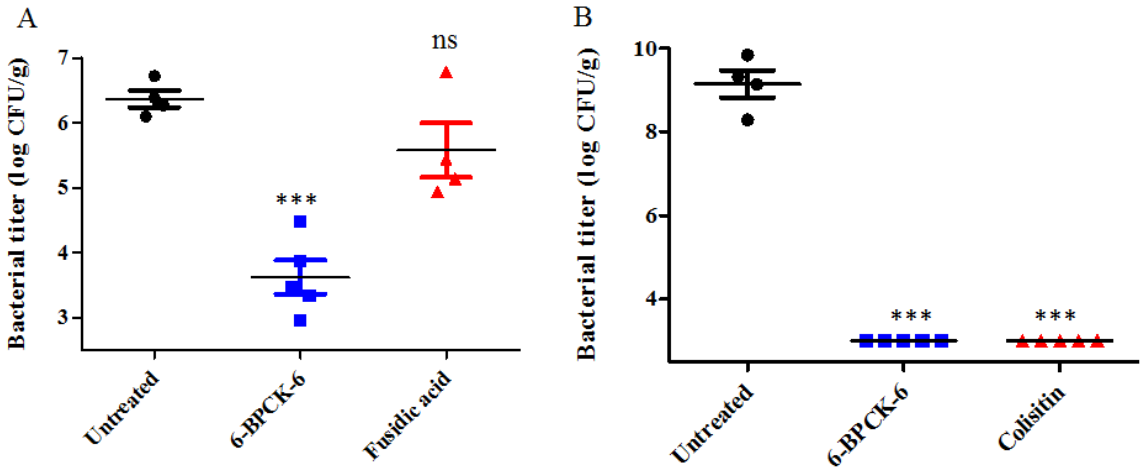


Figure 4.5: *In vivo* activity A) Against MRSA models of skin-infection, 6-BPCK-6 (at 40 mg kg^{-1}) was more effective than fusidic acid (40 mg kg^{-1}). B) Against *A. baumannii* burn infection. 6-BPCK-6 (40 mg kg^{-1}) was as effective as colistin (30 mg kg^{-1}). Statistical analysis was performed using Student's t-test. Differences are considered statistically significant from the untreated group with a value of $P<0.05$ with 95% confidence intervals (*** indicate $P<0.001$).

4.3 Conclusions

In summary, a new molecule 6-BPCK-6 was identified with improved selectivity, over the compounds reported in previous chapters, against planktonic bacteria. In addition to having effect on bacterial membranes, the compound could also inhibit cell wall biosynthesis. An interesting

property of the compound was that it could also inhibit growth of intracellular bacteria. In murine models too, the compound outperformed the compounds reported in the previous chapters.

4.4 Experimental Section

4.4.1 Materials and Instrumentation: All the solvents were of reagent grade and were distilled and dried prior to use wherever required. Chloroform and methanol were supplied by Merck-India. Dimethylformamide, Dicholoromethane, Diethyl ether and other solvents were supplied either by SDFCL (India) or Spectrochem (India). L-Lysine, Di-tert-butyl carbonate, Diisopropylethylamine, HBTU, Butylamine, Hexylamine, Octylamine, Trifluoroacetic acid were purchased from Spectrochem (India). 4,4′-Bis(chloromethyl)-1,1′-biphenyl, Decylamine and Dodecylamine were purchased from Sigma-Aldrich. The rest of the materials and instrumentation were used as described in the previous chapters.

4.4.2 Synthetic procedure and characterization of compounds

4.4.2.1 General procedure for synthesizing secondary amines of biphenyls (1a to 5a): In a typical reaction, 4,4′-Bis(chloromethyl)-1,1′-biphenyl (4 mmols) and alkylamines (8.8 mmols) were dissolved in 20 mL DMF. To that K_2CO_3 (8.8 mmols) was added and stirred for 48 h at 60°C in a pressure tube. Completion of the reaction was confirmed by TLC analysis. After completion of the reaction, DMF was removed under reduced pressure and then the mixture was diluted by addition of ethyl acetate (30 mL). The organic layer was subsequently washed four times water, brine and dried over anhydrous Na_2SO_4. The organic layer was evaporated under reduced pressure to obtain the products in 65-80% yield and characterized by ^{1}H NMR and HRMS. The details of characterization are provided below.

***N,N′*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(butan-1-amine), 1a:** Yield-78%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: ^{1}H NMR ($CDCl_3$) δ/ppm: 7.56-7.54 (t, Ar*H*, 4H), 7.45-7.38 (m, Ar*H*, 4H), 3.82 and 3.61(s, Ar(-*CH*$_2$-NH-R)$_2$, 4H), 2.65 (m, Ar(-CH$_2$-NH-*CH*$_2$-C$_3$H$_7$)$_2$, 4H), 2.5 (m, Ar-(CH$_2$-N*H*-R)$_2$, 2H), 1.67-1.1 ((-NH$_2$-CH$_2$-(*CH*$_2$)$_2$-CH$_3$)$_2$, 8H), 0.82 (m, terminal CH_3 groups, 6H). HR-MS (m/z): $[M+H]^+$ obsd. = 325.2631 (calc. = 325.2644).

***N,N′*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(hexan-1-amine), 2a:** Yield-80%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: ^{1}H NMR ($CDCl_3$) δ/ppm: 7.56-7.54 (t, Ar*H*, 4H), 7.45-7.38 (m, Ar*H*, 4H), 3.82 and 3.61(s, Ar(-*CH*$_2$-NH-R)$_2$, 4H), 2.65 (m, Ar(-CH$_2$-NH-*CH*$_2$-C$_5$H$_{11}$)$_2$, 4H), 2.5 (m, Ar-(CH$_2$-N*H*-R)$_2$, 2H), 1.67-1.1 ((-NH$_2$-CH$_2$-(*CH*$_2$)$_4$-CH$_3$)$_2$, 16H), 0.82 (m, terminal CH_3 groups, 6H). HR-MS (m/z): $[M+H]^+$ obsd. = 381.3264 (calc. = 381.3270).

***N,N′*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(octan-1-amine), 3a):** Yield-78%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: ^{1}H NMR ($CDCl_3$) δ/ppm: 7.56-7.54 (t, Ar*H*, 4H), 7.45-7.38 (m, Ar*H*, 4H), 3.82 and 3.61(s, Ar(-*CH*$_2$-NH-R)$_2$, 4H), 2.65 (m, Ar(-CH$_2$-NH-*CH*$_2$-C$_7$H$_{15}$)$_2$, 4H), 2.5 (t, Ar-(CH$_2$-N*H*-R)$_2$, 2H), 1.67-1.1 ((-NH$_2$-CH$_2$-(*CH*$_2$)$_6$-CH$_3$)$_2$, 24H), 0.82 (m, terminal CH_3 groups, 6H). HR-MS (m/z): $[M+H]^+$ obsd. = 437.3883 (calc. = 437.3896).

***N,N′*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(decan-1-amine), 4a):** Yield-65%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: ^{1}H NMR ($CDCl_3$) δ/ppm: 7.56-7.54 (t, Ar*H*, 4H), 7.45-7.38 (m, Ar*H*, 4H), 3.82 and 3.61(s, Ar(-*CH*$_2$-NH-R)$_2$, 4H), 2.65 (m, Ar(-CH$_2$-NH-*CH*$_2$-C$_9$H$_{19}$)$_2$, 4H), 2.5 (t, Ar-(CH$_2$-N*H*-R)$_2$, 2H), 1.67-1.1 ((-NH$_2$-CH$_2$-(*CH*$_2$)$_8$-CH$_3$)$_2$, 32H), 0.82 (m, terminal CH_3 groups, 6H). HR-MS (m/z)): 493.4511 $[M+H]^+$ obsd. = (calc. = 493.4522).

***N,N′*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(dodecan-1-amine), 5a:** Yield-65%.^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: ^{1}H NMR ($CDCl_3$) δ/ppm: 7.56-7.54 (t, Ar*H*, 4H), 7.45-7.38 (m, Ar*H*, 4H), 3.82 and 3.61(s, Ar(-*CH*$_2$-NH-R)$_2$, 4H), 2.65 (m, Ar(-CH$_2$-NH-*CH*$_2$-C$_{11}$H$_{25}$)$_2$, 4H), 2.5 (t, Ar-(CH$_2$-N*H*-R)$_2$, 2H), 1.67-1.1 ((-NH$_2$-CH$_2$-(*CH*$_2$)$_{10}$-CH$_3$)$_2$, 40H), 0.82 (m, terminal CH_3 groups, 6H). HR-MS (m/z): $[M+H]^+$ obsd. = 549.5115 (calc. = 549.5148).

4.4.2.4 General synthetic procedure for amide coupling reactions: In a typical amide coupling reaction, to a stirred solution of Boc-Lys(Boc)-OH (4.4 mmols) in 20 mL DMF and 10 mL of $CHCl_3$, *N*,*N*-Diisopropylethylamine (DIPEA, 5.49 mmols) was added at 0°C. To the solution HBTU (4.4 mmols) was added to the mixture and stirred for five minutes before the addition of secondary amines of biphenyl (1a-5a, 1.83 mmols). The mixture was stirred at 0 °C for 30 min and subsequently at RT for 24 h typically. At the end, $CHCl_3$ was evaporated under reduced pressure

and the resulting solution was diluted to 2 times its original volume by addition of ethyl acetate. This mixture was subsequently washed with 0.5 M $KHSO_4$, H_2O (thrice), and brine. After passage through anhydrous Na_2SO4, the organic layer was evaporated under reduced pressure and the residue was purified using column chromatography (5% MeOH/$CHCl_3$ mixture) to obtain the product in 50%-68% yield. The purified compound was subsequently characterized using 1H NMR and mass spectrometry.

(*N*,*N'*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(Boc-Lys(Boc)-*N*-butylamide), 1b: Yield-60 %. ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: 7.56-7.50 (t, Ar*H*, 4H), 7.27-7.24 (m, Ar*H*, 4H), 5.5-5.3 (m, N*H* of Boc-Lys(Boc), 4H), 4.9-4.3 (Ar(-*CH*$_2$-NH-R)$_2$ and α-*H* of Boc-Lys(Boc), 6H) 3.4-3.0 (ε-*H* of Boc-Lys(Boc) and Ar-(CH_2-N(Boc_2Lys)- C*H*$_2$-R)$_2$, 8H), 1.9-1.1 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and-[-(C*H*$_2$)$_2$-]$_2$ of R groups, 56H) and 0.88 (m, terminal CH_3 groups, 6H). FT-IR (cm^{-1}): 3330 (carbamate N-H str.), 2984-2863 (sp3 C-H str.), 1710 (C=O str. of carbamate), 1680 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 981.6589 (calc. =981.6640).

(*N*,*N'*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(Boc-Lys(Boc)-*N*-hexylamide), 2b): Yield-61 %. ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: 7.56-7.50 (t, Ar*H*, 4H), 7.27-7.24 (m, Ar*H*, 4H), 5.5-5.3 (m, N*H* of Boc-Lys(Boc), 4H), 4.9-4.3 (Ar(-*CH*$_2$-NH-R)$_2$ and α-H of Boc-Lys(Boc), 6H) 3.4-3.0 (ε-H of Boc-Lys(Boc) and Ar-(CH_2-N(Boc_2Lys)C*H*$_2$-R)$_2$, 8H), 1.9-1.1 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and-[-(CH_2)$_4$-]$_2$ of R groups, 64 H) and 0.88 (m, terminal CH_3 groups, 6H). FT-IR (cm^{-1}): 3330 (carbamate N-H str.), 2984-2863 (sp3 C-H str.), 1710 (C=O str. of carbamate), 1680 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 1059.7058 (calc. = 1059.7086).

(*N*,*N'*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(Boc-Lys(Boc)-*N*-octylamide), 3b): Yield-61 %. ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: 7.56-7.50 (t, Ar*H*, 4H), 7.27-7.24 (m, Ar*H*, 4H), 5.5-5.3 (m, N*H* of Boc-Lys(Boc), 4H), 4.9-4.3 (Ar(-*CH*$_2$-NH-R)$_2$ and α-H of Boc-Lys(Boc), 6H) 3.4-3.0 (ε-H of Boc-Lys(Boc) and Ar-(CH_2-N(Boc_2Lys)C*H*$_2$-R)$_2$, 8H), 1.9-1.1 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and-[-(CH_2)$_6$-]$_2$ of R groups, 72H) and 0.88 (m, terminal CH_3 groups, 6H). FT-IR (cm^{-1}): 3330 (carbamate N-H str.), 2984-2863 (sp3

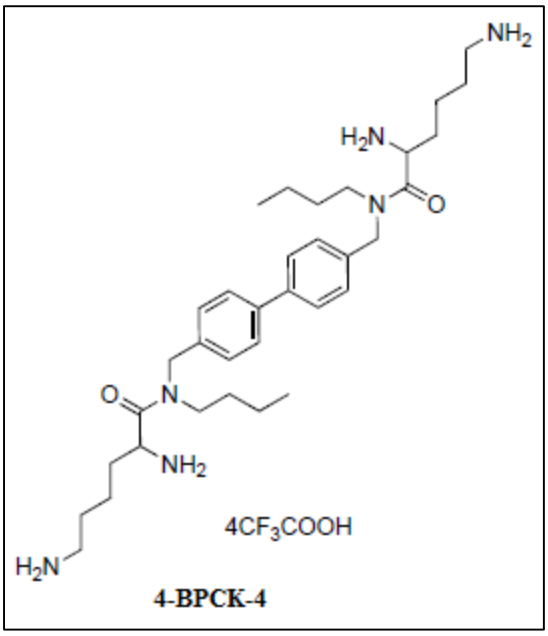
C-H str.), 1710 (C=O str. of carbamate), 1680 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 1093.7881 (calc. = 1093.7892).

(*N*,*N*'-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(Boc-Lys(Boc)-*N*-decylamide), 4b): Yield-50 %.^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: 7.56-7.50 (t, Ar*H*, 4H), 7.27-7.24 (m, Ar*H*, 4H), 5.5-5.3 (m, N*H* of Boc-Lys(Boc), 4H), 4.9-4.3 (Ar(-*CH*$_2$-NH-R)$_2$ and α-H of Boc-Lys(Boc), 6H) 3.4-3.0 (ε-H of Boc-Lys (Boc) and Ar-(CH_2-N(Boc_2Lys)C*H*$_2$-R)$_2$, 8H), 1.9-1.1 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and-[-$(CH_2)_8$-]$_2$ of R groups, 80H) and 0.88 (m, terminal CH_3 groups, 6H). FT-IR (cm^{-1}): 3330 (carbamate N-H str.), 2984-2863 (sp3 C-H str.), 1710 (C=O str. of carbamate), 1680 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 1149.8481 (calc. = 1149.8518).

(*N*,*N*'-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(Boc-Lys(Boc)-*N*-dodecylamide), 5b: Yield-68%.^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: ^{1}H NMR ($CDCl_3$) δ/ppm: 7.56-7.50 (t, Ar*H*, 4H), 7.27-7.24 (m, Ar*H*, 4H), 5.5-5.3 (m, N*H* of Boc-Lys(Boc), 4H), 4.9-4.3 (Ar(-*CH*$_2$-NH-R)$_2$ and α-H of Boc-Lys(Boc), 6H) 3.4-3.0 (ε-H of Boc-Lys (Boc) and Ar-(CH_2-N(Boc_2Lys)C*H*$_2$-R)$_2$, 8H), 1.9-1.1 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and-[-$(CH_2)_{10}$-]$_2$ of R groups, 88H) and 0.88 (m, terminal CH_3 groups, 6H). FT-IR (cm^{-1}): 3330 (carbamate N-H str.), 2984-2863 (sp3 C-H str.), 1710 (C=O str. of carbamate), 1680 (C=O str. of tertiary amide). HR-MS (m/z): $[M+Na]^+$ obsd. = 1227.8914 (calc. = 1227.8964).

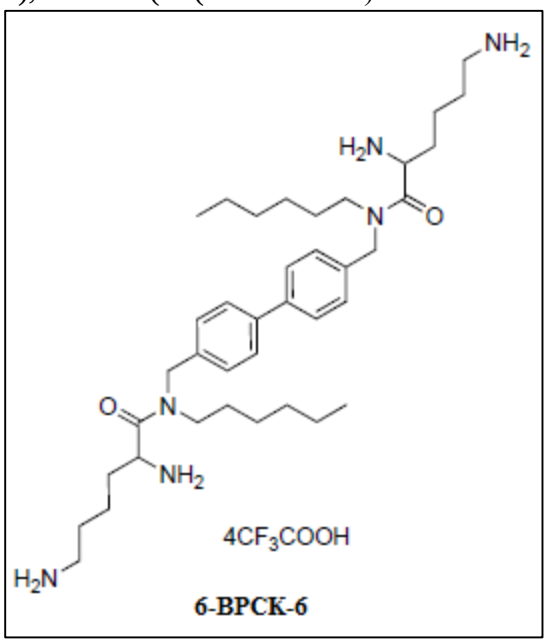
4.4.2.5. Deprotection of Boc groups (1-5): Typically, the compounds (1b−5b, 0.76 mmol) were dissolved in DCM (2 mL) and subsequently CF_3COOH (2 mL) was added and stirred at RT. The reactions were monitored by TLC until complete removal of starting material was observed. All the volatile components were removed and the product was purified by reverse phase HPLC using 0.1% trifluoroacetic acid (TFA) in water/acetonitrile (0−100%) as mobile phase to more than 95% purity. A C18 column (10 mm diameter, 250 mm length) and a UV detector (at 256 nm wavelength) were used. After drying the compounds in a freeze dryer, the compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR and mass spectrometry. The ^{13}C NMR have not been assigned and the spectra are furnished directly.

***N,N'*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(Lys-*N*-butylamide) (4-BPCK-4, 1):** ^{1}H NMR (D_2O, 400 MHz) δ/ppm: 7.7-7.2 (t, Ar*H*, 8H), 4.6-4.3 (Ar(-*CH*$_2$-NH-R)$_2$ and α-H of Lys, 4H) 3.6-3.3 (Ar-(CH_2-N(Lys)C*H*$_2$-R)$_2$, 2H), 3-2.8 (ε of CH_2 of Lys) 2.1-1 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NH_2 of Lys and-[-(CH_2)$_2$-]$_2$ of R groups, 20H) and 0.88 (m, terminal CH_3 groups, 6H). 4Hs have merged with the residual solvent peak of CD_3OD. ^{13}C (DMSO-d^6): 168.71, 168.51, 158.96, 158.62, 158.28, 138.66, 128.14, 127.75, 126. 78, 126.62, 126.56, 49.72,49.48, 47.64, 46.54, 44.76, 40.02, 39.81, 39.60, 39.39, 39.18, 38.97, 38.76, 38.42, 30.60, 30.31, 30.20, 28.45, 27.34, 26.54, 20.98, 20.78, 19.41, 19.25, 13.57, 13.56, 13.39. FT-IR (cm-1): 2963-2853 (sp3 C-H str.), 1679 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 581.4513 (calc. = 581.4543).



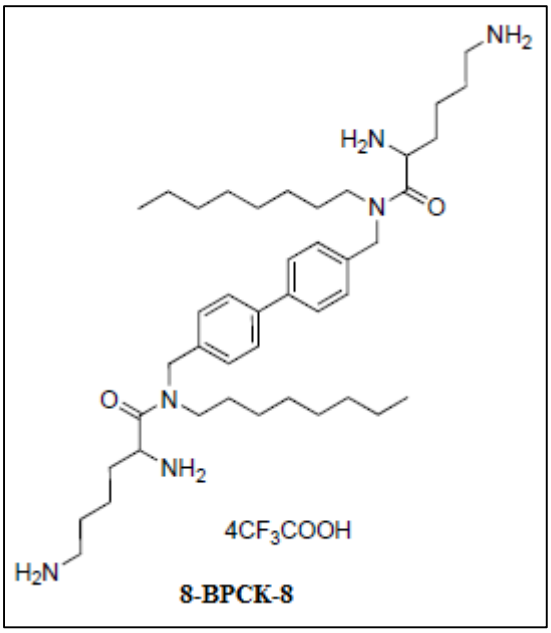
4-BPCK-4

***N,N'*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(Lys-*N*-hexylamide), (6-BPCK-6, 2)**: ^{1}H NMR (CD3OD, 400 MHz) δ/ppm: 7.7-7.2 (t, ArH, 8H), 4.6-4.3 (Ar(-CH2-NH-R)2 and α-H of Lys, 4H) 3.6-3.3 (Ar-(CH2-N(Lys)CH2-R)2, 2H), 3-2.8 (ε of CH2 of Lys) 2.1-1 (-CO-[CH-CH2-CH2-CH2-CH2-NH2 of Lys and-[-(CH2)4-]2 of R groups, 28H) and 0.88 (m, terminal CH3 groups, 6H). 4Hs have merged with the residual solvent peak of CD_3OD. ^{13}C (DMSO-d6): 168.76,168.48,158.63,158.29, 139.11, 138.68, 138.52, 136.70, 136.57, 135.72, 128.17, 127.80, 126.76, 126.60, 126.55, 49.74, 49.50, 47.71, 46.84, 39.97. FT-IR (cm-1): 2963-2853 (sp3 C-H str.), 1679 (C=O str. of tertiary amide). HR-MS (m/z): [M+H]+ obsd. = 637.5145 (calc. = 637.5169).



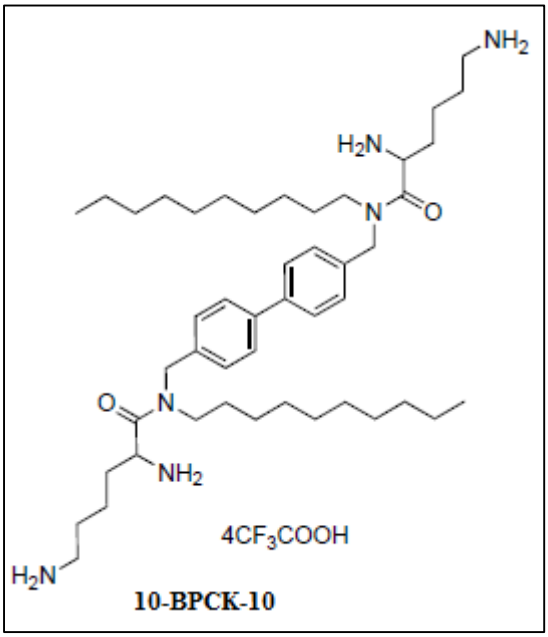
6-BPCK-6

***N,N'*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(Lys-*N*-octylamide), (8-BPCK-8, 3):** ^{1}H NMR (CD_3OD, 400 MHz) δ/ppm: 7.7-7.2 (t, Ar*H*, 8H), 4.6-4.3 (Ar(-*CH*$_2$-NH-R)$_2$ and α-H of Lys, 4H) 3.6-3.3 (Ar-(CH_2-N(Lys)C*H*$_2$-R)$_2$, 2H), 3-2.8 (ε of CH_2 of Lys) 2.1-1 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NH_2 of Lys and-[-$(CH_2)_6$-]$_2$ of R groups, 36H) and 0.88 (m, terminal CH_3 groups, 6H). 4Hs have merged with the residual solvent peak of CD_3OD. ^{13}C-NMR (DMSO-d^6): 170.21, 170.12, 162.65, 162.3, 129.84, 129.74, 128.71, 128.53, 128.2, 40.77, 40.26, 32.92, 32.87, 31.9, 31.74, 31, 30.35, 30.3, 30.25, 30.15, 30.01, 29.73, 28.55, 28.16, 28.06, 27.93, 27.74, 27.44, 23.66, 22.72, 22.62, 14.41FT-IR (cm-1): 2963-2853 (sp3 C-H str.), 1679 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 693.5787 (calc. = 693.5795).



8-BPCK-8

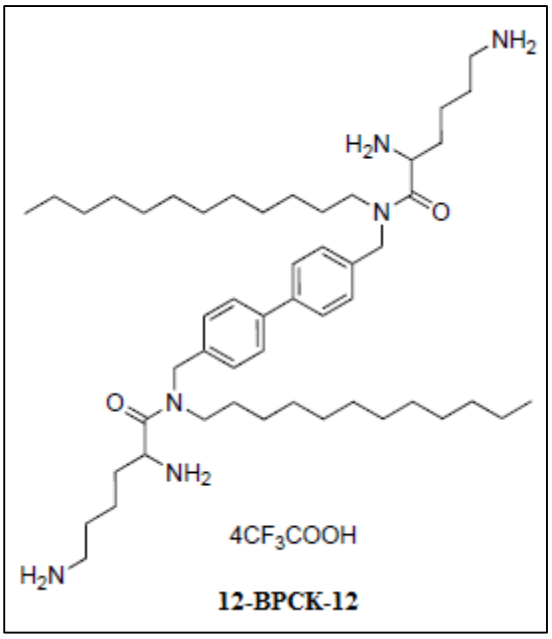
***N,N'*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(Lys-*N*-decylamide), (10-BPCK-10, 4) :** ^{1}H NMR (CD_3OD, 400 MHz) δ/ppm: 7.7-7.2 (t, Ar*H*, 8H), 4.6-4.3 (Ar(-*CH*$_2$-NH-R)$_2$ and α-H of Lys, 4H) 3.6-3.3 (Ar-(CH_2-N(Lys)C*H*$_2$-R)$_2$, 2H), 3-2.8 (ε of CH_2 of Lys) 2.1-1 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NH_2 of Lys and-[-$(CH_2)_8$-]$_2$ of R groups, 44H) and 0.88 (m, terminal CH_3 groups, 6H). 4Hs have merged with the residual solvent peak of CD_3OD. 13C (DMSO-d6): 170.17, 170.06, 163.3, 162.96, 141.7, 141.32, 137.65, 129.78, 128.71, 128.56, 128.21, 119.7, 116.79, 79.6, 79.28, 78.96, 52.03, 51.79, 51.59, 49.68, 49.47, 49.25, 49.04, 48.83, 48.61, 48.4, 47.78, 40.28, 40.24, 33.03, 31.94, 31.78, 30.65, 30.59, 30.41, 30.34, 29.74, 28.22, 28.06, 27.93, 27.75, 23.72, 22.74, 22.63, 14.45 FT-IR (cm-



10-BPCK-10

1): 2963-2853 (sp3 C-H str.), 1679 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 749.6419 (calc. = 749.6421).

***N,N'*-([1,1'-biphenyl]-4,4'-diylbis(methylene))bis(Lys-*N*-dodecylamide), (12-BPCK-12, 5):** ^{1}H NMR (CD_3OD, 400 MHz) δ/ppm: 7.7-7.2 (t, Ar*H*, 8H), 4.6-4.3 (Ar(-*CH*$_2$-NH-R)$_2$ and α-H of Lys, 4H) 3.6-3.3 (Ar-(CH_2-N(Lys)C*H*$_2$-R)$_2$, 2H), 3-2.8 (ε of CH_2 of Lys, 4H), 2.1-1 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NH_2 of Lys and-[-$(CH_2)_{10}$-]$_2$ of R groups, 52H) and 0.88 (m, terminal CH_3 groups, 6H). 4Hs have merged with the residual solvent peaks of CD_3OD. ^{13}C (DMSO-d^6): 158.56, 158.22, 128.19, 126.58, 49.50, 46.84, 40.04, 39.83, 39.62, 39.41, 39.20, 38.99, 38.79, 38.42, 31.23, 30.32, 28.93, 28.83, 28.63, 28.56, 28.13, 26.57, 26.15, 25.95, 22.03, 20.97, 20.78, 13.88 FT-IR (cm-1): 2963-2853 (sp3 C-H str.), 1679 (C=O str. of tertiary amide). HR-MS (m/z): $[M+H]^+$ obsd. = 805.7035 (calc. = 805.7047).



12-BPCK-12

4.4.3 *In vitro* biological assays

4.4.3.1 *In vitro* susceptibility studies: The MIC of the compounds against different bacterial strains were determined using a standardized protocol as described in chapter 2 (Section 2.4.3.1).

4.4.3.2 Hemolytic Activity: Hemolytic experiments were performed using a standardized protocol as described in chapter 2 (Section 2.4.3.2).

4.4.3.3 Bactericidal time-kill kinetics: Kinetics of bactericidal activity with 6-BPCK-6 was performed according to the protocol described in chapter 2 (Section 2.4.3.3).

4.4.3.4 Propensity to induce resistance development in bacteria: This was performed with 6-BPCK-6 following the protocol described in chapter 2 (Section 2.4.3.4).

4.4.3.5 Mechanism of action: The assays for determining the mechanism of action of 6-BPCK-6 were performed according to a standardized protocol as described in chapter 2 (Section 2.4.4) except for leakage of K^+ and inhibition of cell wall biosynthesis which are described below.

4.4.3.5.1 Intracellular potassium ion leakage assay: This was performed using a standardized protocol of the lab.[92] Mid-log phase (grown for 6 h) *S. aureus* cells were harvested, washed twice with 10mM HEPES (pH 7.2) and 0.5% glucose and were resuspended in the same amount of 10 mM HEPES (pH 7.2) and 0.5% glucose (10^8 CFU mL^{-1}). The bacterial suspension (0.2 mL) was placed in a 96 well plate. The fluorescence of the bacterial suspension was measured and allowed to stabilize for 2 min at room temperature before the addition of PBFI-AM dye (2 µM). Data was collected for an additional 2 min to establish a baseline signal before the addition of test compound (6-BPCK-6). The fluorescence signals were collected for each sample over 10 min. The fluorescence of the dye was monitored at excitation wavelength of 346 nm and emission wavelength of 505 nm.

4.4.3.5.2 Intracellular accumulation of UDP-N-acetyl-muramyl-pentadepsipeptide: The experiment was performed as reported earlier.[197] Analysis of the cytoplasmic peptidoglycan nucleotide precursor pool was examined using *S. aureus* grown in 25 mL MHB. First, cells grown to an OD_{600} of 0.6 were incubated with 130 µg mL^{-1} of chloramphenicol for 15 min. Then, test compounds vancomycin (5 µM), compound 2 (15 µM) were added and incubated for another 60 min. Subsequently, bacterial cells were centrifuged and washed with sterile water to remove the antimicrobial agents before being subjected to boiling water treatment for about 30 min. Following the boiling water treatment the cell extract was centrifuged and the supernatant was lyophilized. The lyophilized powder obtained was subsequently dissolved in 2 mL of water and pH was adjusted to 2.0 with 20% phosphoric acid. The UDP-linked cell wall precursors in the solution were then analyzed by RP-HPLC monitoring the UV absorbance peak at 260 nm. The presence of the precursor was confirmed by HR-MS mass spectrometry.

4.4.3.6 MICs of the compounds against several strains of *Clostridium difficile* and its spores: These experiments were performed as described in Chapter 3 (Section 3.4.3.7)

4.4.3.7 Effect of the compounds on human gut normal flora: These experiments were performed as described in Chapter 3 (Section 3.4.3.8)

4.4.3.8 Toxicity against RAW macrophages: Cytotoxicity of the compound 2 was assessed against RAW 264.7 cell line. Briefly, the cells were grown in a 96-well plate in DMEM media (supplemented with 10% fetal bovine serum and 5% penicillin-streptomycin) until they reached around 70-80% confluency. The cells were then treated with 50 μL of serially diluted compound. Two controls were made; one containing no compound (non-treated cells) and the other one was treated with 10 vol % Triton-X 100 solution. The plate was incubated for 1 h at 37 °C under 5% CO_2 atmosphere. After 24 h, the medium was carefully removed and 100 μL of MTT solution (5 mg mL^{-1} concentration) was then added to each well. The plate was incubated for 4 h at 37 °C under 5% CO_2 atmosphere. Then it was centrifuged at 1100 rpm for 5 min and the supernatant was removed. After that 100 μL of DMSO was added to solubilize formazan crystals. The O. D. of the plate was then recorded at 570 nm. Percentage of cell survival was calculated using the following equation: Cell viability (%) = (Atreated-AtritonX-treated)/(Anon-treated-AtritonX-treated)×100. Each concentration had triplicate values and the average of triplicate O. D. values were plotted against concentration followed by fitted with sigmoidal plot. From the curve the values were determined corresponding to 50% cell viability. For bright-field microscopic images, a 40x objective was used and images were captured using a Leica DM2500 microscope.

4.4.3.9 Toxicity against intracellular MRSA within RAW macrophages: The intracellular activity of the test compounds (compound 2 and vancomycin) was determined by modification of a previously published protocol.[3] Briefly, 500 μL complete growth medium (1X DMEM, 10% FBS and 1% Penicillin-Streptomycin) containing ~10^5 RAW 264.7 cells were seeded in 24-well tissue-culture plates at 37 ºC with 5% CO_2 for 12 h to ensure cell adherence. Next, MRSA cells (4×10^5 CFU mL^{-1}) were suspended in complete growth medium during 1 h for opsonization. Then, the growth media of aforesaid 24-well tissue-culture plates containing RAW 264.7 cells was replaced with 500 μL of opsonized bacterial suspension and incubated further for 1 h at 37 ºC in

humidified air containing 5% CO_2 to allow phagocytosis. After incubation, macrophages were washed with 1X PBS (pH ~7.2) twice and treated with gentamicin (50 μg mL^{-1}) for 1 h in complete growth medium to remove any extracellular or non-phagocytic bacteria. Gentamicin was removed by washing the cells with PBS twice. Then compound 2 (15 μg mL^{-1}) and vancomycin (60 μM) in growth medium (500 μL) were added and incubated for 6 h and 24 h at 37 ºC with 5% CO_2. At the end of 6h or 24 h, the supernatant was withdrawn carefully and the adhered cells were lysed with 1 mL of ice cold water for 1 h. Dilutions of lysates in saline were plated on nutrient agar plates. The plates were then incubated at 37 ºC for 24 h, and viable bacterial colonies were counted on the next day. The results furnished are averaged values of two independent experiments.

4.4.4 *In vivo* studies

The mice were housed in individually ventilated cages (IVC) maintained with controlled environment as per the standards. They are housing—pathogen free conventional caging system, bedding material (Corn Cob). The husbandry conditions:-Light: dark cycle- 12:12 hours, Animal Room Temp: 22 ± 2°C, Relative humidity: 30–40%, Access to feed and water: ad libitum and Water: RO Water. Animals were randomly selected, marked to permit individual identification and kept in their cages for at least 5 days before the experiment to allow for acclimatization to the experimental conditions. Animal handling and experimentation protocols were followed according to OECD Guidelines for the Testing of Chemicals (OECD 425). All care was taken to cause no pain to the animals. Humane endpoints were used to avoid unnecessary distress and suffering in animals following an experimental intervention that would lead to death. The animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) and carried out as per the guidelines of Committee for the purpose of Supervision and Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi.

4.4.4.1 Acute dermal toxicity: This experiment was performed with 6-BPCK-6 using the exact protocol mentioned earlier in Chapter 2 (Section 2.4.5.1).

4.4.4.2 MRSA skin infection: The experiment with 6-BPCK-6 was performed exactly as described in Chapter 3 (Section 3.4.5.2).

4.4.4.3 *A. baumannii* burn infection: The experiment with 6-BPCK-6 was performed using the protocol described in Chapter 2 (Section 2.4.5.2) with slight modifications. The strain of *A. baumannii* used was a clinical isolate *A. baumannii* R674 and the concentration of colistin used was 30 mg kg^{-1}.

Chapter 5

Studies against metabolically inactive cells, biofilms and sepsis

Abstract

In the Chapters 2-4, the development of small molecular membrane-active agents with activity against planktonic bacteria were described. However, infections are of a more complex nature and also consist of bacteria in metabolically inactive or less active states. Further, bacteria also survive in stable communities known as biofilms. Biofilms, which are responsible for 65-80% of all infections, are recalcitrant to antibiotic treatment. Another problem associated with bacterial infections, is that of sepsis. Bacteria, when treated with antibiotics, release endotoxins such lipopolysaccharides (LPS), which upon aggregation are recognized by the immune system of the host. This, when overproduced, sometimes leads to multi-organ failure and in severe cases, death. This disease condition, called sepsis, needs urgent attention as no dedicated treatment exists. In this chapter, ability of the previously developed small molecules to tackle the problems of chronic infection and sepsis are presented. The activity of NCK-10 and 6-BPCK-6 against metabolically inactive cells of S. aureus and E. coli have been described, followed by their ability to act on biofilms of drug-resistant bacteria. Then, we probed if NCK-10 was able to inhibit, LPS induced sepsis. Finally, the efficacies of the compounds were validated in murine models of biofilm infection against MRSA and P. aeruginosa.

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(1) Ghosh, C. *et al.* "Aryl-alkyl-lysines: Membrane active small molecules active against murine model of burn infection." *ACS Infect. Dis.*, **2016**, *2*, 111-122.

(2) Ghosh, C. *et al.* "Aryl-alkyl-lysines: Agents that kill planktonic cells, persister cells, biofilms of MRSA and protect mice from skin-infection." *PLoS One*, **2015**, *10*, e0144094.

(3) Ghosh, C. *et al.* "Aryl-alkyl-lysines interact with anionic lipophilic components of bacterial cell envelope to prevent sepsis and chronic *Pseudomonas aeruginosa* infection " (Manuscript to be submitted)

5.1 Introduction

Chronic infections caused by bacteria are recalcitrant to antibiotic treatment.[200] Such infections are associated with stable communities of bacteria called biofilms. Other complications associated with bacterial infection involve the presence of dormant non-dividing cells.[137] Most of the antibiotics require bacteria to be in a metabolically active state to carry out their functions and consequently require a higher concentration to clear chronic infections.[170] During antibiotic treatment, some of the cells can down-regulate their metabolic processes in order to escape antibiotic treatment; these cells can also revert back to being normal after antibiotic stress is removed. These cells, called persister cells, are responsible for severe chronic infections which are very difficult to cure.[139] Thus, it is extremely important to find compounds which are active against such persister cells. Membrane-active agents have been reported to be a solution to stationary phase bacteria and persister cells.[201] Although some studies have been performed with synthetic membrane-active agents, this is one area which is often underexplored.

Bacterial biofilms, established as one of the most stable life forms known, are an eclectic mixture of extracellular polymeric substances (EPS) such as polysaccharides, proteins and extracellular DNA (eDNA). Bacteria can form biofilms on a variety of surfaces including walls, ship hulls and biomedical impants.[131, 132, 202] Biofilms have implications in a variety of chronic diseases such as cystic fibrosis, urinary tract infections, catheter infections, middle-ear infections, formation of dental plaques, gingivitis, skin infections, burn wound infections and so on.[133, 202] Although the matrix of the biofilm and the mechanism of biofilm formation have been studied in detail, treatment against biofilm related infections continues to be difficult.

Several strategies have been used to eradicate biofilms which include quorum sensing inhibitors,[203] metal nanoparticles,[204, 205] combination approaches,[206-208] and so on to inhibit or disrupt biofilm formation. Some small molecules have also been used to target biofilms. The biofilm matrix has several components which are negatively charged and have hydrophobic domains. It was surmised that due to such properties membrane-active compounds can disrupt biofilms. Some designs of membrane-active agents have indeed shown antibiofilm properties but the number is few.

Sepsis is a global problem causing around 8 million deaths per year. New born children are the worst hit, with around 1,90,000 deaths due to sepsis in India. The primary cause of sepsis is release of endotoxins by bacteria (often in response to antibiotic treatment). Ironically, there is no

dedicated treatment for sepsis and antibiotics remain the drugs of choice for treatment. In disease condition, bacteria release endotoxins such as lipopolysachharides (Gram-negative bacteria) or lipoteichoic acid (Gram-positive bacteria) which are recognized by the immune systems and prompts them to overreact. This often results in uncontrolled inflammation leading to multi-organ failure or death. This uncontrolled inflammation is due to an increased production of pro-inflammatory cytokines such as TNF-α (tumor necrosis factor- α), IL-6 (Interleukin-6) from macrophages/monocytes that can cause septic shock. An effective way to treat sepsis would be to develop molecules with anti-endotoxin or anti-inflammatory properties. Certain natural AMPs such as LL-37, Indolicidin, human lactoferrin-derived peptide and Buforin II have been found possess such properties .[209-213] Upon binding to pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), these compounds prevent stimulation of the immune system. It is imperative to understand the mechanism of the processes which lead to sepsis in order to design molecules which might prevent it. The mechanism by which LPS leads to sepsis is briefly described below.

LPS form aggregates in aqueous solution and these aggregates bind to with LPS binding protein (LBP), and then to CD14 (primary receptor of LPS). This LPS-CD14 interacts with transmembrane protein Toll-like receptor-4 (TLR-4) which leads to induction of intracellular signaling and subsequent activation of the NF-κB transcription factor resulting in the secretion of pro-inflammatory cytokines such as TNF-α and IL-6. [148, 149]

Some of the reported AMPs and their synthetic mimics are known disintegrate LPS aggregates thereby preventing recognition by LBP.[213-217] Some other molecules such as Polymyxin B (PMB), $rBPI_{21}$ and ultrashort AMPs have been reported to promote further LPS aggregation.[218-220] As mentioned in Chapter 1, certain small molecules have been shown to possess immunomodulatory and anti-inflammatory properties.

From Chapters 3 and 4 we have identified NCK-10 and 6-BPCK-6 (Figure 5.1) as the most promising compounds. In this chapter, we have first looked at the ability of these two compounds to act on stationary phase and persister cells of *S. aureus* and *E. coli*. Further, we probed if membrane-activity indeed was retained as the primary mechanism of action. Then, the antibiofilm activity of NCK-10 and 6-BPCK-6 have been presented. As model systems, activity against biofilms of MRSA and *P. aeruginosa* have been performed *in vitro* before validation in

murine models of infection. Further, we look at the ability of NCK-10 to interact with aggregates of LPS and prevent LPS-induced stimulation of pro-inflammatory cytokines TNF-α and IL-6.

5.2 Results and discussion

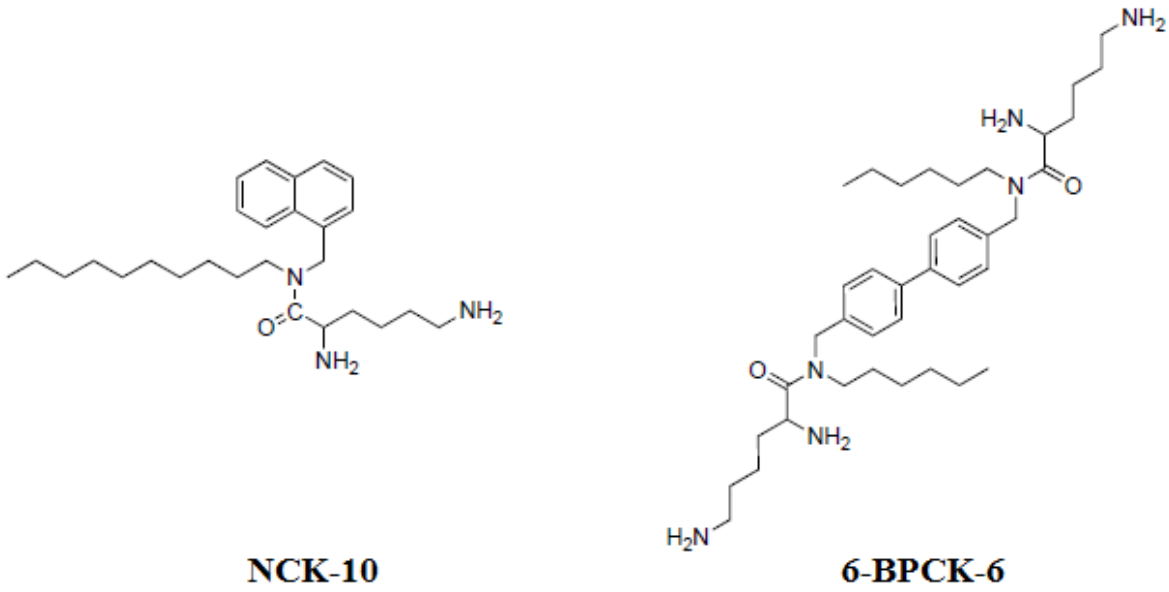

Figure 5.1: Structures of compounds used in the study.

5.2.1 Activity against stationary phase and persister cells of *S. aureus*

Antibiotics are required in very high concentrations to treat stationary phase *S. aureus* cells. Remarkably, NCK-10 when treated against *S. aureus* cells in stationary phase (10^5CFU mL^{-1} in control), showed complete killing at 5 × MIC while, ampicillin, which is active at 0.1 µM against planktonic cells of this strain of *S. aureus* (MTCC 737), was shown to be inactive at even 50 µM Figure 5.2. Likewise, 6-BPCK-6 also lysed the cells at 5× MIC in 2h.

Persister cells of *S. aureus* are often encountered in biofilm conditions and are known to evade treatment with antibiotics. This is an important problem and there is a requirement for compounds which lyse such cells. As expected ampicillin was not able to inactivate the cells. But NCK-10 at 5×MIC was sufficient to completely lyse persister cells of *S. aureus* in comparison to control, where the bacterial count was around 10^5 CFU mL^{-1}. When treated with 6-BPCK-6 too, no

bacterial cells were visible at the end of 2h demonstrating that this class of compounds was effective in treating dormant or non-dividing cells of *S. aureus*.

5.2.2 Activity against stationary phase and persister cells of *E. coli*

NCK-10 when treated against *E. coli* cells in stationary phase, showed complete killing even at its MIC. While in the control, the bacterial cell count was maintained at 6 log CFU, no colonies were observed in NCK-10 (5 µM) treated cultures (the limit of detection in this experiment is 50 CFU mL^{-1}). As can be seen from the Figure 5.2, kanamycin was not effective in eradicating stationary phase cells of *E. coli*. In fact, 6-BPCK-6 was did not lyse the cells even at 5× MIC (20 µM). This shows that 6-BPCK-6 needs *E. coli* to be metabolically active state to carry out its function. Another inference that can be drawn is that membrane-activity is probably not the only mechanism by which 6-BPCK-6 acts against Gram-negative bacteria.

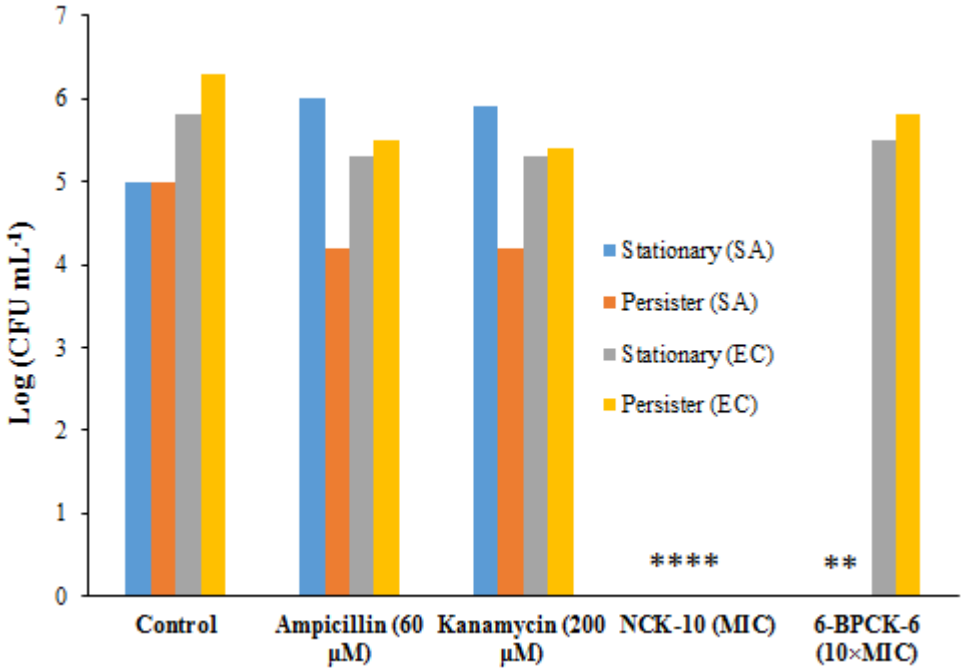

Figure 5.2: Ability of NCK-10 (5 µM) and 6-BPCK-6 (20 µM) to act on stationary phase bacteria and persister cells of *S. aureus* and *E. coli* in comparison to Ampicillin and Kanamycin * indicate no bacteria observed. Detection limit is 50 CFU mL^{-1}.

Persister cells of *E. coli* are also notorious in evading antibiotics in case of chronic infections. Thus, there is a huge demand for compounds which act against such cells. NCK-10, at its MIC was sufficient to completely lyse persister cells of *E. coli* in 2 h unlike kanamycin or 6-BPCK-6 (Figure 5.2). Again, while the untreated control maintained a steady count of cells, no visible colony was observed in persister cells treated with the compound.

It could be concluded that NCK-10 was the more effective agent to treat dormant and slowly-dividing cells of bacteria. Thus, we chose to study the mechanism of action of NCK-10 against persister cells of *S. aureus* and *E. coli*.

5.2.3 Mechanism of action against persister cells of *E. coli*

Membrane active agents are hypothesized to retain similar mechanism of action against persister cells. Herein, we studied the ability of NCK-10 to depolarize and permeabilize the membranes of *E. coli* persister cells. The concentration that was chosen to do the experiment were at MIC (5 μM), 2 × MIC and 5 × MIC. The compound was able to rapidly depolarize the membranes of *E. coli* persister cells (Figure 5.3A). The compound was able to infiltrate the outer-membrane of *E. coli* as well as the inner membrane (Figure 5.3B and 5.3C respectively). However, the ability to permeabilize the inner membrane was greatly diminished at lower concentrations. A similar observation was made when the experiments were done with *S. aureus* persister cells as well.

Although there have been quite a few reports of small molecules which exhibit activity against persister cells, hardly any have probed into their mechanism of action.[221-226] Investigations into the mechanism of action of the compound against the persister cells revealed some interesting results. The ability of the compounds to depolarize the *E. coli* persister cells and act on its outer membrane was as expected. Previous investigations into inner membrane permeabilization of metabolically active *E. coli* always yielded negative results for such compounds. This underscores the difference between metabolically active cells and persister cells; additionally the importance of such membrane active agents against persister cells is also established.

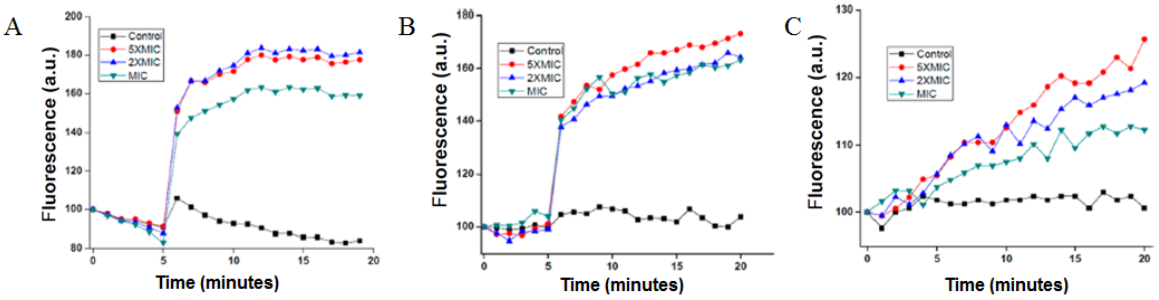

Figure 5.3: Mechanism of action against persister cells. A) Depolarization of the membranes of *E. coli* persister cells. B) Outer membrane permeabilization of *E. coli* persister cells and C) Inner membrane permeabilization of *E. coli* persister cells caused by NCK-10. (MIC is 5 μM).

5.2.4 Ability to lyse cells embedded within biofilms formed by MRSA

S. aureus biofilms are considered to be serious impediments to healthcare officials all over the world. Agents that can disrupt pre-formed biofilms and kill the bacteria dispersed from biofilms are elusive and still represent an unmet need in healthcare. As mentioned earlier, underneath the polymeric matrix of biofilms there is a community of bacterial cells. The ability of NCK-10 and 6-BPCK-6 to disrupt the biofilm matrix and kill the embedded cells were tested. As can be seen from Figure 5.4A, NCK-10 and 6-BPCK-6 were effective in reducing the number of viable cells of 24h pre-formed MRSA (ATCC 33591) biofilms in a concentration dependent manner. At 10×MIC, NCK-10 was effective in reducing the number of bacteria embedded within the biofilm

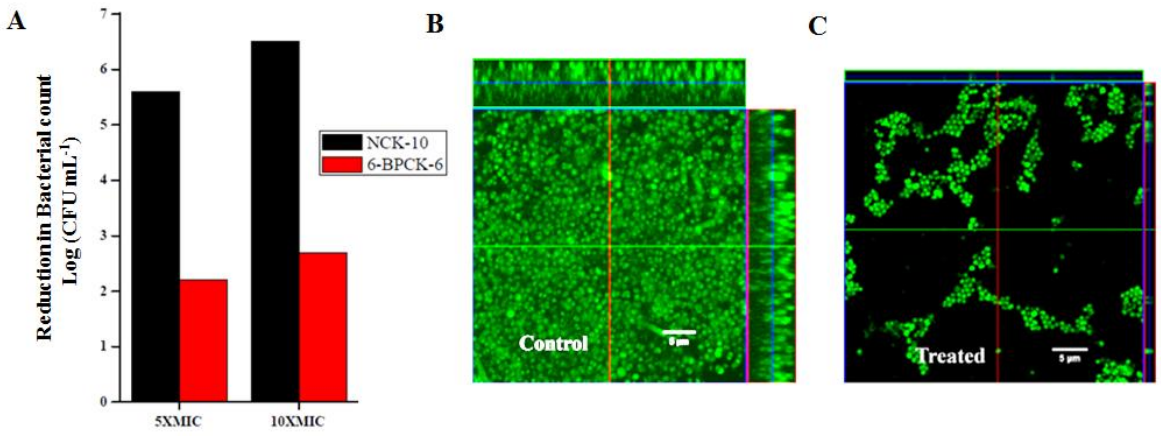

Figure 5.4: A) Ability of NCK-10 and 6-BPCK-6 to act on cells embedded within biofilms of MRSA. Confocal images of biofilms B) Untreated and C) Treated with NCK-10 (10×MIC). MIC considered is 4 μM.

by 6.5 logs, while 6-BPCK-6 at the same concentration could reduce viability by 2.8 logs only. NCK-10 was also effective at lower concentrations unlike 6-BPCK-6. Thus, NCK-10 was much more effective compound in comparison to 6-BPCK-6. We then looked at the extent of disruption caused by NCK-10 using confocal microscopy.

5.2.5 Disruption of MRSA biofilms

Confocal images bear a further proof of the anti-biofilm property of the compound. In this experiment, preformed biofilms of MRSA were treated with NCK-10 at 10×MIC and the resultant biofilms were stained with SYTO-9 and observed under a confocal microscope. As can be seen from Figure 5.4B, in the untreated case, there was a dense biofilm which was 23 μm in thickness, while in the treated case (Figure 5.4C), the thickness of the dispersed biofilm was only 2 μm. A huge reduction in the thickness of the biofilm can also be observed in the images.

5.2.6 Ability to lyse cells embedded within *P. aeruginosa* biofilms

P. aeruginosa is notorious for forming bacterial biofilms. The biofilm matrix of *Pseudomonas sp*. has been studied.[227] It is well accepted that several negatively charged components make up the biofilm matrix such as alginates, LPS, DNA etc. Outer membrane vesicles also contribute to the biofilm matrix.[227-229] Earlier it was shown that these compounds infiltrate the outer membrane of

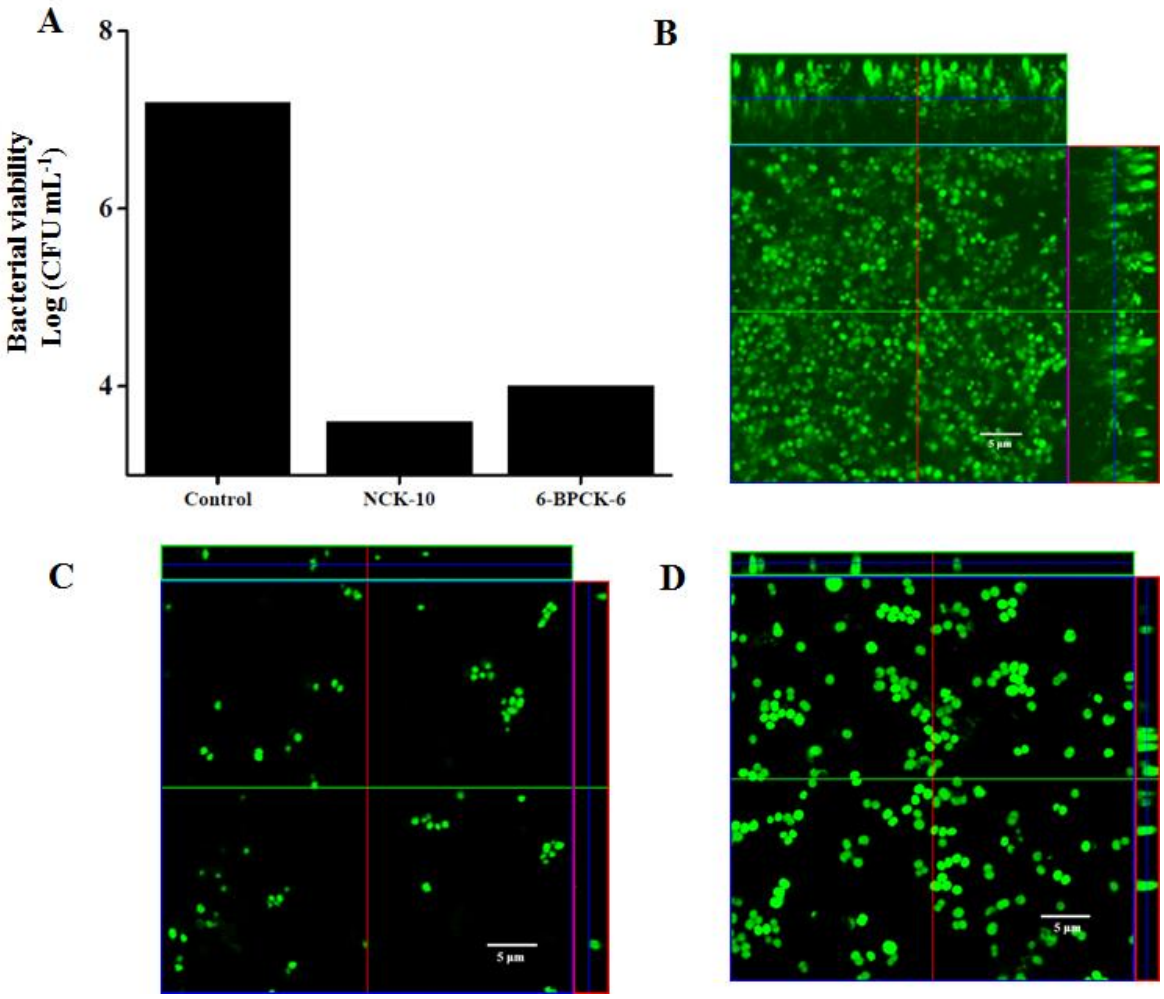

Figure 5.5: A) Ability of NCK-10 and 6-BPCK-6 to act on bacteria embedded within biofilms of *P. aeruginosa* at 50 µM each. Confocal images of biofilms B) Untreated C) Treated with NCK-10 and D) Treated with 6-BPCK-6.

Gram-negative bacteria. Hence, we surmised that NCK-10 and 6-BPCK-6 would be able to disrupt the biofilm matrix of *P. aeruginosa*. In order to do so, we treated 72 h pre-formed biofilms of *P. aeruginosa* with the compounds at 10×MIC and counted the bacterial cells that remained after treatment. As can be seen from the Figure 5.5A, while the bacterial count was 7.3 logs in the untreated case, it was reduced to 3.6 logs upon treatment with 10×MIC of NCK-10, when treated with 6-BPCK-6, the bacterial count was reduced to 4 logs, emphasizing on the efficacy of the compounds in treating biofilms of *P. aeruginosa*.

5.2.7 Disruption of *P. aeruginosa* biofilms

In order to visualize the effect of the compound on the biofilms of *P. aeruginosa*, we treated 72h old biofilm (grown on a glass cover slip) of a clinical isolate of *P. aeruginosa* R590 (described earlier) at concentrations of 50 µM for 24h and subsequently stained the biofilm with SYTO-9. As can be seen from the Figure 5.5(B-D), in the untreated case, the biofilm was thick and uniform whereas, upon treatment with both the compounds, a monolayer of cells remained and were sparsely distributed. This proved, that the compound could indeed demonstrate antibiofilm activity against *P. aeruginosa*.

5.2.8 Ability to interact with LPS

From the experiments and theoretical studies described above, it is clear that NCK-10 interacts strongly with the membranes of bacteria. Since the compound contained two positive charges and one decyl chain, we reasoned that it might interact with other negatively charged hydrophobic molecules such as LPS. As mentioned earlier LPS is a component of Gram-negative bacteria and form aggregates in aqueous solution. These aggregates are recognized by host-cells and a cascade of reactions are initiated upon recognition, which ultimately leads to sepsis. We surmised that if NCK-10 interacts with LPS, it would also prevent inflammatory response and ultimately sepsis.

At first we checked the ability of NCK-10 to interact with BODIPY dye conjugate of LPS. Due to aggregation, the fluorescence of BODIPY-LPS is quenched. However, upon interaction with an amphiphilic molecule, dissociation of the aggregates occur and subsequent increase in fluorescence is observed. As can be seen from Figure 5.6, Sodium dodecyl sulphate (2%) was able to increase the intensity of fluorescence by several orders of magnitude in comparison to control

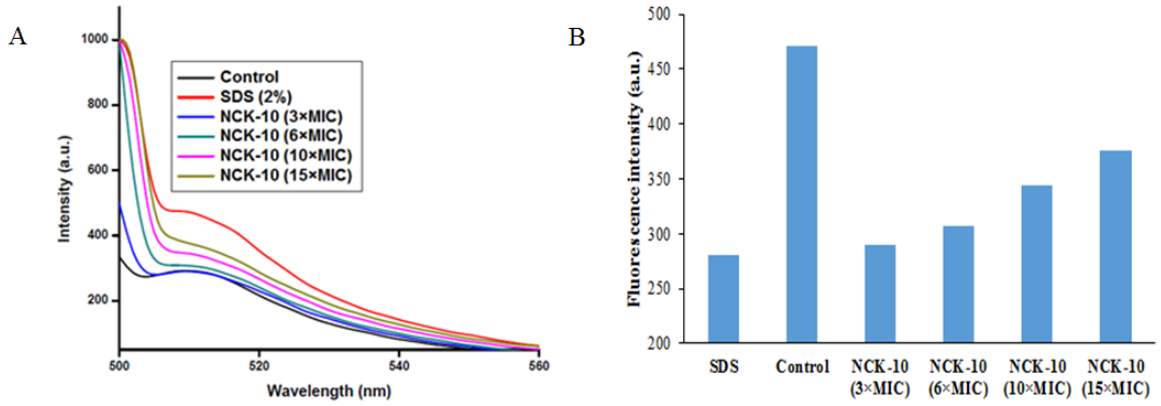

Figure 5.6: A) Ability of NCK-10 to interact with LPS-BODIPY. B) A dose-dependent response can be observed. SDS (2%) was used as a positive control. λmax is 510 nm and the MIC considered is 5 µM.

(blank). NCK-10, was able to interact with LPS at concentrations of 15 µM (3×MIC) and above (Figure 5.6A). With increasing concentration, the response was enhanced at λ_{max} 510 nm (Figure 5.6B). It could, thus be concluded, that like SDS, NCK-10 was able to dissociate the LPS aggregates.

5.2.9 Ability to prevent LPS induced stimulation of pro-inflammatory cytokines TNF-α and IL-6

We surmised that if the compound can dissociate the aggregates of LPS, it can prevent pro-inflammatory response that ultimately leads to sepsis. Thus, we proceeded to check the response of LPS, on immune cells in presence and absence of NCK-10. The experiment was performed with human peripheral blood mononuclear cells (hPBMCs), which are known to circulate in blood and produce inflammatory response upon interacting with bacterial endotoxins such as LPS. hPBMCs were isolates from fresh human blood using Ficoll-Hypaque density centrifugation technique. Then, the hPBMCs were stimulated with LPS (concentration used was 20 ng mL^{-1}) in presence and absence of NCK-10 (3×MIC). The secretion of pro-inflammatory cytokines, TNF-α and IL-6 was quantified using ELISA. As can be seen from Figure 5.7, NCK-10 on its own did not elicit

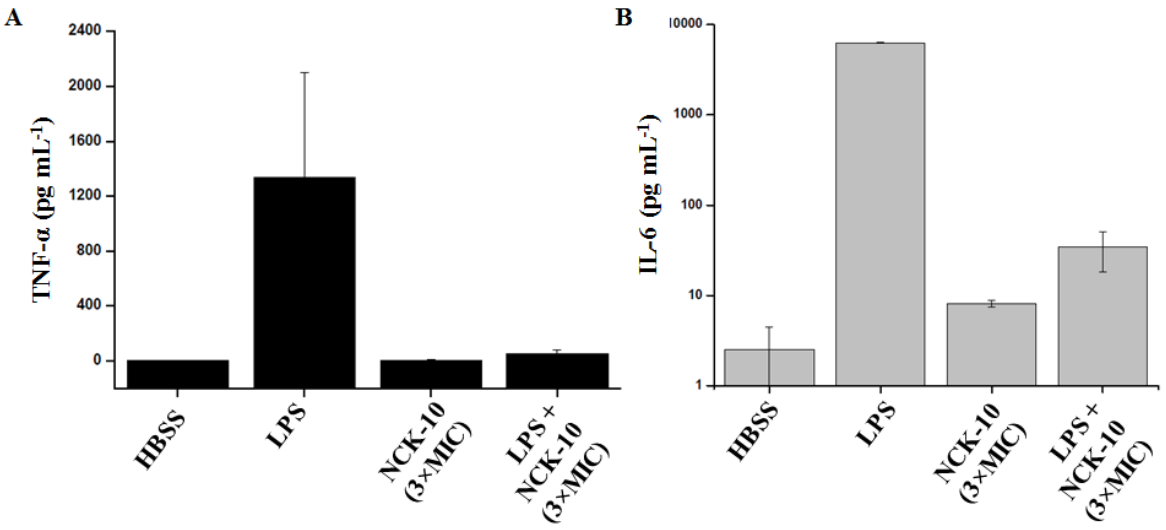

Figure 5.7: Ability of NCK-10 to suppress LPS induced stimulation of A) TNF-α and B) IL-6.

any response. On the other hand, LPS successfully stimulated the production of both TNF-α and IL-6. However, in presence of NCK-10, the pro-inflammatory response of LPS was suppressed. This experiment proved that, NCK-10 could not only treat bacterial infection but also prevent sepsis.

5.2.10 Efficacy studies in murine models of biofilm infection

5.2.10.1 Murine model of skin infection caused by biofilms of MRSA

The activity of NCK-10 was then tested in murine model of MRSA biofilm infection. The experiment was performed like that described in Chapter 2 except that treatment was initiated twenty four hours after infection was set. It has been previously reported that within 24 h of infection bacteria form biofilms. To establish the biofilms in our model, we had separately set infection on three mice, sacrificed them after 24h and analyzed their skin sections under FESEM. It could be observed from Figure 5.8, large portions of the tissue surface were covered by dense

MRSA biofilms. It can be seen that MRSA have already infected the tissues and in certain regions multiple layers could be seen. This clearly indicated that within 24h, biofilms are formed by MRSA on mice skin.

In the MRSA biofilm-infection experiment, fusidic acid was used as a comparator drug. Both NCK-10 and fusidic acid was dosed at 40 mg kg^{-1} (in saline) for 7 days as described above. At the

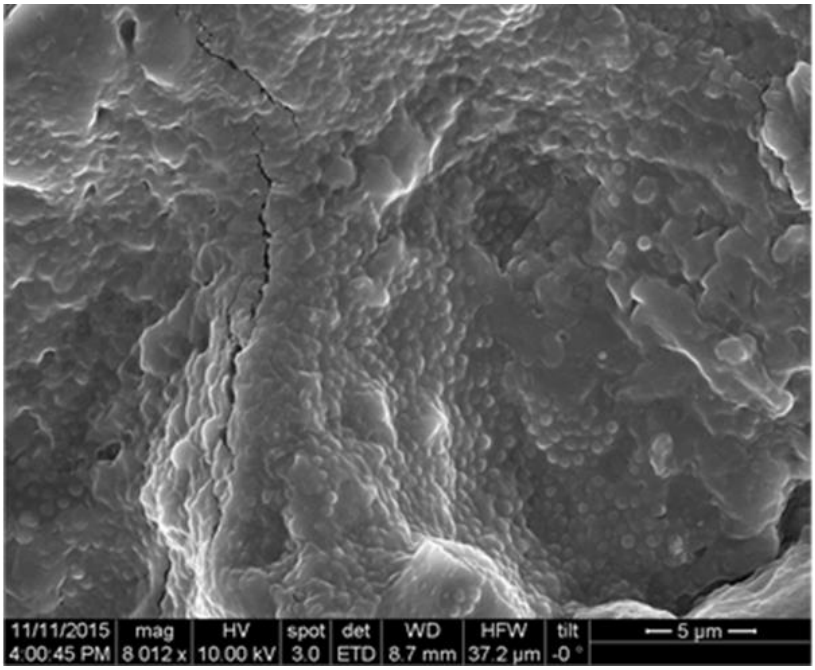

Figure 5.8: Scanning electron micrographs of the wound tissue surface 24 h post inoculation. A carpet of bacteria can be seen on the surface of the skin, indicating biofilm formation.

end of seven days, the mice were sacrificed and their wounded skin sections were analyzed. The skin at the region of infection was excised and a count of bacterial colonies were obtained from their homogenates. The results showed that while fusidic acid was not effective in reducing bacterial burden (p value 0.45), the compound was able to bring about significant reduction (p value 0.0013) in bacterial burden (Fig 5.9A). This result indicated that *in vivo* the compound was able to act on planktonic cells as well was biofilms of MRSA. The fact that it was much more effective in comparison to the approved drug fusidic acid emphasizes on the clinical potential of the compound.

5.2.10.2 Murine model of burn infection caused by biofilms of *P. aeruginosa*

The efficacy of both NCK-10 and 6-BPCK-6 was tested in *P. aeruginosa*-biofilm model of burn infection. Herein, burn wounds were first created in mice and then the wounds were infected with 10^7 CFU mL^{-1} of *P. aeruginosa* R590. In order to facilitate biofilm formation, the wounds were left untreated for 24 h. At the end of 24 h, the wounds were subsequently treated for 6 days with NCK-10 and 6-BPCK-6 at 40 mg kg^{-1} respectively once a day. As a positive control, colistin was used at 30 mg kg^{-1}. As can be seen from Figure 5.9B, in the untreated control, the bacterial count was 8.1 logs while in case of NCK-10 treated mice, the bacterial count was 5.8 logs. 6-BPCK-6 wass more effective and it brought the bacterial burden down to 3.9 log. Although colistin was more efficacious, it has to be kept in mind that colistin is quick to trigger resistance development unlike NCK-10 and 6-BPCK-6. The fact that 6-BPCK-6 significantly outperformed NCK-10 in murine model emphasized that *in vitro* activity is not always reflected *in vivo* conditions.

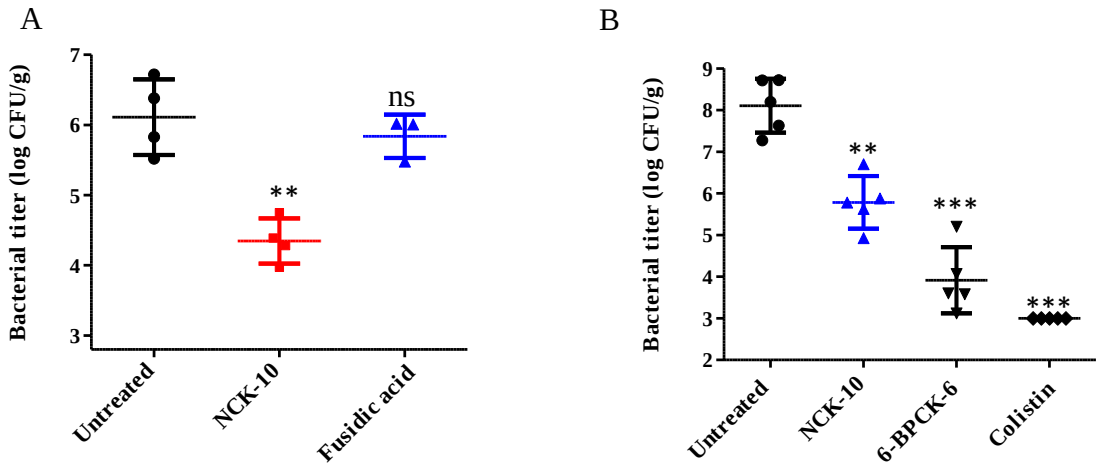

Figure 5.9: A) Ability of NCK-10 to act against biofilms of MRSA in a murine model of skin infection caused by MRSA. Fusidic acid was used as a comparator. B) Activity of NCK-10 and 6-BPCK-6 to act on biofilms of *P. aeruginosa* in a murine model of burn infection. Colistin was used as control.

5.3 Conclusions

From Chapter 5, it can be concluded that membrane-active small molecules (especially NCK-10) can also be used to treat dormant cells of both Gram-positive and Gram-negative bacteria. Indeed, activity on membranes of these cells imparts such potency. In fact, the compounds were also active against biofilms of MRSA and *P. aeruginosa*. NCK-10 could also suppress LPS induced production of TNF-α and IL-6. NCK-10 was active against skin-infection model of MRSA biofilms in mice. Against *P. aeruginosa* model of biofilm infection in mice, 6-BPCK-6 was more efficacious than NCK-10. Overall, the compounds could be used for treatment of chronic bacterial infections.

5.4 Experimental section

5.4.1 Activity against Stationary-phase bacteria (*S. aureus*): This experiment was performed by following a published protocol with little modification. *S. aureus* was grown for 6 h in nutrient broth and contained ~10^9 CFU mL^{-1} (determined through dilution plate technique by spread plate method). This was then diluted to 1000 fold and incubated 37 ºC for 16 h to obtain stationary-phase cultures. At the end of 16h, the cells were centrifuged down, washed twice with Minimum Essential Media (MEM) and resuspended in MEM media at concentration of 10^6 CFUmL^{-1}. 50 µL of the test compound, NCK-10 or Ampicillin was then added to 150 µL of the stationary-phase bacteria (in wells of a 96 well plate) with the working concentrations of 15 µg mL^{-1} and 20 µgmL^{-1} respectively. In the negative control the well contained 200 µL of MEM. The 96 well plate was then incubated at 37 ºC with shaking at 150 rpm. At the end of 2h, 20 µL of aliquots from that solution were serially diluted 10-fold in MEM (GIBCO) media. Then from the dilutions, 20 µL was plated on nutrient agar plates and incubated at 37 ºC. After 24 h the bacterial colonies were counted and results represented in logarithmic scale, i.e. log (CFU mL^{-1}). The results furnished are averages of two independent experiments.

5.4.2 Isolation of Persister cells of *S. aureus* and activity against them: Persister cells of S. aureus were isolated following a previously published protocol. After growing the respective bacteria to their stationary phases using the protocol mentioned above, *S. aureus* cells were then treated with 100 µg mL^{-1} ampicillin for 3h. Then they were centrifuged down, and washed with

MEM twice and resuspended again in the same media. These cells were then diluted to 10^5 cells and treated with the compound (at concentrations of 5×MIC, 2×MIC and MIC) and also with another round of Ampicillin (20 µg mL^{-1}) for 2h or left untreated in wells of a 96 well plate as mentioned above. It was then incubated at 37ºC with shaking at 150 rpm. At the end of 2h, 20 µL of aliquots from that solution were serially diluted 10-fold in corresponding media. Then from the dilutions, 20 µL was plated on nutrient agar plates and incubated at 37 ºC. After 24 h the bacterial colonies were counted and results represented in logarithmic scale, i.e. log (CFU mL^{-1}). The results furnished are averages of two independent experiments.

5.4.3 Activity against Stationary-phase bacteria (E. coli): *E. coli* (MTCC 443), was grown for 6 h in Luria-Bertani broth and contained ~109 CFU mL^{-1} (determined through dilution plate technique by spread plate method). This was then diluted 1000 fold and incubated at 37 ºC for 18 h to obtain stationary-phase cultures. At the end of 18h, the cells were centrifuged down, washed twice with M9 media, resuspended in M9 media and diluted to a concentration of 10^5 CFU mL^{-1}. The test compound, NCK-10 was then added to the stationary-phase bacteria with the working concentrations of 15 µg mL^{-1}. It was then incubated at 37 ºC with shaking at 150 rpm. At the end of 2h, 20 µL of aliquots from that solution were serially diluted 10-fold in saline. Then from the dilutions, 20 µL was plated on nutrient agar plates and incubated at 37 ºC. After 24 h the bacterial colonies were counted and results represented in logarithmic scale, i.e. log (CFU mL^{-1}).

5.4.4 Isolation of persister cells of *E. coli* and activity against them: After growing *E. coli* to their stationary phases using the protocol mentioned above, they were treated with ampicillin (300 µg mL^{-1}) for 3h. Then they were centrifuged down, and washed with M9 media for twice and resuspended again in the same media. These cells were then diluted to 10^5 cells and treated with the compound (at concentrations of MIC, 2×MIC and 5×MIC) for 2h or left untreated. It was then incubated at 37 ºC with shaking at 150 rpm. At the end of 2h, 20 µL of aliquots from that solution were serially diluted 10-fold in corresponding media. Then from the dilutions, 20 µL was plated on agar plates and incubated at 37 ºC. After 24 h the bacterial colonies were counted and results represented in logarithmic scale, i.e. log (CFU mL^{-1}).

5.4.5 Mechanism of action: Persister cells were isolated as described above and diluted to 10^8 cells in respective media. Membrane depolarization studies using $DiSC_3$ (5) dye, membrane permeabilization studies using PI and outer membrane permeabilization studies were all conducted in the same procedure as described in **2.4.4**; the only difference being isolated persister cells were used instead of mid-log phase bacteria. The concentrations used in this study were MIC, 2 × MIC, 5 × MIC.

5.4.6 Biofilm disruption assays

5.4.6.1. Ability to lyse cells embedded within biofilms: Sterilized cover slips were placed in wells of a 6-well plate. 2 mL of Midlog phase (6 h grown) culture of MRSA or *P. aeruginosa* (R590), diluted to approximately 10^5 CFU mL^{-1} in a nutrient broth supplemented with 0.1% NaCl and 0.1% glucose acid, was added to wells containing the cover slips. The plate was incubated under stationary conditions at 33 ºC for 24h (MRSA) or 72 h (*P. aeruginosa*). After 24h (MRSA) or 72 h (*P. aeruginosa*), media was removed and planktonic bacteria were carefully washed with 1X PBS (pH = 7.4) and removed. Biofilm containing cover slips were then placed into another 6-well plate and 2 mL of media containing NCK-10 (5×MIC and 10×MIC) was added to it and allowed to incubate for 24 h. In case of control, 2 mL of complete medium was added without compound. At the end of 24 h, medium was then removed and planktonic cells were removed by washing with 1X PBS. . The disrupted biofilms were then scraped with pipette tip following 5 min-exposure to 100 µL trypsin-EDTA (0.25%) (GIBCO). Cell suspension was then vortexed at high speed for 1 min to break up the clumps. The bacterial counts were assessed by plating serial 10-fold dilutions of biofilm cell suspensions on nutrient agar plates. After 24 h of incubation the plates were counted and cell viability was expressed as log (CFU mL^{-1}) and compared with non-treated control. The experiment was performed twice.

5.4.6.2. Confocal laser scanning microscopy (CLSM): As Cover slips were first sterilized by soaking them in ethanol followed by drying in flame, and then placing in well of a 6-well plate. Midlog phase (6 h grown) culture of *S. aureus* or *P. aeruginosa* was then diluted to approximately 10^5 CFU mL^{-1} in a nutrient broth supplemented with 1% glucose and 1% NaCl and 2 mL of this stock was added to cover slips containing wells. The plate was then incubated under stationary conditions at 37 ºC. After 24 h (MRSA) or 72 h (*P. aeruginosa*), media was removed and

planktonic bacteria were carefully washed out with 1X PBS (pH = 7.4). Biofilm containing cover slips were then placed into another 6-well plates and 2 mL of test compound NCK-10 (10×MIC) was added to it and allowed to incubate for 24 h. In case of control, 2 mL of complete medium was added instead of compound. At the end of 24 h, medium were then removed and planktonic cells were removed by washing with 1X PBS. Coverslips containing biofilm were carefully removed from the well and stained with SYTO-9 (3 μM) and imaged it using a Zeiss 510 Meta confocal laser-scanning microscope.

5.4.7 Ability to bind to LPS-BODIPY: LPS Binding and Dissociation was performed using a previously published protocol.230 Stock solution of BODIPY-LPS (100 μg mL^{-1}), obtained from *E. coli*, Molecular Probes, Life Technologies and diluted to 10 μg mL^{-1} in 1× PBS (pH 7.4). BODIPY-LPS stock solutions were sonicated for 2 min before use. 2 mL of solution of BODIPY-LPS was first sonicated and taken in a quartz cuvette at concentrations of 500 ng mL−1). Subsequently, the test compounds in PBS were added at respective concentrations. Experiments were performed using a λS55 fluorescence spectrophotometer (PerkinElmer) at an excitation wavelength of 485 nm, and emission was collected from 500 to 700 nm (excitation slit width = 5 nm, emission slit width = 15 nm) at room temperature.

5.4.8 Ability of NCK-10 to inhibit LPS-induced stimulation of pro-inflammatory cytokines: This experiment was performed using a previously published protocol.230 Briefly, to freshly isolated human PBMCs, seeded in 24-well plates for 3h (1×10^6 cells in 1 ml of RPMI), 20 ng mL−1 of *E. coli* 0111:B4 LPS (Sigma-Aldrich) was added in either the presence or absence of NCK-10 (10 μg mL^{-1}). Only HBSs and only NCK-10 were also added as controls. The cells were incubated for 18−24 h and then cell culture supernatants were analyzed for cytokines such as tumor necrosis factor (TNF-α) and interleukin- 6 (IL-6) using the human ELISA kits (BD Biosciences) following the manufacturer's instructions.

5.4.9 Animal studies

5.4.9.1 *In vivo* acute skin infection: For this experiment, exactly same protocol was followed as described in Chapter 3, but the dosage with fusidic acid (n=3) and NCK-10 (n=4) was started 24h post infection while in control (n=7) mice were left untreated. To establish the infection, three mice were inoculated with approximately 10^7 CFU of bacteria as described above. After 24 h these mice were sacrificed using isofluorane. Skin portion from the wound site of the infected mice were

severed aseptically, homogenized, serially diluted and plated on agar plates and counted. Small portions of the skin were also imaged under FESEM for visual proof of the formation of biofilm (the experimental protocol is described below). Typically, the count of bacteria 24h after bacterial addition is (6.65±0.03) log. Data was plotted using Graphpad prism software and P value was calculated using unpaired Student's *t* test (2 tailed 1 sample, unpaired distribution) between the control group and the treatment group and a value of $P < 0.05$ was considered significant.

5.4.9.2 *In vivo* chronic biofilm skin infection: This was followed by a modified protocol of previously published papers.[208, 231, 232] Female Balb/c mice (6-8 weeks, 22-25 g) were anesthetized with ketamine-xylazine cocktail, their dorsal surface shaved and cleansed. Around 6 mm burn wounds were created by applying a 120 s heated brass bar for 10s. Immediately after injury, burn wounds were infected with a mid-log phase bacterial inoculum of 10^7 CFU of *P. aeruginosa* (R590) prepared in PBS. Treatment was started 24 h post infection, to allow sufficient time for biofilm formation. Burn wounds were treated every 24 h for 5 days. NCK-10 (at concentrations of 20 mg kg^{-1} and 40 mg kg^{-1}) and colistin (30 mg kg^{-1}) was dissolved in saline and burn wounds were treated with 40 µL of these solutions. Only saline was used as untreated control. Mice were euthanized 6 days post-injury; the wounded muscle tissue was excised, weighed, and homogenized in 10 mL of PBS. Serial homogenate dilutions were plated on MacConkey agar (Himedia, India) and the results were stated as log (CFU g^{-1}) of tissue. P value was calculated using unpaired Student's t test (2 tailed 2 samples assuming equal variances) between the control group and the treatment group and a value of $P < 0.05$ was considered significant.

Chapter 6

Antifungal properties of aryl-alkyl-lysines

Abstract

Mortality due to pathogenic fungi has been exacerbated by the rapid development of resistance to frontline antifungal drugs. Fungicidal compounds with novel mechanisms of action are urgently needed. Aryl-alkyl-lysines, which are membrane-active small molecules, were earlier shown to be broad-spectrum antibacterial agents with potency in vitro and in vivo. In this chapter, the antifungal properties of aryl-alkyl-lysines have been reported. After identification of the most active compound (NCK-10), its activity against a panel of clinically relevant pathogenic fungi was tested. NCK-10 was capable of inhibiting growth of various species of fungi (including Candida spp., Cryptococcus spp., and Aspergillus fumigatus) at concentrations similar to antifungal drugs used clinically. It was observed that polarization and permeability of the fungal cell membrane was compromised upon addition of NCK-10, indicating its mechanism is disruption of the fungal cell membrane. In addition to interfering with growth of planktonic fungi, NCK-10 demonstrated the ability to both inhibit biofilm formation and reduce the metabolic activity of cells in C. albicans biofilm. Additionally, the compound was capable of crossing the blood-brain-barrier in an in vitro model, expanding the potential antifungal applications for NCK-10. Overall, aryl-alkyl-lysines were found to be excellent compounds that warrant further investigation as novel antifungal agents.

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(1) Ghosh, C. *et al.* "Aryl-alkyl-lysines: Membrane-active fungicides that act against biofilms of *Candida albicans*". *ACS Infect. Dis.*, **2017**, (DOI: 10.1021/acsinfecdis.6b00192)

6.1 Introduction

Invasive fungal infections afflict millions of patients annually resulting in more than 1,350,000 deaths. Despite the fact that infections caused by pathogenic fungi lead to significant mortality and morbidity, the threat of these pathogens is often overlooked.[233] Only four classes of antifungal drugs are currently available to clinicians: polyenes, azoles, echinocandins and 5-fluorocytosine.[234] While azoles inhibit the biosynthesis of ergosterols, polyenes bind to ergosterol, form pores, and induce leakage of intracellular ions.[234, 235] In contrast, echinocandins and 5-fluorocytosine exert their antifungal effect by a different method. Echinocandins inhibit fungal cell wall biosynthesis while 5-fluorocytosine inhibits both protein synthesis and DNA synthesis in fungi.[236] There are reports of resistance to each antifungal agent mentioned above; this makes it imperative to design and develop novel antifungal agents.[237]

In additional to targeting fungal viability, identifying novel antifungals capable of interfering with key fungal virulence factors (such as biofilm formation) is highly desirable. Similar to bacteria, fungi are capable of forming stable communities within biofilms that are extremely difficult to treat.[238] Only recently have therapeutic agents against antifungal biofilms gained more prominence in the research community.[239, 240] Thus design of novel antifungal agents which can act against both planktonic and biofilm forms of pathogenic fungi is highly desirable.

Antimicrobial peptides and their mimics have gained popularity among researchers as next generation therapeutic agents, primarily because of their broad spectra of activity.[17, 20] Several antimicrobial peptides possess notable antifungal activity. This has prompted additional research in peptidomimetic designs with antifungal properties.[161, 241, 242] Although, none of the peptidomimetic designs have progressed to preclinical studies to date, they bear much promise as potential drugs for the treatment of pathogenic fungi.

In this chapter, the antifungal properties of aryl-alkyl-lysines have been described. In Chapters 3 and 5 their efficacy as antibacterial and antibiofilm agents both *in vitro* and *in vivo* were described. Herein, the activity of compounds from the NCK series, containing naphthalene rings, and the BCK series, containing benzene rings, against clinically-relevant pathogenic fungi has been furnished. After identification of the most active compound (NCK-10), its antifungal mechanism of action was confirmed. Furthermore, the ability of the compound to inhibit *C.*

albicans biofilms was also examined. Finally, the ability of NCK-10 to passively permeate across the relatively impermeable blood-brain-barrier was studied in an *in vitro* model. The results garnered provide a valuable foundation for further investigation for potential clinical applications of NCK-10 as a novel antifungal agent.

6.2 Results and Discussion

6.2.1 Antifungal activity

The antifungal activity of the six compounds presented in Figure 6.1 were initially screened against two fungal strains, *C. albicans* SC5314 and *C. neoformans* H99, in YPD media. The synthesis and antibacterial activity of the compounds have been described in Chapter 3. The MIC and MFC of the compounds is presented in Table 6.1. NCK-6 lacked antifungal activity against *C. albicans* SC5314. The MIC for NCK-8 was 40 µM and correlated to the MFC, indicating this compound is fungicidal at this concentration. From the NCK series, NCK-10, was the most potent antifungal compound identified, as it inhibited growth of *C. albicans* SC5314 at a concentration of 20 µM. In the BCK-series, only BCK-12 showed potent activity inhibiting fungal growth at 40 µM and demonstrating a fungicidal effect at 40 µM. Although the decyl analogue BCK-10 was slightly active against *C. albicans* (MIC of 40 µM), BCK-14 was inactive even at a concentration of 50 µM. Against *C. neoformans* H99, however, all the compounds (except for NCK-6) inhibited growth at 5 µM. From this screen, NCK-10 emerged as the most active compound against both *C. albicans* and *C. neoformans*. Thus, we moved to further explore the spectrum of antifungal activity of NCK-10.

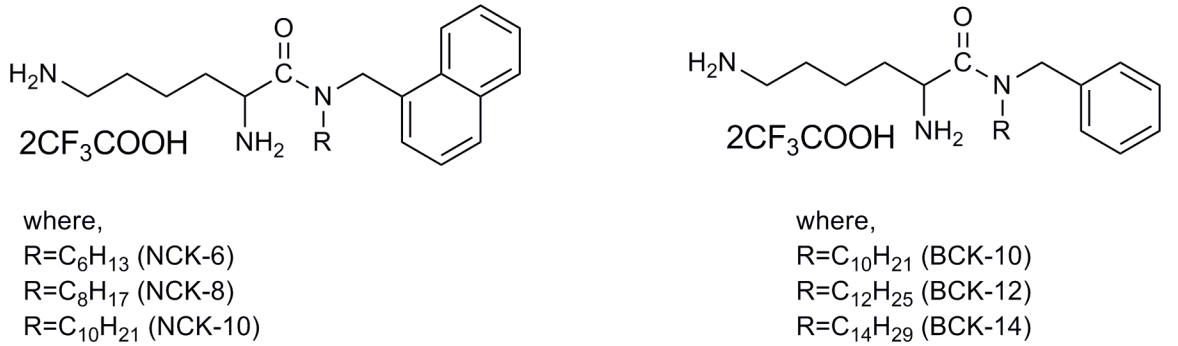


Figure 6.1: Structures of the compounds tested.

Table 6.1: Antifungal activity of BCK and NCK compounds against *Candida albicans* and *Cryptococcus neoformans*.

Compounds	*C. albicans* SC5314		*C. neoformans* H99	
	MIC (μM)	MFC (μM)	MIC (μM)	MFC (μM)
NCK-6	>50	>50	42	42
NCK-8	40	40	5	5
NCK-10	20	40	5	5
BCK-10	40	80	5	5
BCK-12	40	40	5	5
BCK-14	>50	>50	9	9

Values are the averages of at least two experiments, each done in triplicate (error <5%)

The susceptibility of cationic antimicrobial agents often vary in different media.[243] We have reported similar observations against bacteria previously.[172, 244] In order to test if YPD media had an adverse effect with respect to susceptibility, we determined the MIC and MFC of NCK-10 in RPMI medium as well. Interestingly, a significant decrease in the MIC value was observed. The MIC of the compound against *C. albicans* SC5314 in RPMI media was found to be 2.4 μM. Thus, we chose to carry out further susceptibility testing using RPMI medium. NCK-10 was subsequently tested against additional clinical fungal species including *C. albicans, C. parapbsilosis, C. glabrata, C. tropicalis, C. gattii, C. neoformans* and *A. fumigatus* (Table 6.2). *Candida parapsilosis* has been gaining notoriety as an invasive fungus responsible for the most infections caused by *Candida sp.* after *C. albicans*.[245] Furthermore, excessive use of immunosuppressive agents and broad-spectrum antimycotic agents has led to emergence of

pathogenic strains of *C. glabrata*, especially for patients suffering from HIV and diabetes.[246] A major problem with this microorganism is that some of the strains are inherently resistant to fluconazole therapy. *Candida tropicalis* is another major problem in India, wherein it is the most dominant contributor to candidiasis caused by Candida-non-albicans (CAN) species. Cryptococcosis and cryptococcal meningitis are potentially fatal diseases caused by *Cryptococcus sp.* Although *Cryptococcus neoformans* mostly affects immunocompromised individuals (particularly patients infected with HIV), *Cryptococcus gattii* has been reported to cause illness in immunocompetent individuals.[247] Drugs against such pathogens are medically important, particularly in Sub-Saharan Africa where the largest population of AIDS patients resides. Susceptibility of nine strains of *C. albicans* to NCK-10 was examined. Out of these nine strains, two strains (NR-29351 and NR-29365) were susceptible to fluconazole. Against NR-29351, NCK-10 was active at 2 µM while amphotericin B was active at 1 µM. Against NR-29365, however, NCK-10 (MIC 1 µM) was two-fold more active than amphotericin B (MIC 2 µM). Against the remaining seven strains of *C. albicans*, fluconazole was not active up to a concentration of 200 µM. Against *C. albicans* NR-29448, NCK-10 matched the potency of amphotericin B, inhibiting fungal growth at a concentration of 1 µM. Against *C. albicans* ATCC MYA A573, NCK-10 (MIC 1 µM) was as active than amphotericin B. When examined against *C. albicans* NR-29438, NR-29366, NR-29367, ATCC 26790 and ATCC 64124, amphotericin B inhibited growth at 1 µM which was similar to the MIC for NCK-10 (3 µM).

Against *C. parapsilosis* ATCC 22019, NCK-10 had MIC and MFC values of 1 µM and 2 µM respectively, which is comparable to the control antifungals amphotericin B (MIC of 1 µM and MFC of 2 µM) and superior to fluconazole (MIC and MFC of 7 µM). Against two strains of *C. glabrata* (ATCC MYA-2950 and ATCC 66032), which were resistant to fluconazole (MFC >200 µg mL^{-1}), NCK-10 was active at 2 µM (MIC and MFC). These strains were susceptible to amphotericin B (MIC 1 µM). We tested the activity of NCK-10 against two species of *C. tropicalis* ATCC 1369 and ATCC 13803. Against both strains, amphotericin was active at 1 µM. NCK-10 was also active at less than 1 µM against ATCC 1369 while fluconazole was active at 7 µM. Against ATCC 13803, fluconazole was active at 2 µM while NCK-10 was active at 2 µM. Overall,

Table 6.2: Activity of NCK-10 against clinical isolates of pathogenic fungi in comparison to amphotericin B and fluconazole.

Fungal strains	NCK-10		Amphotericin B		Fluconazole	
	MIC (µM)	MFC (µM)	MIC (µM)	MFC (µM)	MIC (µM)	MFC (µM)
Candida albicans NR-29448	1	2	1	1	>200	ND
Candida albicans NR-29351	2	3	1	1	2	25
Candida albicans NR29438	3	3	1	2	>200	ND
Candida albicans NR-29366	3	3	1	1	>200	ND
Candida albicans NR-29367	3	3	1	2	>200	ND
Candida albicans ATCC *26790*	3	3	1	1	>200	ND
Candida albicans NR-29365	1	1	2	2	2	>200
Candida albicans ATCC MYA573	1	1	1	1	>200	ND
Candida albicans ATCC 64124	3	3	1	1	>200	ND
Candida parapbsilosis ATCC22019	1	2	1	2	7	7
Candida glabrata ATCC MYA-2950	2	2	1	1	100	>200
Candida glabrata ATCC 66032	2	2	1	2	50	>200
Candida tropicalis ATCC 1369	1	1	1	1	7	>200
Candida tropicalis ATCC 13803	2	2	1	2	2	>200
Cryptococcus gattii NR-43208	2	2	1	2	7	7
Cryptococcus gattii NR-43209	2	2	1	4	25	25
Cryptococcus neoformans NR41294	3	3	1	1	13	25
Cryptococcus neoformans NR-41296	3	3	1	1	13	25
Cryptococcus neoformans NR-41295	3	3	1	1	50	100
*Cryptococcus neoformans*NR-41292	2	2	1	17	25	25
Aspergillus fumigatus NR-35308	6	6	0.3	0.03	>200	ND
Aspergillus fumigatus NR-35304	6	6	≤0.0625	0.3	>200	ND
Aspergillus fumigatus NR-35303	6	6	≤0.0625	0.1	>200	ND
Aspergillus fumigatus NR-35301	6	6	0.3	0.3	>200	ND
Aspergillus fumigatus NR-35302	3	3	0.5	0.5	>200	ND*

Values are the averages of at least two experiments, each done in triplicate (error <5%)

than, the clinical antifungals amphotericin B and fluconazole.

Against two strains of *C. gattii* (NR-43208 and NR-43209), NCK-10 and amphotericin B exhibited MIC values of 2 µM and 1 µM respectively, whereas the MIC of fluconazole was 7 µM and 25 µM respectively. Against four strains of *C. neoformans* (NR-41292, NR-41294, NR-41295 and NR-41296), the MIC of fluconazole varied from 13 µM to 50 µM. The antifungal activity of NCK-10 against these same strains was superior (MIC ranged from 2 µM to 3 µM) and was comparable to the activity of amphotericin B (MIC 0.0624 µM-0.5 µM).

Similar to fungal pathogens like *C. neoformans*, *Aspergillus fumigatus* severely affects immunocompromised individuals including patients with leukemia, cystic fibrosis, human immunodeficiency virus, and patients undergoing organ and stem cell transplants; mortality rates associated with this pathogen are as high as 90%.[23] We tested NCK-10 against five strains of *A. fumigatus* (NR 35301, NR 35302, NR 35303, NR 35304 and NR 35308) which were all resistant to fluconazole (MIC >200) but susceptible to amphotericin B (MIC 1 µM). Against all strains of *A. fumigatus* that were tested, NCK-10 was fungicidal at 6 µM.

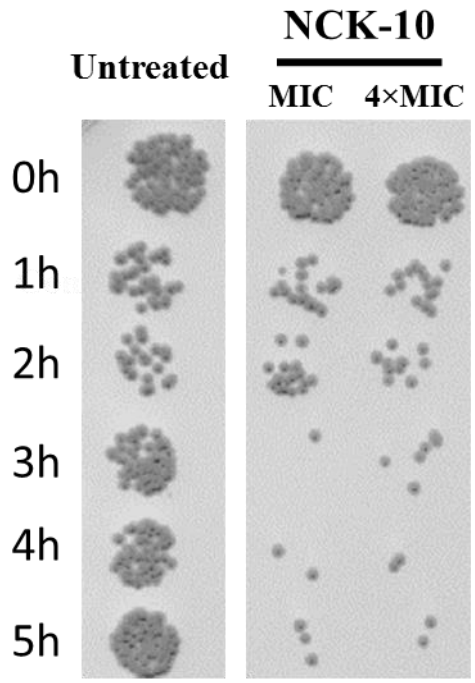


Figure 6.2: Kinetics of fungicidal action of NCK-10

6.2.2 Kinetics of fungicidal action

Per the MFC data, NCK-10 appears to be a fungicidal agent against all fungal species tested. We moved to confirm this observation via a standard time-kill assay. The growth of *C. albicans* SC5314 in the presence of NCK-10 (at concentrations equivalent to the MIC and 4×MIC) was observed over a five-hour period. As depicted in Figure 6.2, NCK-10 was effective in diminishing the fungal count within the first hour. At the end of three hours >90% was observed. Although, a few colonies were observed thereafter, the ability of the compound to lyse fungal cells was evident.

6.2.3 Mechanism of action

We previously confirmed NCK-10 exerted its antibacterial effect by infiltrating the cell membrane.[17-18] Thus, we postulated that NCK-10 would behave in a similar manner against fungi. To confirm this, the compound's ability to depolarize and permeabilize the fungal cell membrane of *C. albicans* (SC5314) was examined (Figure 6.3). The ability of NCK-10 to perturb the polarization across fungal membranes was studied using the membrane potential sensitive probe, $DiSC_3(5)$. The fluorescence intensity of the dye is enhanced upon perturbation of the membrane potential. NCK-10 at concentrations equivalent to its MIC and 2 × MIC was able to depolarize the fungal membrane within one minute (Figure 6.3A), in a dose-dependent manner.

We then checked if the compound was able to compromise the membrane to permit influx of external substances. The external agent used was propidium iodide (PI), which cannot enter the cell unless the cell membrane is compromised. Upon binding to nucleic acids, there is an enhancement in the fluorescence signal emitted. Upon addition of the compound, PI found rapid entry inside the fungal cell as presented in Figure 6.3B. These results support our hypothesis that NCK-10 exerts its antifungal effect by rapidly permeabilizing the fungal cell membrane.

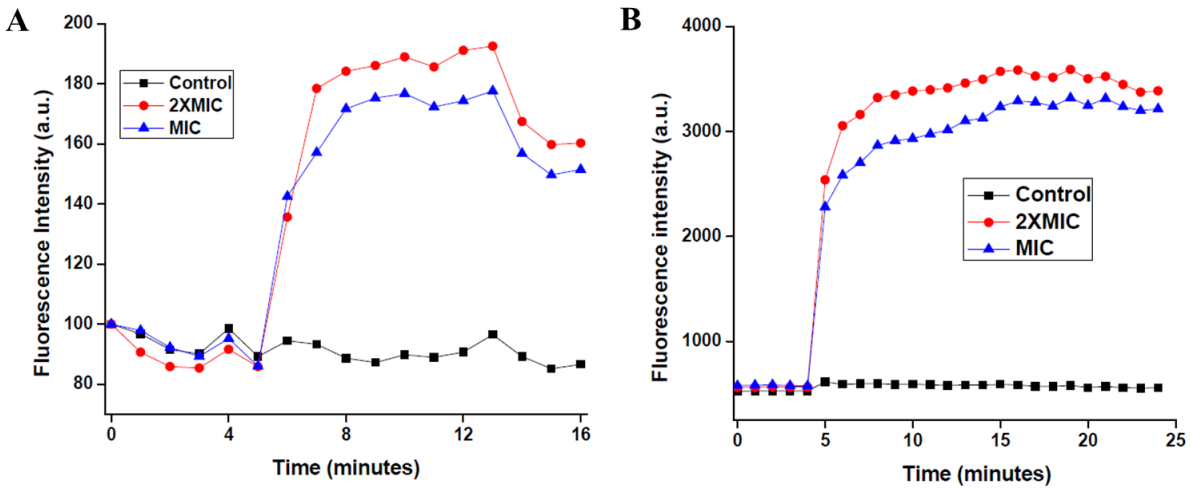


Figure 6.3: Ability of NCK-10 to infiltrate the cell membrane of *C. albicans* A) Depolarization of fungal cell membrane B) Permeabilization of the cell membrane. MIC considered is 20 µM.

6.2.4 Microscopic observation of cell viability

In order to further confirm the proposed antifungal mechanism of action, NCK-10's ability to compromise the fungal cell membrane was observed microscopically. In this experiment, two dyes SYTO-9 and PI were used to differentiate between live and dead/membrane-compromised cells. SYTO-9 gains entry both in intact and membrane-compromised cells. However, as mentioned earlier, PI can only enter membrane-compromised cells. As can be seen from Figure 6.4, in the untreated control, only uptake of SYTO-9 by *C. albicans* SC5314 was observed, indicating that the cell membrane was not compromised. However, upon treatment of *C. albicans* SC5314 cells with NCK-10 (at its MIC)), PI entered the cells easily. In the merged image, one can see the predominance of the red dye (PI) in comparison to the untreated control, further confirming the membrane-disrupting ability of NCK-10.

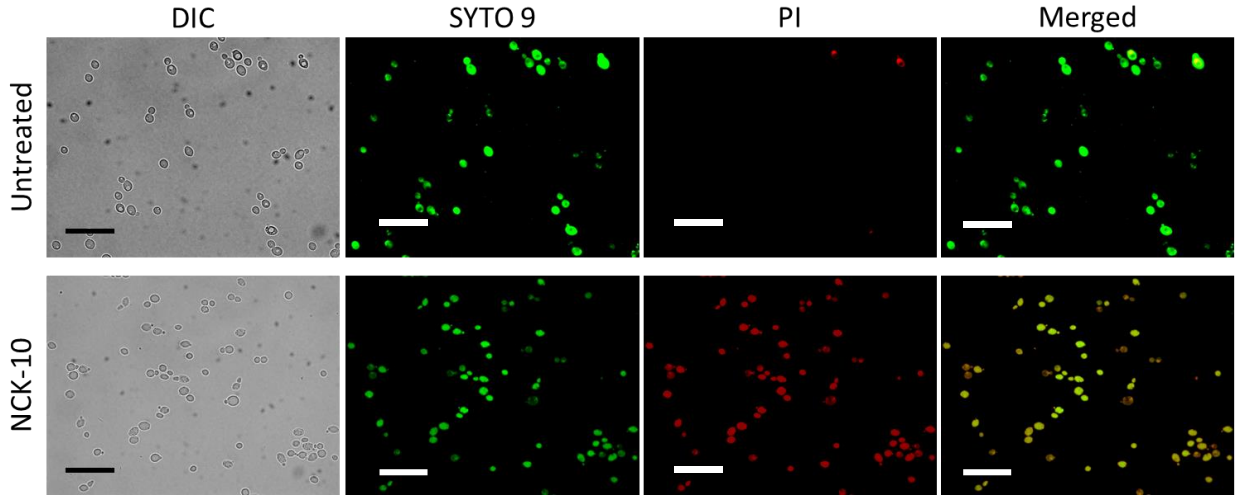


Figure 6.4: Fluorescence microscopy images of *C. albicans* after staining with SYTO 9 and PI in absence (control) or presence of NCK-10 (2 × MIC). Scale bar, 5 μm. MIC considered is 20 μM.

6.2.5 Antibiofilm properties

In addition to the challenge posed by free-floating, planktonic fungal cells, fungal biofilms pose a significant additional concern. These biofilms are capable of forming on medical devices (such as indwelling catheters) leading to recurring infections that are challenging to treat.[248] Understandably, several recent studies have focused on identifying agents that are effective at attacking biofilms of pathogenic fungi.[239, 240, 249, 250]

The ability of the compound to both prevent and disrupt biofilms of *C. albicans* SC5314 was tested. To examine inhibition of biofilm formation, we incubated NCK-10 at different sub-MIC concentrations (ranging from MIC to MIC/32) and allowed the fungal biofilms to grow for three days. Subsequently, the metabolic activity of biofilm that was formed was quantified using the MTT assay. As presented in Figure 6.5A, with respect to the control, the compound was able to inhibit formation of biofilms in a concentration-dependent manner, At MIC/32, more than 30% inhibition of fungal biofilm formation was observed. At MIC/4 the compound produced an 80% inhibition of biofilm formation, relative to the untreated control. The effect was most pronounced at MIC/2 and MIC/4 concentrations.

We next investigated the efficacy of the compound to disrupt pre-formed fungal biofilm. *C. albicans* SC5314 biofilms were grown for 48 hours before subjecting them to compound treatment. The concentration of the compound tested ranged from MIC/2 to 4 × MIC. The metabolic activity of the biofilm was significantly diminished by 25% even at MIC/2. No antibiofilm activity was observed for NCK-10 at MIC/2 (Figure 6.5B). At concentrations equal to the MIC and beyond, more than 80% reduction in the metabolic activity of cells in the biofilm was observed, relative to the untreated control.

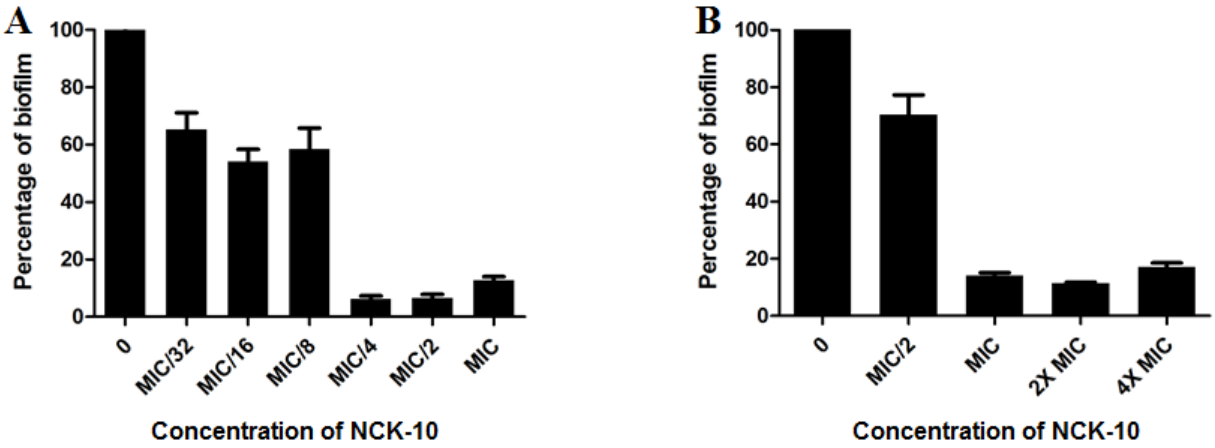


Figure 6.5: Ability of the compound NCK-10 to A) inhibit the formation of *C. albicans* biofilm and B) Disruption of pre-formed *C. albicans* biofilm. MIC considered is 20 µM.

6.2.6 Parallel artificial membrane permeability assay for ability to cross the blood brain barrier

Certain fungal infections, particularly *Cryptococcal meningitis* and *Rhinocerebral mucormycosis*, necessitate that a viable therapeutic agent be capable of crossing the blood-brain barrier in order to reach the target site of infection. Thus we evaluated the ability of compounds NCK-10 to penetrate the BBB utilizing PAMPA-BBB (Table 6.3). Although the value obtained for NCK-10 was not as high as that of the highly permeable drug verapamil, it was found to be superior to the low permeability control drug, atenolol, indicating NCK-10 is capable of passively traversing across the barrier imposed by the blood-brain barrier. Traditionally, compounds possessing a mean permeability coefficient less than 2×10^{-6} cm sec^{-1} will experience difficulty in effectively crossing the BBB. As NCK-10, possesses a mean permeability coefficient (Table 6.3) of 2.87×10^{-6} cm

Table 6.3: Effective permeability coefficient of NCK-10 and control drugs obtained from the PAMPA-BBB assay.

Test Agent	Mean P_e (cm/sec)	Inference
NCK-10	2.87×10^{-6}	Good permeability
Atenolol	0.000013×10^{-6}	Low permeability control
Verapamil	14.6×10^{-6}	High permeability control

sec^{-1}, this indicates that NCK-10 would be a good candidate for investigation of treatment of fungal infections impacting the brain (namely meningitis).

6.3 Conclusions

Overall, present study demonstrates the promise of aryl-alkyl-lysines, specifically NCK-10, as prospective antifungal agents. NCK-10 possesses excellent efficacy *in vitro* against various species of clinically-relevant fungi and is also capable of interfering with biofilms formed by *C. albicans*. The compound exerts its antifungal effect by targeting the cell membrane of fungi, inducing rapid lysis. The next step towards validating the therapeutic potential of these compounds as antifungal agents is testing their efficacy in suitable animal models of infection. Given fungi are responsible for various infections of the body, it will be worthwhile to investigate whether NCK-10 is able to treat invasive fungal infections (e.g. candidiasis), brain infections (caused by Cryptococcal meningitis), skin infections (onchomycosis) and pulmonary infections. At present, NCK-10 represents a good lead for design of a new generation of antifungal agents.

6.4 Experimental Section

6.4.1 Materials and Media: Yeast extract peptone dextrose (YPD) was obtained from HIMEDIA, India. RPMI purchased from GIBCO, Amphotericin B, fluconazole, atenolol, verapamil, HEPES,

DiSC3 (5), MTT and Propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO). SYTO 9 dye was purchased from Invitrogen. For optical density (OD) and fluorescence measurement, Tecan Infinite Pro series M200 Microplate Reader was employed. An Olympus microscope (Model BX51) and Olympus DP71 camera were used for the fungal imaging.

6.4.2 Initial fungal susceptibility assay: The antifungal activity of the compounds was initially tested against *Candida albicans* SC5314 and *Cryptococcus neoformans* H99 using a previously described 96-well plate assay, with few modifications.16 Briefly, the fungal strains were grown overnight in 5 mL YPD (1% yeast extract, 2% peptone and 2% dextrose). Fungal growth was measured via a spectrophotometer (OD_{600}) and cells were diluted in fresh media (either in YPD or RPMI) to get the required concentration of 1.3×10^5 cells mL^{-1}. An aliquot (150 µL) of the culture dilution (equivalent to 2×10^4 cells) was added to wells containing 50 µL of water or the compounds, at different concentrations. The plates were incubated at 30 °C for 20 hours with shaking (180 rpm) to allow sufficient growth of fungal cells. The growth of the fungi was measured (OD600) using media alone (YPD/RPMI) as a blank. Simultaneously, 3 µL of the culture from each well was taken and spotted onto an YPD agar plate to determine the minimum fungicidal concentration (MFC). The concentration where the optical density of the test well was close to the value of the blank well value was categorized as the minimum inhibitory concentration (MIC).

6.4.3 Antifungal activity against clinical isolates: The minimum inhibitory concentration (MIC) of the most potent compound, NCK-10, was determined against several clinical strains of Candida species, Cryptococcus species and one strain of *Aspergillus fumigatus. C. albicans, Cryptococcus spp., A. fumigatus* (tested from 64 µg mL^{-1} down to 0.5 µg mL^{-1}) were transferred to a 96-well plate and incubated at 37 ºC for 48 hours (or 72 hours for *C. neoformans*) .The MICs reported represent the lowest concentration of each compound necessary to inhibit fungal growth, by visual inspection. For determination of the minimum fungicidal concentration, aliquots (5 µL) were transferred from wells with no growth onto YPD agar plates. Plates were incubated at 37 ºC for 48 hours before MFC results were recorded. Amphotericin B and fluconazole were used as control antifungals.

6.4.4 Kinetics study: In order to confirm if NCK-10 was fungistatic or fungicidal, a time-kill experiment was conducted. Fungal cells were grown overnight and added to a 96-well plate as described above. Starting at the initial time point, a small aliquot (3 µL) of cells was taken and

spotted onto an YPD plate every hour (for five hours). The plates were then incubated at 30 °C for 24 hours to score for viable cells.

6.4.5 Mechanism of action

6.4.5.1 Depolarization of fungal cell membrane: Briefly, 10^7 CFU mL^{-1} of *C. albicans* SC5314 was washed and resuspended in 5 mM glucose, 5 mM HEPES buffer and 100 mM KCl solution in a 1:1:1 ratio. To this solution $DiSC_3(5)$ dye (obtained from Sigma-Aldrich) was added to a final concentration of 2 µM. The fungal suspension containing the dye (200 µL) was preincubated for 20 min in a black 96-well plate with a transparent bottom. The fluorescence of the fungal suspension was measured (excitation wavelength: 622 nm; emission wavelength: 670 nm) and allowed to stabilize at room temperature before the addition of 2 µL of NCK-10 (final concentration was equivalent to the MIC (12.5 µg mL^{-1}) or 2 × MIC (25 µg mL^{-1})). After addition, the fluorescence intensity was measured every minute for 20 minutes.

6.4.5.2 Permeabilization of fungal cell membrane: Propidium iodide (PI) dye (15 µM, final concentration) (obtained from Sigma-Aldrich) was added to a fungal suspension containing *C. albicans* SC5314 (~10^7 cells mL^{-1}) in 5 mM HEPES and 5 mM glucose (pH 7.4). The suspension containing the dye (200 µL) was then added to the well of a 96-well, clear-bottom black plate. After four minutes, NCK-10 (2 µL) was added to the solution to a final concentration equivalent to the MIC (12.5 µg mL^{-1}) or 2 × MIC (25 µg mL^{-1}). The fluorescence intensity was measured at excitation wavelength of 535 nm (slit width: 10 nm) and emission wavelength of 617 nm (slit width: 5 nm). The uptake of PI was detected by the increase in fluorescence for 10 minutes; this correlates to permeabilization of the inner cell membrane.

6.4.6 Microscopy assay for viability: *C. albicans* SC5314 was grown in a 96-well plate for six hours in the presence or absence of NCK-10 (12.5 µg mL^{-1}). The cells were harvested, washed once with sterile water, and resuspended in 50 µL of sterile water. The cell suspension was incubated with propidium iodide (PI, 15 µM final concentration) and SYTO9 (2 µM final concentration) at room temperature for 30 minutes. The cells were then observed under a fluorescence microscope. A bright-field filter was used for differential interference conference (DIC) images whereas green and red emission filters were used to capture SYTO 9 and PI

fluorescence, respectively. The images were captured using an Olympus microscope (Model BX51) and an Olympus DP71 camera. The images were further processed using Image Pro-Plus software and ImageJ.

6.4.7 Inhibition of *C. albicans* biofilm formation: *Candida albicans* SC5314 cells were grown overnight in YPD, pelleted and washed with 1 × phosphate buffered saline (PBS). Washed cells were resuspended in RPMI media to reach a final concentration of 6.7×10^5 cells mL^{-1}. Compound NCK-10 (50 µL) and 150 µL of cell suspension was mixed together and added into wells of a 96-well plate. Wells lacking either compound or cell suspension were utilized as positive and negative controls. The plates were incubated at 37 °C for 72 hours in stationary condition. Inhibition of biofilm was then quantified using the MTT assay, as follows. The wells were washed twice with 1 × PBS. MTT solution (100 µL of 5 mg mL^{-1} in 1× PBS) was subsequently added to wells and incubated at 37 °C for four hours. The MTT solution was discarded and wells were washed once with 1 × PBS. DMSO (100 µL) was added into wells and further incubated at 37 °C in the dark for an hour. The solution was then transferred to a fresh plate and the optical density was measured at 590 nm to quantify the extent of biofilm inhibition compared to untreated wells.

6.4.8 Disruption of pre-formed *C. albicans* biofilms: *Candida albicans* SC5314 cells were grown overnight in YPD, pelleted and washed with 1 × PBS. Washed cells were resuspended in RPMI medium to a final concentration of 1×10^6 cells mL^{-1}. An aliquot of cell suspension (100 µL) was added into wells and incubated at 37 °C for 24 hours. The non-adhered cells were removed from the wells and 50 µL of fresh RPMI medium along with 50 µL of varying concentrations of the compound were added into the cells. As a control, 100 µL of RPMI media alone was added. These plates were further incubated at 37 °C for 48 hours. Inhibition or disruption of biofilm was then quantified using the MTT assay, as described above.

6.4.9 Parallel artificial membrane permeability assay for examining ability to cross the blood brain barrier: NCK-10 and control drugs (atenolol and verapamil) were dissolved in transport buffer (pH 7.4) to a final concentration of 10 µM. The filter membrane was coated with 4 µL of a 20 mg mL^{-1} porcine brain lipid in dodecane. An aliquot (300 µL) of the compound solution was added to the donor well. The acceptor well was filled with 200 µL of transport buffer. The acceptor filter plate was carefully placed on to the donor plate and was left undisturbed for 18 hours.

Samples of the donor and acceptor wells were analyzed, in quadruplicate, by LC-MS/MS and the effective permeability (Pe) was calculated as follows:

$$\log (P_e) = \log \{- \frac{Vd * Va}{(Vd + Va) A * t} \ln(1 - \frac{[drug\ ]A}{[drug\ ]E})\}$$

Where P_e is the permeability, V_d and V_a are the volumes of the donor and acceptor compartments, A is the area of the membrane, t is the incubation time, and A and E subscripts on the drug concentration terms refer to the acceptor and equilibrium concentrations, respectively.

Chapter 7

Antimalarial properties of aryl-alkyl-lysines

Abstract

Despite decline in the number of cases, malaria, continues to take numerous lives all across the globe. However, the development of resistance against the recently introduced therapies such as artemisinin derivatives has raised serious concern. Thus, there is a steady need for new antimalarial drugs. Here, we report a new class of water soluble, non-toxic compounds, aryl-alkyl-lysines, with potent activity against Plasmodium falciparum. Preliminary investigations show that these compounds acted by arresting the development of parasites at the ring stage inside the erythrocytes. It has been shown that the compounds had an ability to perturb the plasma membrane potential and the digestive vacuole of parasites. In the murine model of malaria (Plasmodium bergheii ANKA) the optimal compound was able to increase the survival of mice by at least 5 days by intra-peritoneal route. Further, the compounds showed no apparent toxicity to mice at the concentration tested. Thus, this class of compounds bear potential to be developed as novel antimalarial drugs.

(1) Ghosh, C. *et al.* "Aryl-alkyl-lysines: Small Molecular Membrane-active Antiplasmodial Agents". *Med.Chem.Comm.*, **2017**, *8*, 434-439

7.1 Introduction

Although the World Health Report on Malaria has documented substantial decrease in mortality due to malaria, the disease still poses a huge threat considering the rapid rate at which resistance is being developed against the frontline antimalarial drugs. WHO recommended artemisinin based combination therapies only in 2005, but already resistance against artemisinin has been reported in South-east Asia.[251] Since, vaccine against malaria parasite is yet to reach the clinics, much emphasis is still stressed on the use of drugs.[252] Thus, there is a constant need for drugs with novel mechanism of action.

The first "soldiers" that an invading pathogen encounters upon infecting a host are antimicrobial peptides (AMPs).[17, 20] These are small protein molecules, mostly cationic in nature, bear facial amphiphilicity which helps them to interact with the membranes of microorganisms.[23] AMPs have been found to possess activity against bacteria, fungi, viruses, parasites in addition to having immunomodulatory properties.[17, 20, 147]

Several natural AMPs, such as magainin,[253] dermaseptin derivatives,[254] NK-2,[255] scorpine,[256] cecropin-melittin[257] and gramicidin[258] possess potent activity against *Plasmodium falciparum.*[259, 260] Although the mechanism of action is not clearly understood, it is believed that membrane activity remains the primary feature of such antiplasmodial peptides.[260] It has been fairly well established that the membranes of parasite-infected erythrocytes (RBCs) differ from their uninfected counterparts, as there are increased levels of phosphatidylinositol and phosphatidic acid, and decreased contents of sphingomyelin.[261] Moreover, translocation of anionic phosphatidylserine happens from the inner leaflet to the outer leaflet of the infected RBCs.[262] These two phenomenon, make the infected RBCs more anionic compared to their uninfected counterparts, which explains the initial interaction between cationic peptides and such cells.[259] Several other peptides and synthetic mimics of AMPs have been prepared which show potent antiplasmodial activity.[156] Oligoacyl lysines, which possess a variety of biological functions also showed potent antimalarial activity *in vitro* but were found to have significant toxicity *in vivo*.[157] Some small non-peptidic mimics of host-defense peptides showed excellent activity both *in vitro* and *in vivo*.[158]

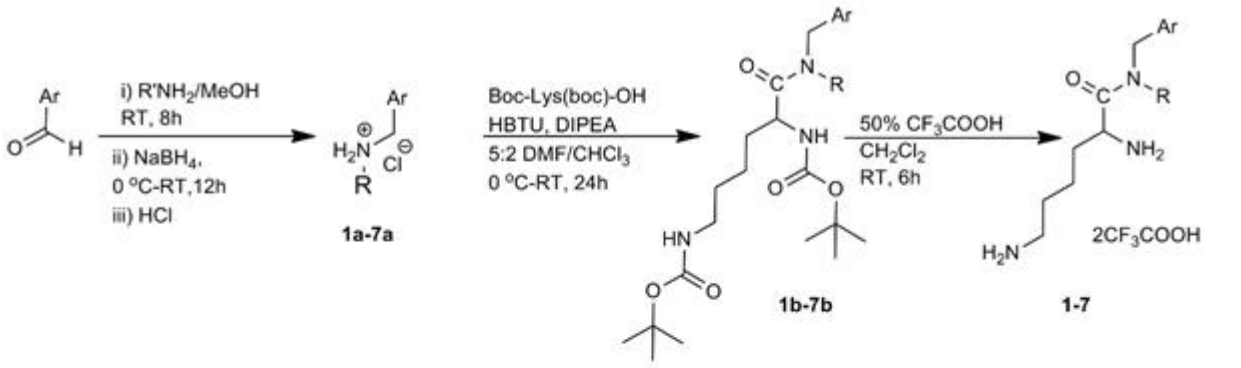
In this chapter, we describe the antiplasmodial properties of aryl-alkyl-lysines. We have investigated the activity of the compounds against 3D7 strains of *P. falciparum* and the activity of the best compound against a chloroquine-resistant strain *in vitro*. The stage of parasite

development, at which the compound was most active was also studied. The plausible mechanism of action has also been investigated. *In vivo* studies of the best compound revealed good activity in murine model of cerebral malaria with negligible toxicity.

7.2 Results and Discussion

7.2.1 Synthesis

The synthesis of the aryl-alkyl-lysines (compounds 1-4) were carried out following the protocol previously described in Chapter 3 (Section 2.5.1). Compounds 5-7 are being reported for the first time here. The synthetic scheme for the preparation of the compounds has been presented in Scheme 1. In the first step, aromatic aldehydes (benzaldehyde, 1-naphthaldehyde, 4-quinolinecarboxaldehyde, 2-naphthaldehyde and biphenyl-4-carboxaldehyde) were reacted with aminoalkanes (aminohexane or aminooctane) to obtain Schiff's bases. Reduction of the Schiff's bases with sodium borohydride yielded secondary amines (1a-7a), which were precipitated out as



1a, 1b and 1: Ar = Ben, R = C_6H_{13}; **2a, 2b and 2:** Ar = Ben, R = C_8H_{17}; **3a, 3b and 3:** Ar = 1-Nap, R = C_6H_{13}; **4a, 4b** and **4**: Ar = 1-Nap, R=C_8H_{17}; **5a, 5b and 5:** Ar = Qnl, R = C_6H_{13}; **6a, 6b and 6:** Ar = 2-Nap, R = C_6H_{13}; **7a, 7b and 7:** Ar = Bip, R = C_6H_{13};

Scheme 7.1: Chemical structures of the compounds used in the study.

their HCl salts. These compounds were then coupled to Boc-Lys(Boc)-OH using HBTU coupling chemistry which were purified using column chromatography. Finally, deprotection of the Boc groups yielded the final compounds: BCK-6 (**1**), BCK-8 (**2**), NCK-6 (**3**), NCK-8 (**4**), 4-QCK-6 (**5**), 2-NCK-6 (**6**) and 4-BipCK-6 (**7**). All the compounds were purified to more than 95% purity using reverse phase HPLC. The compounds were subsequently characterized using NMR, IR and Mass Spectrometry. The details of the synthesis and characterization of the compounds have been given in the Experimental section.

7.2.2 Antimalarial activity and selectivity

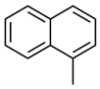
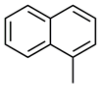
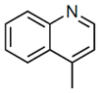
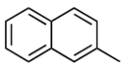
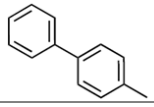
For an effective antiplasmodial candidate, solubility in water and toxicity are important criteria. In this preliminary study, we chose the relatively non-toxic but highly water soluble compounds. Thus, we chose to study the antiplasmodial properties of the benzene and naphthalene based compounds which bore hexyl and octyl chains. The IC_{50} of the compounds, which is defined as the concentration that inhibits the growth of 50% of the total number of parasites, was determined by the SYBR green assay and also validated microscopically.[263] The antiplasmodial activities of the compounds have been reported in Table 7.1. Compound 1 (BCK-6), which contained a benzene core and a hexyl chain was not active till 5 μM (in order to be a potent drug the IC_{50} of the compound should be less than 5 μM). Compound 2 (BCK-8) containing a benzyl core and an octyl chain, was active at IC_{50} value of 4 μM. The compounds of the 1-substituted naphthalene series (compounds 3 and 4) exhibited potent antimalarial activity. Compound 3 (NCK-6) displayed impressive activity at an IC_{50} of 1 μM while compound 4 (NCK-8) was active at 3 μM. From this initial screening, compound 3 emerged to be the best compound.

Most of the 4-aminoquinoline and 8-aminoquinoline drugs possess some structural similarity with compound 3 (NCK-6). The naphthalene core closely resembles the quinoline moiety. This observation led us to substitute the naphthalene moiety with a quinoline moiety. Thus we carried out the synthesis of compound 5 (4-QCK-6) with 4-quinolinecarboxaldehyde. In order to identify a more potent compound we carried out a structure-activity-relationship study wherein we varied the aromatic core keeping the chain length and lysine moiety constant. In order to understand if the configuration was important for the study, we used 2-naphthaldehyde for synthesis instead of 1-naphthaldehyde to obtain compound 6 (2-NCK-6). Subsequently, we

replaced the naphthaldehyde with biphenyl-4-carboxaldehyde to obtain compound 7 (Bip-CK-6). However, in this structure-activity-relationship study, no significant increase in activity was achieved. For example, compound 6 was as active as compound 3 with IC_{50} value of 1.2 µM whereas the compounds 5 and 7 had IC_{50} values of 2.5 µM each.

The hemolytic property of an antimalarial drug is an important parameter that needs to be considered while taking the compounds to murine models. It is imperative to have drugs which are non-hemolytic at the concentration at which they are active on parasites. The haemolytic activity of the compounds have been represented as their HC50 value, which is the concentration at which they show 50% haemolysis. The HC_{50} values of the compounds have been furnished in Table 1. The HC_{50} values of the compounds varied from 96 µM to >1000 µM. The HC_{50} value of compound

Table 7.1: Antimalarial activity and haemolytic acitvityi of the compounds.

Compound	Ar	R	R'	IC_{50} (µM)	HC_{50} (µM)	HC_{50}/IC_{50}
BCK-6 (1)		C_6H_{13}	H	>5	>1000	-
BCK-8 (2)		C_8H_{17}	H	3.4±2	564	165
NCK-6 (3)		C_6H_{13}	H	1±0.1	850	850
NCK-8 (4)		C_8H_{17}	H	3±1	96	32
4-QCK-6 (5)		C_6H_{13}	H	2.3±0.7	>1000	>400
2-NCK-6 (6)		C_6H_{13}	H	2±1	620	516
BipCK-6 (7)		C_6H_{13}	H	2±1	144	57.6

Values are the averages of at least two experiments, each done in triplicate (error <10%)

3 was 850 μM, which was excellent while its IC_{50} value was 1 μM. In order to gauge the toxicity of the compounds towards plasmodial cells over mammalian cells we had looked at the selectivity ratio of all the compounds. Selectivity ratio (S.R.) is defined as the ratio between HC_{50}/IC_{50}, which emphasizes on the ability of the compounds to act specifically on the plasmodial cells. The selectivity of compound 3 was the best in the series with a S.R. of 850. Most of the other compounds containing a hexyl chain exhibited impressive selectivity, for example compound 5 (S.R. >400) and compound 6 (S.R. of 516). Although the 4-quinoline derivative (compound 5) exhibited HC_{50} values much higher than 1000 μM, it was less potent than the naphthalene derivative (compound 3). Even the biphenyl derivative (compound 7) although potent was significantly more toxic. All the other studies were thus conducted with compound 3.

7.2.3 Stage of action

In order to ascertain the mechanism of action of the compounds, we performed several assays using the most active compound 3. We first wanted to determine in what stage the compound acted on the parasites. The malarial parasites have three distinct stages in their asexual life cycle, rings, trophozoites and schizonts. In order to understand the effect of the compound on the different stages, first the parasites were tightly synchronized, grown to their respective stages and then the compound was added. The compound was found to be most active on the ring stage. In 48 h, in the untreated cases, the rings had grown to late trophozoites or schizonts of the same cycle. Upon treatment with compound 3 at concentration of $2 \times IC_{50}$, the growth of the rings were found to be arrested and the few parasites that remained were either in the ring stages or early trophozoite stage (Figure 7.1). The parasites were also observed to be severely stressed. The compound did not seem to have significant activity on the trophozoite stage or the schizont stage since in both control as well as compound treated case, the parasites had developed normally with hardly any change in parasitemia. Thus it was concluded that the compound specifically stalled the development of the parasites at the ring stage.

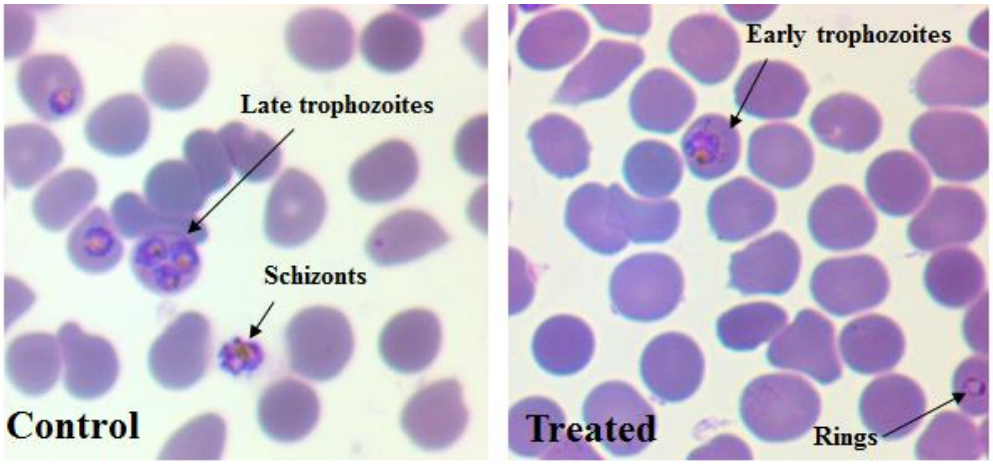


Figure 7.1: Compound 3 (2 μM) can stall the development of the parasites in ring stages.

7.2.4 Mechanism of action

Furthermore, we conducted fluorescent dye based experiments to understand whether the compound had any effect on the parasite plasma membrane or the digestive vacuole of the parasite. The assay to probe if the compound perturbs the plasma membrane potential of the parasite was done using Rhodamine 123. This dye is known to fluoresce when the membrane potential is maintained, however, if the membrane potential is perturbed, the dye loses intensity.[158] When the dye is used at a concentration of 1 μM, the effect on the membrane potential of the plasma membrane of the parasite can be monitored. [158] From Figure 7.2A it is clear that upon treatment with the compound 3, the plasma membrane of the parasite is perturbed. There is significant loss in fluorescence compared to the control.

The digestive vacuole of the parasite is acidic in nature, and the acid sensitive dye, Lysotracker green stains the intact digestive vacuole green. However, if the digestive vacuole is perturbed, the dye stains the cytoplasm and there is a significant decrease in fluorescence. intensity.[158] Compound 3 was found to act on the parasite digestive vacuole and cause significant damage. There was decrease in fluorescence and the dye stained the cytoplasmic components of the parasites (Figure 7.2B).

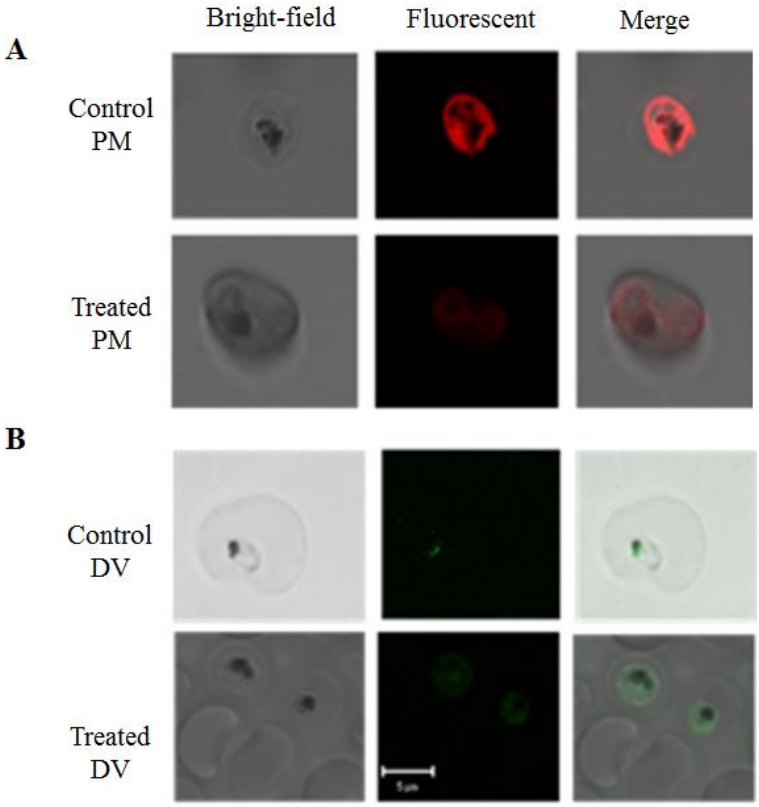


Figure 7.2: Perturbation of membrane potential of the parasite membrane (PM) and that of digestive vacuole (DV). Compound 3 at 2 μM (treated PM) causes greater perturbation of the membrane potential comparison to untreated (control PM). B) The intensity of the fluorescence exhibited by the dye Lysotracker Green is localized in the DV while in the treated case the dye has diffused all over the cytoplasm.

7.2.5 *In vivo* toxicity

Next we investigated the profile of compound 3 in mice. Initially we determined the LD_{50} of the compound in mice and determined sub-chronic toxicity of the compound. For determining the LD_{50} of the compound, we chose to inject different concentrations of the compound, intravenously into mice via the tail-vein. The compound was injected at concentrations of 175 mg kg^{-1}, 55 mg kg^{-1}, 17.5 mg kg^{-1} and 5.5 mg kg^{-1} and checked the survival of the mice for fourteen days. We followed the Spearman-Karber method to determine the LD_{50} of the compound. The LD_{50} of the

compound was determined to be 98 mg kg^{-1}. When injected with 20 mg kg^{-1} the mice exhibited no significant toxicity to any of the biochemical parameters of the blood, 48 h after injection (Figure 7.3). Specifically, we checked the change in sodium, potassium, chloride (measure of electrochemical balance of the blood), alanine transferase (ALT, biomarker for liver health), aspartate aminotransferase (AST, biomarker for liver and heart health), urea nitrogen (biomarker for kidney and liver health) and creatinine (biomarker for kidney health). From the data presented in Figure 7.3 (A-C), there has been no statistically significant imbalance in any of the parameters.

7.2.6 *In vivo* antimalarial activity

We, then investigated the ability of the compound 3 to reduce parasitemia and increase the survival of mice afflicted with malaria. We introduced cerebral malaria in Swiss-Harlan mice by injecting them with *P. bergheii* ANKA strain. Peter's Four day test was followed for determining the *in vivo* efficacy of the compound. Specifically, we treated the mice for four consecutive days with compound 3 at concentration of 20 mg kg^{-1}.The compound was able to reduce parasitemia in the all the mice significantly as observed from Giemsa stained blood smears from mice five days after injection of parasites. Although the mice were not completely cured of parasites, the percentage of parasitemia was lowered by more than 60% in the treated case. The survival of the infected mice were also increased due to the treatment of the compound (Figure 7.3D). While all the mice in the control succumbed to death within 7 days of injection, the mice treated with the compounds survived till the 12th day.

7.3 Conclusions

Aryl-alkyl-lysines represent simple designs with multifaceted activity. This chapter reports the activity of aryl-alkyl-lysines against malarial parasites. The most active antiplasmodial agent, compound 3, was effective in stalling the development of the parasites inside the RBCs and was more active on the ring stage of the parasite. This phenomenon has also been reported in other examples of synthetic membrane active peptides or their mimics. Although from the studies conducted with fluorescent dyes Rhodamine 123 and Lysotracker green emphasize on the membrane damaging nature of the compound, alternative mechanisms of action might play a role.

Well tolerated in mice, the compound exhibited significant therapeutic efficacy at 20 mg kg^{-1} in murine model of cerebral malaria. Although, these compounds are not ideal drug candidates for treating malaria, they do represent a starting point for development of more effective antiplasmodial agents.

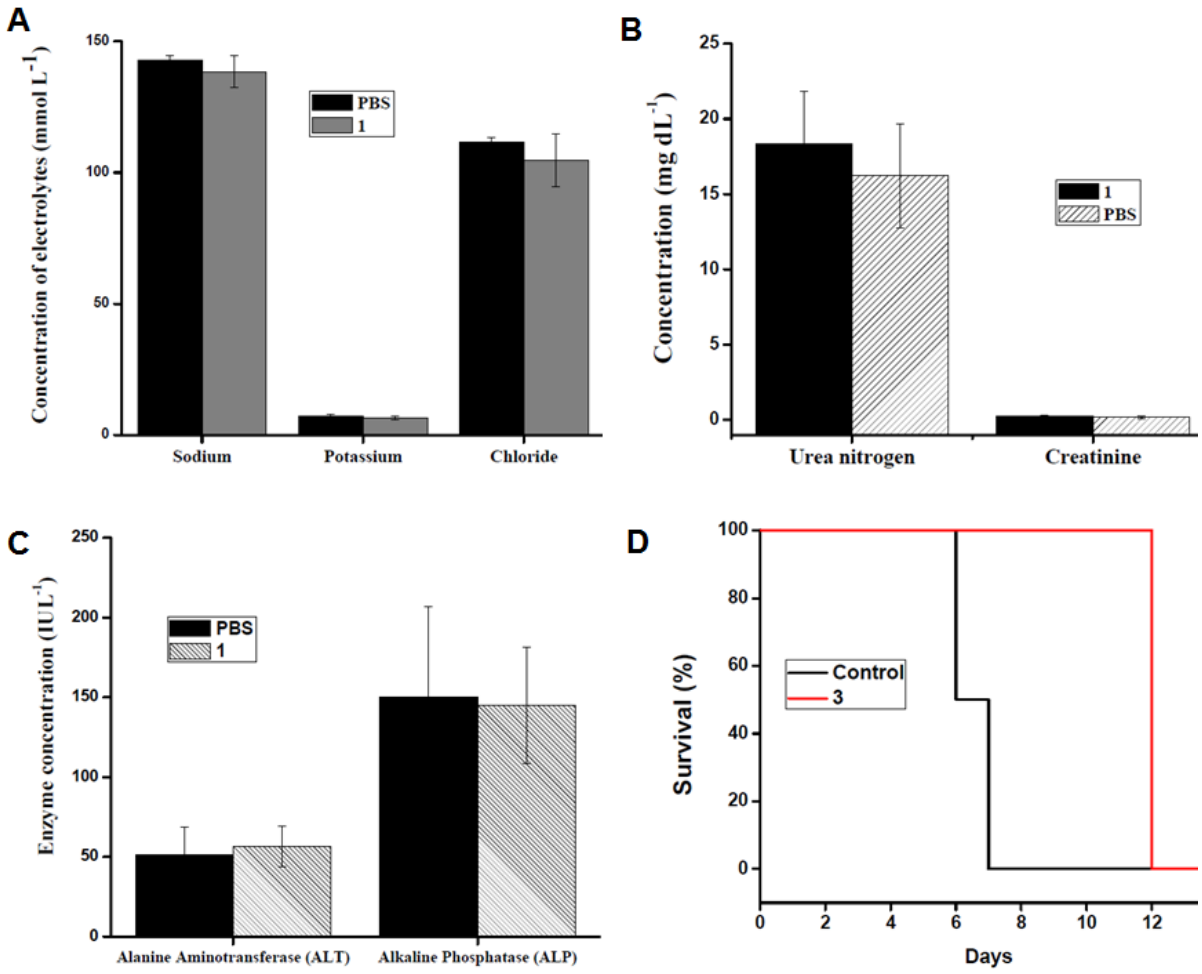


Figure 7.3: *In vivo* studies with 20 mg kg^{-1} of compound 3. (A-C) Acute toxicity of the compound 3 in comparison to vehicle (PBS). No observed toxicity with respect to A) electrochemical balance B) kidney functions C) liver functions. D) Survival of mice treated with compound 3 in comparison to vehicle.

7.4 Experimental Section

7.4.1 Synthesis and characterization: Synthesis of the secondary amines, HBTU coupling and deprotection of Boc groups were carried out as described in Chapter 2 (Section 2.4.2). The characterization data have been furnished below.

***N*-(quinolin-4-ylmethyl)hexan-1-aminium chloride (5a):** Yield-70%. ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 9.2 (Ar-CH_2-*NH_2*-C_6H_{13}, 2H), 8.4-8 (Ar-H, 6H), 5.1 (s, Ar-*CH_2*-NH_2-, 2H), 3.3 (t, -NH_2-*CH_2*-C_5H_{11}, 2H), 1.8 (m, -NH_2-CH_2-*CH_2*-C_4H_9, 2H), 1.5-1.3 (m, -NH_2-C_2H_5-(*CH_2)$_3$*-CH_3, 6H), 0.82 (t, -NH_2-C_5H_{12}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 243.1853 (calc. = 243.1861).

***N*-(naphthalen-2-ylmethyl)hexan-1-aminium chloride (6a):** Yield-85%. ^{1}H NMR ($CDCl_3$) δ/ppm: 9.9 (s, Ar-CH_2-*NH_2*-C_6H_{13}, 2H), 8.1-7.51 Ar*H*, 7H), 4.5 (s, Ar-*CH_2*-NH_2-, 2H), 2.75 (t, -NH_2-*CH_2*-C_5H_{11}, 2H), 1.85 (q, -NH_2-CH_2-*CH_2*-C_4H_9, 2H), 1.2 (m, -NH_2-C_2H_5-(*CH_2)$_3$*-CH_3, 6H), 0.82 (t, -NH_2-C_5H_{12}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 242.1889 (calc. = 242.1903).

N-([1,1'-biphenyl]-4-ylmethyl)hexan-1-aminium chloride (7a): Yield-76%. ^{1}H NMR ($CDCl_3$) δ/ppm: 9.9 (m, Ar-CH_2-*NH_2*-C_6H_{13}, 2H), 7.7 (d, Ar*H*, 2H), 7.6 (d, Ar*H*, 2H), 7.51 (m, Ar*H*, 2H), 7.44-7.3 (Ar*H*, 3H) 4.0 (s, Ar-*CH_2*-NH_2-, 2H), 2.8 (t, -NH_2-*CH_2*-C_5H_{11}, 2H), 1.87 (m, -NH_2-CH_2-*CH_2*-C_4H_9, 2H), 1.3-1.17 (-NH_2-C_2H_5-(*CH_2)$_3$*-CH_3, 6H), 0.82 (t, -NH_2-C_5H_{12}-*CH_3*, 3H). HR-MS (m/z): $[M+H]^+$ obsd. = 268.2059 (calc. = 268.2065).

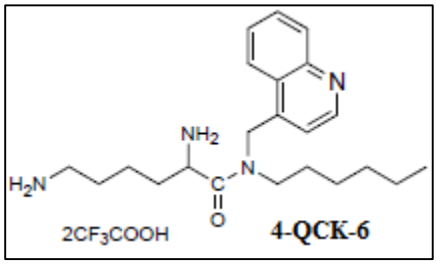
Boc-Lys(Boc)-*N*-(quinolin-4-ylmethyl)hexan-1-amide (5b): Yield-72%. ^{1}H NMR ($CDCl_3$) δ/ppm: 8.15-7.9 (Ar*H*, *2*H), 7.8-7.5 (Ar*H*, *2*H), 7.1 (Ar*H*, 1H), 5.2-4.9 (Ar-C*H*^{1}H^2-N(R)Lys(boc)$_2$ and α-N*H*-Boc of Lys(boc)$_2$, 2H), 4.75-4.40 (Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$,Lys (ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 3H), 3.40-3.0 (δ-*CH_2* of Lys(boc)$_2$ and Ar-CH_2-N(-C*H_2*-C_5H_{11})Lys(boc)$_2$, 3H), 1.72-1.12 (-CO-[CH-*CH_2*-*CH_2*-*CH_2*-CH_2-NH-COO-C(C*H_3*)$_3$]-NH-COO-C(C*H_3*)$_3$ of Lys(boc)$_2$ and –CH_2-*$(CH_2)_4$*-CH_3 of R group, 32H), 0.84 (m, Ar-CH_2-N(-C_5H_{10}-C*H_3*)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3331 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1709 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): $[M+H]^+$ obsd. = 570.3954 (calc. = 570.3907).

Boc-Lys(Boc)-*N*-(naphthalen-2-ylmethyl)hexan-1-amide (6b): Yield-80%. ^{1}H NMR ($CDCl_3$) δ/ppm: 7.86-7.74 (Ar*H*, 3H), 7.62 (s, ArH, 1H) 7.5-7.42 (Ar*H*, 2H), 7.32-7.3 (Ar*H*, 1H), 5.4 (α-

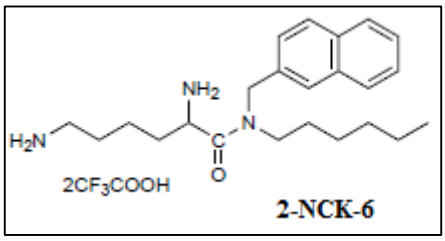
N*H*-Boc of Lys(boc)$_2$, 1H) 5.0-5.0 (Ar-C*H*^{1}H^2-N(R)Lys(boc)$_2$ and α-N*H*-Boc of Lys(boc)$_2$, 2H), 4.8-4.40 (Ar-C*H*^{1}H^2-N(R)Lys(boc)$_2$, Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$,Lys (ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 4H), 3.50-2.9 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-C$_5$H$_{11}$)Lys(boc)$_2$, 4H), 1.76-1.15 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH*$_2$*)*$_4$-CH$_3$ of R group, 32H), 0.84 (m, Ar-CH$_2$-N(-C$_5$H$_{10}$-C*H*$_3$)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3331 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1709 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): [M+H]$^+$ obsd. = 570.3954 (calc. = 570.3907).

Boc-Lys(Boc)-*N*-([1,1'-biphenyl]-4-ylmethyl)hexan-1-amide (7b): Yield-76%. ^{1}H NMR (CDCl$_3$) δ/ppm: 7.64-7.5 (Ar*H*, 4H), 7.48-7.38 (Ar*H*, 2H), 7.38-7.3 (Ar*H*, 1H), 7.3-7.2 (Ar*H*, 2H) 5.38 (α-N*H*-Boc of Lys(boc)$_2$, 1H), 4.87-4.37 (Ar-C*H*^{1}H^2-N(R)Lys(boc)$_2$, Ar-CH^1*H*^{2}-N(R)Lys(boc)$_2$,Lys (ε-N*H*-Boc)-α-NH-boc and α-C*H* of Lys(boc)$_2$, 4H), 3.50-2.9 (δ-*CH*$_2$ of Lys(boc)$_2$ and Ar-CH$_2$-N(-C*H*$_2$-C$_5$H$_{11}$)Lys(boc)$_2$, 4H), 1.76-1.19 (-CO-[CH-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH-COO-C(C*H*$_3$)$_3$]-NH-COO-C(C*H*$_3$)$_3$ of Lys(boc)$_2$ and –CH$_2$-*(CH*$_2$*)*$_4$-CH$_3$ of R group, 32H), 0.84 (m, Ar-CH$_2$-N(-C$_5$H$_{10}$-C*H*$_3$)Lys(boc)$_2$, 3H). FT-IR (cm^{-1}): 3331 (carbamate N-H str.), 3085 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1709 (C=O str. of carbamate), 1643 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): [M+H]$^+$ obsd. = 570.3954 (calc. = 570.3907).

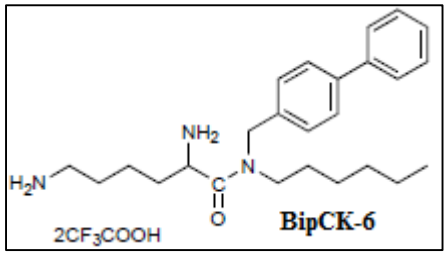
Lys-*N*-(quinolin-4-ylmethyl)hexan-1-amide trifluoroacetate (5): ^{1}H NMR (400MHz, D$_2$O) δ/ppm: 8.9 (ArH, 1H) 8.3-7.8 (Ar*H*, 4H) 7.5 (Ar*H*, 1H), 5.5-5.1 (Ar-C*H*$_2$-N(R)Lys, 2H), 4.7 (m, α-C*H* of Lys, 1H), 3.8-2.7 (Ar-CH$_2$-N(*CH*$_2$(CH$_2$)$_2$CH$_3$)Lys and ε-C*H*$_2$ of Lys, 4H), 2.1-1.19 (β-C*H*$_2$ of Lys, γ-C*H*$_2$ of Lys, δ-C*H*$_2$ of Lys and Ar-CH$_2$-N(CH$_2$*CH*$_2$C$_3$H$_7$)Lys and Ar-CH$_2$-N(C$_2$H$_4$*C*$_3$*H*$_6$CH$_3$)Lys, 14H), 0.85 (m, Ar-CH$_2$-N(C$_5$H$_{10}$*CH*$_3$)Lys, 3H). FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): [M+H]$^+$ obsd. = 371.2795 (calc. = 371.2811).



Lys-*N*- *N*-(naphthalen-2-ylmethyl)hexan-1-amide trifluoroacetate (6): ^{1}H NMR (D_2O) δ/ppm: 7.9-7.7 (Ar*H*, 4H) 7.7-7.37 (Ar*H*, 3H), 4.72-4.27 (Ar-C*H*^{1}H^{2}-N(R)Lys, Ar-CH^{1}*H*^{2}-N(R)Lys, 1H and α-C*H* of Lys, 3H), 3.4-2.7 (Ar-CH_2-N(*C**H*$_2$(CH_2)$_2$$CH_3$)Lys and ε-C*H*$_2$ of Lys, 4H), 1.94-1.19 (β-C*H*$_2$ of Lys, γ-C*H*$_2$ of Lys, δ-C*H*$_2$ of Lys and Ar-CH_2-N(CH_2*C**H*$_2$$C_3H_7$)Lys, 8H), 0.85 (m, Ar-$CH_2$-N($C_2H_4$*C*$_3$*H*$_6$$CH_3$)Lys, 6H), 0.65 (m, Ar-$CH_2$-N($C_5H_{10}$*C**H*$_3$)Lys, 3H). FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): [M+H]$^+$ obsd. = 370.2844 (calc. = 370.2822).



Lys-*N*-([1,1'-biphenyl]-4-ylmethyl)hexan-1-amide trifluoroacetate (7): ^{1}H NMR (D_2O) δ/ppm: 7.37-7.12 (Ar*H*, 4H) 7.12-6.87 (Ar*H*, 5H), 4.5-4.1 (Ar-C*H*^{1}H^{2}-N(R)Lys, Ar-CH^{1}*H*^{2}-N(R)Lys and α-C*H* of Lys, 3H), 3.1-2.74 (Ar-CH_2-N(*C**H*$_2$(CH_2)$_2$$CH_3$)Lys and ε-C*H*$_2$ of Lys, 4H), 1.94-1.19 (β-C*H*$_2$ of Lys, γ-C*H*$_2$ of Lys, δ-C*H*$_2$ of Lys and Ar-CH_2-N(CH_2*C**H*$_2$$C_3H_7$)Lys, 8H), 0.8 (m, Ar-$CH_2$-N($C_2H_4$*C*$_3$*H*$_6$$CH_3$)Lys, 6H), 0.5 (m, Ar-$CH_2$-N($C_5H_{10}$*C**H*$_3$)Lys, 3H). FT-IR (cm^{-1}): 3414 (primary amine N-H str.), 3089 (sp^2 C-H str.), 2967-2867 (sp^3 C-H str.), 1678 (C=O str. of tertiary amide), 1517-1450 (aromatic C=C str.). HR-MS (m/z): [M+H]$^+$ obsd. = 396.2995 (calc. = 396.3015).



7.4.2 Biological assays

Plasmodium falciparum culturing. *Plasmodium falciparum* 3D7 was cultured in human O$^+$ erythrocytes in complete RPMI 1640 (Sigma Aldrich) medium supplemented with 0.5% albumax II (Invitrogen), 0.2% NaHCO3, 0.2% Glucose, 200μM Hypoxanthine and 5μg/L Gentamycin. At every 24 h, the spent medium was removed and replenished with fresh medium. The culture was scaled up by splitting it, once the parasitemia had increased up to 10%. A fresh O-positive human erythrocytes was added for the newer infection.

7.4.2.1 *In vitro* antimalarial activity: For *in vitro* antimalarial screening, SYBR green I based fluorescence assay was used as reported by Smilkstein et al. with a minor modification.263

Parasites were synchronized to ring stages (haematocrit: 2%, parasitaemia: 1%, 100 µl) by sorbitol treatment. The compounds were serially diluted in complete RPMI media. Parasite cultures (50µL) were incubated with increasing concentrations of compounds in media (50µL) in a 96 well plate. The highest concentration tested was 10µM. CQ was used as positive controls and absence of compound was considered to be negative control. After 48 h of incubation 100 µL of lysis buffer containing SYBR Green I was added to the 96 wells. The composition of the lysis buffer per mL is as follows: 0.2 µl of 10,000 X SYBR Green I (Sigma), Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%; wt/vol), and Triton X-100 (0.08%; vol/vol). This solution (200µL) was then mixed gently with multi-channel pipette and incubated in dark at 37°C for 1 h. At the end of the incubation period, the solutions were transferred to black-round bottomed 96 well plates. Fluorescence was measured using a Tecan plate reader with excitation and emission wavelengths centred at 485 and 530 nm respectively. The fluorescence intensities were plotted against increasing compound concentrations and IC_{50} (the 50% inhibitory concentration) was determined by analysis of sigmoidal curves. Giemsa-stained smears of compound-treated parasite cultures were also done in order to validate the results.

7.4.2.2 Haemolytic activity: Hemolytic experiments were performed as described in Chapter 2 (Section 2.4.2).

7.4.2.3 In vitro stage dependence of action: The stage dependence of action of the compounds were determined by addition of the compounds at specific stages of parasite development: ring, trophozoite and schizonts. To achieve a synchrony in stage development of *P. falciparum*, selective destruction of trophozoite and schizont stage erythrocytes by 5% sorbitol was used. The parasites were tightly synchronized in the ring stages using two rounds of sorbitol synchronization spaced 4h apart. This culture was divided into three groups. Each group was subdivided into two groups. In the subdivision, one served as treated while the other as positive control. The three groups were divided as rings, trophozoites and schizonts. Compounds were added at concentrations twice their IC_{50} values to the ring stage culture after second synchronization. In case of trophozoites the culture was allowed to grown for 24 h before addition of compounds. In case of schizonts the compounds were allowed to grow for 38 h before addition of compounds. Rings, trophozoites and schizonts were allowed to grow for 48h, 24h, and 12h respectively before Giemsa-stained smears from the assays were analyzed microscopically. The ability of the

compound to inhibit parasite development at different stages was gauged by comparing the numbers with untreated controls.

7.4.2.4 Mechanism of action (Microscopy): For these experiments two dyes were used Rhodamine 123 and Lysotracker Green. Briefly, In a total volume of 200 µL, Parasite-infected erythrocytes (2% haematocrit and 3% parasitemia) were incubated at 37°C for 30 min with 1 µM rhodamine 123 (for parasite plasma membrane potential), or 10 nM LysoTracker Green (for DV potential) before incubation with the test compound for 4 hr. Untreated cultures served as negative controls. These were then transferred onto a cover slip, which was fixed using paraformaldehyde. The fixed coverslips were then visualized under a confocal microscope.

7.4.3 *In vivo* studies

7.4.3.1 *In vivo* toxicology: The *in vivo* toxicology study was performed at JNCASR following the institute guidelines. For conducting *in vivo* toxicology studies, the compounds (in PBS) were injected intravenously into female Balb/c mice (6-8 weeks old).

Systemic toxicity and LD_{50} determination: Mice were treated with decreasing concentrations of compound 3 (in PBS) intravenously. The concentrations used were 175mg kg^{-1}, 55 mg kg^{-1}, 17.5 mg kg^{-1} and 5.5 mg kg^{-1}. Survival of mice were observed and the parameters were fit into Spearman-Karber's equation to obtain the LD_{50} values of the compounds. Systemic toxicity was also examined after i.v. injection of compounds. Animals were directly inspected for adverse effects for 4 h, and mortality was observed for 14 days.

Acute toxicity: For the evaluation of the acute toxicity, two groups of 10 mice each received intravenous injection of NCK-6 at 17.5 mg kg^{-1} in 0.2 ml of sterilized PBS. 10 mice were sacrificed at 48 h and the rest mice at 14 days to collect blood samples for analysis of biochemical parameters such as alanine transaminase (ALT), urea nitrogen, creatinine, sodium ion, potassium ion and chloride ion levels. Blood samples were analyzed at Gokula Metropolis clinical laboratory, Bengaluru, India.

7.4.3.2 *In vivo* activity: The *in vivo* activity study was performed at Indian Institute of Science following the institutional guidelines. *P. bergheii* ANKA Mouse Studies: 6-8 weeks old Swiss mice (female) were first injected with 10^7 *P. bergheii* ANKA parasitized RBCs via IP administration. Two hours after the infection, mice (n=4) were given dosages of NCK-6 (20 mg/kg) or PBS (Control). These dosages were continued every 24 hours for four days. Parasitemia were determined on day 5 via Giemsa-stained smears of blood drawn from the tail of the mice. Survival of the mice were monitored till they were dead. Mice experiments were performed at Indian Institute of Science following the guidelines of the institute.

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List of publications:

1. **C. Ghosh,** V. Yadav, W. Younis, H. Mohammad, Y. A. Hegazy, M. N. Seleem, K. Sanyal and J. Haldar. Aryl-alkyl-lysines: Membrane-Active Fungicides That Act against Biofilms of *Candida albicans*. *ACS Infect. Dis.*, **2017**, (DOI: 10.1021/acsinfecdis.6b00192).

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6. **C. Ghosh** and J. Haldar; "Membrane active small Molecules: Designs inspired from Antimicrobial peptides" *ChemMedChem*, **2015**, *10*, 1606-1624

7. **C. Ghosh**, G.B. Manjunath, P. Akkapeddi, V. Yarlagadda, J. Hoque, D.S.S.M. Uppu, M.M. Konai, and J. Haldar; "Small Molecular Antibacterial Peptoid Mimics: the simpler the better ! ", *J. Med. Chem.*, **2014**, *57*, 1428–1436.

8. **C. Ghosh**, P. Sarkar, S. Samaddar, M.M. Konai, D.S.S.M. Uppu and J. Haldar; "L-lysine based lipidated biphenyls as agents that inhibit intracellular bacteria and disrupt biofilms" (under revision).

9. **C. Ghosh**, A. Ray, N. Harmouche, B. Bechinger, S. Pati and J. Haldar, "Understanding the effect of aryl-alkyl-lysines on the components of bacterial cell envelope using simulation and experiments for treating bacterial infection " (Manuscript to be submitted)

Other publications

1. J. Hoque, P. Akkapeddi, **C. Ghosh**, D.S.S.M. and J. Haldar. "A Biodegradable Polycationic Paint that Kills Bacteria *In Vitro* and *In Vivo*." *ACS Appl. Mater. Interfaces*, **2016**, *8*, 29298–29309.

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Patent:

Antimicrobial compounds, their synthesis and applications thereof

C. Ghosh, Goutham B Manjunath, Padma Akkapeddi and Jayanta Haldar. National phase applications have been filed USA, Canada, China, India, Australia, Japan and Europe.

WO2014097178 (A1), US2015329478 (A1), JP2016504330 (A), EP2934563 (A1), EP2934563 (A4), CN104981249 (A), CA2894202 (A1), AU2013365769 (A1)

Awards

1. **Winner: Antimicrobial Resistance Diagnostic Challenge Autumn School (2015)** held at University of Edinburgh, Edinburgh, Scotland.
2. **Gandhiyan Young Technological Innovation Award (2015)** under "SRISTI Technological Edge/Strategic innovation" category at Rashtrapathi Bhavan (President of India), New Delhi.
3. **Best Oral Presentation Award at** 4th International Meeting on Antimicrobial Peptides (2014), Lorient, France.
4. **International Travel Support (2014)** Science and Research Board, Department of Science and Technology, Government of India.
5. **Best Oral Presentation award at** Chemical Frontiers (2013), Goa, India.
6. **Best Poster award** at In-house Symposium (2013), JNCASR