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Development and validation of thiolactone peptide mimics to antagonize agr-quorum sensing system in *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus variants evolved via a group specific/ non-group specific interaction of a two component system, histidine-kinase receptor, AgrC and autoinducing peptide, AIP. In consistent to this report, our prime interest was to develop and validate a global inhibitor against these variants using peptidomimetic approach. As one of the AIP variant (AIPII) also reported to show weak activator of its own and cross inhibitor of the others, its response domain (macrocyclic ring) was theoretically substituted with the aminoacids (glycine, phenylalanine, isoleucine, tyrosine) that had earlier showed significant in inhibiting virulence effect of *S.aureus*keeping constant the conserved residue, cysteine. The AIP-II mimics, mAIP-II [1] and mAIP-II [2] that showed the best glide score on docking with the protein AgrC variants individually were synthesized to confirm its biological competitive mode of action in the presence of 100nM of natural AIP of its respective groups using reporter strains. The mAIP-II[1] was further validated in i*nvivo* rat protection test and the histopathological studies showed the infiltration of neutrophils in the infected region among diseased groups, where in treated groups the region consists of predominantly lymphocytes and angiogenesis, a clear confirmation of healing process. Thus we conclude the peptidomimetic approach would be a promising approach to design a global inhibitor to inhibit virulence genes among *S.aureus* variants.
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KEYWORDS

Staphylococcus aureus;
Peptidomimetics;
Competitive inhibition;
Neutrophils;
Lymphocytes.

INTRODUCTION

Staphylococcus aureus, a gram-positive, human pathogen, is responsible for nosocomial infections. Its infection ranges from local colonization to metastatic infection, such as endocarditis, septic arthritis, and toxic shock syndrome etc. *S.aureus* infection involves primarily production and secretion of toxins that damages or completely lyse the host tissue. They also produce enzymes which interfere with the host immune system e.g. adenosine synthase (AdsA) responsible for the conversion of adenosine monophosphate to adenosine, as it helps *S.aureus* to evade phagocytic clearance of host immune system[1,2] cell surface adhesion proteins, "Microbial surface components recognizing adhesive matrix molecules" (MSCRAMM) help them to

attach to and protect themselves against the host[3,4]. The need of a new therapeutics against *S.aureus* infection is due to its increase resistance to antibiotics, so targeting virulence genes or biofilm could be a more potent and promising way of treating this nosocomial infections. Targeting *S.aureus* infection using anti-virulent drug strategy could be primarily accomplished on blocking the global regulator *agr,* activated in response to secreted autoinducing peptide (AIP). The autoinducing peptide serves as ligand, in turn regulates the expression of virulence factors on its interaction to the signal receptor, AgrC. The Receptor-Ligand interaction initiates and drives the classical Two-component signalling pathway to elicit the signal response to establish pathogenesis. In *S.aureus* the inter strains receptor-ligand i.e. (receptor-auto inducer) pair show considerable sequence variations, as it is classified into four groups[5]. Surprisingly, homologous interaction of AIP – receptor induces *agr* response, whereas heterologous interaction inhibit *agr* gene expression[6] as it involves. Thus inhibition involves repression of certain virulence genes of the groups without affecting the growth, termed as bacterial interference.

The *agr* locus contains two divergent promoters, P2 and P3. The P2 operon consists of four genes (*agrA-D*) encoding cytosolic, transmembrane and extracellular components of the two component system. The *agrD* genes encodes a propeptide, processed and secreted out extracellularly as mature auto inducing peptide(AIP) through AgrB, *an integral membrane protein.* The binding of AIP to the AgrC, histidine-kinase receptor, a transmembrane protein, results in autophosphorylation, subsequently trans-phosphorylation of AgrA occurs, that in turn activate the transcription from the promoters P2/P3 promoter[7,8]. The P3 operon consists of RNAIII,

effector of *agr* and RNAIII transcript is responsible for the up regulation of virulent genes and down regulation of surface proteins[7,9].

Structural activity analysis had revealed that derivates of Autoinducing peptides, lactone or lactam analog of AIP-II formed by replacing the thiolactone bond changed the intragroup activation retaining its cross inhibitory activity to other groups[6,10] and less modification or complete removal of the tail part of AIP can lead to the generation of global inhibitor of the *agr* response. In the existing report of our laboratory, that aminoacids such as glycine, phenylaniane, isoleucine, and tyrosine had some adverse effect on inhibition of *S.aureus* growth and virulence[11]. Henceforth in this study we attempted a peptidomimetic strategy, modifying the AIP-II in substituting those aminoacids at all possible combination to elucidate the mechanism of competitive inhibition with natural AIP. The competitive peptide mimic will be validated through *invitro and invivo studies*, to identify a global anti-virulent agent for *S.aureus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

S.aureus groups I (RN6390B), II (SA502A), III (RN8463), each consist of *agr* P3-*blaZ* (β-lactamase) fusion plasmid and *agr* Null (RN6911) were gifted by Dr.Richard. P.Novick and are listed in TABLE 1. Cells were grown in tryptic soya broth (TSB), with shaking at 37 ºC. Overnight cultures were routinely maintained in TSB and the slants were prepared using TSB supplemented with 1.5% of agar.

TABLE 1 : *S.aureus* strains

Strain	Description	Ref or source
RN6390B	*agr* group I with P3-*blaZ* (β-lactamase) fusion plasmid	[10,19]
SA502A	*agr* group II with P3-*blaZ* (β-lactamase) fusion plasmid	[10, 19, 20]
RN8463	*agr* group III with P3-*blaZ* (β-lactamase) fusion plasmid	[10,19]
RN6911	RN6390B with *agr* locus replaced with tetM.	[10,19]

Computational studies

Protein prediction and modelling

Sequences of the proteins *agr*C1, *agr*C2, *agr*C3 and *agr*C4 were retrieved from NCBI (*agr*C1 - YP_001332979; *agr*C2 - YP_001442613; *agr*C3 - YP_044054; *agr*C4 - ABB29292) in FASTA format. The retrieved sequences were compared to study the positions of aminoacids using alignment software. NCBI BLAST was performed to search of similar sequences with crystal structure. Since the crystal structures for those sequences of search were not available in the protein databank, threading was preferred over homology

modelling. The number and lengths of the various loops and spanning segments were predicted using the trans-membrane prediction software's TMHMM-TOP 2.0 and OCTOPUS. The sequences were given to the LOMETS site that collects high scoring target to template alignments from 10 threading programs namely PPA, SAM-TO2, SP3, PRC, MUSTER, HHsearch, PROSPECT2, FFAS, FUGUE and SPARKS. The model with the highest confidence score of the 10 and satisfying the alignment requirements as per the trans-membrane software prediction (OCTOPUS) was chosen.

Ligand preparation

The 604 AIP-II mimic sequences were formulated through permutations and combinations in partially and completely substituting the four amino acids with glycine, tyrosine, phenylalanine and isoleucine at all positions other than the cysteine residues (conserved) of the AIP-II thiolactone ring removing the tail portion[12]. The structures were drawn using ACD Chemsketch software and the 3D structure of ligand were prepared using LigPrep product of Schrödinger.

Docking studies

The prepared ligands were docked into the active site of the protein models (receptors) of S*taphylococcus auerus agr*C1, *agr*C2, *agr*C3, and *agr*C4 using GLIDE (Grid-based Ligand Docking with Energetics) module of Schrödinger[13,14]. The grids were generated around the residual amino acids from 101-109 in the second loop of *agr*C1, *agr*C3 and *agr*C4, and from 42-50 residual amino acids in the first loop of *agr*C2, defines the space for the ligand to get bound with the protein model. The prepared ligands (604) were split into 6 sets, and each set was docked against the modelled *agr*C1 using HTVS docking (High Throughput Visual Screening), which was used for screening a very large number of ligands. Based on their best glide score top ten ligands from each set were shortlisted, then the shortlisted molecules were docked using SP docking (Standard Precision Docking). The glide score obtained from SP, scrutinized the top five ligand molecules that was further analysed by eXtra Precision (XP) Docking.

Synthesis of AIP-II mimics

AIP-II mimic peptides were synthesized by a standard solid-phase Fmoc (N-(9-fluorenyl) methoxycarbonyl) method using a ThuradMed TETRAS synthesizer (GenScript, USA). Fmoc solid-phase peptide synthesis was employed to build linear peptides. Peptide synthesized from its C-terminus by stepwise addition of aminoacids. Initially, the first Fmoc-amino acid was attached to an insoluble support resin via an acid labile linker. After deprotection of Fmoc on treatment with piperidine, the second Fmoc-amino acid is coupled utilizing a pre-activated species or *in situ* activation, after the desired was synthesized, the resin bound peptide was deprotected and cleaved.

Activation and inhibition assay

Preparation of AIP containing supernatant

S.aureus strains (RN6390B, SA502A, and RN8463) were grown in Tryptic soya broth with shaking 37 ºC for 9 hours starting with an inoculum of approximately 1×10^9 cells/ml in 100 ml of broth. Cells were removed by centrifugation at 4 ºC, and the supernatant was filtered (0.22µm filter, Millipore)[12]. The filtrate was lyophilised and then dissolved in phosphate buffer of pH 6.4, stored in -80 C and used as a source natural AIP for competitive inhibition study.

β – Lactamase activation assay

Assay were performed using the groups I (RN6390B), II(SA502A), III(RN8463) where these reporter *S.aureus* strains each containing an *agr* P3-*bla*Z (β-lactamase) fusion plasmid. The cells were grown on Tryptic soya broth to mid-exponential phase[10]. To the cultured cells varying concentration of AIP supernatant or varying concentration of AIP-II mimic peptide alone or varying concentration of AIP-II mimic peptide in presence of fixed concentration of 100nM of AIP containing supernatant were added. Incubate for 180minutes and β-lactamase assay was performed[15]. EC_{50} and IC_{50} values are determined using sigmoidal dose-response curve using Prism (Graph pad). All assays were performed at least in triplicates.

In vivo wistar rat protection assay

The AIP-II mimic peptides (mAIP-II [1], mAIP-II [2]) that showed competitively inhibition of its own and other strains with respect to its natural AIP was further validated through *invivo* rat protection test. Mid-exponential phase cultures of either RN6390B (*agr I*)

or SA502A (*agr II*) or RN6911 (*agr* null) grown in Tryptic soya broth centrifuged, and the bacteria were resuspended in physiological saline+10mM sodium phosphate (pH7.4) at approximately 10^9 cells per ml and stored on ice until use. Wistar rats (6 to 8 weeks old) were injected S.C in the flank area with approximately 10^9 colony-forming units[10]. A synthetic preparation of the AIP-II mimic peptide ($4\times IC_{100}$) diluted in phosphate buffer pH 6.4 were simultaneously injected with the bacteria for five of the 10RN6390B animals and five of the 10 SA502A animals. Animals injected with saline and RN6911 (*agr* Null) were kept as control. The animals are observed for ten days. After ten days haematology, histopathology and biochemical parameter were analyzed.

Haematology

The Blood samples were collected by retro orbital puncture in heparinized tubes and immediately used for determination of White Blood cells. The total WBC count was estimated using haemocytometer (Neubauer Chamber). For differential counting, a small drop of blood was applied on one end of a clean glass slide using a dry micropipette. A second slide was kept over it at 45º and the blood drop is smeared over the length of the glass slide. Then it was dried before staining with Leishmann stain and the slide was washed gently in running water, dried and examined under microscope, the cells were counted and differentiated.

Biochemical parameters

Blood samples were collected in test tubes once it got clotted, the serum was separated by centrifugation at 2500rpm, 4 ºC and analysed for various biochemical parameter (Albumin, AST, ALP-AMP, ALT, Glucose, Creatinine, Total Protein, Urea, Uric acid and Triglycerides) to elucidate the significant changes in an automated biochemical analyser (Biosystem A15).

Statistical analysis

For comparison of total WBC, Lymphocytes, Neutrophils and Biochemical parameters among the various diseased (Control) and treatment groups, the Mean ± SEM were obtained for each group in experiment, two way analysis of variance with Turkey's multiple comparison were applied.

RESULTS AND DISCUSSION

Structure activity studies on the AIP-II mimics group specific interaction with AgrC

604 AIP-II mimics were formulated using four amino acids Glycine, Phenylalanine, Isoleucine and Tyrosine as reported to have anti-virulent property[11]. Docking the mimics against the protein models AgrC1, 2, 3 and 4 revealed that mAIP-II [1] and mAIP-II [2] have an appreciable binding, were selected based on their docking score in comparison with the natural AIPs of their respective groups and with natural AIP-II (Figure 1). The interaction pattern (Figure 2) showed the amino acid residues of AgrC1 (Ile-200, Phe-167) and AgrC2 (Phe-41, Leu-44, Arg-202, Ala-203, Leu-265) form hydrogen bond with mAIP-II [1], whereas the amino acid residues of AgrC1 (Lys-105, Thr-197, Ile-200, Gln-202) and AgrC2 (Asp-196, Asp-197, Asn-264, Leu-265) form hydrogen bond to bind mAIP-II [2]. In AgrC3 (Tyr-102, Tyr-168, and Glu-207) and AgrC4 (Tyr-102) forms hydrogen bond with mAIP-II [1], whereas in AgrC3 (Tyr-102, Tyr-200) and AgrC4 (Tyr-102, Val-104) forms hydrogen bond with mAIP-II [2].

Biological activity studies

Biological activity studies were carried out using the synthesized peptides mAIP-II [1] and mAIP-II [2] where the activity of the drugs were correlated with the expression of β-lactamase reporter gene in AgrC variants. Studies conducted on AgrC 1,2,3 strains using mAIP-II [1] showed (Figure 3) that the level of expression of β-lactamase induced in each strain in the presence of 100nM natural AIPs of respective strains is low when compared to β-lactamase expression induced by mAIP-II [1] alone in each strain. In AgrC1 strain, we observed that the level of expression of β-lactamase induced by mAIP-II [1] had better inhibitory activity against activation by natural AIP I. However, in AgrC2 and AgrC3 strains, the individual effect of mAIP-II [1] on expression of β-lactamase was low, with increase in the concentration of mAIP-II [1]; its level of expression is high when compared to the expression of β-lactamase induced by their respective natural AIPs. Considering the level of expression of β-lactamase was low in the presence of 100nM of natural

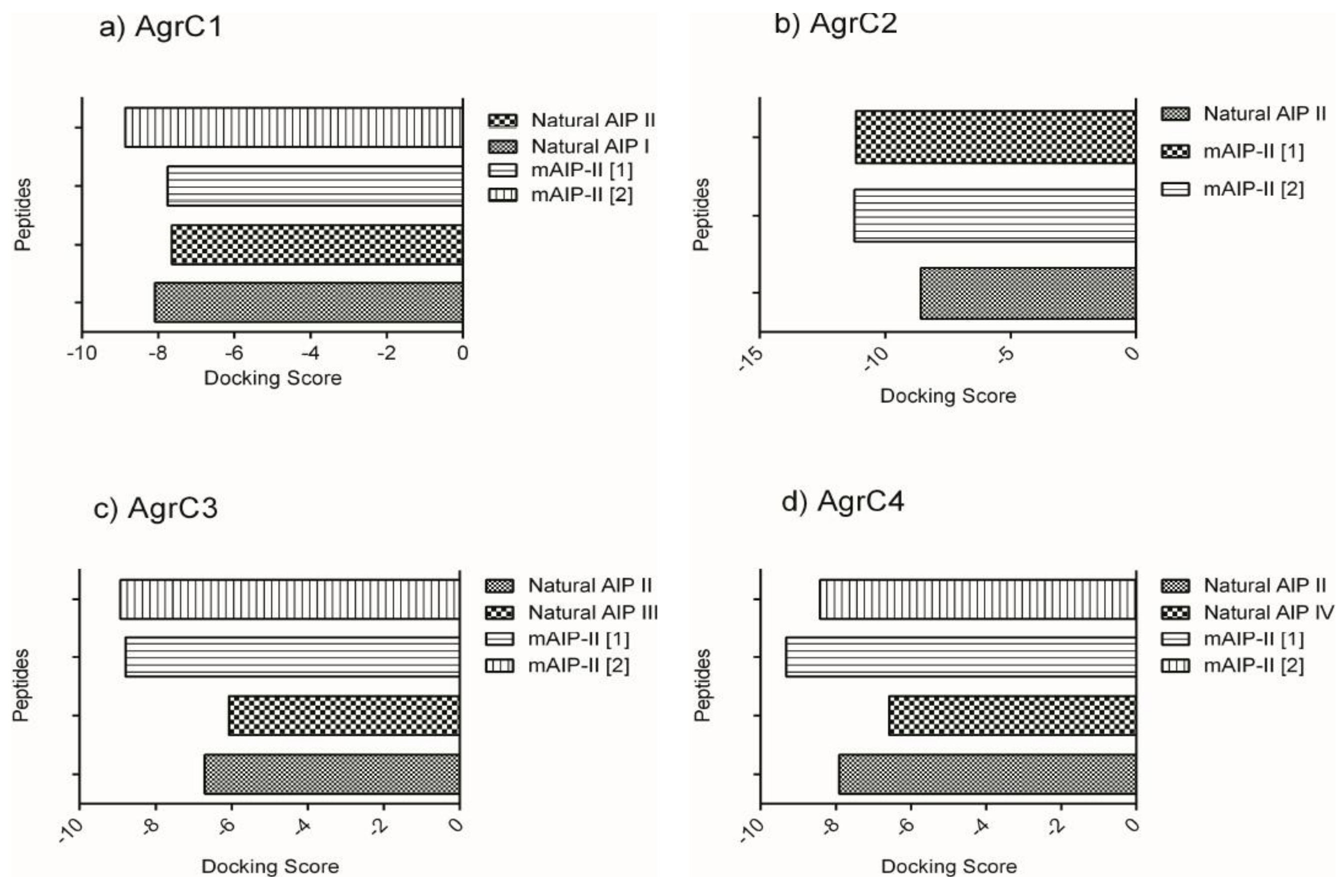

Figure 1 : Comparison of the docking score of peptides against their group specific interaction of histidine kinase receptor (AgrC)

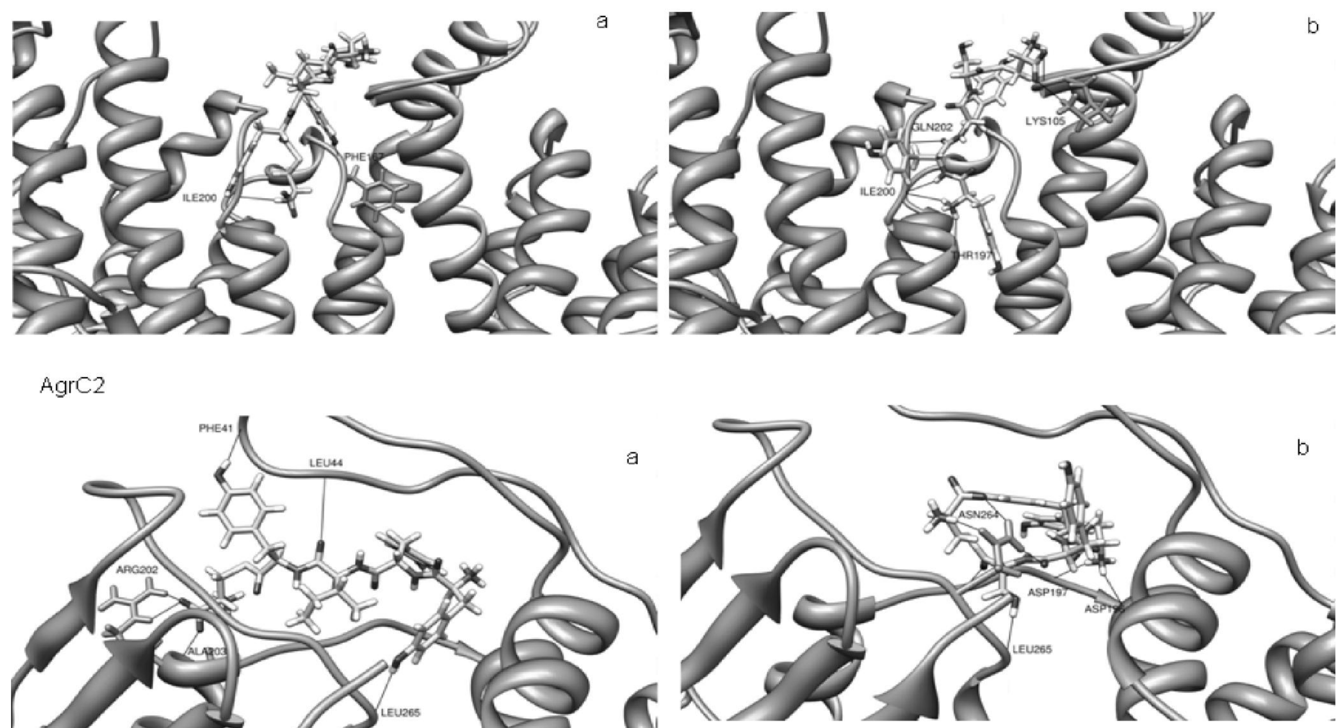

Figure 2 : Interaction of AIP-II mimics group specifically against AgrC1(Top) and AgrC2(Bottom) a)mAIP-II [1] and b)mAIP-II [2]

AIP II and natural AIP III of their respective strains, thus we could prove that mAIP-II [1] has some appreciable inhibitory effect towards AgrC2 and AgrC3 strains. Similarly, studies conducted with mAIP-II [2] also showed similar results where the level of expression of β-lactamase in AgrC1 and AgrC2 in the presence of 100nM of respective natural AIP had better inhibitory activity whereas in AgrC3, the level of expression was high when compared to the effect produced by Natural AIP III, although increase in concentration of mAIP-II [2] decreases the β-lactamase activity to some extent. The IC_{50} values of mAIP-II [1] and mAIP-II [2] in the presence of 100nM Natural AIP's of respective strains were determined and tabulated (TABLE 2). Since mAIP-II [1] seems to have better inhibitory activity in all three variants of *S.aureus* compared to mAIP-II [2], that has only towards AgrC1 and AgrC2 variants, further *invivo* studies were performed with mAIP-II [1].

Animal studiesm

Animal studies were conducted using control animals treated with saline; diseased models were generated by injecting with approximately 10^9 cells of AgrC1 and AgrC2 variants and treatment group injected with variants of *S.aureus* along with mAIP-II [1]. After 10 days, the body weights were calculated and the animals were sacrificed, the spleen weight of each animal

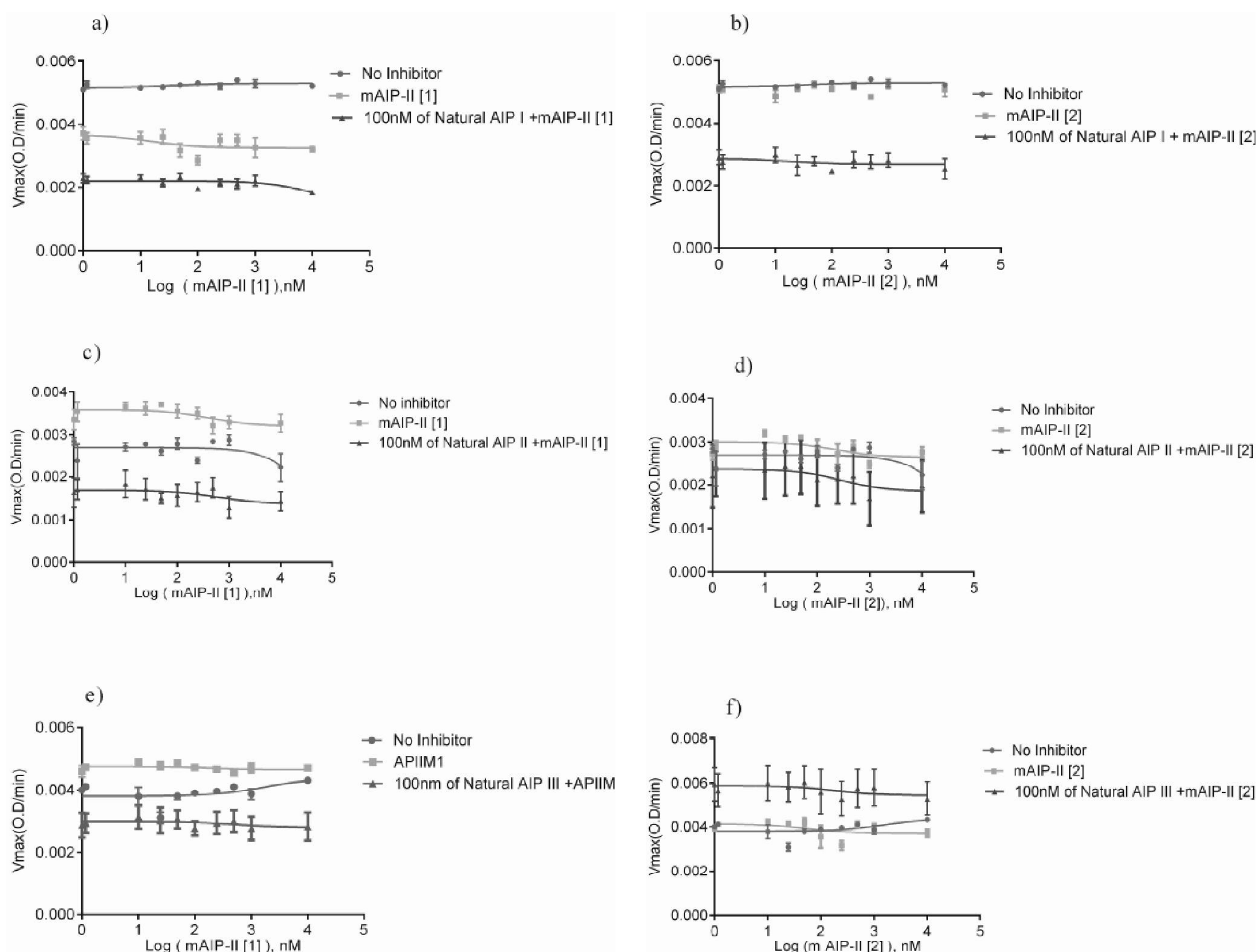

Figure 3 : Dose response curve for activation of b-lactamase reporter gene in AgrC Group cells. a), c) & e) are AgrC I, II & III cells were incubated with varying mAIP-II [1] alone or varying mAIP-II [1] and a fixed concentration of Respective AIP at 100nM. b), d), & f) are AgrC I, II & III cells were incubated with varying mAIP-II [2] alone or varying mAIP-II [2] and a fixed concentration of respective AIP at 100nM The Natural AIP concentration-response curve is shown for comparison (No Inhibitor) in each respective AgrC groups. Data were collected as b-lactamase activity (VmaxOD/min), and are shown at each concentration ± SEM. unless otherwise visible; error bars are contained within the confines of the symbol

TABLE 2 : Activation and Inhibition by AIP's and AIP-II mimics

Peptide	EC_{50} Activation			IC_{50} Inhibition		
	S.aureus Group			*S.aureus* Group		
	I	II	III	I	II	III
mAIP-II [1]	---	---	---	12μM	550nM	476nM
mAIP-II [2]	---	---	---	18nM	276nM	157nM

was noted. The Total WBC count data (Figure 4) showed that there is an increase in WBC level among diseased group and the treated group shows lesser WBC count. This data is corroborated with the relationship between mean body weight and spleen index of each group (Figure 5), shows an increase in the spleen weight among the diseased group (AgrC Null, AgrC1 and AgrC2). In accordance to this data, the diseased models showed an elevate in the WBC count, whereas the treatment group (AgrC1+mAIP-II [1] and AgrC2+mAIP-II [1]) showed low spleen weight with low WBC count. Neutrophils plays an important role in amplifying and sustaining neutrophils response in the site of infection to promote abscess formation and bacterial clearance[16]. Study conducted[17] in murine model of infectious dermatitis, induced by *S.aureus*, revealed the importance of neutrophils, that neutrophils depleted mice developed ulceration that was not healed and in control minor skin lesion was observed. In this study all normal group animals treated with saline had no tissue reaction, whereas among diseased groups, chronic-active inflammation (moderate to severe tissue) reaction was observed, that predominantly consists of neutrophils (Figure 6a) and plasma cells, which form micro abscess (Figure 7). This is a clear indication of active immune response to the foreign body injected (induction of disease). Usually neutrophils are predominant during the early hours of disease and their life time will be within a few days to weeks[18]. Animal groups treated with mAIP-II [1] showed chronic inflammation – (moderate tissue reaction), which is dominated by lympho-

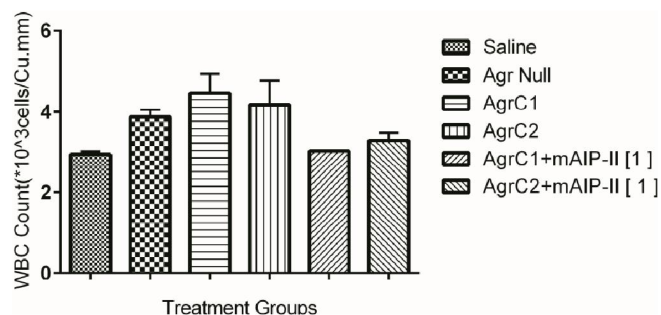

Figure 4 : Variation in WBC count among Treatment Groups

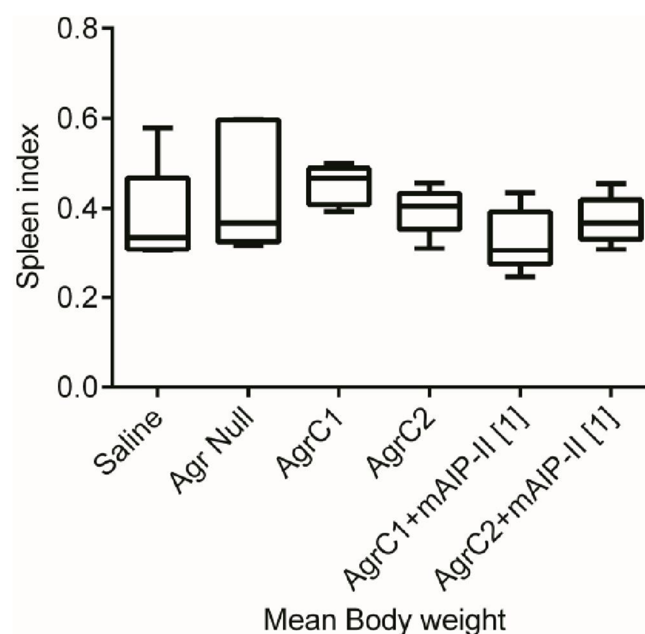

Figure 5 : Relationship between Mean Body weight and Spleen Index of Wistar Rats after Treatment. Values are Mean±SEM (n=5).

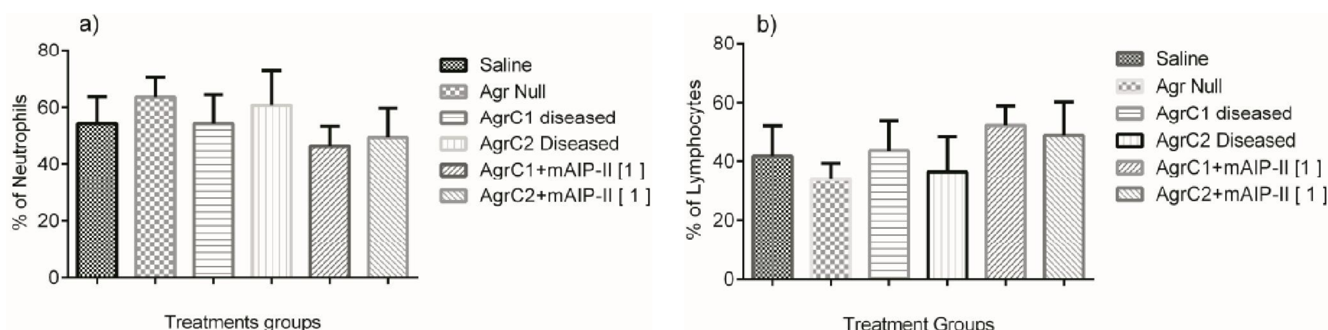

Figure 6 : Differential count of Blood samples collected from treatment a) % Neutrophils b) % Lymphocytes

Figure 7: Photomicrograph shows the inflammatory infiltration in skin of *S.aureus* control a) AgrCI and b) AgrC II abscess with predominant neutrophils (N), c) and d) are treated groups shows predominant lymphocytes (L) and Angiogenesis (A)

cytes (Figure 6b) and macrophages, and granulation tissue with angiogenesis (Figure 7), with macrophages playing the role of mediator. The formation of granulation tissue with angiogenesis is the hallmark of the healing process, in which proliferation of new blood vessels from existing vessels are seen with the presence of fibroblasts and collagen particles[18]. Thus mAIP-II [1] had suppressed the virulence of *S.aureus* variants, as the treated groups were healed at the early stage than the diseased groups. Biochemical parameter analysis of serum samples of the normal, diseased and mAIP-II [1] treated group animals (Figure 8) showed that there is no significant difference in the liver marker levels (ALP-AMP, AST, ALT, and Albumin), kidney marker levels (Creatinine, uric acid and urea) glucose and triglyceride levels, a sign of no kidney and liver damage and the proven the drug is effective and do not elicit any toxic effect on the hosts. As the *invitro* and *invivo* result are in consistent to

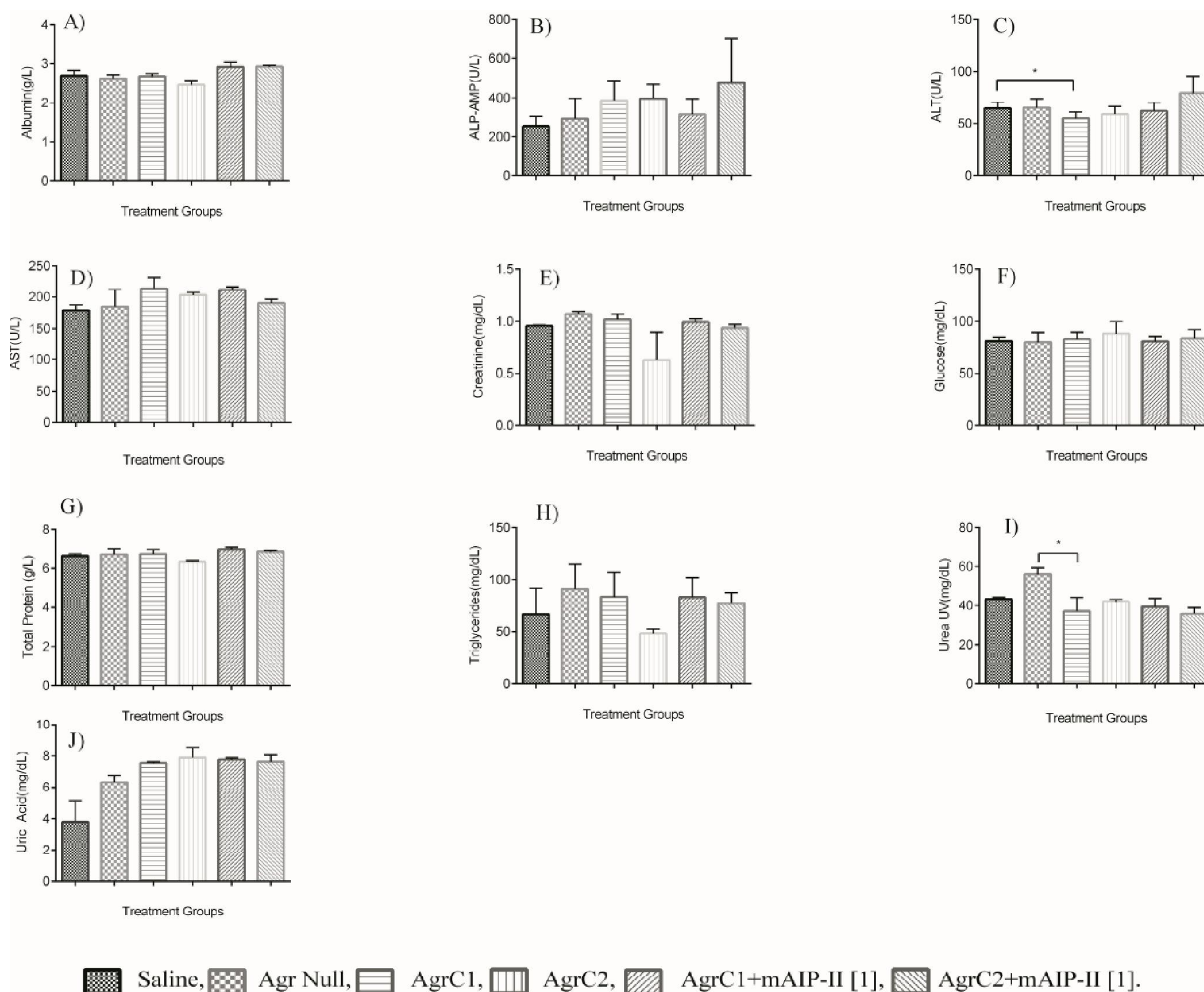

Figure 8 : Comparison of Serum Biochemical Parameters of Wistar rats in treatment groups. Values are Mean ± SEM (n=3) Difference between * Vs Saline, # Vs Null are significant (P<0.05)

prove that mAIP-II [1] would be a better molecule to down regulate the global regulator to dodge the virulence effect influenced by *S.aureus* in diseased host.

CONCLUSION

The peptidomimetic approach of designing potential competitive inhibitor, mAIP - II [1] to globally dodge virulence has provided a new approach in the treatment of *S.aureus* infection evidenced through our *invitro* and *invivo* studies to globally inhibit the *S.aureus* variants.

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