*Chapter I*

# INTRODUCTION

## 1.1 Quorum Sensing

Microorganisms communicate with each other via a very unique mechanism known as quorum sensing (QS). It is a cell-to-cell communication language, involving some specific chemical molecules called autoinducers (AI). A bacterium produces autoinducers at a minimal quantity and the concentration of AI gradually increases as bacterial population density increases. As the concentration of AIs reaches a threshold level, bacteria are able to detect and respond collectively to signal molecules (AIs). At this threshold level, signal molecules diffuse through membranes and bind to an active receptor which passes through the autophosphorylation process. The phosphorylated response regulator in turn modulates gene expressions that are beneficial for the survival of bacterial community[1-6].

Acyl-homoserine lactones (AHLs), autoinducing peptides (AIPs), and autoinducer-2 (AI-2) are three major significant QS signals. They play key roles in the secretion of bacterial virulence factors and regulation of bacterial pathogenesis[7-9]. Gram-negative bacteria mostly produce AHLs as signaling molecules whereas Gram-positive bacteria excrete AIPs as signaling molecules. AI-2 signals are produced by Gram-negative and Gram-positive bacteria both. The pathways of QS are different for Gram-negative and Gram-positive bacteria[10, 11] (**Fig. 1.1**).

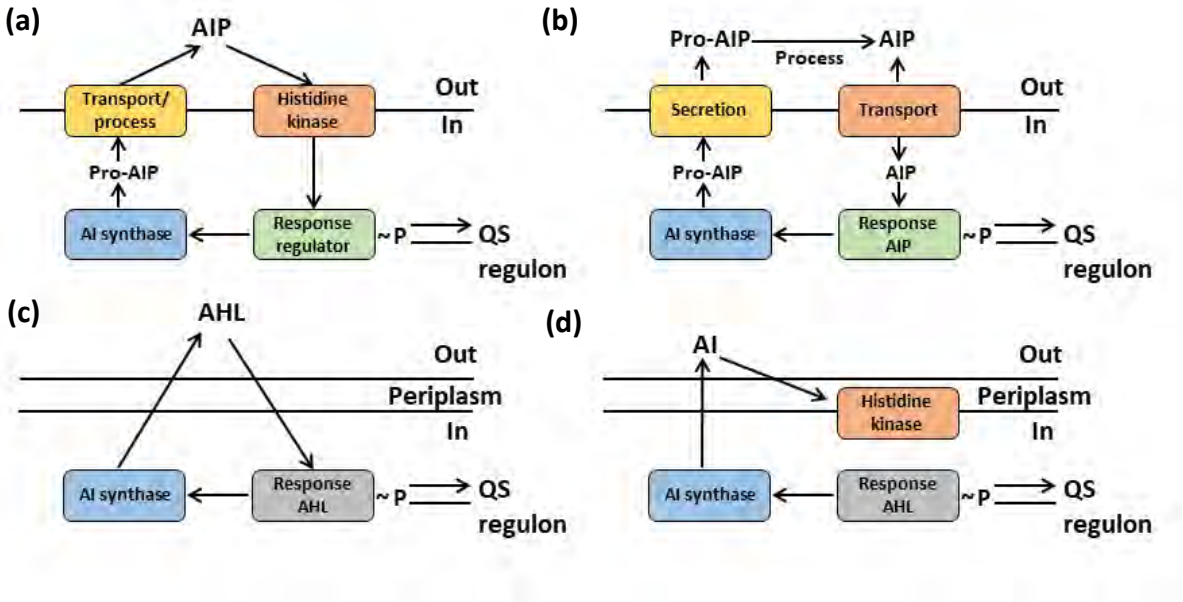


**Fig. 1.1:** Canonical bacterial quorum-sensing (QS) circuits. Autoinducing peptide (AIP) QS in gram-positive bacteria by (A) two-component signaling, or (B) an AIP-binding transcription factor. Small molecule QS in Gram-negative bacteria by (C) a LuxI/LuxR-type system, or (D) two-component signaling[10].

### 1.1.1 QS System in Gram-Negative Bacteria

The most common class of AIs that are found in Gram-negative bacteria are Acyl-homoserine lactones (AHLs). AHLs consist of an N-acylatedhomoserine-lactone ring as the core and a 4– 18 carbon acyl chain containing modifications[12, 13]. LuxI and luxMclass of enzymes are the major producers of AIs. Freely diffusible AHLs in the cytoplasm are detected by the LuxR type receptors. LuxR-AHL complexes bind to DNA while unbound LuxR proteins are degraded rapidly[1, 14]. Combined receptor protein complexes work as cytoplasmic transcription factors to regulate gene expression that affects biofilm formation, virulence, and other biological processes in bacteria. This happens in a cycle called the autoinduction process which in turn promotes synchronous gene expression in the bacterial population[15].

### 1.1.2 QS System in Gram-Positive Bacteria

In case of Gram+ bacteria autoinducers are oligopeptides (AIPs) which involve two kinds of canonical AIP-QS circuits. In one of these circuits, AIPs that are encoded as precursors from the QS operon are secreted outside the cell by special transporters. Amino acids in the AIPs can be linear or cyclized and lie in the range from 5 to 17[1, 16-18]. In the case of other canonical AIP-QS circuits, secretion and processing of AIPs are done by extracellular proteases such as neutral protease B (NprB). Secreted AIPs bind to transcription factors and regulate DNA expression through the oligopeptide permease system (Opp)[19, 20]. In addition, autoinduction triggered by the transcription of the QS operon synchronizes the QS response[19, 20].

### 1.1.3 Inter-Species QS System

New discoveries indicate that some molecules like AI-2 have the potential to enable inter-species communication[21] in bacteria. Three specific receptors: the 1st one LuxP which is found in Vibrionales[22], the 2nd type LsrB in Salmonella typhimurium, Bacillus cereus, and Escherichia coli (E. coli)[23] and the third receptor RbsB which exists in A. actinomycetemcomitans and Haemophilus influenza strain 86-028NP[24]; can recognize extracellular communication signal in bacteria kingdom: AI-2[25].

### 1.1.4 Biofilm Formation and Virulence Factors

Bacteria can exist by attaching themselves and grow upon living and inanimate surfaces such as medical devices, pathological tissues, heart valves, lung tissues, etc**.** The characteristic of this attached growth state is that the cells develop a biofilm which is necessary requirement for bacterial adhesion and growth[26, 27]. A number of studies have shown that bacterial quorum sensing (QS) signaling plays a key role in biofilm formation[27-29].

Bacteria produce virulence factors to evade host's immune response system which is crucial for the pathogenesis of infections[30-32]. Production of virulence is regulated by the bacterial QS signaling systems[33, 34]. Virulence factors differ widely across different strains. As for examples, Gram-negative Pseudomonas aeruginosa produces virulence factors, such as pyocyanin, elastase, lectin, and exotoxin A[35, 36] whereas Gram-positive *Staphylococcus*

*aureus* produces virulence factors such as fibronectin binding protein, hemolysin, protein A, lipase, and enterotoxin[37, 38].

- Quorum sensing is responsible for widespread microbial infections. Henceforth, an effective strategy is to be taken to disrupt bacterial QS circuits by the use of quorum sensing inhibition agents sensibly. This way one can control the production of bacterial virulence factors and biofilm formation. Novel therapeutic strategies can be developed by disrupting the bacterial QS system through a quorum sensing inhibition mechanism, avoiding harmful side effects of traditional therapy involving only antibiotics.

## 1.2 Quorum Sensing Inhibition

Antibiotics which saved human life from many life threatening diseases were discovered in the beginning of 20th century[39]. Within a century of this remarkable discovery, excessive and unscientific usage of this life saving drug has led to the beginning of multiple drug resistant (MDR) bacterial strains[40]. One of the major global public health concerns of twenty-first century is due to this antimicrobial resistance (AMR)[41-43]. A recent study showed that by 2050 human death toll would be 10 million, unless a global step towards the problem of AMR was wisely taken[44]. Treatment based on quorum sensing inhibition technique may show the right pathway to alternative approach of modern therapy with no side effects.

QS system can be retarded in a number of ways, out of which following four methods are widely followed[41, 45-49]: (i) suppressing of the production of signal molecules; (ii) degrading of signal molecules by using proper enzyme; (iii) restricting signal molecules in binding to receptor sites; and (iv) blocking the binding of signal molecules to gene promoters and thereby inhibiting gene expression. Generally method (ii) where enzyme inactivate QS signals, is known as quorum quenching (QQ) and other methods (i, iii, iv), where chemicals interrupt QS pathways and weaken the expression of QS-controlled genes are termed as quorum sensing inhibitors (QSIs)[50, 51]. QSIs are of two types: synthetic or natural. Natural QSIs are found from terrestrial, marine, or freshwater ecosystems[52].

### 1.2.1 Examples of QS Inhibitors

#### 1.2.1.1 Natural QSI

A large no of organisms are hosts of QS bacteria. Their coexistence for many years in natural ecosystem capable them to develop a mechanism to inhibit QS to reduce colonization and pathogenic activity[53]. QSIs can be extracted from the following natural resources.

**A. Prokaryotic QSI**

Many organisms produce various types QQ enzymes. There are 4 types of enzymes which cause the degradation of QS signals. AHLs that hydrolyse lactone ring are AHL-lactonase, and decarboxylase and AHL that cleaves the acyl side chain are AHL-acylase and deaminase. Enzymes and their corresponding source are show in T**able 1.1**[54].

**Table 1.1:** Bacterial enzymes as quorum sensing inhibitors[54].

| Source of quorum sensing inhibitor | Enzyme | Degraded quorum sensing signal |
|---|---|---|
| Acinetobacter sp. strain C1010 | Lactonase | AHLs |
| Agrobacterium tumefaciens | AHL-Lactonase | AHLs |
| Arthrobacter sp. IBN110 | AHL-Lactonase | AHLs |
| Alteromonas sp. strain 168 | Acylase | C4HSL and 3OC12-HSL |
| Bacillus sp.strain 240B1 | Lactonase | AHLs |
| Bacillus thuringiensis | Lactonase | AHLs |
| Burkholderia strain GG4 | AHL — oxidoreductase | 3OC6HSL |
| Bacillus megaterium | AHL-oxidase | C4HSL and 3OC12HSL |
| Bacillus circulans strain 24 | Different from Lactonase | C4HSL and 3OC12HSL |
| Bacillus pumilus S8-07 | AHL-acylase | 3OC12HSL |
| Halomonas sp. strain 33 | Lactonase | AHLs |
| Hyphamonas sp. DG895 | Acylase/Lactonase | C4HSL and 3OC12-HSL |
| Oceanobacillus strains 30, 172, and 97-2 | Lactonase | AHLs |
| Pseudomonas aeruginosa PAO1 | AHL-acylase | Long chain AHLs |
| Ralstonia sp. XJ12B | AHL-acylase | Long chain AHLs |
| Stappiaa sp. strains 5, 176 and 97-1 | Lactonase | AHLs |
| Tenacibaculum discolor strain 20J | Acylase/Lactonase | AHLs |

### B. Animal Based QSI

Mice, rat and zebra fish are three important sources of QQ enzymes. Acylase is effective to regulate the formation of biofilm in Pseudomonas putida and A. hydrophila[55]. QS signal-3OC12HSL is found inactivated in serum of many mammals like mouse, goat, bovine horse, and rabbit[56]. Home-made cheeses, beef steak and fatty acids derived from poultry meat have strong inhibitory effect[57].

### C. Plant Based QSI

Plant extract like pyrogallol from Emblicaofficinalis[58], GABA from tumefaciens[59], curcumin from Curcuma longa[60], capable of degrading signal receptor (LasR/ LuxR) can act as QSI[61, 62]. Flavonoids obtained from different plant parts like leaves, flower, fruit and bark of Combretam albiflorum, Laurusnobilis, and Sonchusoleraceus play the role as anti-oxidant, anti-inflammatory, and anticancer agents. Flavonoid, flavan-3-ol catechin, found from the bark of Combretum albiflorum decreases the production of QS-controlled virulence factors pyocyanin, elastase and biofilm formation by P. aeruginosa[63]. Grape and garlic are rich source of QSIs. Biofilm formation by E. coli is resisted by grape-fruit juice (Furocoumarins)[64] and in case of P. aeruginosa it is restricted by rosmarinic acid found in the roots of Ocimumbasilicum (Sweet Basil)[65]. Some potent QSIs of the viable plant extracts are listed in **Table 1.2**[54].

**Table 1.2:** QSI potential of the tested plant extracts[54].

| Plant | Part used | Anti-QS zone (mm) | Anti- QS potential |
|---|---|---|---|
| *Anethumgraveolens* | Fruits | - | - |
| *Cucumismelo* | Seeds | - | - |
| *Carumcarvi* | Fruits | - | - |
| *Citrus sinensis* | Seeds | 20 | ++ |
| *Pimpinellaanisum* | Fruits | - | - |
| *Foeniculumvulgrae* | Fruits | - | - |
| *Trigonellafoenumgraecum* | Seeds | - | - |
| *Coriandrumsativum* | Fruits | 10 | ++ |
| *Laurusnobilis* | Leaves | 10 | ++ |
| *Psidiumguajava* | Leaves | 3 | + |
| *Allium cepa* | Outer scales | 20 | ++ |
| *Eugina aromatic* | Flowers | - | - |
| *Menthalongifolia* | Aerial part | 5 | + |
| *Elettariacardamomum* | Seeds | 10 | ++ |
| *Senna italic* | Aerial part | - | - |
| *Valantiahispida* | Aerial part | - | - |
| *Tephrosiapurpurea* | Aerial part | - | - |
| *Teucriumpolium* | Aerial part | - | - |
| *Commophoramolmol* | Bark | - | - |
| *Tribulusarabicus* | Aerial part | - | - |
| *Allium sativa* (positive control) | Bulbs | 10 | ++ |

+: Moderate antiquorum sensing activity ++: Potent antiquorum sensing activity

### D. Marine Organism Based QSI

A lot of studies indicate that marine organisms are potent source of QSIs. Furanone, produced by Deliseapulchra can inhibit QS aided activity in bacteria[66-69 70]. Bromoperoxidase enzymes perform like QSI to limit the signal of AHL (3OC6HSL) by oxidation[71]. In P. aeruginosa, 8 - epi-malygamide and Malyngamide C inhibit the QS activity[72]. Oroidin, a pyrrole-2-aminoimidazole alkaloid, derived from the marine sponge Agelasoroides, suppresses the ATPase activity of the Heat shock protein 90 (Hsp90)[73].

### E. Fungus Based QSI

Fungi produce secondary metabolites similar to antibiotic. Penicillin extracted from Penicillium spp. has most successful history to curb bacterial infections[39] since its century old discovery. 33 type of Penicillium spp produce patulin and penicillin as QSIs[74]. Through lactonases activity, rhizosphere plant and fungi together degrade association of C6HSL and 3OC6HSL[75]. Auricularia auricular, a natural pigment, regulates production of violacein in C. viola[76].

### F. Antibody Based QSI

Among eukaryotes, the immune systems of mammals produce antibodies with QQ characteristics in response to allergens[61]. Out of a vast no of antibodies, the most effective one, secreted by P. aeruginosa, is XYD-11G2 which is quite effective in quenching 3OC12HSL[77]. In many cases, AHL-antibody prevents cell death in primary bone marrow derived from murine macrophages[78].

#### 1.2.1.2 Synthetic QSIs

Gram-negative bacteria mostly use AHL as QS-signal. Depending upon their structural features, analogues of AHL may act as antagonist or agonist to receptor protein and can be used as effective QSI. In 2005, Persson et al. successfully introduced sulphur in the acyl side chain in place of C-3 atom to develope AHL analogue that was able to inhibit expression in both LuxR and LasR controlled QS reporters. LuxR-regulated 3-oxo-C6 HSL also shows inhibiting property when substituted by the aryl group at the end of the side chain. Aryl compounds binds to AHL receptor and together they stop the receptor from producing response[79]. Antagonist aryl-AHL complex becomes very effective QSI if C-1 carbonyl group of the side chain is replaced by a sulphonyl group[80]. AHL analogues function as a agonist when extra moieties are present on the C-3 ring[81]. Usually AHLs are modified by following three procedures: (1) substitution in the acyl side chain keeping the lactone ring unchanged, (2) substitution in the lactone ring leaving the acyl side chain unchanged and finally (3) extensive modifications of both acyl side chain and lactone ring[53]. **Table 1.3** displays some major synthetic QS-inhibitors[82, 83].

**Table 1.3:** Synthetic QS inhibitors[82, 83].

| No. | Synthetic compound | QS inhibition |
|---|---|---|
| 1 | *N*-(4-bromo-phenylacetanoyl)-l-HSL; *N*-(indole-3-butanoyl)-L-HSL (AHLs) | AHLs antagonist (*las* QS system) |
| 2 | 3-oxo-C12-cyclohexanone (AHLs) | AHLs antagonist (*las* QS system) |
| 3 | C10-cyclopentylamide (AHLs) | *las* and *rhl* QS system |
| 4 | Furanone C-30 and C-56 (Furanone) | *las* and *rhl* QS systems |
| 5 | *S*-phenyl-L-cysteine sulfoxide (Cysteine sulfoxide alliin) | *las, rhl* QS systems |
| 6 | Diphenyl disulfide (Disulfide derivatives of the alliinase mediated reactions) | *las* QS system |
| 7 | Azithromycin (Erythromycin) | *gacA, las* and *rhl* QS systems |
| 8 | Biaryl hydroxyketones F1, F12 and F19 | *agr QS system* |
| 9 | Savirin | *agr QS system* |
| 10 | Oxacillin | *agr QS system* |

### 1.2.2 QS Inhibition Mechanism

In most of the QSI studies, usually four methods are followed as working principle. a) QS signals degradation, (b) Biosynthesis of inhibited QS signal, (c) Detected QS signal inhibition, and (d) Antibiotics as QS inhibitors[50] (**Fig. 1.2**).

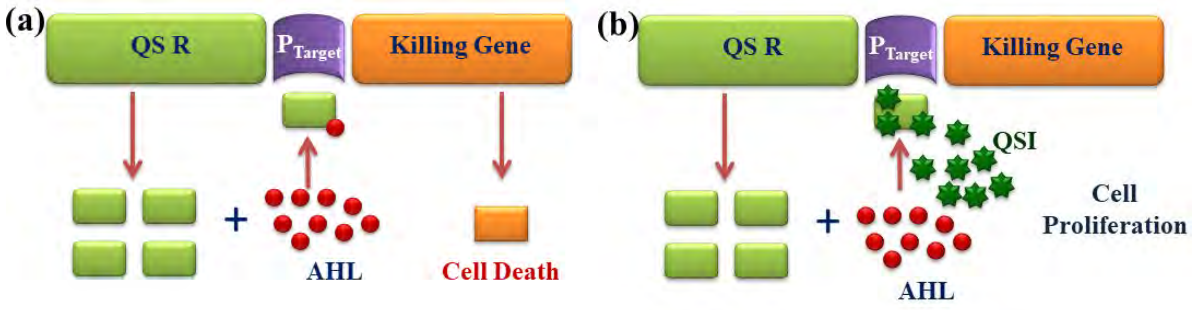


**Fig. 1.2: Layout of the QSIs screening systems:** (a) An AHL receptor/response regulator is activated by exogenously supplied AHL. The activated LuxR homologue (QS R) upregulates expression from a QS-regulated promoter (P Target) which controls expression of a gene (killing gene) encoding a toxic protein leading to growth arrest or cell death. (b) If a QSI compound is present, reception of the AHL signal is blocked and expression of the killing gene is prevented, allowing for growth of the screening bacterium[54].

**a. QS Signals Degradation:** Degradation of QS signal may be realised by enzymatically or non-enzymatically. AHL-lactonase, AHL-acylase and AHL-oxidoreductase are three major types of enzyme used for degradation of AHL[50, 84]. Catalytic antibodies are also capable of hydrolysing AHL signal, like lactonase enzyme [85].

**b. Biosynthesis of Inhibited QS Signal:** Theoretically, production of AHL may be suppressed by anyone of the mechanism: obstruction of acyl-ACP generation, hampering of

SAM biosynthesis, or inactivation of synthase enzyme[50]. Inhibition of enzyme FabI may prevent biosynthesis of acyl-ACP, thereby production of AHL[50].

**c. Detected QS Signal Inhibition:** QS signal inhibition can be achieved if binding site of LasR becomes non-productive[50]. Itc-11 and Itc-12 are two such isothiocyanate-based probes that covalently modify the nucleophilic cysteine containing ligand-binding pocket of LasR, and thereby prevent QS[86].

**d. Antibiotics as QS Inhibitors:** Some species are found to secret target oriented antibodies which reduce the expression of QS[50]**.** In case of *P. aeruginosa*, elastase, proteases, DNase, leukocidin, and phospholipase C inhibit QS without affecting the growth of the bacteria[50, 87, 88].

- QS regulation has vast area of application. Besides healthcare system, QSI technique may be applied in industrial membrane bioreactors for waste water treatment, biotransformation in biorefineries, aqua culture and crop production[89, 90]. On the other hand, QS-disruption strategy faces challenge if bacterial species exhibit multiple QS pathways. For example the two AHL-QS systems in P. aeruginosa, aid them to survive in changing environmental stress condition[91, 92]. In such cases combination effect of QQE and QS proved to be fruitful[93]. Primary objective of our research is to investigate some potent natural QSIs targeting different components of the QS and their applications in medical fields. The aim of this study is to explore a novel technique to regulate bacterial infections without stressing the bacterial cells and reducing the role of antibody development.

## 1.3 Quorum-Sensing Regulation in *Staphylococcus aureus*

*Staphylococcus aureus* (*S. aureus* ), a member of the genus Staphylococcus, usually colonize human epithelia and mucous membranes[94]. It is a gram positive opportunistic pathogen which causes plethora of infections in humans, mostly skin and nosocomial infections[95, 96]. Methicillin-resistant *S. aureus* does not develop diseases without penetrating the epithelial barrier of the skin or mucosal surfaces but as it gets inside the human body causes severe infections that range from abscesses and furuncles to scalded skin syndrome, sepsis, and toxic shock syndrome through wide variety of virulence factors including exotoxins, superantigens, exfoliative toxins, hemolysins, Panton–Valentine leukocidin and shock syndrome toxin[97, 98]. Moreover, *S.aureus* is also accountable for community-acquired (especially from healthcare institutions and medical equipment) infections and becomes more challenging when the bacterium achieves the ability to resist multiple antibiotics[99, 100]. Modern studies of drug research showed that inhibition of virulence factors and biofilm formation would be more beneficial to control the spreading of bacterial infections rather than killing of the bacterium itself.

Among the regulatory mechanisms quorum sensing is one of the most studied and outstanding mechanism to control pathogenesis. Quorum-sensing is population dependent gene regulated cell-cell communication that improves the regulation of numerous colonization and virulence factors of bacteria[11]. There exist two regulatory systems in staphylococci, the accessory gene regulator (*agr*) system and the LuxS system. The *agr* QS system exploits oligopeptides as signaling molecules to regulate expression of a series of toxins and virulence factors and their interaction with the innate immune system[18]. Furthermore, *agr*-QS system reduces the expression of several cell surface proteins and enhances the expression of many secreted virulence factor during transition[101, 102]. Different *agr* groups are expressed in different diseases including murine subcutaneous abscesses[103], arthritis[104], rabbit endocarditis[105], and apoptosis of epithelial cells[106]. Two primary transcripts RNAII and RNAIII, are generated by the *agr* locus and originated from the P2 and P3 promoters, respectively[107]. The RNAII locus contains four genes, *agrB, agrD, agrC and agrA encoded by* the P2 operon whereas operon P3 activates *agr*-mechanism and increases the levels of intracellular RNAIII that increases the transcription of numerous secreted virulence factors[2] (**Fig. 1.3**).

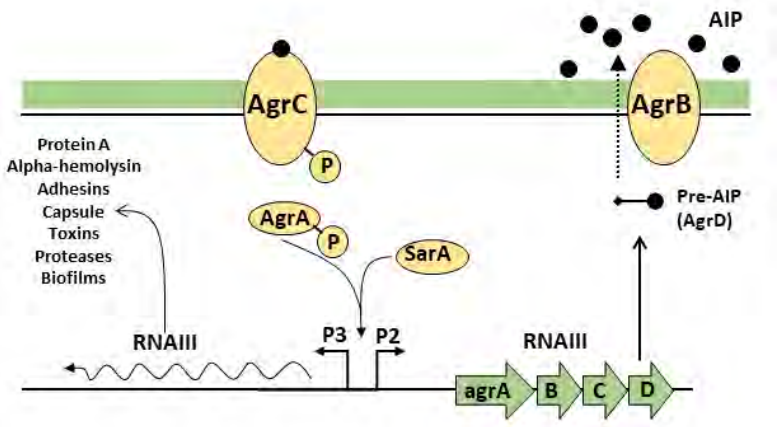


**Fig. 1.3**: The accessory gene regulator (*agr*) quorum sensing system of *S. aureus.*

Nowadays, biofilm formation has been recognised as one of the most significant virulence determinants of several bacterial pathogens[108]. Recent studies estimated that 60% of all microbial infections arise due to biofilms[109]. It appears that biofilm-associated *S. aureus* involves in many diseases, including endocarditis, osteomyelitis, and foreign-body related infections[110], and shows innate resistance to host defences, disinfectants and antibiotics[111, 112]. Biofilm formation comprises mainly three stages: (1) attachment of microorganisms to abiotic or host matrix protein-coated surface, (2) proliferation/maturation, and (3) detachment/dispersal[113]. A divergent relation exists between *agr* expression and *S. aureus* biofilms and it depends upon conditions under which the biofilm is grown. Expression of *agr* is necessary for biofilm configuring and the spreading of biofilm related infections on contrary, in some cases repression of *agr* stimulates extensive biofilm formation, which may

be beneficial for the bacteria to survive[114, 115]. Biofilm formation and antibiotic susceptibility also connected to AI-2 regulation in *S.*aureus[116, 117]. The conserved *luxS*-QS system employs an autoinducer AI-2, as signaling molecules and modulates capsular polysaccharide synthesis and virulences in *S. aureus.* The *luxS* gene impact the expression of a number of genes, including biofilm exopolysaccharide biosynthesis[118] and it is found that *S. aureus luxS* strain regulates biofilm growth through the *icaR* locus[116].

Many plant-based bioactive compounds possess excellent inhibition properties against QS-associated virulence factors and biofilm formation. Compounds such as quercetin, catechin, rosmarinic acid, limonoid, ichangin, apigenin, kaempferol, and naringenin exhibit a role against biofilm-associated infections[119] and can be used to develop antipathogenic drugs and reduce the development of antibiotic resistance. The phytochemical hamamelitannin (20,5-di-O-galloyl-D-hamamelose) (**Fig. 1.4**) extracted from American witch hazel, called Hamamelis virginiana is believed to target *agr* system of *S. aureus* and could be used as antimicrobial agents to counter methicillin-resistant *Staphylococcus aureus* (MRSA) infections[120]. Hamamelitannin (HAM), a non-peptide analogue of RNAIII inhibiting protein (RIP), may be used to inhibit RNAIII activating protein RAP/TRAP[121-123] and restrain biofilm formation[124]. In our research work, using computational method fourteen best derived compounds of HAM has been designed and could be considered for the development of anti-staphylococcal drugs.

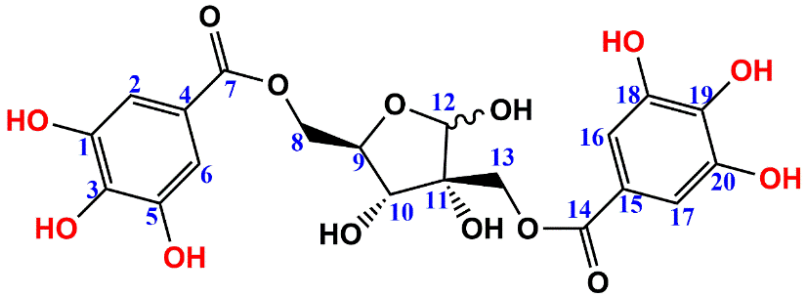


**Fig. 1.4:** Structure of hamamelitannin.

## 1.4 Heat Shock Protein (HSP)

Heat shock proteins (HSP) are a group of proteins released by cells under stressful condition such as heat, cold, UV light, wound healing or tissue modeling, infection, inflammation etc[125-128]. They were first discovered in cells exposed to high temperature and being present in those cells they protect them from severe heat stress. HSP can initiate the production of a small number of proteins and on the other hand restricts the production of other proteins. Due to its wide presence, ranging from bacteria to human and for its biological importance, nowadays HSPs are immensely studied. These proteins are categorized into five major families according to their molecular weight such that HSP100, HSP90, HSP70, HSP60 and small HSP (sHSP)[129].

Heat shock proteins are referred to as stress proteins as expression of HSPs are triggered by environmental stress condition and their upregulation are described as part of the stress response that leads to gene transcription, induced primarily by heat shock factor (HSF)[130, 131]. Many member proteins of HSP family behave as molecular chaperones, which play a regulatory role in the folding of proteins, intracellular transport of proteins in cytosol, endoplasmic reticulum and mitochondria; repair or degradation of proteins and refolding of misfolded proteins[132]. Heat shock proteins are also expressed under non-stressful condition. Their functions are to monitor the cell proteins such as carrying old protein to the recycle bin of cell i.e proteasome and help newly synthesised proteins in proper folding. Self-degradation occurs in HSPs due to slow proteolytic action on themselves[133].

HSP90 is one of the most studied proteins which contain 1-2% of total proteins of eukaryotic cells under non-stress condition and essential for cell survival. HSP90 controls gene expression, proliferation, cellular signal transduction and transcription networks of many proteins and stabilizes them under stress. On the other hand in many cases, HSP90 promotes cell homeostasis, misfolding and overexpression of client proteins which are responsible factors for the development of refractory diseases including cancer, inflammation, neuro-degeneration and viral infection[134, 135]. HSP90 client proteins play a key role in the development, proliferation and survival of several types of cancer. Both induction and inhibition of this chaperon have strategic importance in combating various diseases.

N-terminal domain containing ATP-binding site, the Bergerat-fold and recently discovered C-terminal domain of this chaperon shows us the new possibilities for the inhibition of this protein to target the tumor[136, 137]. Some natural products and their derivatives target HSP90-ATP binding site, thereby exhibit antitumor activity. Oroidin, a pyrrole-2- aminoimidazole alkaloid (**Fig. 1.5**), derived from a marine sponge *Agelas oroides*, is an example of small molecule that inhibits the actions of HSP90, could be a promising candidate for the treatment of various cancers including adenocarcinoma, pheochromocytoma, multiple myeloma, lymphoblastic leukaemia and lung cancer[138-141].

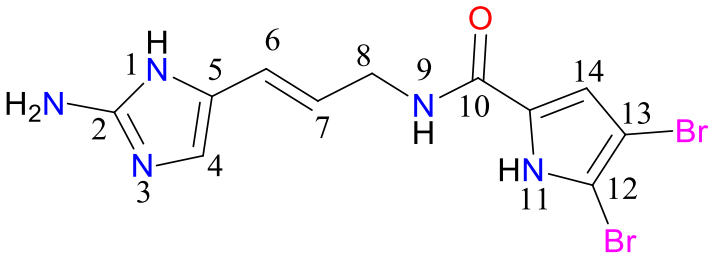


**Fig. 1.5:** Structure of oroidin.

## 1.5 Background of QSAR

Quantitative structure-activity relationship is a computational method that correlates structural or property descriptors of a compound with its activities. The QSAR modeling attempts to derive empirical models that relate chemical structure of the compounds to their biological activity.

In the year of 1863 A.F.A. Cros established a relationship between the toxicity of primary aliphatic alcohols and their water solubility[142]. Crum-Brown and Fraser (1968) was the first to introduce an idea that the biological activity of alkaloids (Φ) was a function of molecular constitution (C)[143]:

$$\Phi = f(C) \quad (1)$$

$$\text{and } \Delta\Phi = f(\Delta C) \quad (2)$$

The first equation served as the first general formulation of a Quantitative structure activity relationship. In the second relation ΔΦ are the differences in biological activity values and ΔC are their corresponding changes in the chemical and especially the physicochemical properties.

In the year of 1893, Richet established that toxicities of alcohols, ethers, and ketones were inversely related to their water solubility[144].

Louis Hammett studied reaction mechanisms involving two parameters, namely the (i) substituent constant and the (ii) reaction constant. In 1935 he put forward a linear relation that was expressed as:

$$\text{Log } K/Ko = \rho \text{ Log } K'/K'o = \rho\ \sigma \quad (3)$$

Ko and Ko' are equilibrium constants for unsubstituted compounds whereas K and K' stand for substituted compounds. Depending upon substituents the other two constants (ρ & σ) may be positive or negative[145, 146]. The above mechanism laid the basis of the development of the famous Hansch parabolic equation[147] which was expressed as:

Log 1/C = a (hydrophobic parameter) + b (electronic parameter) + c (steric parameter) + constant

Where C represents the molar concentration producing the biological effect; a, b and c are the regression coefficients. The success of these models led to remarkable development in QSAR analysis and related approaches.

In the same year, along with the Hans approach (1964), Free and Wilson established a structure activity-based methodology where biological activity was the sum of contributions from different substituents[148]:

$$BA = \Sigma a_i x_i + u \quad (4)$$

where BA expresses biological activity, u represents the average contribution of the parent molecule, and $a_i$ denotes the contribution of each structural feature; $x_i$ means the presence ($x_i = 1$) or absence ($x_i = 0$) of a particular structural fragment.

The use of quantum-mechanical descriptors in QSAR started in the early 1970s[149]. Balaban[150], Randić[151], Kier and Hall[152] developed QSAR approach based on topological indexes. The next decade saw the development of the 3D-QSAR, on the basis of geometrical aspect of molecular structures. Shadow indices[153], charged partial surface area descriptors[154], WHIM descriptors[155], gravitational indices[156], EVA descriptors[157], 3D-MoRSE descriptors[158], and GETAWAY descriptors[159] were some major Geometrical descriptors which were obtained from the 3D spatial coordinates of a molecule.

Cramer and Milne, in 1978, had suggested a new methodology to describe the molecular properties by aligning molecules in the space and by mapping their molecular field into a 3D grid[160]. After 10 years in 1998, Cramer proposed a new concept of three dimensional molecular parameters in the field of QSAR. The method was labelled as Comparative Molecular Field Analysis (CoMFA) and later was developed as 3D-QSAR[160]. A representative structural group, involved in 3D QSAR model is known as pharmacophore[161, 162]. Comparative Molecular Similarity Indices Analysis (CoMSIA)[162] or Self Organizing Molecular Field Analysis (SomFA)[163] are other major 3D-QSAr methods which involve comparisons of different sets of molecular descriptors.

Golbraikh et al. used a method involving two-dimensional (2D) molecular descriptors and k-nearest neighbours (kNN) QSAR method for the investigation of several datasets. The important conclusion of their study was the high value of LOO $q^2$ is necessary but not sufficient for the model to have a high predictive power. Although, they could not formulate any correlation between the values of $q^2$ for the training set and the predictive ability for the test set for any of the datasets and could not establish further validation by LOO as well as by random trials[164].

## 1.6 Phosphodiesterase-4 (PDE4)

Inflammation protects our body form infection, injury or other diseases and acts as a first line of defense. Inflammatory response is necessary for healing purpose but uncontrolled and unresolved acute inflammation results in chronic inflammatory diseases such as cancer, diabetes, CRDs and many more[165]. Chronic respiratory diseases (CRDs) usually affect airways and other parts of human respiratory system. The most common CRDs are asthma, pulmonary hypertension and occupational lung diseases which may develop cardiac malfunction[166]. CRDS are the side effects of massive modernization, smoke and air pollution. Nowadays millions of people are suffering from these diseases globally and the trend is increasing with alarming rate. Various inflammatory mediators and enzymes like chemokines which lower pulmonary function are released during CRDs[167, 168].

Cyclic adenosine monophosphate (cAMP), a cyclic nucleotide, is an important second messenger that regulates a vast number of physiological processes such that as gene transcription, cell migration, mitochondrial homeostasis, cell proliferation and cell death[169]. In addition, growing concentration of cAMP in the airway tissues and cells reduces the inflammatory responses and that is beneficial for treatment of asthma and chronic obstructive pulmonary diseases (COPD)[170]. Phosphodiesterase (PDEs) are key enzymes that are able to degrade cAMP. PDEs catalyze the decomposition of cAMP into AMP and as a result cAMP level inside the cell increases. PDE enzymes are superfamily of 11 PDE families (PDE1-PDE11)[171]. Among them only PDE4, PDE7 and PDE8 are cAMP-specific[172].

PDE4 enzymes hydrolyze cAMP and encode four distinct genes (PDE4A--PDE4D). These isoforms exhibit distinct and specific cell-type patterns of expression and distribution, and they play significant roles in regulating various physiological activities[172, 173]. PDE4A is reported to be highly expressed in brain, cardiovascular tissues, and small intestine cells[174, 175]. PDE4B and PDE4D are highly expressed in immune cells whereas expression of PDE4C is low in the lung tissues[176, 177]. All the isoforms of PDE4 family, hydrolyzes the second messenger and lowers the concentration of cAMP that suppresses inflammatory cell activity[178]. They are considered to be a therapeutically potent target in treatment of neurological, psychiatric disorder, respiratory and other inflammatory diseases including chronic obstructive pulmonary disorder (COPD)[179].

PDE4 inhibitors bind to active site of PDE4 enzyme and prevent cAMP degradation. The outcomes of this inhibition are growing concentration of cAMP, activation of PKA and then phosphorylation of the transcription factor cAMP-response element-binding protein (CREB).This cascading effect decreases the production of TNF-α and finally inhibits the pro-inflammatory properties of PDE4 enzyme. Inhibition of PDE4 has been attracting significant attention of pharmaceutical companies over the last two decades. Although most of the inhibitors show side effects like emesis and nausea, numerous PDE4 inhibitors are under or ready to start clinical trial for COPD treatment. Despite the failure in many cases, several small molecules have been approved by the market and patented over the last 10 years as PDE4 inhibitors[180, 181].

Roflumilast was the first to get approved in 2011 and next was the apremilast in 2014. Both are pan-inhibitors of PDE4A—4D and being used for the treatment of COPD[182, 183]. CHF 60001, most significant member of the different series of 1-phenyl-2-pyridinyl alkyl alcohols has promising future in asthma and COPD treatment[184]. Heterocyclic compounds, such as 7,8-dihydro-1,6-naphthyrydin-5(6H)-one derivatives, 5-substituted-1,4-benzodiazepines, benzo-fused heterocycles, triazolopyridazines, triazolopyridines, pyrimidinone derivatives, or biaryls are some potent inhibitors which can be used as alone or combining with other known drugs. It is reported that they are able to decrease TNF-α levels and also beneficial in the treatment of numerous diseases, such as asthma, COPD, rheumatoid arthritis, autoimmune pathology and neurological disorders as like depression, Parkinson's and Alzheimer's disease[185]. The 8-biarylnaphthyridinone called MK0952 has the ability to infiltrate into the blood brain barrier and is being applied for the treatment of Alzheimer's disease. In case of atopic dermatitis problem, boron-containing small molecule AN2728 that diminishes the production of TNF-α by suppressing PDE4, is proved to be fruitful.

The aim of our study is to develop subtype selective inhibitors that maximize the therapeutic benefits but produce no adverse side effects. Here we have followed cost effective computer aided drug design (CADD) method to predict the potent molecules. A QSAR model is developed to envisage the activity of a newly designed pyrazolo-oxazine, and imidazo-pyridazine derivatives (**Fig. 1.6**) as potent PDE4A inhibitors and to indicate the interaction between the inhibitor molecules and PDE4A protein.

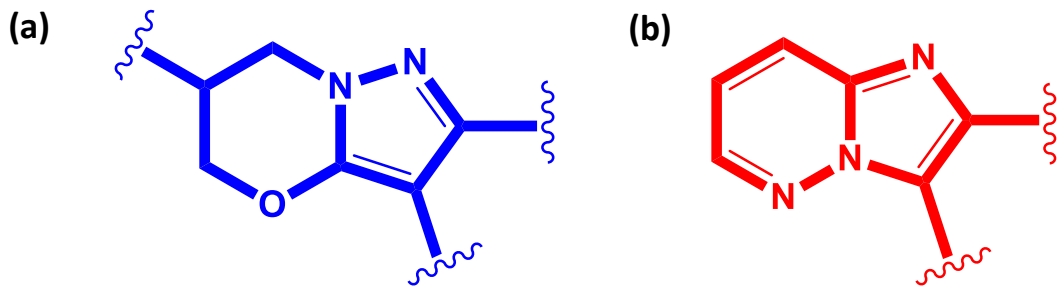


**Fig. 1.6:** 2D structures of (a) pyrazolo [5,1-b][1,3] oxazine, and (b) imidazo [1,2-b] pyridazine scaffolds.

## 1.7 Lysine-Specific Demthylase 1 (LSD1)

Histones ((H2A, H2B, H3, and H4)) are a family of basic proteins that unite with DNA in nucleus of eukaryotic cells and play a key role in chromatin regulation. DNA winds around histone, forming a structural unit called nucleosome which in turn wrapped into a condensed form known as chromatin[186, 187]. Histones help chromatins to pack together tightly into chromosomes and thereby protect DNA from tangling and damage. In addition, they have significant role in DNA replication and gene regulation[188]. Post translational modifications (PTM) of lysine or arginine residues of histones are epigenetic modifications that regulate chromatin structure without altering DNA sequence. Histone modifications work in diverse biological developments such as gene regulation, DNA repair, chromosome condensation (mitosis) and spermatogenesis (meiosis)[189-191].

The genetic irregularities that push tumorigenesis are usually initiated due to aberrant histone modifications including histone lysine methylations. Prior to discovery of histone lysine demethylase LSD1 (also known as KDM1A ) in 2004, it was thought to be an irreversible process and since then histone methylation has become a centre of attention in epigenetics and cancer research[192]. Histone methylations play a key role in regulation of gene expression and genetic stability, and obviously, dysregulation of this process may result in various cancers[193]. Methyl transferases and demethylases are two types of enzymes that are associated with histone methylation. Methyl transferases are responsible for inclusion of methyl groups, whereas, demethylases are able to remove methyl groups not only from histones but other proteins as well[194, 195]. On the basis of demethylation mechanism there exist two main classes of histone lysine demethylases that are: flavin adenine dinucleotide (FAD)-dependent amine oxidases and α-ketoglutarate-dependent hydroxylase. Demethylase proteins include array of domains that are accountable for histone recognition. Methylation of lysine residues of Histone H3 and H4 may initiate either transcriptional repression or activation depending on the methylation site and degree of methylation[195]. Lysin can be mono-, di-, tri-methylated with diversified functionality, e.g. di- and tri methylation at H3K4 is coupled with improved gene expression whereas methylation at H3K9, H3K27 and H4K20 is related with repression[196]. There exist two histone demethylases families: amino oxidase homolog lysine demethylase 1 (KDM1: KDM1A and KDM2A) and JmjC domain-containing histone demethylases (KDM2–KDM8)[197, 198]. Number of studies reported that these enzymes and their homologue are overexpressed in various cancer cells. Lysine-specific demethylase (LSD1) may initiate undesired gene expressions in various diseases including tumorigenesis [199], stem cell biology [200], neurodegenerative disorders [201, 202], viral infection [203-205], diabetes [206], and fibrosis [207]. According to many studies abnormal expression of LSD1 is related to the development of malignant tumours, including prostate, gastric, breast, lung, and blood cancers. Recent studies confirmed that small molecule-LSD1 inhibitors can prevent cell proliferation, malignant transformation, and the epithelial-mesenchymal transition (EMT) pathway in tumour cells[208] and effectively inhibit cancer cell differentiation, proliferation, invasion, and metastasis, as well as tumour formation, in several animal studies [209-211]. Demethylation of K370me2 by LSD1 blocks the interaction of p53 with p53 binding protein 1 (53BP1), which silences the gene p53 and initiates apoptosis[212]. KDM1A prevents

differentiation process in MLL-AF9 leukemia and inhibition of it reactivates the differentiaon pathway in acute myeloid leukemia[213, 214]. LSD1 decreases the expression of gene p21 that leads tumorigenesis. Because of its diverse biological roles, LSD1 inhibitors are considered as promising anticancer agents[215]. In recent years several types of LSD1 inhibitors are reported in different scientific journals and exhibited excellent efficacy in clinical trials till date. Tranylcypromine, containing cyclopropyl group, is the most significant irreversible LSD1 inhibitor[216, 217]. IMG-7289, GSK-2879552, and ORY-1001 are three LSD1 inhibitors undergoing clinical trials[218]. TCPA-based inhibitor, ORY-1001 is undergoing phase II clinical trials for handling acute myelogenous leukemia (AML) and the inhibitor GSK2879552 (**Fig. 1.7**) is taken into the first phase of clinical trials for treatments involving lung cancer (SCLC) [219, 220]. Following QSAR study, our aim is to provide a direction in the development of novel LSD1 inhibitors as potent anticancer drugs.

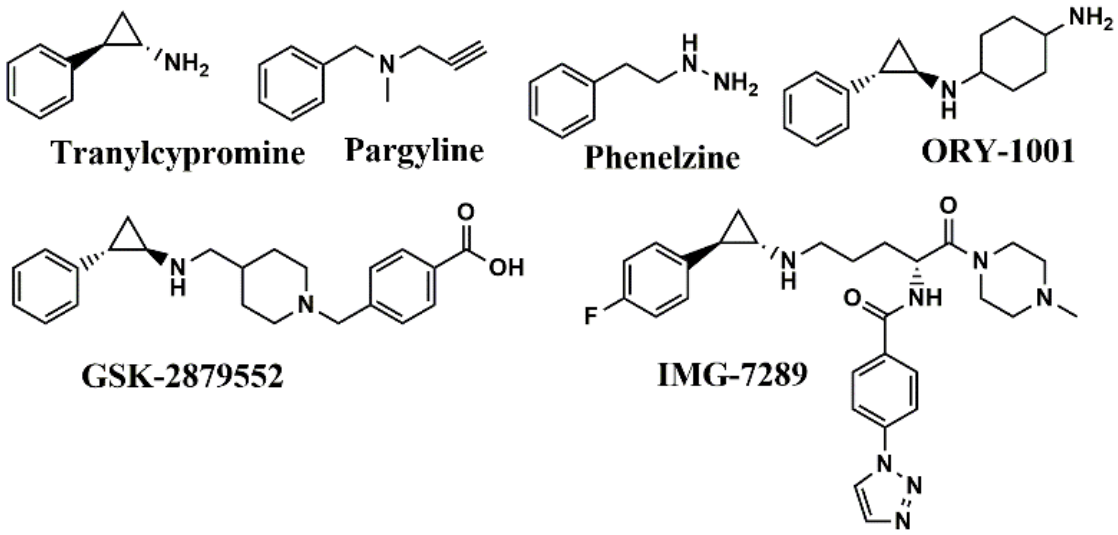


**Fig. 1.7:** Structures of several LSD1 inhibitors.

## 1.8 Aims and Objectives

The primary goal of this study is to select the potent natural compounds and design derivatives of them as novel inhibitors for different key human proteins including Aurora kinases, Heat Shock protein, DNA binding protein (PDB ID: 4AE5, 4G4K and 2FNP), Phosphodiesterase-4 (PDE4) and. Lysine-specific demethylase (LSD1). Owing to have less or minimum side effects and risk of toxicities, natural compounds and their derivatives are identified as QS inhibitors which may lead to the discovery of promising alternatives of unsafe traditional antibiotic therapy.

The detailed objectives are discussed below:

1. To design a library of derived compounds of selected natural inhibitors (*Rosmarinic acid, Oroidin, Hamimalitinin, LSD1 & PDE4*).
2. Geometric optimization of the molecules by performing DFT analysis.
3. Screening of natural QS inhibitors, etc. and their derivatives through docking and molecular dynamics simulation studies.
4. To study ADMET (absorption, distribution, metabolism, excretion and toxicity) properties of the lead compounds.
5. To evaluate the quantum mechanical properties of the inhibitors through DFT analysis.
6. 2D-QSAR study to obtain the best model equation by which one can easily predict IC50 value of newly designed molecules.

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