Awwa, Monaf

Research Strategy

Although mankind has made massive efforts in diminishing the mortal danger of microbial organisms, the existing strategies devised are becoming obsolete due to antibiotic resistance[1]. This is not surprising in light of natural selection, which favors pathogens that can overcome the intense selection pressure of bactericidal agents such as penicillin. Nevertheless, the phenomenon of antibiotic resistance has created an urgent need for new drugs to combat infectious organisms such as *M. tuberculosis* and *Pseudomonas Aeruginosa[2]*. The death toll from these organisms will only rise if no new antibacterial agents are designed. A highly promising novel drug target in *Pseudomonas Aeruginosa* is the quorum sensing gene regulatory network, which activates transcription of particular operons based on the cell density of *P. Aeruginosa*. In order to develop novel, non-toxic inhibitors, computational methods can be applied to screen for compounds with desirable properties [3] [4]. We hypothesize that if an antagonist to the *LasR* transcriptional factor can be developed, the antagonist will act synergistically with traditional antibiotics to improve patient outcomes via biofilm dispersal. To this end, the current project has three main goals.

Aim 1. Identify non-toxic antagonists of quorum sensing transcriptional factor LasR of *Pseudomonas Aeruginosa*.

Preventing and dissolving biofilms are important steps in maximizing the effectiveness of traditional antibiotics. The objective of the aim is to perform an *in silico* virtual screen of a diverse array of drug libraries, in particular libraries with easily modifiable functional groups , via the ZINC database[5] to find compounds predicted to work as inhibitors of *LasR*. This objective will be accomplished using the program UCSF DOCK version 6.6 to evaluate the electrostatic and van der Waal binding energy between the drug library and the crystal structure of *LasR* (PDB ID:2UV0[6]) [7]. The footprint scoring and pharmacophore scoring functions of UCSF DOCK 6.6 will be applied to refine the top 20 scoring compounds to help select compounds with similar binding characteristics as the natural agonist. Only compounds with drug like characteristics and easily modifiable functional groups will be selected for *in vitro* and *in vivo* assays.

Aim 2. Perform *In vitro* assay of Las operon expression via real time quantitative PCR

The las operon has a positive autoregulatory network motif. This means messenger RNA levels of LasR, a transcriptional factor, are proportional to ligand binding and dimerization of LasR previously expressed. In order to observe the effect of inhibitor concentration on LasR expression, an *in vitro* quantitative PCR experiment will be performed[8]. The experiment will be controlled for native agonist by knocking out the LasI gene from the Las operon. LasI, the synthase of the LasR agonist, will nullify the cell-density dependent expression of LasR. Previous work has demonstrated LasI is necessary for quorum sensing[9]. Thus the only LasR ligands will be controlled for. The *P. Aeruginosa* strain PAO1 is commercially available provided adequate facilities exist for managing the pathogen and recombinant strains can be engineered via CRISPR technology advances. The experimental design is based on varying concentrations of the native agonist against various concentration of lead compounds. Performing real-time quantitative PCR on the *in vitro* bacterial cells will yield estimates of how effective the inhibitors are as competitive inhibitors of LasR against its native agonist since LasR mRNA levels are proportional to its capacity to act as a promoter of its own expression. The goal of the current aim is to apply novel advances in quantitative PCR to track the shifts in LasR mRNA expression levels across varying concentrations of native agonist and lead compounds elucidated via UCSF DOCK 6.6. *[10] [11]*

Aim 3. Conduct biofilm assays on *P. Aeruginosa* to determine anti-quorum sensing agents

A reliable proxy of anti-quorum sensing effects of a molecule can be extrapolated from a biofilm assay. When *P. Aeruginosa* normally grow in a petri dish with adequate nutrients, they will construct a biofilm in regions of high cell density. However, addition of anti-quorum sensing agents will inhibit the production of biofilm. Although this is not a quantitative experiment, it is a validated qualitative experiment based on the connection between quorum sensing regulation and small molecules. The *P. Aeruginosa* strain PAO1 is commercially available provided adequate facilities exist for managing the pathogen. Its genome has been fully sequenced,

enabling a robust insight into the relation between mRNA expression levels via GeneChip^{tm} analysis. The other requirements for a biofilm assay such as the buffers, salts, and nutrient media are also commercially available through vendors such as Sigma-Aldrich. Concentration of molecules will be varied across several orders of magnitude to provide a rough idea of the IC_{50} value of each molecule. The negative control will be an assay with no predicted inhibitor, and the positive control will be an assay with cinnamaldehyde, a known inhibitor.

The seemingly robust system of quorum sensing provides several innovative antimicrobial drug targets due to the reliance on chemical messengers, or more precisely hormones, for quorum sensing activation [12]. The structures of chemical messengers differ between bacteria, however *P. Aeruginosa* uses two distinct chemical messengers in order to control multiple quorum sensing operons; acylhomoserine lactones and 4-quinolones [12, 13]. Genes controlled by the quorum sensing hierarchy include biofilm and exotoxin production, which are known to greatly complicate infections *in vivo*[14] [15]. This system is a clever evolutionary design because *P. Aeruginosa* can operate as a commensal bacterium and avoid immune responses when isolated, yet also take advantage of immunocompromised individuals such as AIDS patients or cystic fibrosis sufferers and grow exponentially[16]

An operon of great interest for drug design is the Las operon, which directly controls proteins involved in biofilm assembly and exotoxin production [6]. The operon serves as a hub of network regulation for virulence factors, demonstrated visually by figure 2 below. LasR directly upregulates Rhl, which encodes for rhamnolipid synthase genes rhlA and rhlB. Rhamnolipids form the bulk material of a biofilm, thus it is not surprising that both rhl knockouts and las knockouts each lack biofilm production[17]. The Pqs operon, which stands for *Pseudomonas* quinolone signal, encodes genes for synthesis of phenazines which function to cause oxidative stress to host tissue. The toxic nature of the signal molecule is very interesting, and the Pqs Regulatory (PqsR) transcription factor has also been proposed as a valuable drug target for quorums sensing inhibition.

The Las operon encodes for two proteins which are validated drug targets; the acylhomoserine lactone synthase, designated LasI, and the regulatory DNA binding protein designated LasR [18]. One previously explored method of interfering with quorum sensing is by inhibiting the acylhomoserine lactone synthase [18]. This method has been explored with some success. It is based on the lack of quorum sensing in LasI *P. Aeruginosa* knockout strains. Experiments on mouse models of *Pseudomonas Aeruginosa* pulmonary infection showed outcomes were greatly improved when antibiotics were used in conjunction with quorum sensing inhibitors[19]. However, mathematical models of the effect of quorum sensing inhibitors show that, for a vigorous therapeutic effect, inhibitors at both loci of quorum sensing regulation, LasI and LasR, are required[20]. Therefore, an inhibitor of LasR is almost certainly required for effective therapy.

LasR requires acylhomoserine lactones to bind to a ligand site to dimerize, thereby allowing LasR to bind to DNA at promoter sequences. Inhibitors of LasR dimerization have been known for at least a decade, with one of the first discovered being a brominated furanone very similar in structure to the LasR agonist, depicted below[19]. The mechanism of inhibition is rationalized by a conformational shift preventing the dimerization of LasR and alignment of the DNA binding domain. By leaving the LasR monomers in inactive conformational arrangements, the expression of las operon will be strongly inhibited.

The LasR transcription factor was crystallized with its autoinducer and characterized via X-ray crystallography in 2007 by Bottomley et al. The las operon is part of a larger regulatory network of over 350 genes which function to deal with both environmental stressors such as low iron or phosphate concentration, and efflux of virulence factors[21]. The PqsR transcription factor was crystallized in 2011 and has also been subject to inhibitor design. The purpose of targeting these proteins is to shift the phenotype of *P. Aeruginosa* to abolish pathogenicity, however the current proposal is only focused on design of a LasR inhibitor.

PCR is a technique that has long been in use in chemical biology, however recent technological advances have exponentially augmented the precision of instruments. The current proposal aims to take advantage of these improvements to observe how the LasR transcriptional factor mRNA varies with inhibitor and native agonist concentrations. Since LasR upregulates its own expression, mRNA levels will be directly proportional to native agonist binding. If an inhibitor has higher affinity or a high activation energy of binding, LasR will not be able to dimerize, and thus not produce mRNA encoding more LasR. Quantitative PCR (qPCR)

is thus a powerful tool, however there are special considerations. Since LasR can bind to promoter sequences other than the one for the Las operon, the relation between mRNA and inhibition may not be directly proportional. The second aim will thus also highlight the preference of LasR binding to the Las operon promoter versus all other promoter regions. This relation can be quantified based on qPCR mRNA of other genes known to be upregulated by LasR. This method may even elucidate new virulence genes. Vendors for qPCR instruments include bio-equip.cn which provide reagents for running the reaction. If the experiment matches the theoretical background, it will undoubtedly demonstrate interesting relationships between promoters and gene expression.

Biofilm production is a major complication in *P. Aeruginosa* infection, causing persistent infections that have augmented resistance to antibiotics[22] [23]. Biofilms also generate a physical barrier between the pathogen and the immune response, allowing the bacteria to flourish regardless of host defenses[24]. This is key since experimental evidence of mammalian epithelial cells strongly supports the idea that host immune responses disrupt the quorum sensing response by metabolizing chemical messengers [25]. Thus, inhibiting quorum sensing is an extension of a natural mechanism of preventing pathogenicity of *P. Aeruginosa*. Quorum sensing genes control production of toxic immune evasion molecules such as phenazines, rationalizing the selective pressure for innate defenses [26]. These features support the notion that the las operon holds the key to developing effective quorum sensing inhibitors[27].

There are several other natural quorum sensing inhibitors such as cinnamaldehyde and zingerone [11] [28]. Diketopiperazines have also emerged as quorum sensing inhibitors, however it is strongly suggested that they function as analogues to the 4-quinolone signal molecule of the pqs operon so their mechanism of action is likely to be a different target [29]. Figure 4 visually represents the backbone of diketopiperazines. Another avenue of inhibitor design is via analogues of the native agonist, such as the compounds synthesized by O'Loughlin et al. The scaffolds all have phenyl groups similarly to the natural product inhibitors, and the lactone ring and amide group are conserved with the natural agonist for specificity.

In vitro experiments of quorum sensing inhibitors consistently demonstrate biofilm dispersal and increased susceptibility to antibiotics. However, most inhibitors elucidated so far have serious drawbacks including cytotoxicity and a lack of understanding of the precise mechanism of action[17]. Specificity is also a major issue, since most natural products have affinity for a diverse array of proteins in both eukaryotes and prokaryotes. It is even plausible that inhibiting quorum sensing at the level of the Las operon does not mean that other operons will not compensate in virulence phenotypes. It has been demonstrated that LasR mutants only delay the activation of the Pqs operon rather than totally abolishing its transcription[13]. This leads to another key caveat; it is likely that over time resistance to anti-quorum sensing agents will arise even if it does not affect growth of *P. Aeruginosa*. Therefore, drug discovery projects should focus on inhibitors with the capacity for medicinal modifications akin to the evolution of penicillin derivatives.

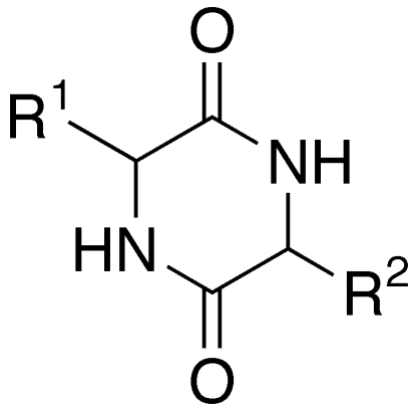

Figure 1. Diketopiperazine backbone. Biosynthesis of amino acids via Claisen condensation yields R1 and R2 groups that are amino acid derivatives, demonstrated by Degrassi et al.

There are several challenging components to the current research strategy. It is plausible that a virtual screen will score promiscuous aggregators highly, or that molecules with high predicted affinity will function as agonists rather than antagonists of LasR. It is also possible that many high scoring compounds will not be available for purchase, or would be difficult to synthesize or modify. In this case, the virtual screen can be expanded to include alternative libraries such as natural products, existing drugs, or even cytotoxic molecules. The benefit of a virtual screen is the cost effective selection of molecules to further analyze through more demanding and time consuming methods such as biochemical assays. Since the success of computational drug design is definitively stochastic, adding more compounds to the screen is an imperfect but adequate solution. With respect to the specific system of quorum sensing, a competitive inhibitor is likely to have several properties in common with the native agonist, which could be a major issue for specificity since so many metabolic intermediates have a hydrophobic and amphiphilic moiety. This also relates to the notion that diketopiperazines, natural inhibitors of quorum sensing in general, are formed by condensation of amino acids. The difficulty of designing a drug to target LasR can partially be attributed to these observations. The scenario would illustrate the limitations of computational drug design with respect to the quorum sensing transcription factor network.

The quantitative PCR reaction can also have several complications. Since relative fluorescence of the cDNA of the LasR gene between samples is the experimental observation, the actual difference in expression level may be misleading. This depends on the threshold number of PCR cycles until detectable fluorescence. Since PCR duplicates DNA to the power of cycles, stochastic effects may exaggerate or underestimate the differences in mRNA levels. The fact that all bacterial cells will have the LasR gene should not interfere with accurate measurement of relative mRNA expression levels. In order to reduce stochastic effects, qPCR analysis will be performed on entire colonies of bacteria under the same experimental conditions. All in all, aim two is the least conventional and is geared towards innovating new methods of analyzing positive autoregulation in gene regulatory networks. Aim three is more straightforward, however quorum sensing can be inhibited by several mechanisms and is thus less specific.

If the biofilm assays demonstrate that the top 20 compounds from the virtual screen all have anti-quorum sensing effects, then there may be an alternative mechanism in play. This is based on the notion that only a few of the top 20 compounds will be able to effectively diffuse, bind, and reside persistently in the LasR active site. This is a caveat that is unlikely, however it would indicate that the pharmacophore properties of the molecules are extremely important to inhibitor design. This would lead to the conclusion that all future inhibitors should have key conserved structural motifs. Another caveat of the current proposal is elucidation of the mechanism of inhibition. It is possible a lead compound will have high binding affinity to multiple quorum sensing transcription factors such as PqsR or QscR. The mechanism of action may or may not involve binding to those proteins, each of which is directly implicated in the progression of infection.

Kinetics of inhibitor design are a relevant caveat to consider. Currently, robust *in silico* methods of estimating kinetics of drug binding are not as thoroughly developed as thermodynamic parameters. This is a major limitation of existing *in silico* methods which cannot be determined though quantitative PCR or biofilm assays either. Kinetics are highly relevant to quorum sensing inhibitors, which are likely to diffuse at a slow rate through well-developed biofilms in patients within late stages of bacterial infection. A slow onset, long residence time inhibitor would be ideal for LasR, preventing even high concentration of native agonist irrelevant to increasing pathogenic gene expression. Biofilm assays may nevertheless provide a rough idea of how rapidly a lead compound could diffuse through to the bacterial cell based on the time it takes for the biofilm to disperse.

The impact of these three aims extends beyond antibacterial therapies. Creating more robust *in silico* methods of drug design requires consideration of the physiological goal of a project. The quorum sensing system is particularly important in this respect since if a lead compound causes strong inhibition of LasR but cannot diffuse across both a gram negative bacterial membrane and a dense biofilm, it may be an unreliable drug candidate. The combined aims of the current proposal will realistically provide a new chemical biology tool in the form of an inhibitor of quorum sensing, and more optimistically lead to a drug with lifesaving potential.

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