Quorum quenching as a therapeutic strategy to combat bacterial infections

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The increase in antibiotic resistance makes it difficult to combat bacterial infections. These had become much less lethal and dangerous when antibiotics arrived on the scene. Antibiotics were one of the most greatest pharmaceutical innovations in the history of medicine. No more would so many people die from relatively simple infections, no longer were wounds nothing more than a postponed death sentence due to infection. No longer would millions die from tuberculosis, indeed millions, perhaps even billions of lives have been saved by the various antibiotics however that great era seems to be coming to an end. Now many pathogens have gained resistance to many different antibiotics and our arsenal is running out. We are to blame for this, the enormous quantities of antibiotics we use in our daily lives and agriculture have led to emergence of antibiotic resistance on an unprecedented scale. This increase in antibiotic resistance has led to a search for new ways to combat pathogens to give us a more varied arsenal to protect ourselves from them. Many of these focus on new and more indirect ways to combat disease than simply killing the pathogens. One of these targets is their intercellular signaling based on population density which they use to regulate their pathogenicity. This mechanism known as quorum sensing can be blocked making it easier to combat the diseases caused by these bacteria. This blocking is called quorum quenching and might prove to be a new and powerful weapon in the eternal struggle to keep us healthy.

In order to see the future prospects for the therapeutic use of quorum quenching it is important to first look at the phenomenon of quorum sensing to gain understanding about where it can be blocked. Using that knowledge as a foundation it then becomes possible to look at the different modes of quorum quenching that have been discovered, both those based on small molecules and those based on proteins. Using that knowledge one can look at the world and see how much quorum quenching occurs to see if there is much left to be discovered. After all, nature is so incomprehensibly vast that if quorum quenching as a broad strategy is viable it would occur at the very least in some organisms. Using the knowledge from these first steps we can look at an overview and from there determine where the research into quorum quenching is headed and how viable it is in the forms found so far. In short, there are a few questions that I shall attempt to answer in this report. The first is What types of quorum sensing occur in nature? The second question is how quorum quenching works and where is it found? Finally and perhaps most importantly comes the third question. How can quorum quenching be used as part of a therapy?

Quorum Sensing

Euprymna scolopes or the Hawaiian bobtail squid is a species of bobtail squid notable for a special tactic it uses to camouflage itself from predators. The squid can emit light of the same intensity and wavelength as that of the down-welling light in its surroundings from its ventral region. This makes it much harder to detect by predators as the silhouette of the squid becomes invisible. It was known that scolopes contains a light organ covered by the ink sac which can adjust the light's wavelength. Bacteria of the species *Vibrio fischeri* were discovered to live in the light organs. These bacteria occur in low quantities in the oceans but are much more frequently found inside light organs. These organs contain specialized cells which bind *V. fischeri* and die off when the organ is sufficiently colonized. At dawn the squids vent these organs removing most of the bacteria from the light organ and grow new cells in order to attract new bacteria.(1)

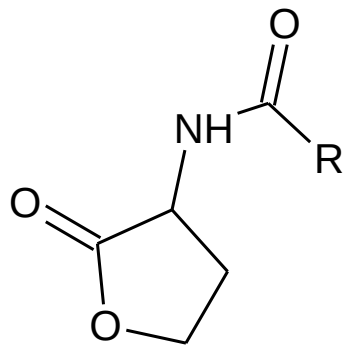

Figure 1, an AHL

While free-living *V. fischeri* emits no light, only when inside the light organ and when there is a sufficient amount of cells present do they start to express luciferase, the enzyme responsible for bioluminescence. For a long time is remained a mystery how this could be achieved. This led to the discovery of quorum sensing, the ability of a bacterium to detect how many other members of its own species are in the vicinity. It was discovered that this is due to an autoinducer. A molecule excreted in small amounts by the bacterium which can bind to receptors on other bacteria of *V. fischeri*.

Quorum sensing

Quorum sensing works by molecules excreted by a bacterium which are detected by other bacteria of the same species. When a sufficient concentration of these molecules has been achieved, which happens when there are enough bacteria in the vicinity to produce this amount, an action is triggered. This action can take many forms as will be illustrated by the examples given later on in this report.(6) Quorum sensing was at first thought to be a relatively uncommon system but it has been discovered in a wide array of different species so far. But why would it be that common? To be used in many different species it would have to lead to a significant increase in fitness. But what would that increase in fitness be? Let us start with the example in *V. fischeri*. What does it have to gain from emitting light? The advantage it gives the squid is simple. The squid is camouflaged from predators, but why would the bacterium help the squid? In this case it is quite clear, the bacterium is given sustenance by the squid making it a kind of symbiosis. It gives the light in trade for food, but only when there are enough of them around to prevent expending too much energy without gaining any increase in fitness in trade for it.

Other species use quorum sensing as well, here too it is used to regulate actions which are only useful when a whole group takes these action. Biofilm formation is one of these actions. It is useless to form a biofilm when a bacterium is alone. But when in a group of sufficient size forming a biofilm will lead to a large increase in fitness.

AHL-based quorum sensing

In *V. fisheri* the responsible molecule was N-(3-oxohexanoyl)-3-aminodihydro-2(3H)-furanone, an N-acyl homoserine lactone or AHL.(2) Luciferase is in an operon regulated by two proteins, LuxR and LuxI. LuxR is activated by an AHL to increase operon transcription while LuxI is responsible for the production of the AHL.(3) This AHL-based system is used in many other gram-negative bacteria as well.

Peptide-based quorum sensing

Many gram-negative bacteria used AHL's and the LuxI/LuxR system as described for *V. fischeri. B*ut in gram-positive bacteria peptides are commonly used as the signaling molecule.(5) These peptides are

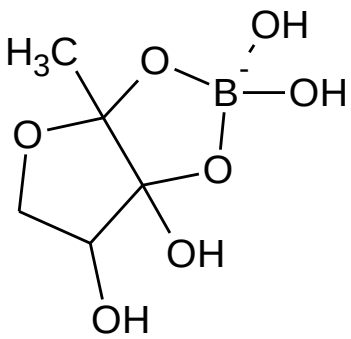

Figure 2, AI-2

excreted and bind to receptors leading to phosphorylation cascades and transcription. (5)

AI-2 quorum sensing

In both gram-positive and gram-negative species AI-2 or autoinducer 2 is used. AI-2 is one of the few biological molecules containing boron. It is extremely common, receptors have been found in almost half of all investigated bacterial genomes.(6) These bacteria use LuxS to produce AI-2. Different genera of bacteria use different detecting enzymes. In *Vibrio* AI-2 is detected by LuxP, in *Salmonella* and *Escherichia* AI-2 binds to LsrB and subsequently transported into the cell by the lsr ABC transporter. Inside the cell AI-2 is phosphorylated and in this modified form binds lrsR which regulates the lsr operon. (7,8)

Detecting other species

Some bacteria do not produce autoinducers but instead detect those produced by other species and react to their presence. Another example of this are those bacteria which lack LuxI but do have proteins with partial homology with LuxR called SdiA. This allows them to detect the number of bacteria of another species in the vicinity. But they themselves do not contribute. SdiA's can detect multiple different AHL's which is a major difference between them and LuxR which can only detect one specific AHL. (4)

Pathogenicity

Some bacteria use quorum sensing to coordinate pathogenicity and other strategies which can damage our health. One example of this is P*seudomonas aeroginosa* which is increasingly immune to many antibiotics. In addition to infections of wounds, the ear, the internal coats of the eye(mostly after eye surgery), the endocardium, meningitis, the urinary tract and the blood, this bacterium infects the lungs causing cystic fibrosis.(14) The infection leads to lung damage causing loss of lung function and later on death. *P. aeroginosa* forms biofilms in the lungs making them highly resistant to antibiotics making even long, vigorous treatments become useless. The biofilm formation is mediated by quorum sensing. Biofilm formation is a multistep process which is mediated by multiple quorum sensing AHL's. (9) *P. aeroginosa* uses quorum sensing to regulate other behaviours as well, such as swarming motility. Swarming is the rapid, coordinated motion of a population of bacteria over a semisolid surface. Swarming requires the cells to overcome the surface tension of the medium which is usually done by the production of biosurfactants*. P. aeroginosa* uses a mixture of compounds, a variety of rhamnolipids. The production of these biosurfactants is regulated by quorum sensing.(47,48)

EHEC and AI-3 quorum sensing

Another example is EHEC, the shorthand name for enterohemorrhagic *E. coli* which gained worldwide fame after thousands became ill after eating sprout in Germany. Dozens of people died. An important part of the disease is regulated by quorum sensing. The bacteria use the presence of AI-3 and Adrenaline/noradrenalin to regulate the expression of pathogenicity genes.(17) The bacterium caused haemorrhagic Colitis which is commonly complicated with Hemolytic-Uremic syndrome(HUS) or thrombotic thrombocytopenic purpura. In a few percent of cases this leads to chronic kidney disease.(10) In this disease antibiotics have little effect, they are associated with longer lasting diarrhea, a higher risk for HUS and even a higher mortality.(18)

Dental plaque

Quorum sensing has also been found to be involved in dental plaque. Here biofilms are formed under the influence of AI-2 making the involved bacteria more resistant to antibiotics and sometimes even increasing their virulence leading to periodontal disease. In many cases there are multiple species involved which coordinate their cooperation with AI-2. *Streptococcus Mutans*, a gram-positive bacterium which is commonly found in the mouth and plays in important role in tooth decay, can detect both AI-2 and a quorum-sensing peptide called CSP which plays a role in biofilm formation and increases acid tolerance of cells in the biofilm. In periodontal species these quorum sensing molecules also increase competence or the uptake of DNA from the environment making horizontal gene transfer more common.(11)

Quorum quenching

Quorum sensing has been discovered in more and more species of bacteria. Amongst them pathogenic ones where quorum sensing is involved with their pathogenicity. This has led to research into the disruption of quorum sensing because it could become a valuable new tool in our arsenal against disease. Especially in the case of antibiotic resistant bacteria it could be very useful. The disruption of quorum sensing is called Quorum Quenching. In this section I shall attempt to first describe the general mechanisms used for quorum quenching. Then examples will be given of quorum quenching in nature. Finally I shall attempt to show the future prospects of quorum quenching in therapy.

Quorum quenching is a logical reply to competitors or pathogens that use quorum sensing to gain an advantage. This can be compared to the antibiotics produced by many species. These natural quorum quenchers tend to be proteins but small molecules are in use as well. Especially plants and bacteria tend to produce quorum quenchers. (19,20)

Quorum quenching can be done in multiple ways. One simple broad strategy is the disruption of quorum sensing by removing the quorum sensing molecules such as the AHL's. This can be done by making sure that the quorum sensing molecules are not produced at all, by modifying or destroying them or by binding them to take them out of the surrounding. Another general strategy is to prevent their binding to the receptors by blocking receptor binding sites with other molecules.

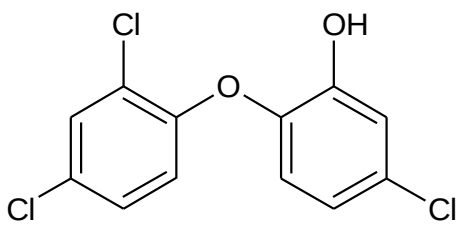

Figure 3, Triclosan

Preventing the quorum sensing molecules from being formed is one of the strategies in use in nature. This is done by targeting key enzymes in the biosynthesis of the quorum sensing molecule with an inhibitor. This has been shown to work in *P. aeruginosa* where triclosan has been used as an inhibitor for the Enoyl-Acyl Carrier Protein Reductase(ENR) which is involved in the biosynthesis of

Acyl-ACP, an important precursor for the AHL used by *P*. *aeruginosa*.(12) Another molecule targeting the same protein is green tea epigallocatechin gallate(EGCG) which has a higher affinity for ENR making it a better quorum quencher. (13) ENR is a part of the type 2 fatty acid biosynthesis pathway making these agents harmless to humans and other animals who use the type 1 pathway. Many other potential small-molecule quorum quenchers targeting the biosynthesis of AHL's have been identified as well. However these suffer from either being unstable or even worse, being toxic to humans and other animals.(15,16)

Removing the quorum sensing molecules is the strategy which so far shows the most promise. Many organisms produce enzymes which can quorum quench by targeting the responsible molecules and modify them in some was in order to prevent them from being detected by the target bacterium.

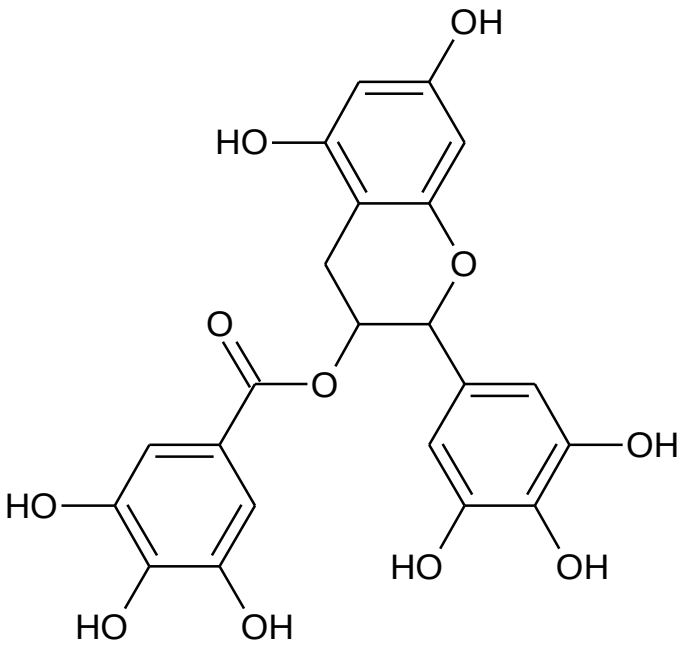

Figure 4, green tea epigallocatechin gallate

AHL's are known to hydrolyze at high pH, raising the pH however is not an all too useful method for therapeutic quorum quenching as a high pH is hard to generate inside the body and is impossible to generate without causing extreme tissue damage. A raised temperature has the same effect but shares the problems of a high pH. Although it is not as hard to generate, one would only have to heat the surroundings of the patient to a sufficient level without allowing him or her to cool down. It would cause severe, lethal damage preventing the therapy from working as we would like it to. Quorum quenching therapy is not a short duration therapy, the bacteria do not die without quorum sensing. The only effect is that they become less dangerous pathogens. Short-time heating of the patient is already a dangerous undertaking but long-term heating would only cause death.(27)

AHL quorum quenching

AHL quorum sensing can be quenched by three different classes of enzymes, AHL-lactonases, AHL-acylases and AHL-oxidoreductases. The first quorum quenching enzyme that was discovered was an AHL-lactonase in *bacillus sp. 240B1*.(21)

AHL-lactonases

The AHL-lactonases work using the mechanism shown in image 5. This class of enzymes uses hydrolysis catalyzed by the two zinc atoms and an acid group to open the lactone ring converting it

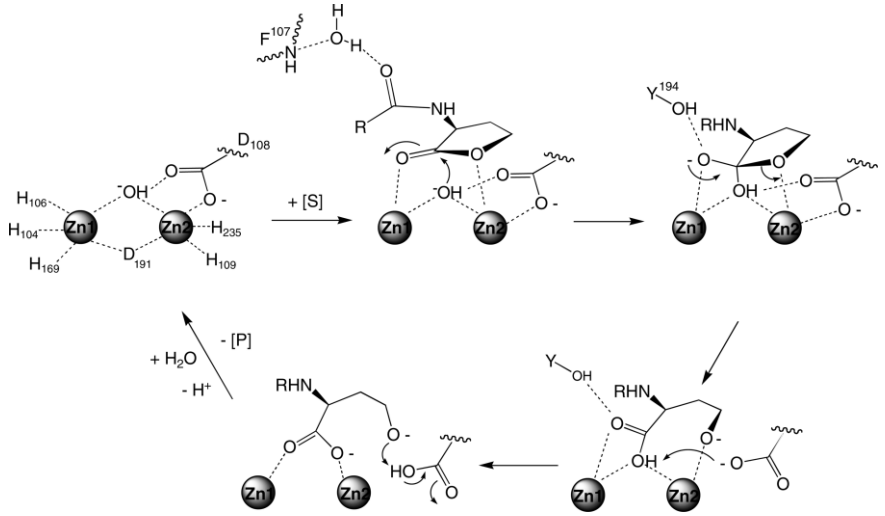

Figure 5, the mechanism of *Bacillus thuringiensis* AHL-lactonase(AiiA). Image taken from (22)

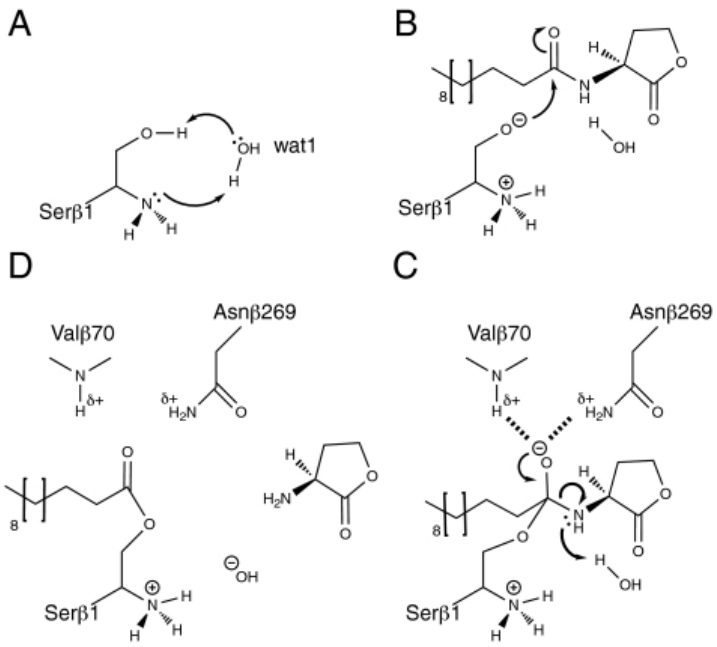

Figure 6, the mechanism of PvdQ, an AHL-acylase from *P. Aeruginosa*. Taken from(26)

into an acyl-homoserine that cannot be detected by LuxI.(22)

AHL-acylases

The AHL-acylases remove the acyl-tail from the AHL by attacking the carbonyl carbon, splitting the AHL into a fatty acid and a homoserine lactone. They were first discovered in *V. paradoxus,* at least, this type of quorum quenching activity was discovered. The enzyme which is responsible for the quorum quenching remains unknown. (23)But soon more quorum quenching enzymes of this type were discovered, such as PvdQ from *P. aeruginosa* and AiiD from *R. eutropha*.(24,25). The full mechanism is shown in image 6. This is another efficient way to disrupt quorum sensing, the AHL is permanently disabled from participation in quorum sensing reactions as it is cut to pieces. The acylases are special in one regard, they are not only used as quorum-quenching enzymes but some species use them to process AHL's as a carbon and energy source. (25) This makes them common in soil bacteria who use it as another nutrition source when quorum-sensing species are in the surroundings and the other sources of food become scarce as bacterial populations grow.

AHL-oxidoreductases

A third AHL-modifying strategy is used by AHL-oxidoreductases which was first discovered in *R. erythropolis.* This species has multiple AHL-degrading enzymes, the before mentioned AHL-oxidoreductase and an AHL-acylase which it uses to access its food source, AHL's. In this case it uses 3-oxo-AHL's as substrate for its oxidoreductase converting it into 3-hydroxy-AHL's. (28) AHL-oxidoreductases have not been found as often as the other types of quorum quenchers. This is probably because they require more specific target AHL's which need the 3-oxo group. This makes them less effective at disrupting many different quorum sensing systems at once. This makes it a less likely candidate for therapeutic use unless the invading pathogen is discovered to use quorum sensing using 3-oxo-AHL's.

AHL receptor inhibitors

Removing the quorum sensing molecules from the environment however is not the only option for quorum quenching. It is also possible to use inhibitors of LuxR and SdiA proteins. One example of this are AHL-analogs derived from furanone which bind to the receptor without leading to signaling.(31) Another such quorum quencher is patulin, which can block the AHL-receptor but which has the unpleasant property of being a genotoxin.(32) These have the advantage of being small molecules and therefore not as horribly expensive as proteins. They however have a tendency to be poisonous, another disadvantage is their lack of specificity, many of these compounds can affect multiple other systems than the targeted

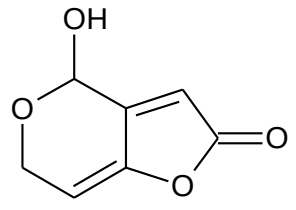

Figure 7, patulin

system. Another option is to use AHL's with a different chain length, even a slight extension of this chain significantly reduces activity while still binding to the receptor. Shorter chain lengths have a large effect as well but the appears to be a minimum chain length before binding is accomplished making longer AHL's more effective. Increasing de degree of unsaturation greatly diminishes the binding and therefore makes this useless for therapeutic purposes.(36,38)

AI-2 quorum sensing can be inhibited in the same basic ways as AHL quorum sensing. AI-2 synthesis can be blocked, an example of this is quercetin from broccoli extract. Broccoli extract also contains a so far unidentified inhibitor of the AI-3 pathway which prevents AI-3 and adrenaline/noradrenalin from having their effect on the bacterial cell, such as EHEC.(33)

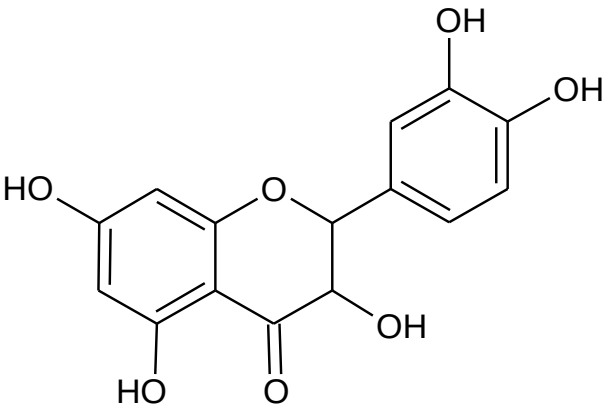

Figure 8, quercetin

Peptide-based quorum sensing in *S. aureus* has been blocked using monoclonal antibodies. These antibodies bind the signaling peptide effectively removing it from the environment. This seems to be an effective way of combating the quorum sensing of methicillin-resistant *Staphylococcus aureus* (MRSA) which regulates its pathogenicity. (34,35)

There are also many cases where a quorum quenching effect has been observed but the working mechanism is not yet known. One example is the use of garlic on patients with cystic fibrosis. In a pilot randomized control trial garlic extract which showed a small effect on quorum sensing.(29)In garlic extract the responsible molecule remains unknown but it is suspected that it is a LuxR inhibitor. (30)

Ethnopharmacy

These and many more strategies are used to block quorum sensing and the search continues for ever more quorum quenchers. A promising source for new quorum quenchers is the kingdom of plants. Plants produce a huge variety of metabolites, of which a number has quorum quenching activity. Ethnopharmacy and ethnobotany give pointers about which plants to look at first. Many medicinal plants produce substances that can inhibit quorum sensing, of course the people using those medicinal plants did not know about quorum sensing at all but they did find plants containing substances which can inhibit it.

One example of this can be found in a recent Indian study where twenty-four medicinal plants were investigated for an effect on *Chromobacterium violaceum* quorum sensing. Six of them did have an effect on the quorum sensing showing that these plants produce some sort of quorum quencher.(40) The same type of experiment was done with traditional Chinese medicinal plants as well. Ten plants were tested, eight of which had a quorum quenching effect.(19) This shows once more that there is a crenel of truth in everything, even in ancient herbal medicine. Quorum quenching activity has also been found in essential oils. Rose, geranium, lavender and rosemary oils have been found to be

effective against quorum sensing. In another experiment twenty-one essential oils were tested, four of these had an effect on quorum sensing of which one had a large effect.(41,42)

In all these plants it however remains unknown which compound is responsible for the activity. In *Medicago tranculata* the metabolome has been analyzed and at least fifteen different compounds were found that had an effect on quorum sensing, both stimulating and quenching in *Vibrio harveyi*. These stimulating mimics are used to attract certain bacteria and regulate symbiosis, most importantly in the case of nitrogen-fixating species.(39) Pea, or *Pisum sativum* contains this same type of mixture, compounds that minic AHL's to activate quorum sensing and compounds that inhibit quorum sensing in other species. (43)

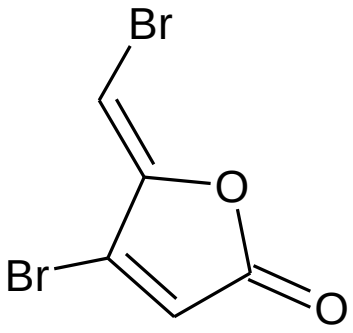

Figure 9, an example of a halogenated furanone.

Marine plants are vulnerable to bacteria because of their lack of an advanced immune system. However *Delisea pulchra* attracted attention because its surface is not covered by microorganism but is clean instead. It produces a variety of halogenated furanone compounds which have an antimicrobial effect. Surface colonization of an underwater surface usually starts with the formation of a bacterial biofilm to which other organisms then attach themselves. (44) This biofilm formation which is mediated by quorum sensing based on AHL's. furanones displace AHL at the binding site of LuxR inhibiting quorum sensing.(45)

Phytochemicals from fruits and spices have been tested as well. Raspberry, blueberry and grape were the only fruits that were found to have quorum quenching activity. Many herbs and spices have quorum quenching effects as well, most notably basil, thyme, turmeric, ginger and rosemary. This once more shows that the best place to look is indeed nature, where quorum quenching has been an important part in many organism's arsenals for millions of years.(46)

Biologics are drugs produced by organisms, also called biopharmaceuticals. They tend to be gene products. First generation biopharmaceuticals are unengineered, mostly murine antibodies and human proteins. The second generation consists of modified proteins from all kinds of species. Most quorum quenching biopharmaceuticals tend to belong to this second generation.(49) The problem of biologics is that they are horribly expensive to produce in sufficient amounts. The alternative for biopharmaceuticals are the more traditional small molecules which are synthesized. Almost all of the drugs in use are small molecules. They have the advantage of being a lot cheaper. However they also tend to have a more general effect.(50)

There are no pharmaceuticals using quorum quenching on the market at this time. There however is much research being done which hopes to make quorum quenching useful as a tool against disease. This mostly focuses on compounds from traditional herbal therapy on one hand and quorum quenching enzymes produced by a wide variety of organisms on the other.

Conclusion

From the evidence gathered so far it seems that quorum quenching occurs in many varieties. It is probable that within the next few years the first quorum quenchers will pass clinical trials and will be put on the market. These first approved quorum quenchers will probably be small molecules which are analogues of the signaling molecules in use by the targeted pathogen. Later on monoclonal antibodies and other biopharmaceuticals might come in therapeutic use but before that will happen the cost for producing them will have to fall significantly, otherwise they are simply too expensive to come in common use and to replace antibiotics or to be used alongside them in a supporting role. For them to be viable it needs to be discovered how these proteins can be given the specific selectivity. And perhaps even more importantly new ways have to be devised to produce and deliver them. These will probably only be used against truly life threatening diseases because of the large cost involved in their production.

Many of the small molecules that will see use will probably be derived from secondary metabolites in use by plants as quorum quenchers. This would be a vast source of new molecules. However, using these small molecule quorum quenchers has its risks as well as advantages. When they see increasingly common use resistance to them will be developed taking us back to zero. This will not happen nearly as fast as with the antibiotics but it is something that needs to be kept in mind.

In order to make small molecule quorum quenchers a common part of antibacterial therapies many more will need to be discovered, they will probably come from medicinal plants. However before that can happen much more research will be needed to discover which compounds cause the effect and after that will come extensive trials before they will be allowed on the market.

Quorum quenching is not yet in use but in the coming years is almost certain to make an impact which might prolong the age that was started with the discovery of antibiotics, the age where people live longer and healthier lives than was imagined to be possible in the preceding centuries. Chances are that quorum quenching will do much to help humanity in the never-ending struggle against disease.

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