***cis*-2-Alkenoic acids as promising drugs for the control of biofilm infections**

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Abstract

Microbes attached to surfaces and form biofilms where they are difficult to eradicate. Here they are embedded in a complex matrix of polymers and are much less sensitive against antibiotics or the immune system. This is a growing problem, especially for implants; hence, novel approaches are urgently needed to control biofilm infections. Many of these approaches interfere with the communication between the microbial cells required for biofilm formation and maintenance, a process known as quorum sensing. But microbes have also several mechanisms to disperse their own biofilms if conditions become unfavourable. Recently, it has been found that the pathogen *Pseudomonas aeruginosa* disperses its mature biofilms using *cis*-2-decenoic acid. This fatty acid belongs to a group of *cis*-2-alkenoic acids which are known from several bacteria and are also triggering the communication between different species. In this review the biosynthesis of these compounds, their signal transduction and their role in species-species communication are presented. Examples are discussed where *cis*-2-alkenoic acids have been used to eradicate biofilms and enhance the sensitivity of pathogens against antibiotics, either alone or in combination with antibiotics. Although this presents an interesting approach for the control of biofilm infections it is still in its infancy and a much broader characterization of the effects of *cis*-2-alkenoic acids are needed before moving to any medical application.

Keywords: biofilm, quorum-sensing, diffusible signal factor (DSF), quorum-quenching, host-pathogen interaction, *cis*-2-alkenoic acids, antibiotics

Pathogens organized in biofilms are difficult to eradicate

Microorganisms can attach to surfaces where they form biofilms, microbial aggregates embedded in a complex and structured matrix of polymers [1]. In human infections they are found on many implants but also on teeth [2] or the mucosa, e. g. in the cystic fibrosis lung [3], the skin [4] or the middle ear [5]. These aggregates are structurally complex, dynamic systems with a multitude of interactions between the community members and the host [6]. Biofilm formation is a key factor for the survival of microbes but biofilms have also the ability to disperse under unfavourable conditions to colonize new niches [7]. Biofilm infections cause many deaths and high health costs worldwide, e. g. in the U. S. alone 32% of all healthcare-associated infections are urinary tract infections, 22% are surgical site infections and 14% are blood stream infections causing costs between \$5000 and \$34,000 per infection and summing up to more than \$5 billion in added medical costs per annum [8].

The main problem comes not so much from the human pathogens causing the infections but from their organization in biofilms. Biofilm are formed for the protection of the cells against hostile environmental conditions. As a consequence, biofilm infections are difficult to eradicate because of much better protection against antibiotics compared to free living cells [9]. It has been reported that the eradication of pathogens from biofilms required more than 1000 times higher antibiotic concentrations than for the same strain living in planktonic form in the serum [10]. The reasons are manifold, e.g. in biofilms many genes are differently expressed, bacteria in biofilms grow slower and with the reduced metabolic activity they are less prone against most antibiotics [11], antibiotics are complexed by components of the biofilm matrix [12] or actively pumped out of the cell [13].

The vast majority of biofilm studies were carried out on single isolates. However, in nature most biofilms are comprised of consortia of species that interact with each other in complex networks [14]. This is further complicated by increasing antibiotic resistances of pathogens [15] and the huge diversity and flexibility of microbial physiology [16], urgently demanding novel targets to control biofilm infections [17]. Cell communication leading to biofilm formation and their role for the survival of bacterial pathogens in humans will be outlined here with a special focus on the application of medium sized unsaturated fatty acids, especially *cis*-2-alkenoic acids, for the control of biofilm infections.

To form biofilms microbes have to communicate with each other

To organize in biofilms, a certain number of cells is needed for survival, therefore, bacteria have to determine their cell density in order to coordinate their population behaviour for the formation of a biofilm. This is achieved by means of several small extracellular signal molecules released into the environment. If the cell is alone their concentrations are low but as soon as more cells are around their concentrations increase. When a certain threshold value is reached these molecules induce a signalling cascade leading to the induction of several genes. These signalling molecules act therefore also as autoinducers to start genetic programs [18]. As a result cells attach to the surface, form a biofilm and start the production of virulence factors. This intercellular communication principle is used by a large number of bacterial species to monitor cell density, hence quorum-sensing (QS), allowing individual cells to behave as a community [19]. But quorum-sensing is not only necessary to start the formation of biofilms it is also required to maintain them.

To date about 100 different autoinducers mediating quorum-sensing have been described. The best studied autoinducers, also called autoinducer-1 (AI-1), are *N*-acyl-L-homoserine lactones **1** – **3** which are only known from Gram-negative bacteria but related compounds have also recently been reported from an archaeon [20]. The acyl side chain of AI-1 is between 4 and 18 carbons long and usually saturated, although some mono-unsaturated side chains are also known. Many AI-1 compounds also carry a hydroxyl or oxo-function at C-3 [21]. Another group of autoinducers, autoinducer-2 (AI-2), was detected both in Gram-positive and -negative bacteria. The most widely distributed AI-2 compound was found in *Vibrio harveyi* and is the cyclic boronic ester **4** [22] but the epimer **5**, lacking the boronic ester, was also described from *Salmonella typhimurium* [23]. Because AI-2 is produced and sensed by many bacteria it is regarded as autoinducer for interspecies communication between bacteria [24]. Recently it has been shown that in the gut *Escherichia coli* strains overproducing AI-2 **4** favour after application of the antibiotic streptomycin bacteria of the phylum *Firmicutes* against *Bacteroides* [25]. This is one of the few reports where the formation of AI-2 changes the composition of complex bacterial communities. However, the exact mechanism of action of AI-2 in bacteria is still unclear and a non-quorum sensing role in most bacteria has been also suggested [26].

Acyl homoserine lactones (AHL) are not known from Gram-positive bacteria, here small peptides function as autoinducers, the autoinducer peptides (AIP). They are transformed by posttranslational processing of larger precursor peptides to the mature oligopeptide autoinducers. The peptide is actively transported out of the cell and the sensory information triggers a phosphorylation cascade leading to altered gene expression [27]. While in *Streptococcus* species linear peptides [28] are autoinducers in most *Staphylococcus* species including the human pathogens *S. aureus* and *S. epidermidis* cyclic peptides have this role.

Since most bacteria live in microbial communities the quorum-sensing signals not only serve for quorum-sensing by cells of the same species but also influences foreign species [29] as has been shown for example for *Escherichia coli* [30]. While the pathogen *Burkholderia cepacia* reacts to AHLs produced by another pathogen, *Pseudomonas aeruginosa*, the reverse is not the case [31]. This probably explains why *B. cepacia* always occurs together with *P. aeruginosa* in the cystic fibrosis lungs while *P. aeruginosa* is often found without *B. cepacia* [32]. Another autoinducer signal interaction has been described between the oropharyngeal flora and *P. aeruginosa* in cystic fibrosis. The autoinducer AI-2 **4** is produced only by the oropharyngeal flora, whereas *P. aeruginosa* reacts to AI-2 without producing it [33].

These examples show only the tip of the iceberg in the highly diverse kingdom bacteria. AHLs, AIP and most other autoinducers are species-specific and often several autoinducers are produced by the same cell enabling the fine-tuning of signalling [34]. Furthermore, we still know only for a small number of bacteria species their quorum-sensing autoinducers and even for those a number of other autoinducers have been described. Among them are diketopiperazines **6** - **9** formed by two condensed amino acids [35], 2-alkyl-4-quinolones, e.g. the *Pseudomonas aeruginosa* quinolone signal (PQS) **10**, in *Pseudomonas aeruginosa* [36], farnesol **11** in several fungi [37] or the unique bradyoxetin **12** [38] (Figure 1). However, for diketopiperazines their role remains unclear as there are some contradicting reports on their effects [39].

The obvious way for the control of biofilm infections is blockage of the quorum-sensing signalling cascade and many studies were directed towards this goal. Bacteria, however, cannot only form biofilms, they

also have to have mechanisms to disperse their biofilms and to convert the cells back to planktonic cells. This is important if unfavourable conditions occur and living in a biofilm is becoming a disadvantage. Still, very little is known about the routes taken by different pathogens to disperse their biofilms. One principle was discovered when *Pseudomonas aeruginosa* biofilms were treated with cell-free medium from old cultures and responded by the self-destruction of the biofilm. The responsible compound triggering this event was found to be *cis*-2-decenoic acid **13** [40]. Homologues were known for many years as diffusible signalling factor (DSF) in several bacteria.

Figure 1: Various types of autoinducers known from bacteria and fungi

Diffusible signal factors are different fatty acids acting as the signalling molecules between bacteria species regulating virulence factors of the recipient [41]. They are long known from *Xanthomonas* species where they mediating the communication with plants. But these signalling factors are more widespread and are also used by human pathogens. Because they pose another dimension of communication between bacteria and the

host this review will focus on these diffusible signalling factors and discuss their occurrence, regulation, biosynthesis and potential applications in medicine.

Interspecies communications seems to be the prime target of DSFs

When the plant pathogen *Xanthomonas campestris* was studied for factors regulating extracellular degradative enzymes and extracellular polysaccharide (EPS) virulence factors it was found that mutants lacking these factors could be restored by components extracted from the supernatant [42]. These components were termed diffusible signaling factors. Chemical analysis identified 11-methyl-*cis*-2-dodecenoic acid **16** as the responsible compound. Later on similar compounds, **13** – **23**, were found also in *Xylella fastidiosa*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, several *Burkholderia* species, including the only known double unsaturated DSF **23**, [43] and some other bacteria (Figure 2) suggesting a more widespread occurrence of this type of signaling [44].

Figure 2: Diffusible signal factors and analogues

The *cis*-unsaturated double bond at the 2-position is a key structural feature for activity and its hydrogenation leads to products of lower activities of 1-2 orders. Hence, it can be seen as the signature for DSFs. For *Xanthomonas* species it was also shown that the methyl branch increases the activity 60- to 120-fold compared to the unbranched *cis*-2-dodecenoic acid and *cis*-2-tridecenoic acid. Therefore, chain length and methyl-branching modulate the activity of the different DSFs but they do it in a species-specific way.

Synthesis and perception of DSF in *Xanthomonas campestris* require products of the *rpf* gene cluster (for regulation of pathogenicity factors). Similar gene clusters have also been found in many but not all DSFs producing bacteria [45]. Only recently the biosyntheses of the DSFs has been elucidated [46]. For *Pseudomonas aeruginosa* the involvement of the putative enoyl-coenzyme A hydratase DspI showing significant homologies to the protein RpfF of *Xanthomonas campestris* has been identified [47]. *cis*-2-Dodecenoic acid of *Burkholderia cenocepacia* is synthesized from the acyl carrier protein (ACP) thioester of 3-hydroxydodecanoic acid which is then converted by the enzyme RpfF first to *cis*-2-dodecenoyl-ACP. But RpfF catalyzes not only dehydration of 3-hydroxydodecanoyl-ACP to *cis*-2-dodecenoyl-ACP, it also cleaves the thioester to release the free acid [48]. However, the mechanisms by which DSFs are degraded are still unknown.

Most genes of the *rpf* gene cluster, however, are involved in signal perception and transduction. This is achieved by sensor kinases anchored in the cytoplasmic membrane [49]. Here again the sensor kinase RpfC of *Xanthomonas campestris* is the best understood system. It consists of a membrane spanning sensory domain, a histidine kinase domain, an ATP kinase binding domain, followed by a receiver domain and a histidine phosphotransfer domain. The structure of the corresponding *Burkholderia cenocepacia* sensor kinase RpfR is similar but in *B. cenocepacia* there are only two transmembrane helices instead of the 5 helices in *Xanthomonas campestris*. In addition, *B. cenocepacia* harbours a second sensor kinase, BCM0227, and *Xanthomonas campestris* pv. *campestris* also has a second one, RpfS, controlling the expression of a subset of genes [50]. The sensor kinase in *Pseudomonas aeruginosa* has also 5 transmembrane helices but the histidine phosphotransfer domain is missing. After binding of DSF the histidine kinase domain is autophosphorylated requiring ATP. The phosphorelay signalling then leads to the transfer of the phosphate to the receiver domain and then to the histidine phosphotransfer domain. From here the cyclic di-GMP phosphodiesterase RpfG is phosphorylated causing changes in the level of cyclic di-GMP in the cell which in turn affects, e.g. the synthesis of virulence factors or causes biofilm dispersal [51]. The sensor kinase RpfC of *Burkholderia cenocepacia* controls only a subset of DSF-regulated genes and another regulation mechanism was identified. Upon binding of the DSF of *Burkholderia* RpfR becomes a potent c-di-GMP phosphodiesterase leading to the control of several DSF-influenced genes [52]. The analysis of 82 *Stenotrophomonas maltophilia* clinical isolates revealed the presence of two distinct classes of *rpfC-rpfF* genes. While only one class was able to form DSF the other one seemed to repress permanently DSF-formation [53]. Obviously, at least *S. maltophilia* can infect humans even without the DSF-regulation system.

DSFs have a multitude of effects within the cell and they interfere with other quorum-sensing systems of the cell [54, 55]. In *Burkholderia cenocepacia*, *cis*-2-dodecenoic acid **14** increases motility and biofilm formation, but also virulence [56]. In *Stenotrophomonas maltophilia* 11-methyl-*cis*-2-dodecenoic acid **16** and in *Burkholderia cenocepacia* *cis*-2-dodecenoic acid **14** stimulate the secretion of outer membrane vesicles. These small vesicles contain large amount of outer membrane proteins which are involved in virulence and biofilm formation. They usually also contain metallo- β -lactamase and serin- β -lactamase, responsible for the resistance against certain antibiotics but when induced by the DSF the β -lactamases are absent [57]. Interestingly, the AHL-quorum sensing system and that of DSF seem to interact [58]. Comparative transcriptome analysis in *Pseudomonas aeruginosa* demonstrated a large number of genes being differently regulated by *cis*-2-decenoic

acid **13** [59], however, a clear pattern explaining the altered phenotype could not be deduced from these experiments.

As in *Xanthomonas campestris* these DSFs mediate the interaction between the producing bacteria and other organisms but are also involved in biofilm formation, production of virulence factors or stress tolerance [60]. From the opportunistic pathogen *Stenotrophomonas maltophilia* several fatty acids (**14** - **21**) have been identified which facilitate movement of its cells [61] and mediate the communication between *Stenotrophomonas maltophilia* and the pathogen *Pseudomonas aeruginosa*. The DSFs produced by *S. maltophilia* cause in *P. aeruginosa* increased stress tolerance including higher resistance against cationic antimicrobial peptides [62]. Interestingly, 12-methyl-tetradecanoic acid **22** not found in *Stenotrophomonas maltophilia* blocked swarming motility completely at 10 $\mu\text{g mL}^{-1}$ in *Pseudomonas aeruginosa* and led to reduced biofilm formation by 31 % [63]. Another DSF, *cis*-2-dodecenoic acid **14**, enabled bacteria-fungus interaction, e.g. between *Burkholderia cenocepacia* and *Candida albicans* [64]. The same fatty acid, *cis*-2-dodecenoic acid, down-regulates in *Pseudomonas aeruginosa* biofilm formation and inhibits its type-III secretion system [65]. Members of the Gammaproteobacteria are also affected by this DSF as has been shown for the genus *Francisella*. *Francisella tularensis* is a highly infectious pathogen causing tularaemia in humans. The closely related *Francisella novicida*, often used a model organism to study this pathogen, reacts to *cis*-2-dodecenoic acid by the dispersion of its biofilm. This effect is also influenced by chitinase as experiments with chitinase inhibitors could show. In addition to biofilm dispersal the DSF also led to increased siderophore production [66]. 11-Methyl-*cis*-2-dodecenoic acid **16** of *Stenotrophomonas maltophilia* blocks the yeast to hyphal transition of *Candida albicans* and kills the fungal cells [67]. All these examples show the broad diversity of species-species interactions mediated by DSFs.

Prevention and dispersal of biofilms using *cis*-2-alkenoic acids or their derivatives

The identification of *cis*-2-decenoic acid as the active compound dispersing preexisting biofilms of *Pseudomonas aeruginosa* opened novel possibilities to eradicate biofilms and initiated a number of studies for the elucidation of the mechanism and effects [68]. Marques *et al.* studied the effect of the compound on the metabolic status of *Pseudomonas aeruginosa* and *Escherichia coli* and found that it could convert persister cells from a dormant to a metabolically active state, making them more sensitive to antibiotic treatments. *cis*-2-Decenoic acid increased the respiratory activity caused by changes in protein abundances. As a consequence these cells became more sensitive against antibiotics and the number of surviving cells decreases in biofilm treated with such a combined therapy [69]. When methicillin-resistant *Staphylococcus aureus* (MRSA) were exposed to 125 $\mu\text{g mL}^{-1}$ *cis*-2-decenoic acid the formation of biofilms and at >500 $\mu\text{g mL}^{-1}$ growth was inhibited. This characteristic was used in a drug release experiment where a chitosan sponge was loaded with *cis*-2-decenoic acid and the concentration of this DSF was high enough over 5 days to prevent biofilm formation [70].

cis-2-Alkenoic acids do not only interfere with the biofilm formation of bacteria but also of fungi. *Cis*-2-dodecenoic acid **14** at 90 μM reduced the ability of *C. albicans* to adhere to plates and catheters by 4 and 25 fold, respectively, disrupting the formation of a biofilm. This DSF reduced the level (4 and 0.25 fold) of expression from some adhesion genes (*ALSI* and *EAP1*) and increased the level of YWP1 (4 fold), a protein that had an antiadhesive activity [71]. Furthermore, **14** showed only activity in the initial adhesion process, because

when the yeast had germinated and formed the normal hyphae, these were unaffected by subsequent addition of **14** [72].

cis-2-Alkenoic acids are relatively simple molecules which facilitates the generation of analogues. However, only few analogues of *cis*-2-alkenoic acids have yet been tested for their effect on biofilms of pathogens. 4,5-disubstituted-2-aminoimidazole-triazole conjugates, e.g. **36**, inhibited biofilm formation of methicillin-resistant *Staphylococcus aureus* and *Acinetobacter baumannii* and resensitized them against antibiotics. When the activity of these compounds were compared with the one of *cis*-2-decenoic acid they were found to be more active [73]. However, since no attempt was made to elucidate the mechanism of action it can only be speculated that they act similar to *cis*-2-decenoic acid. Gutierrez *et al.* found that some 2-bromoalkanoic acids reduced the capability of *Pseudomonas aeruginosa* to form biofilms. Treatment of *Pseudomonas aeruginosa* PAO1 with 5 mM 2-bromohexanoic acid **37**, 2-bromooctanoic acid **38** and 2-bromodecanoic acid **39** resulted in a reduction of biofilm formation by 29%, 40% and 69%, respectively, over 48 h (Figure 3). Although the exact mode of action of these compounds on the biofilm was not investigated it could be shown that this effect was independent of the inhibition of the formation of rhamnolipids. The same compounds with 2 mM concentration caused after 120 h a reduction of rhamnolipid formation by 89%, 71% and 29%, respectively, showing just the reversed order of activity of the bromoalkanoic acids compared to biofilm inhibition and pointing to a mechanism independent from biofilm formation [74].

Figure 3: Some possible analogues of *cis*-2-alkenoic acids

Because the main effect of *cis*-2-decenoic acid **13** on various biofilms is their dispersion the idea is obvious that a combination with disinfectants or antibiotics could be a promising approach to control biofilm infections. The success of such an approach has been demonstrated for *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella enterica* and *Escherichia coli*. When pre-established biofilms of these four bacteria were treated with 310 nM *cis*-2-decenoic acid **14** and disinfectants or antibiotics an 80 % reduction of the biofilm mass was observed [75]. This works not only with pure cultures but also with mixed communities and biofilms of

Escherichia coli growing together with *Klebsiella pneumonia* could be reduced by 78 % when treated with 310 nM *cis*-2-decenoic acid and ciprofloxacin or ampicillin. Moreover, most of the cells remaining in the biofilm were also killed [76]. The same treatment where the antibiotic has been replaced by a disinfectant could also reduce significantly biofilms of *Streptococcus mutans*, a pathogen of dental plaques [77]. *cis*-2-Decenoic acid **13** seems to be much more active than the homologous *cis*-2-dodecenoic acid **14**. When using 50 μ M *cis*-2-dodecenoic acid **14** instead of *cis*-2-decenoic acid **13** and gentamycin a similar, although much weaker, effect could be seen for *Bacillus thuringiensis*, *Staphylococcus aureus*, *Neisseria subflava* and *Pseudomonas aeruginosa*. In the same study the effects of homologues of *trans*- and *cis*-2-alkenoic acids in combination with three different antibiotics on *Bacillus cereus* have been determined. It was found that in general longer fatty acids were more active than shorter ones but the configuration of the double bond and the added antibiotics were even more important for the activity. While, e.g. *trans*-2-tetradecenoic acid **29** was the most active *trans*-fatty acid in this study in combination with gentamycin, the most active *cis*-fatty acid was *cis*-2-pentadecenoic acid **34**. *cis*-2-Tetradecenoic acid **15** was less active than the homologous *cis*-2-pentadecenoic acid **34** when applied together with gentamycin but equally active when combined with kanamycin (Figure 4) [78]. At least for *Bacillus cereus* there seems to be not a single optimal *cis*-2-alkenoic acid and it is reasonable to assume that for other bacteria an even different pattern of activity will be found.

Figure 4: Increase of sensitivity of *Bacillus cereus* against antibiotics caused by various DSFs. Note that the enhancement of the activity of the antibiotic does not only depend on the configuration and the chain length of the 2-alkenoic acid but also on the co-administered antibiotic. Compound numbers are those from Figure 2, data taken from [78].

Discussion and outlook

The discovery of *cis*-2-decenoic acid **13** from *Xanthomonas* species and its role in bacteria-plant communication had no relevant impact on medicinal chemistry. This changed dramatically when the same compound from *Pseudomonas aeruginosa* was found to disperse various biofilms. Immediately, several applications could be imagined and the dissection of its biosynthesis, regulation and mode of action started. Now we know that *cis*-2-decenoic acid belongs to a group of bioactive *cis*-2-alkenoic acids produced by several pathogens often also for the purpose of inter-species interactions. This characteristic made *cis*-2-alkenoic acids interesting candidates for the modulation of pathogenic biofilm communities. Because several *cis*-2-alkenoic acids can disperse established biofilms and increase their sensitivity towards antibiotics approaches combining them with antibiotics or disinfectants have been investigated. The first results were rather promising, although these studies are only the beginning. We still do not fully understand the effects of *cis*-2-alkenoic acids on the bacterial cells, their entire circuits of regulation, their structure activity relationships, or their mode of action on biofilms, to name only a few open questions. We also still do not know their pharmacokinetics and their effects on complex microbial communities in biofilms or in animal infection models. This all will be studied in the coming years but the expectations are high that *cis*-2-alkenoic acids will develop into an important tool for the control of biofilm infections.

Many characteristics of *cis*-2-alkenoic acids are still not clear, e.g. Davies *et al.* [40] reported dispersion of *S. aureus* biofilms with 10 nM of *cis*-2-decenoic acid **13** but Su *et al.* [73] needed about 200 μ M to achieve this effect. For the characterization of effects of *cis*-2-alkenoic acids we need strictly controlled protocols which will allow the comparison of results between different compounds, different pathogens and different laboratories. These data are essential for the establishment of QSAR required for the search for compounds with highly specific activities. After finding such compounds their efficiencies in host-pathogen models have to be demonstrated. For the application in drug release systems on implants it is essential to know that they are active but also that they do not hinder the integration of the implant. At least for *cis*-2-decenoic acid no effect on osteoblast differentiation has been detected [79]. If in animal experiments these positive results can be confirmed several combinations of antibiotics with *cis*-2-alkenoic acids can be the answer to specific biofilm infections of the host. Applications of *cis*-2-alkenoic acids are still in its infancy but the results obtained so far are very promising for the development of drugs against biofilm infections.

Conflict of interest

The authors have no conflict of interest

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