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The quorum quenching antibody RS2-1G9 protects macrophages from the cytotoxic effects of the *Pseudomonas aeruginosa* quorum sensing signalling molecule *N*-3-oxo-dodecanoyl-homoserine lactone

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Abstract

The Gram-negative bacterium *Pseudomonas aeruginosa*, an opportunistic human pathogen, uses acylhomoserine lactone-based quorum sensing systems to control its pathogenicity. One of its quorum sensing factors, *N*-3-oxo-dodecanoyl homoserine lactone, has been shown not only to mediate bacterial quorum sensing but also to exert cytotoxic effects on mammalian cells. The monoclonal antibody RS2-1G9 generated against a 3-oxo-dodecanoyl homoserine lactone analogue hapten was able to protect murine bone marrow-derived macrophages from the cytotoxic effects and also prevented the activation of the mitogen-activated protein kinase p38. These data demonstrate that an immunopharmacotherapeutic approach to combat *P. aeruginosa* infections might be a viable therapeutic option as the monoclonal antibody RS2-1G9 can readily sequester bacterial *N*-3-oxo-dodecanoyl homoserine lactone molecules, thus interfering with their biological effects in prokaryotic and eukaryotic systems.

1. Introduction

Cell-to-cell communication is used by prokaryotic and eukaryotic single-cell organisms to coordinate their behaviour allowing them to adapt to changing environmental conditions and to compete with multicellular organisms. This exchange of information among single-cell organisms using small chemical molecules has been termed “quorum sensing” (QS) (Engebrecht et al., 1983; Fuqua et al., 1994). Examples of QS-controlled microbial processes include biofilm formation, virulence factor expression, and bioluminescence. These processes

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are advantageous to a bacterial population only when they are carried out simultaneously by all or, at least, by a large percentage of the population.

N-acyl homoserine lactones (AHLs) are produced by over 70 species of Gram-negative bacteria, and vary in the length and oxidation state of the acyl side chain. Upon reaching a critical threshold concentration, the AHLs bind to their cognate receptor proteins, e.g. LuxR in Vibrio fischeri, LasR and RhlR in Pseudomonas aeruginosa, inducing receptor oligomerization, thus enabling DNA binding of the ligand-receptor and triggering target gene expression. AHLs are known to play an important regulatory role in bacterial infections. The Gram-negative bacterium *P. aeruginosa* is an example of a human bacterial pathogen utilizing AHL-based QS to control its virulence factor expression and biofilm formation (Fuqua and Greenberg, 2002). Over the past 20 years, significant progress has been made in elucidating the molecular mechanisms underlying *P. aeruginosa* pathogenicity (Smith and Iglewski, 2003a). Two different AHLs, *N*-3-oxo-dodecanoyl homoserine lactone (3-oxo-C_{12}-HSL) synthesized by LasI and N-butyrylhomoserine lactone (C_4-HSL) synthesized by RhlI, have been identified as AHL autoinducers used by *P. aeruginosa*. Most prominent is the role of *P. aeruginosa* in patients suffering from cystic fibrosis (CF) and in burn victims.

Several studies have reported on the biochemical effects of AHLs on mammalian cells (Kravchenko et al., 2006; Ritchie et al., 2003; Smith et al., 2002a; Smith et al., 2002b; Telford et al., 1998). Notably, 3-oxo-C_{12}-HSL specifically promotes induction of apoptosis in macrophages and neutrophils (Tateda et al., 2003). In fact, antivirulence therapies have attracted attraction as a new strategy to combat microbial infections and, thus, bacterial QS represents a promising therapeutic target (Cegelski et al., 2008; Clatworthy et al., 2007). A plethora of small-molecule discovery efforts have been reported (Geske et al., 2007; Hjelmgaard et al., 2003; Muh et al., 2006a; Muh et al., 2006b; Smith et al., 2003). However, our group recently pioneered an immunopharmacotherapeutic approach via the generation of anti-AHL monoclonal antibodies (mAbs) (Kaufmann et al., 2006). The mAb RS2-1G9 demonstrated potent inhibitory activity of 3-oxo-C_{12}-HSL-based QS in *P. aeruginosa* in vitro. Intrestingly, the crystal structure of RS2-1G9 in complex with its hapten was solved and provided insight into the immune system's ability to generate immunoglobulin against small hydrophobic molecules (Debler et al., 2007). An advantage of mAbs is that by scavenging soluble molecules they also neutralize the cytotoxic AHL effects on host cells, in analogy to antibodies against other bacterial toxins. Notably, an active immunization using an AHL-based hapten coupled to a carrier protein has shown promise in *in vivo* infection models (Miyairi et al., 2006). To gain greater insight into the molecular details of antibody-mediated protection, we investigated mAb RS2-1G9's ability to neutralize 3-oxo-C_{12}-HSL and protect murine macrophages against the cytotoxic effects *in vitro*.

2. Materials and Methods

2.1. Reagents

N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL, C12) was synthesized and purified as described (Kaufmann et al., 2005). The purity was greater than 99% as confirmed by HPLC/mass spectrometry analysis. 3-oxo-C_{12}-HSL was dissolved in DMSO (Sigma-Aldrich, St. Louis, MS, USA) at 200 x of the desired concentration and aliquots were stored at -20 °C.

The murine mAbs RS2-1G9 and RS3-6D9 were produced and purified at the antibody core facility at The Scripps Research Institute.

2.2. Cell culture

RAW 264.7 cells (ATCC, Manassas, VA) were cultured in T75 culture flasks (Corning Inc., Corning, NY, USA) in DMEM (4.5 g/L glucose) supplemented with 10% FBS (HyClone, Logan, UT, USA), L-glutamine, penicillin/streptomycin and nonessential amino acids (Invitrogen, Carlsbad, CA, USA). Bone marrow-derived macrophages (BMDM) were prepared by using standard protocols and cultured in 70% DMEM (vide supra) and 30% L929 conditioned medium (DMEM+L929 medium = growth medium GM). All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 and maintained according to ATCC protocols.

2.3. Cytotoxicity assay

For the cytotoxicity assay $5x10^5$ BMDM cells were seeded in each well of a 96-well tissue culture microtiter plate in 100 μL GM and incubated 48 h. The medium was changed on the second day and the cells were incubated for an additional 4 h. Afterwards, 20 μL of medium were removed, followed by the addition of 20 μL of the appropriate antibody (in GM) and 0.5 μL of 3-oxo-C_{12}-HSL (10 mM stock in DMSO) were added. The BMDM were incubated for 20 h at 37 °C in a humidified atmosphere with 5% CO_2. Cell viability was determined by XTT assay according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MS, USA).

2.4. Western Blot analysis

BMDM (10^6 cells) were seeded in 24-well plates in 500 μL of GM. After 48 h, the medium was changed; 4 h later 250 μL of the medium were removed and 250 μL of GM containing the anti-AHL antibody at the appropriate concentrations were added followed by the addition of 2.5 μL of 3-oxo-C_{12}-HSL (10mM stock in DMSO). The cells were incubated for 1 h, washed and lyzed. The protein extract was boiled for 10 min, spun down, and stored at -20 °C. Equal amount of total protein were loaded on a 10% gel and analyzed by SDS-PAGE. The proteins were transferred onto PVDF membranes and all subsequent steps followed standard Western blot protocols. Anti-PARP and anti-P-p38 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and used in the Western blot analyses as recommended by the manufacturer.

3. Results

3.1. Protection of murine bone marrow-derived macrophages from the cytotoxic effects of 3-oxo-C_{12}-HSL by mAb RS2-1G9

As discussed (vide supra), 3-oxo-C_{12}-HSL not only controls bacterial behaviour, but also induces distinct biochemical changes in and exerts cytotoxicity on mammalian cells. As a logical progression of our work on the quorum quenching abilities of RS2-1G9, we evaluated this mAb for its ability to protect mammalian cells from the AHL-induced cytotoxic effect. Macrophages are important cells of the host innate immune system and are first-line defenders against bacterial invaders. The murine macrophage-like bone marrow RAW 264.7 and primary mouse bone marrow-derived macrophages (BMDM) were chosen for our assays. The mAbs RS2-1G9 and the isotype (γ1/λ) control RS3-6D9, raised against a different AHL analogue hapten, were tested for their ability to protect the macrophages from 3-oxo-C_{12}-HSL. A concentration of 50 μM 3-oxo-C12-HSL was chosen as we had previously demonstrated that this concentration induces apoptosis in macrophage in a highly reproducible fashion (Kravchenko et al., 2006). Viability assay demonstrate that RS2-1G9 confers protection to the macrophages in a concentration-dependent manner (Figure 2A and B). The EC_{50} of RS2-1G9 is approximately 20 μM, which corresponds to an approximate 1:1 molar ration of 3-oxo-C_{12}-HSL and antibody binding sites, i.e. every IgG molecule has two antigen binding sites. The data also show that once the antibody concentration falls below a protective threshold

concentration cell death in the macrophages occurs rather efficiently, suggesting that a lower 3-oxo-C_{12}-HSL concentration than the 50 μM used in our assays is indeed needed to induce apoptosis in macrophages. These findings suggest that in a therapeutic setting, the anti-AHL antibody has to be used in concentrations that result in at least a 1:1 molar ratio between 3-oxo-C_{12}-HSL and the antibody binding sites.

3.2. RS2-1G9 prevents p38 activation and PARP cleavage in BMDM in response to 3-oxo-C_{12}-HSL

Previously, we have reported that 3-oxo-C_{12}-HSL can induce the phosphorylation of the mitogen-activated protein (MAP) kinase p38 in mammalian cells. This represents a biochemical marker of the cytotoxic effects induced by *N*-3-oxo-acyl homoserine lactones (Kravchenko et al., 2006). In our biochemical assays, the caspase-mediated cleavage of the enzyme Poly(ADP-ribose)polymerase (PARP) serves as a surrogate marker of apoptosis. To investigate the underlying molecular features of the protection mechanism, BMDM were incubated with 50 μM 3-oxo-C_{12}-HSL for 1h in the presence of absence of varying concentrations of RS2-1G9 or RS3-6D9 and performed Western blot analysis of cell lysates. Gratifyingly, the mAb RS2-1G9 was able to prevent the induction of apoptosis, as evident from the inhibition of PARP cleavage, as well as inhibition of p38 phosphorylation and, thus, protect the cells from the cytotoxic 3-oxo-C_{12}-HSL effects (Figure 3). As anticipated, these data clearly show that once the antibody concentration falls below the concentration of approximately 20 μM, the protection of the macrophages from the cytotoxic effects significantly is reduced as evident by the appearance of cleaved PARP as well as the phosphorylation of p38, which could represent an attempt by the cells to avoid apoptosis as p38 activation as been implied in anti-apoptotic mechanisms.

Taken together, our data demonstrate that mAb RS2-1G9 can readily sequester bacterial 3-oxo-C_{12}-HSL signal molecules and efficiently protect mammalian cells against the cytotoxic effects of the AHL as long as the molar ratio of antibody binding sites and AHL are approximately 1:1.

4. Discussion

The two main strategies in anti-infective therapy are either to eradicate the invading microorganism, or to attenuate it such that the establishment of an infection is prevented and the bacteria are readily cleared by the host's immune system (Finch et al., 1998). The discovery that QS signalling is a widespread mechanism for controlling gene expression in many pathogenic Gram-negative and Gram-positive bacteria may provide new targets for the latter antimicrobial strategy (Finch et al., 1998; Hentzer et al., 2003; Otto, 2004; Smith and Iglewski, 2003b; Suga and Smith, 2003; Williams, 2002). Recently, we have reported the successful application of an immunopharmacotherapeutic approach in the AIP-based QS system in the Gram-positive bacterium *S. aureus* (Park et al., 2007).

Chemical synthetic efforts focused on targeting the *P. aeruginosa* 3-oxo-C_{12}-HSL-based QS system have resulted in the discovery of a number of small molecule signalling antagonists. Alternatively, microbial QS systems represent an excellent target for antibody-based anti-infective therapy given the evolutionary highly conserved components and extracellular distribution of the QS signalling molecules. Furthermore, another advantage of therapeutic mAbs is the predictability of their pharmacodynamic (PD) and -kinetic (PK) properties, a critical aspect in drug development (Reichert, 2003; Reichert and Valge-Archer, 2007). In light of the potent 3-oxo-C_{12}-HSL-mediated cytotoxicity, it might be appropriate to think of it as a bacterial toxin and mAbs have successfully been used as neutralizing agents for a variety of bacterial toxins (Casadevall et al., 2004; Nowakowski et al., 2002; Zhou et al., 2007). Here, we have demonstrated that the quorum quenching antibody RS2-1G9 can efficiently protect

murine macrophage from the detrimental effects of the *P. aeruginosa* quorum sensing molecule 3-oxo-C_{12}-HSL. The antibody also prevents the activation of cellular stress kinase pathways, indicating that the sequestration of 3-oxo-C_{12}-HSL is complete. Our data also suggest that the protection achieved by the active vaccination approach reported by Tateda and co-workers might be due to the elicitation of 3-oxo-C_{12}-HSL-neutralizing antibodies (Miyairi et al., 2006).

In the case of *P. aeruginosa* infections, one could envision the application of anti-AHL mAbs, maybe in an IgA format, via inhalation into the lung of in cystic fibrosis patients and carriers of the CFTR mutation at young age to prevent the establishment and onset of infections. It is important to point out that, due to absence of direct cytotoxicity towards the bacterial organism, any anti-infective therapy targeting bacterial QS signalling will most likely be used in a prophylactic manner to prevent establishment of infectious organisms rather than to treat acute and chronic already established bacterial infections. However, this absence of inherent bactericidal activity and, thus, lack of selection pressure for resistant escape mutants, suggest that quorum quenching therapeutic approaches will not promote the appearance or spread of "superbugs".

QS-deficient *P. aeruginosa* strains show a clear reduction in overall pathogenicity (Christensen et al., 2007; Mittal et al., 2006). It has also been shown that in clinical *P. aeruginosa* isolates various components of the QS circuit have been lost due to mutations. These findings indicate that QS signalling for the control of virulence factor expression in *P. aeruginosa* is essential for the establishment of infection in the patient, but might be dispensable for the maintenance of the infection and thus, is lost during the course of chronic infections. However, in the case of the 3-oxo-C_{12}-HSL/LasI/LasR QS system, these mutations quite frequently occur in the receptor gene *lasR* rather than the synthase gene *lasI*, suggesting that 3-oxo-C_{12}-HSL molecules might indeed have another important biological function besides their importance in QS signalling (Cabrol et al., 2003; Favre-Bonte et al., 2007; Heurlier et al., 2006; Lee et al., 2005; Salunkhe et al., 2005; Schaber et al., 2004).

In summary, 3-oxo-C_{12}-HSL does not only regulate the expression of virulence factors in *P. aeruginosa*, but also contributes significantly to the pathogenesis via exertion of cytotoxicity on host cells. This is an important hallmark of 3-oxo-C_{12}-HSL and necessitates potentially more complex therapeutic strategies that go beyond simple signalling antagonism. This is the first report of a potentially therapeutic agent that not only inhibits AHL-based QS signalling but also protects mammalian cells from the cytotoxic effects and, thus, gives further credence to our overall strategy of using immunopharmacotherapy as a prophylactic quorum quenching approach.

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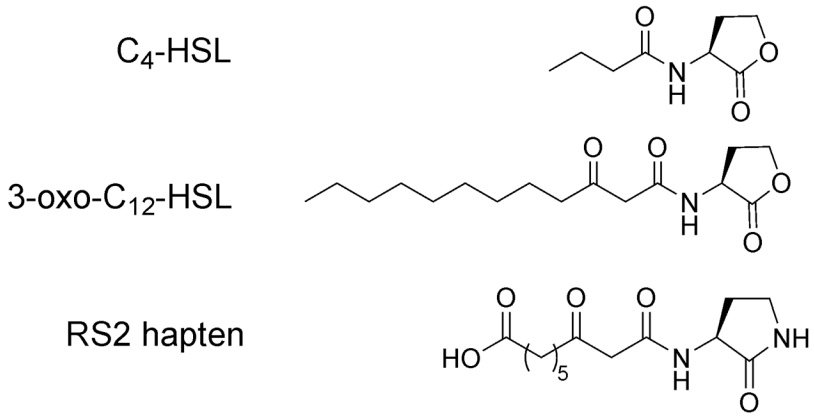

Figure 1.
Chemical structures of the AHLs used by *Pseudomonas aeruginosa* and the AHL analogue hapten used to generate the mAb RS2-1G9.

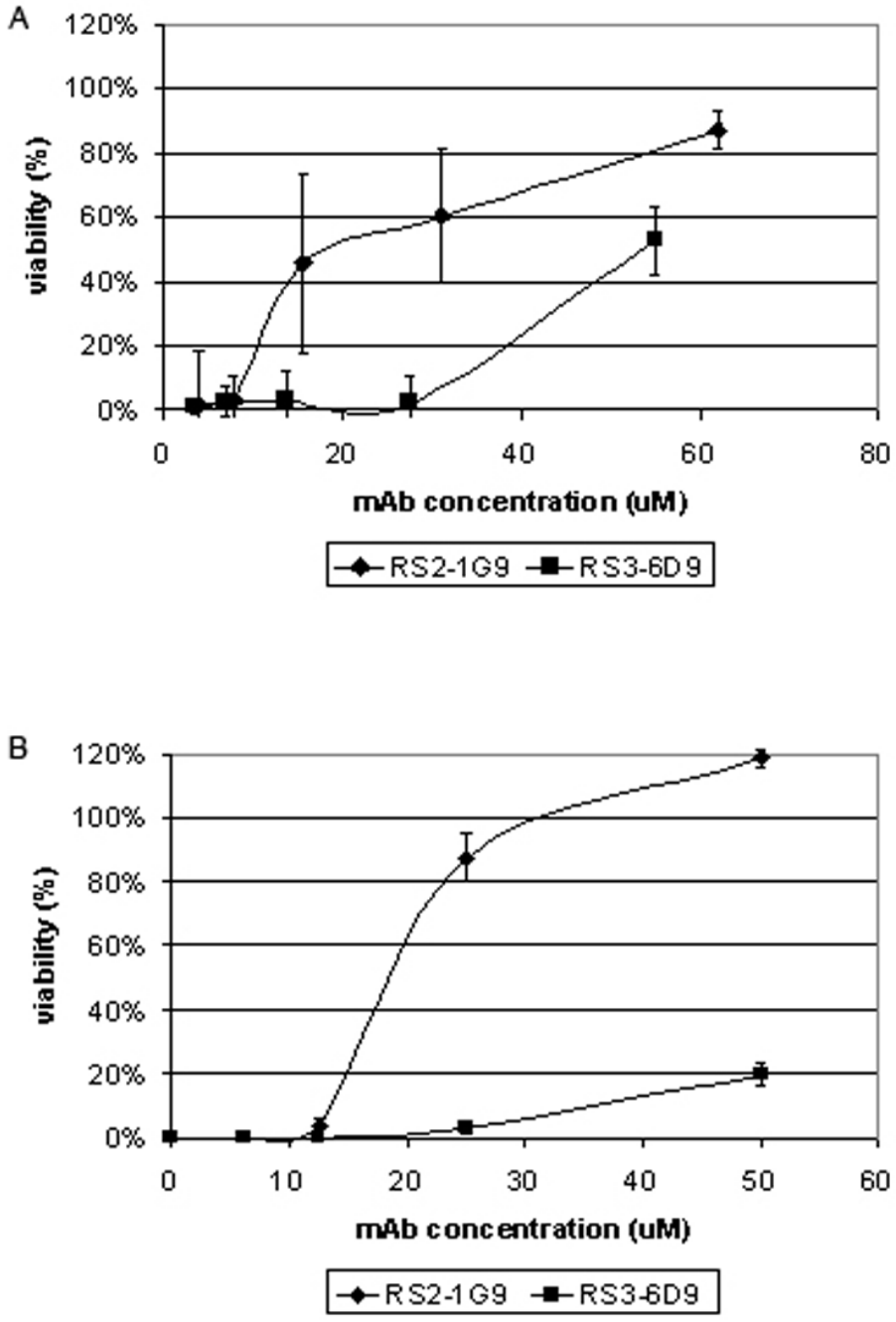

Figure 2.
Protection of RAW cells (A) and BMDM (B) from the cytotoxic effects of 3-oxo-C_{12}-HSL. Results are expressed as percentages of cells still viable relative to non-treated control cells.

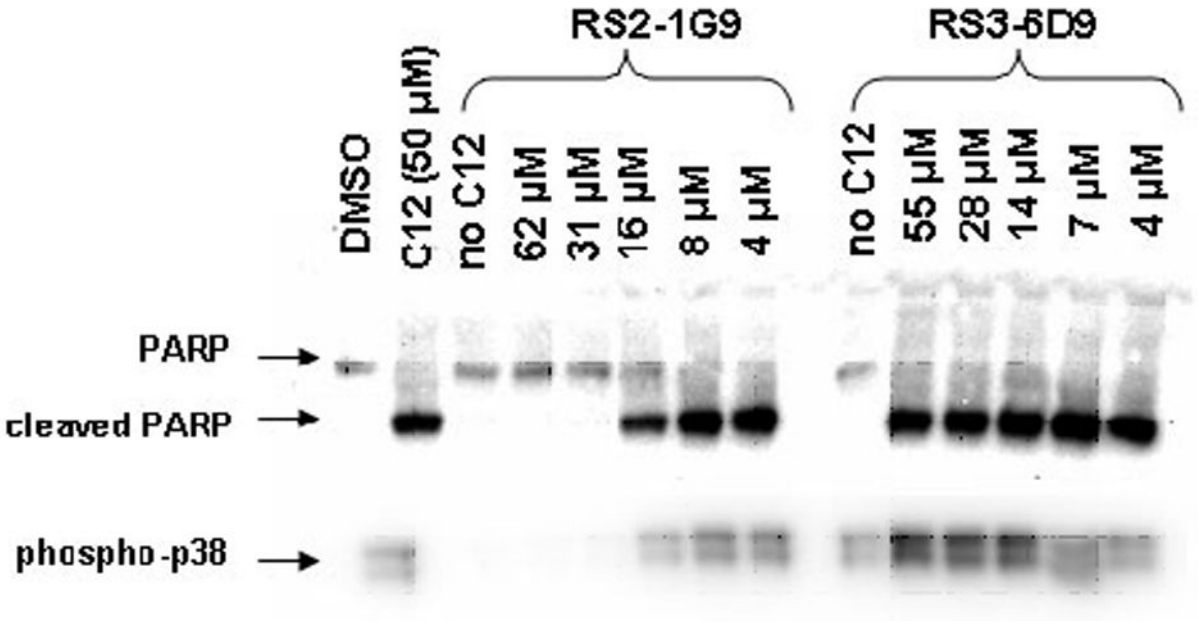

Figure 3.
Western blot analysis of BMDM protection from cytotoxic effects of 3-oxo-C_{12}-HSL by mAb RS2-1G9