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(RESEARCH ARTICLE)

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Assessment of *Pseudomonas aeruginosa* Las autoinducer and OdDHL protein conjugate on viability and functional ability of various cells *in vitro*

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Abstract

Pseudomonas aeruginosa, a major cause of nosocomial infections, has the ability to produce number of factors responsible for its virulence as well as biofilm formation which are regulated by Las and Rhl QS system. The autoinducer OdDHL of las system, play crucial role in pathogenesis of P. aeruginosa. It is involved in induction of factors responsible for the virulence of the organism as well as modulate the host immune responses. In the present study, the effect of KLH OdDHL and BSA OdDHL protein conjugate on various cells in terms of cytotoxicity and phagocytosis was assessed. Concentration of 1µM showed maximum viability of macrophages. Macrophages also showed accelerated phagocytosis, as observed by neutral red assay, which was attributed to the immune-modulatory stimulation provided by the OdDHL conjugate. Other cells like Uroepithelial cells and lymphocytes also showed no significant decrease in cell viability in presence of lower concentrations of conjugated OdDHL. Our Data suggested that interaction of OdDHL conjugated to any of the carrier protein KLH / BSA, with various cells not only improved cell viability but also improved the phagocytic ability of macrophages.

Keywords: OdDHL; Conjugation; Immunomodulation; Cell viability; Pseudomonas aeruginosa

Highlights

- Protein conjugated Las autoinducer significantly increased the cell viability of various cells in vitro
- KLH/BSA Conjugated OdDHL significantly decreased the adhesive ability of *P. aeruginosa* to uroepithelial cells
- Functionalized OdDHL increased the susceptibility of P. aeruginosa towards phagocytosis

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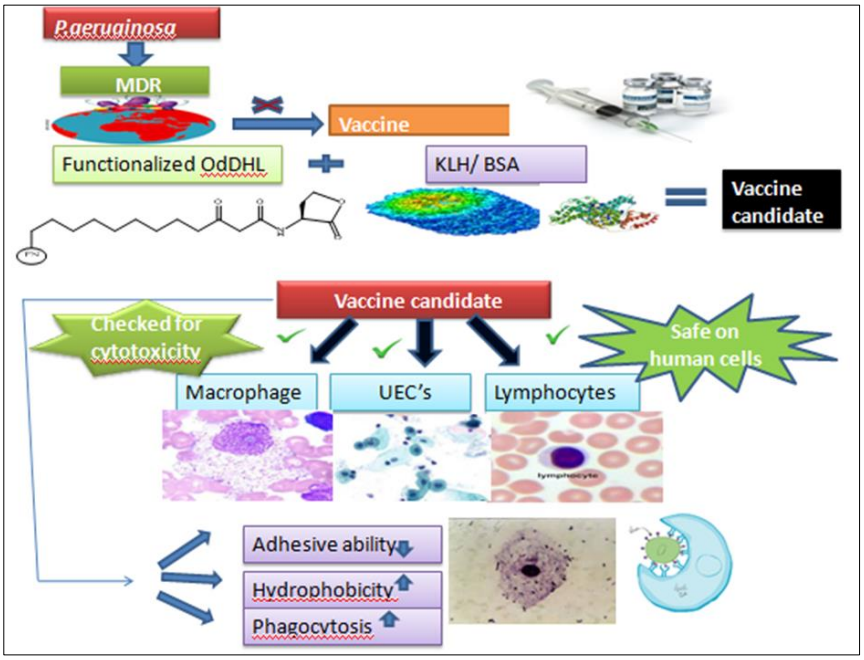
Graphical Abstract



1. Introduction

Pseudomonas aeruginosa is a nosocomial pathogen that causes various acute as well as chronic infections, such as sepsis, burn wound and pulmonary infections (Richard et al. 1999). In large number of cases, it has been seen that these infections may lead to the high degree of mortality due to systemic sepsis. *Pseudomonas* is known to produce a variety of factors responsible for the virulence such as Adhesins, Alginate, Pilli, Flagella, Lipopolysacchride (cell associated factors) Elastase, Pigment, Exoenzyme S, Endotoxin A , Hemolysin , iron binding proteins(extracellular factors) which helps in immune system evasion, tissue destruction and bacteremia (Drake and Montie 1988; Galloway 1991; Konig et al.1996; Shi and Sun 2002; Hentzer 2003). Las and Rhl are the two prime quorum sensing systems, and this communication network enables bacteria to systematically turn on and turn off by the production of small diffusible molecules called autoinducer i.e, N-3-oxo-dodeconoyl homoserine lactne (3-oxo-C12-HSL) and N-butyryl-L-homoserine lactone (C4-HSL) in a density – dependent manner (Pearson et al. 1994).

Stimulation with OdDHL and expression of the factors responsible for virulence induce production of IL-8 (chemokine interleukin) in fibroblast and bronchial epithelial cells of human lungs indicating the vital role of OdDHL's in inflammation (Smith et al.2001; Smith et al. 2002). Therefore, OdDHL is also known to exhibit immunostimulatory activities. Also, 3-oxo-C_{12} HSL in addition to 3-oxo-C_{10} HSL can act as chemoattractant for human neutrophils and can induce their migration to the site of AHL in dose dependent manner. Acyl homoserine lactones can control expression of pro-inflammatory mediators, cytokines, chemokines, enzymes, and interferones through multiple signaling pathways. The apoptotic induction of host cells mediated by OdDHL occurred through calcium dependent signalling pathway, while the proinflammatory effect of OdDHL takes place through calcium independent and peroxisome proliferator-activated receptor mediated pathway (Smith et al. 2002; Shiner et al. 2006; Kravchenko et al. 2008; Mayer et al. 2011; Karlson et al. 2021a; Glucksam-Galnoy et al. 2013).

When the recognition of OdDHL's by mammalian cells takes place, the phospholipids in model membrane systems and in T-cell membranes may directly interact with this lipophilic molecule with long acyl chain and an intact homoserine lactone ring (Davis et al. 2010). They interact with various *in vitro* eukaryotic cells and can modulate immune responses (DiMango et al. 1995; Saleh et al. 1999; Telford et al. 1998). Since these mediators are involved not only in harmonisation of innate immune response but also in employing the effector cells of adaptive immune system at the infection site to encounter the bacteria, hence blocking auto inducers as a immunoprophylaxis approach can be an encouraging strategy for the treatment of *P. aeruginosa* infections (Miyairi et al. 2006)

The treatment of the infections caused by the ESKAPE pathogens i.e. *Pseudomonas aeruginosa*, which has ability to develop resistance rapidly to multiple classes of antibiotics, is a growing challenge for intensivists (Lister et al. 2009). Quorum sensing signal molecules (QSSMs) have emerged as a new candidate for vaccine development (Smith and

Iglewski 2003b; Suga and Smith 2003). Active or passive immunization with these vaccine candidates may serve as a upcoming strategy for blocking *P. aeruginosa* pathogenicity during various infections. The progress in field of quorum sensing has demonstrated that *P. aeruginosa* OdDHL's at the site of infection interact with various immune cells and cause immunomodulation and immunostimulation (16). OdDHL has a low molecular weight (300Da) therefore it has to be conjugated with protein (KLH and BSA) so as to increase its stability and to make it immunogenic. Since all body cells may be exposed to quorum sensing signals, influence of the conjugate directly on cells was a matter of concern. Therefore, investigating the potential of conjugate to interact with the cells *in vitro* becomes imperative for furtherance of research.

2. Material and methods

2.1. Preparation of protein- 3-oxo-C_{12} HSL Conjugate

Synthesis of 12-succinyl-3-oxo-C_{12} HSL was carried out as reported by Horikawa et al. 2006 (Horikawa et al. 2006). Further conjugation with BSA and KLH protein was carried out by the synthetic method using N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride as reported previously in literature (19). Conjugates were found to be stable after one month. These were then checked for the presence of endotoxin and they were found to be endotoxin free.

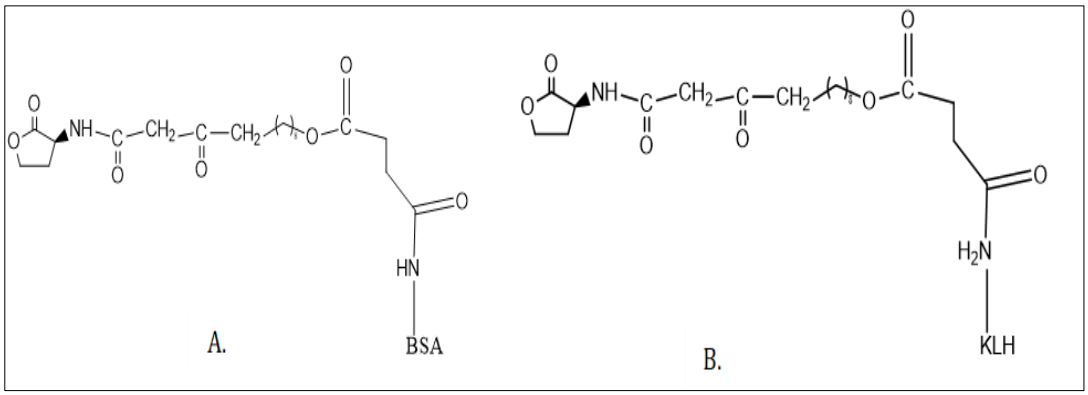


Figure 1 Structure of the 3-oxo-C_{12}-HSL- Carrier protein conjugate (A.) BSA (B) KLH

2.2. Growth Profile of P.aeruginosa

To study the effect of synthetic OdDHL and OdDHL conjugates on the growth of *P.aeruginosa* early log phase cells of *P.aeruginosa* were inoculated in luria broth containing synthetic 3-oxo-C_{12}-HSL (50µM) and different concentrations of KLH OdDHL and BSA conjugated OdDHL. Luria Broth inoculated with *P. aeruginosa* served as control. All the flasks were incubated at 37°C under shaking conditions. To study the growth kinetics, 1ml aliquots were removed at different time intervals (0, 2, 4, 6, 8, 10, 12 and 24 h) and absorbance was taken at 595nm.

2.2.1. Mice and ethics statement

Female 6–8 weeks old BALB/c mice (weighing 20 to 30 g), bred and procured from the Central Animal House, Panjab University, Chandigarh, India were used in the present study. Mice were housed in polypropylene cages, bedded with dry and clean husk with proper ventilation of the room. Animals were fed on antibiotic-free synthetic diet in the form of pellets (Hindustan Levers Ltd., Mumbai, India) and *ad libitum water*. The ethical clearance to perform experiment was approved by Institutional Animal Ethics Committee of Panjab University, Chandigarh, India (Approval No. IAEC/119). All the experimental protocols were done according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.3. Cell cultures

2.3.1. Macrophages

Macrophages were derived from peritoneal cavity. After swabbing the abdomen, 5ml of ice cold RPMI along with 10% FCS was injected into the peritoneal cavity. After injection peritoneum was massaged to dislodge any attached cells into the RPMI. Peritoneal fluid was collected which was pooled and centrifuged at 1800rpm for 10min at 4°C. Cells were washed twice with PBS and count of macrophages was adjusted to 10^5 cells/ml.

2.3.2. Uroepithelial cells cultures

Normal human urine was collected and pooled. Urine was centrifuged at 5000rpm for 10 minutes at room temperature and pellet was washed twice with sterile PBS (0.01 M, pH 7.2) Number of live uroepithelial cells was adjusted to 10^5 cells/ml. Percentage of viable cells was determined by dye exclusion method using 0.01% trypan blue (dead cells appeared stained).

2.3.3. Lymphocytes

Human blood in EDTA was collected and plasma was separated. Lymphocytes were collected by overlaying 6ml of plasma on 3ml isopaque ficoll column and Centrifuging at 1800rpm for 30 minutes in cold centrifuge (4°C). The interface layer containing cells was collected in another tube and washed twice by the addition of RPMI. Supernatant was discarded and cells were resuspended in 3 ml of RPMI. Number of live cells was adjusted to 10^5 cells/ml. The percentage of viable cells was determined by dye exclusion method by using 0.01% trypan blue (dead cells appeared stained).

2.4. Cell viability assays

2.4.1. [MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide)] Assay

MTT assay was performed by the method of Mosmann, 1983; Wilson, 2000 (Mosmann 1983; Wilson 2000)**.** Viability of cells treated with 3-oxo-C_{12}-HSL and conjugates was estimated using MTT assay. Macrophages were seeded in 96 well plate at a density of 2× 10^5 cells/ml, and cultured overnight and incubated in presence and absence of synthetic 3-oxo-C_{12}-HSL (50µM) and 1µM , 10 µM & 100µM of KLH OdDHL and BSA OdDHL at 37º C for 2-24 hrs in CO_2 incubator. 50µl of MTT solution (5mg/ml) was added to each well. Viable cells were stained with MTT dye for 4 h. Media was removed and the formazan crystal was dissolved by adding 200µl of 100% ethanol. Absorbance at 490nm was recorded. Percent viability of cells was calculated by comparison to that of untreated control cells.

2.4.2. SRB (Sulphorhodamine B) Assay

The method of Skehan, (1990) was employed to check the viability of cells (Skehan et al. 1990). Viability of cells treated with 3-oxo-C_{12}-HSL and conjugates was measured using SRB assay. Macrophages were seeded in 96 well plate at a density of 2× 10^5 cells/ml, and cultured for overnight and then incubated in the presence and absence of synthetic 3-oxo-C_{12}-HSL (50µM) and 1µM , 10 µM & 100µM of KLH OdDHL and BSA conjugated OdDHL at 37º C for 2-24 hrs in CO_2 incubator. 25µl of 50% TCA (Trichloro acetic acid) was added over conjugates to give overall concentration of 10%. Plates were incubated for 1h at 4^0 C. Supernatant was discarded and wells were washed five times with tap water. Air dried plates were stained with 100 µl SRB (sulphorhodamine B) using stock solution (0.4%) for 30 minutes at room temperature. Unbound dye was removed by adding 1% acetic acid and washed 3-4 times and air dried. 100µl of 10mM Tris base was added to solublize the dye. Absorbance at 540nm was monitored. The percent viability of cells was calculated in comparison to that of untreated control cells.

2.4.3. Uroepithelial Adhesion Assay

Uroepithelial cells (UECs) were isolated by method of Mittal et al., 2008a (Mittal et al. 2008a).

In the adhesion assay, mixture consisted of 1 ml each of bacteria grown in presence of synthetic 3-oxo-C_{12}-HSL (50µM) and 1µM, 10 µM & 100µM of KLH OdDHL and BSA OdDHL and UECs. These were then incubated at 37°C for 1 h in a shaking water bath. Tubes were centrifuged and cells were washed three times with PBS to remove any unattached bacteria. Smears of the cells were prepared, fixed with methanol and stained with Giemsa stain. Bacteria adhering to 30 UECs were counted and the average number of bacteria adhering per UEC was calculated.

2.4.4. Cell Surface Hydrophobicity Assay

The method of Rosenberg et al., (1986) was used to measure cell surface hydrophobicity. Planktonic cells (10^8cfu/ml) were centrifuged at 10,000 rpm, washed and resuspended in 3ml of PBS (pH 7.2) (Rosenberg et al. 1986). Optical density was measured by spectrophotometer at 600nm and taken as initial OD. $_{(i)}$ Cells were vortexed for 1 minute with 0.35ml of xylene and left for 30 minutes to allow phase separation. O.D of the aqueous phase was again measured at 600nm and expressed as final O.D. $_{(f)}$ Surface hydrophobicity of each strain was expressed as percent hydrophobicity.

2.4.5. Neutral Red Uptake Assay

The method of Yang et al., (2013) was used to perform neutral red uptake assa**y.** Macrophages were cultured in 96 well microtitre plate (Yang et al. 2013). Synthetic OdDHL and KLH as well as BSA conjugated OdDHL were added to wells.

DMSO was taken as control. After incubation of 2 hours, 200µl of neutral red solution was added and incubated for 1 hour. The supernatant was discarded and the cells in 96-well plates were washed twice with PBS to remove the neutral red dye that was not phagocytized by the cells. Then cell lysate (ethanol and 0.01% acetic acid at the ratio 1:1200 µl/well) was added to lyse the cells, incubated at 4°C overnight and Optical density at 490nm was measured by a microplate reader.

3. Results

3.1. Growth curve analysis

Growth profile of *P.aeruginosa* was carried out at different time intervals in the absence and the presence of 1µM, 10µM and 100µM KLH and BSA OdDHL conjugate. Results indicated that the growth of *P.aeruginosa* was not altered in the presence of different concentrations of conjugated OdDHL .(Fig. 2a,2b).

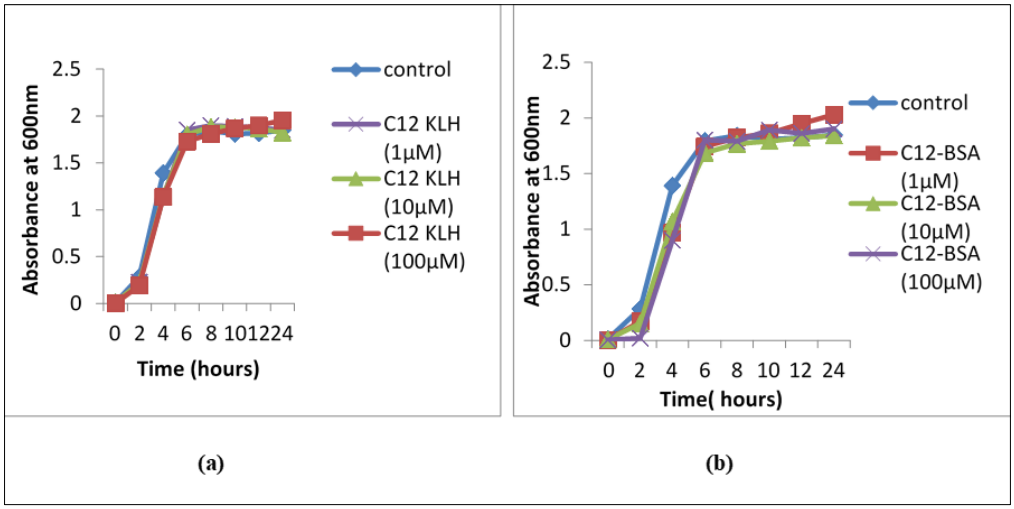


Figure 2 Growth profile of Pseudomonas aeruginosa in absence and presence of different concentrations of conjugates (a) KLH OdDHL Conjugate (b)BSA OdDHL Conjugate

3.2. Cell viability assays

3.2.1. L oss of viability of macrophages caused by synthetic as well as conjugated OdDHL.

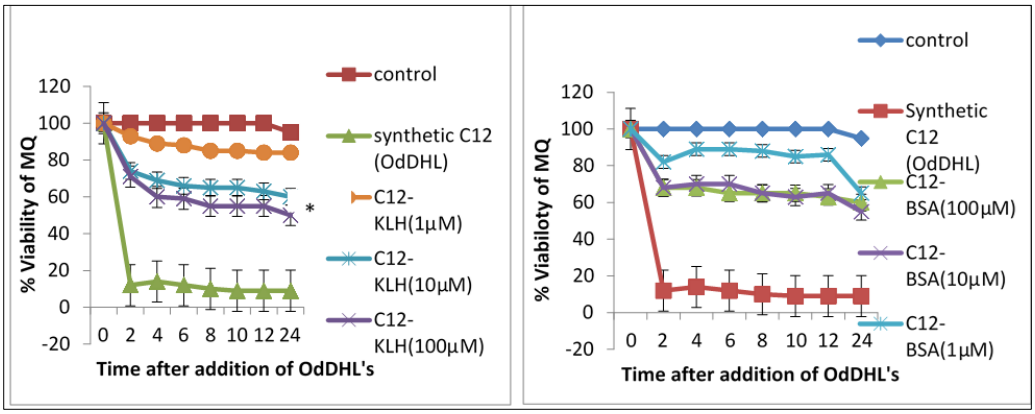


Figure 3 Effect of synthetic OdDHL, BSA and KLH conjugated OdDHL on the viability of macrophages at different time intervals by MTT assay. *p<0.001; **p<0.05

Loss of cell viability was performed through MTT assay. KLH conjugated OdDHL and BSA conjugated OdDHL were incubated with macrophages for 2-24 hours. At 2 hours of incubation, macrophages showed surprisingly good survival rate in time dependent manner in presence of low concentrations of KLH OdDHL (1µM & 10µM) ($p<0.05$)(Fig. 3) as compared to the BSA OdDHL. Best survival rate of 92% was found in presence of 1µM concentration of KLH conjugated

OdDHL which then decreased to 80% after 24 hours of incubation. However synthetic OdDHL(<20%) which was taken as control showed drastic loss of viability ($p<0.001$) (Fig. 3)

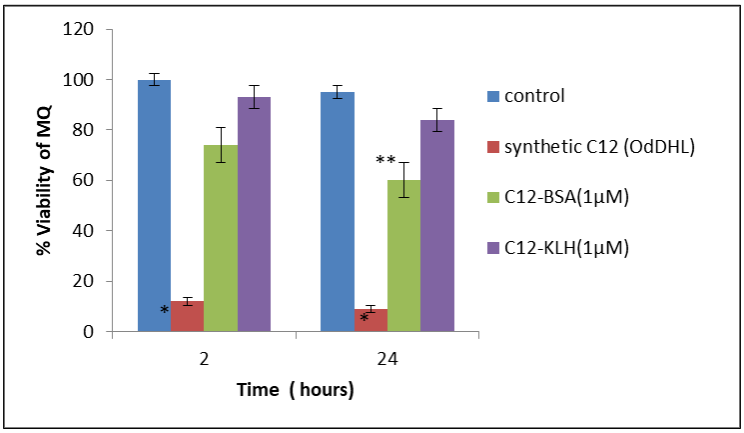


Figure 4 Effect of synthetic OdDHL and BSA & KLH conjugated OdDHL on the viability of macrophages by MTT assay. *p<0.001, **p<0.05

3.3. Comparative cell viability of macrophages by MTT and SRB

Cell viability was also checked with the SRB assay. The SRB assay showed comparative results with MTT assay (Fig. 5). 93% and 80% cell viability was obtained when macrophages were incubated with C12-KLH and C12-BSA and tested by MTT assay whereas SRB results indicated cell viability of 90% & 70% at 2 hr time interval.

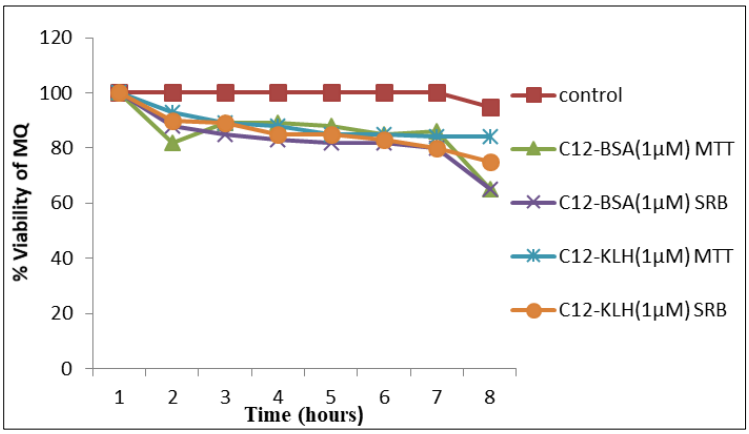


Figure 5 Comparative cell viability of macrophages in the presence of 1µM of BSA and KLH conjugated OdDHL by MTT and SRB assay

3.4. Senstivities of various kinds of cells to synthetic, KLH and BSA conjugated OdDHL's.

Uroepithelial cells and lymphocytes were collected from human urine and blood and were incubated with 50 µM synthetic OdDHL and 1µM BSA as well as KLH- conjugated OdDHL for 2 and 24 h to examine whether the effects of OdDHL's on cell viability is specific to macrophages or to other types of cells as well. Cell viability was compared with that of the same type of cells, incubated without OdDHL. It was observed that synthetic OdDHL induced significant loss of viability in uroepithelial cells as well as lymphocytes ($p<0.001$). In contrast, the uroepithelial cells and lymphocytes showed good survival rate of 65- 85% in presence of KLH and BSA conjugated OdDHL till 12 hr. ($p<0.05$) (Fig. 6)

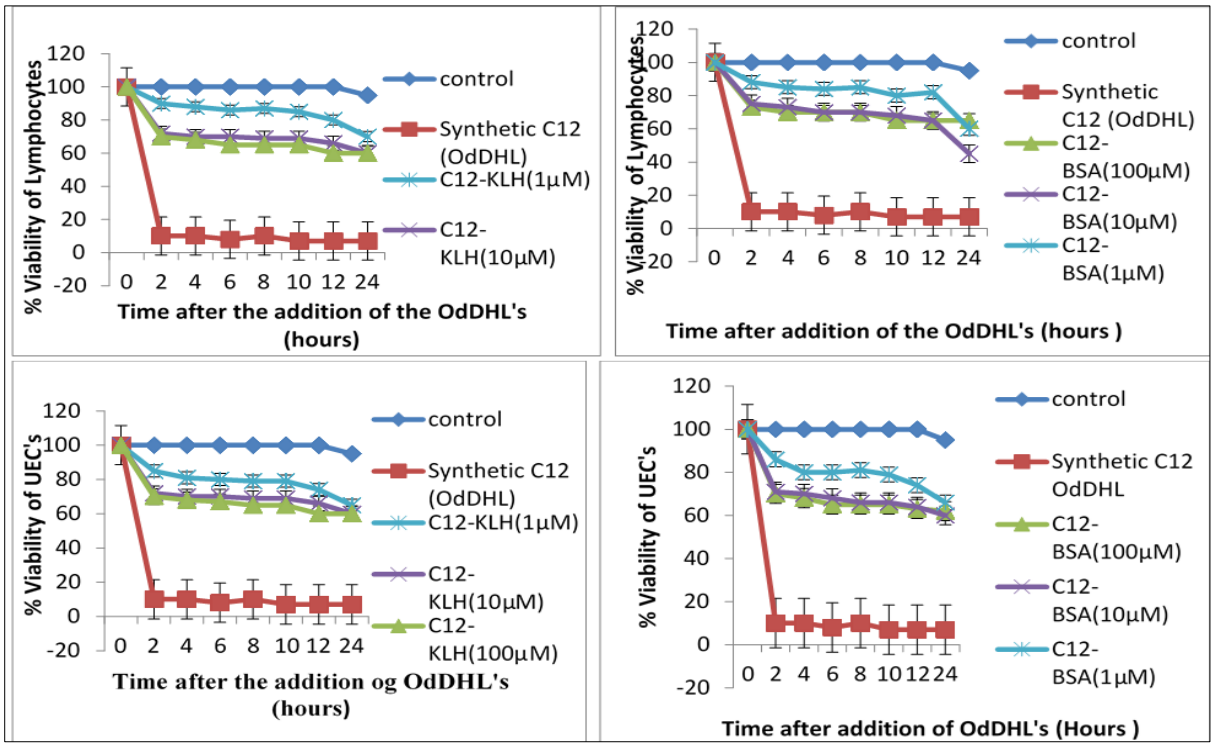


Figure 6 Effect of synthetic , BSA and KLH conjugated OdDHL on the viability of UECs & lymphocytes at different time intervals by MTT assay. *p<0.001; **p<0.05

3.5. Adhesive ability of *P.aeruginosa*

Ability of PAO1 to adhere to uroepithelial cells was determined by employing the method of Sharma *et al* ., (1987). It was observed that on an average a total of 18 and 16 bacteria adhered to each uroepithelial cell in case of control and in presence of synthetic OdDHL, whereas in case of PAO1 grown in presence of 1µM of BSA and KLH conjugated OdDHL, significant reduction (p< 0.001) in adherence of *P.aeruginosa* to UEC's was observed. Apparently KLH OdDHL reduced the number of PAO1 adhering to UEC's approximately by 50% (Fig. 7).

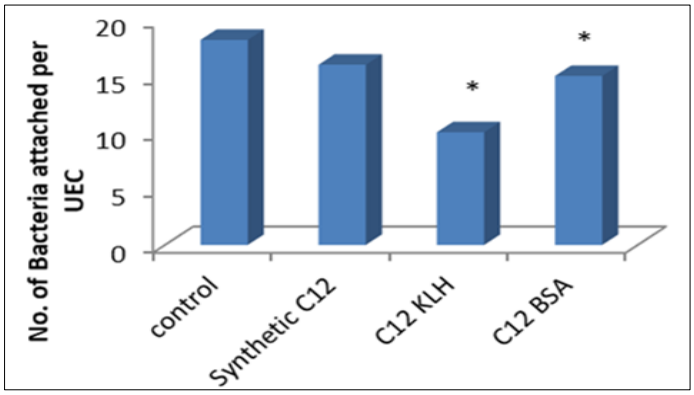


Figure 7 Uroepithelial cell adherence of *P.aeruginosa* in presence and absence of Synthetic and conjugated OdDHL. *p<0.001

3.6. Cell surface hydrophobicity

Cell surface hydrophobicity was performed. Control *P.aeruginosa* showed hydrophobicity of 55%. In presence of Synthetic OdDHL, hydrophobicity of *P.aeruginosa* decreased to 53%. Despite of the less adhesive ability, increased hydrophobicity of *P.aeruginosa* was observed in presence of KLH OdDHL and BSA OdDHL where the cell surface hydrophocity was found to be 63% and 64% (p<0.05).(Fig. 8).

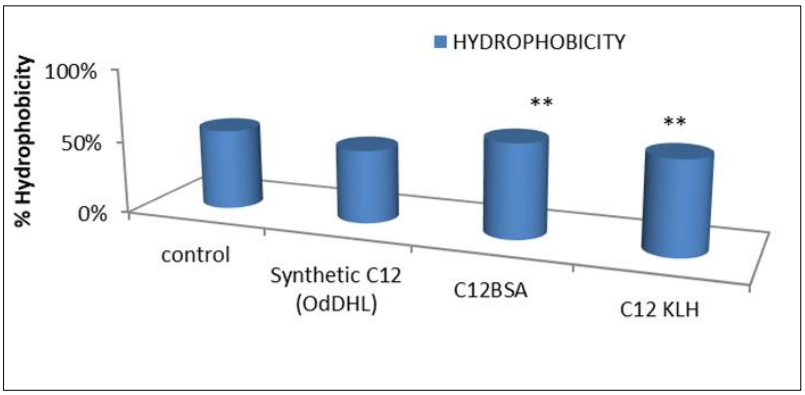


Figure 8 Cell surface hydrophobicity of *P.aeruginosa* in absence and presence of synthetic OdDHL and conjugated OdDHL. **P<0.05

3.7. Neutral red uptake assay

The phagocytic ability of macrophages was measured by neutral red uptake assay.. The Phagocytosis of macrophages was decreased significantly in presence of synthetic OdDHL ($p<0.05$). Significant increased phagocytosis by neutral red uptake assay was observed in presence of 1µM of BSA and KLH conjugated OdDHL as compared to control ($p<0.05$)(Fig. 9a , 9b).

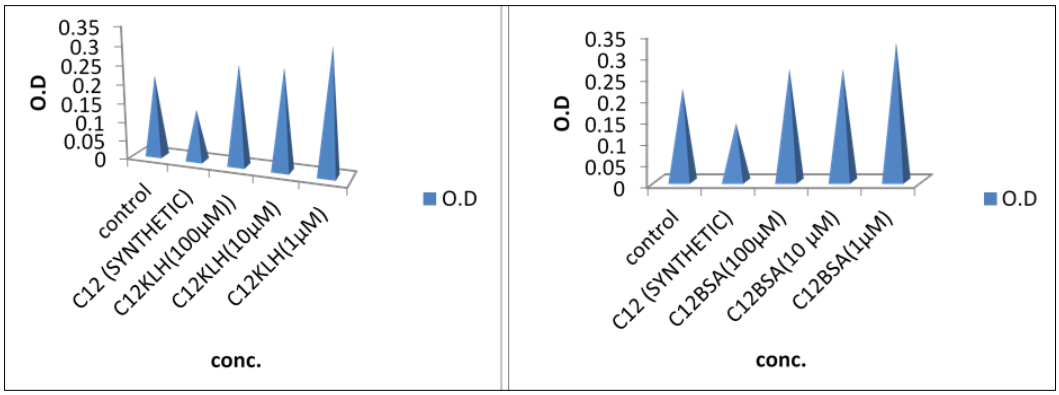


Figure 9 Effect of (a.) C12-KLH and (b.) C12-BSA on the phagocytosis measured by neutral red uptake assay. **p<0.05

4. Discussion

The results of the present study, for the first time, have demonstrated the ability of OdDHL to interact with host cells including epithelial cells and cells of non-specific and specific immune responses. OdDHL represents the AHL molecule produced by the las quorum sensing system. Since Cell to cell signaling cascade has Las system as central and main signaling system which plays a vital role in pathogenesis of *P.aeruginosa*, hence OdDHL was selected for this study. Recently it has become quite apparent that OdDHL is important for regulation of bacterial genes responsible for virulence. These studies further reinforce the current concept that *Pseudomonas* AHLs are not only crucial in the regulation of the expression of genes responsible for virulence but also can modulate immune responses. However, not many studies are available relating to its interaction with eukaryotic cells and the host immune system.

Since the molecular weight of OdDHL is approximately 300Da, making it a hapten, natural antibodies to this molecule may not be produced in animals and human, even in individuals infected with *P.aeruginosa*. Therefore, in the present study, the conjugation reaction between hapten and protein was carried out by activated ester method as previously describe (Hasoda et al. 1979). BSA as well as KLH was bound to C12 position of acyl chain. KLH (Keyhole limpet hemocyanin) is a copper containing protein that is found in Arthropods, and is known to act as ideal carrier for use in mammalian host because it has higher immunogenicity. Although the solubility in water is limited but turbidity doesn't affect immunogenicity resulting in suspension which can be successfully used for immunization of mice. BSA (BOVINE SERUM ALBUMIN) is a plasma protein in cattle that is one of the most stable and soluble albumins available. BSA is also

a popular carrier protein of MW 67×10^3 Da including 59 lysines, out of these approx. 30-35 are accessible for use in linker conjugation for weakly antigenic compounds. Studies have shown that this position of conjugation of carrier protein to OdDHL might be important for maximum efficacy to induce specific antibody in the pathogenesis of *P.aeruginosa* (Rambaugh et al. 1999; Singh et al. 2000; Donabedian 2003). Protein conjugation not only increases the molecular weight of OdDHL, but can also result in deviation of the target in the binding site in cells. These findings indicate that if this conjugate is used *in vivo*, this could have protective effect. Moreover, there can be antibodies against the conjugate *in vivo* leading to enhancement in protection (Miyairi et al. 2006).

In the present study, first of all, the effect of different concentrations of BSA conjugated OdDHL and KLH conjugated OdDHL was checked on the growth of *P.aeruginosa*. All the three concentrations (1µM , 10µM , 100µM) did not have any effect on the growth profile of the organism .To the best of our knowledge , the effect of OdDHL on growth of *P.aeruginosa* has not been investigated previously. However, some reports are available on the effect of supernatant of *P.aeruginosa* on the growth of other bacteria in planktonic state (Qin et al. 2009). The results showed that growth of *S. aureus* was inhibited in a concentration dependent manner in the presence of OdDHL , an AHL produced by *P. aeruginosa* (Qazi et al. 2006). This was observed at a concentration of 30µM or above and growth was completely inhibited at 75µM indicating that OdDHL of *P.aeruginosa* confers a competitive advantage to *P.aeruginosa* in host tissues that are also infected by *S.aureus*.

Once inside the host tissue, various cells can be the targets of P. aeruginosa induced apoptosis where AHLs can be the active molecules (Hauser et al. 1999; Bruno et al. 2000; Kaufman et al. 2000). It has been seen that patients suffering from cystic fibrosis, colonized with *P.aeruginosa*, showed presence of OdDHL in the sputum. In some areas of site of infection, concentration equivalent to or higher than 12-50µM/L can be found (Erickson et al. 2002). Another study has shown that biofilms of *P.aeruginosa* can produce up to 300-600µM OdDHL, which is significantly higher than that observed in planktonic cells indicating their active participation in diseases pathogenesis (Charlton et al. 2000). In the present study, the effect of 50µM synthetic OdDHL and different concentrations of BSA conjugated and KLH conjugated OdDHL was studied on various cells. The concentration of dose of synthetic OdDHL was selected based on the studies available in literature. Wide range of concentration was employed for conjugates since none of the concentration was affecting the growth of *P. aeruginosa*. Effect of synthetic OdDHL was studied on the viability of macrophages which showed only 10% viability indicating that OdDHL was responsible for the cell death of macrophages.Tateda et al. (2003) have reported that when bone marrow derived macrophages were incubated with synthetic OdDHL, significant loss of viability was observed in concentration (12µM-50µM) and time (1-24 hr) dependent manner (Tateda et al. 2003). Also, the morphological alterations, indicative of apoptosis, were observed. However, in the present study, KLH conjugated OdDHL (1µM) showed maximum viability of macrophages (92%). BSA conjugated OdDHL on the other hand showed the viability of 85%. Protein conjugation not only increased the molecular weight of OdDHL but also could have resulted in deviation of the binding site as target in the cells. These results indicate that if this conjugate is used *in vivo*, this can have protective effect on macrophages. Moreover, *in vivo* there can be antibodies against the conjugate which can lead to further enhancement in protection of the host against *P.aeruginosa* infection. The response was found to be concentration dependent.

Macrophages play a significant role in innate immune responses. They are scattered throughout the mammalian organs and vary in their morphology depending on their state of activity (Elhelu 1983). Induction of cell death may thus be a powerful weapon used by bacteria as evasion strategy of host defenses (Tateda et al. 2003). Obtained data demonstrated that the structure of OdDHL, in addition to its backbone and side chain length may be crucial for the maximal activity, suggesting that there is a specific binding molecule or site in cells which is a target for OdDHL. Hence identification of a eukaryotic molecular target for OdDHL can be a promising research option. In addition to macrophages, other cells like epithelial cells and lymphocyres are eukaryotic targets of *P.aeruginosa* induced apoptosis (Hauser et al. 1999; Bruno et al. 2000; Kaufmann et al. 2000). The present study revealed that macrophages, uroepithelial cells and lymphocytes showed good survival rate in the presence of KLH as well as BSA conjugated OdDHL as compared to synthetic OdDHL (50µM) which showed reduction in cell viability. Tateda et al (2003) have also reported the reduction in viability of various cells *in vitro* in the presence of 50µM of synthetic OdDHL (Tateda et al. 2003). The results indicated that conjugation of OdDHL with carrier protein KLH/BSA improved the cell viability. Specific ability of OdDHL to induce apoptosis in certain types of cells (macrophages and neutrophils) can provide advantage to the invading pathogen and can act as their survival strategy. Direct evidence of the involvement of QS signal molecule in neutrophil influx at the site of infection through stimulation of 1L-8 production and MIP-2 production exists in literature (DiMango et al. 1995).

The first step in bacterial infection is adherence and colonization of bacteria to the surface . So it is very important to study the adhesive ability of *P.aeruginosa* in presence of synthetic and conjugated OdDHL. Effect of synthetic as well as conjugated OdDHL on adhesive ability of *P.aeruginosa* to uroepithelial cells was determined in the present study. It was

observed that the conjugated OdDHL (1μM concentration) was able to reduce the number of adhering bacteria to uroepithelial cells by approximately 50%. However, to the best of our knowledge, no study regarding the attachment of the organism in the presence of conjugated OdDHL is available. Present study signifies that conjugated OdDHL not only prevented cell cytotoxicity but also decreased the adhesive ability of *P.aeruginosa* to epithelial cells. Alteration in the adherence potential of Gram negative organisms to eukaryotic cells , observed in the present study, may be due to alteration in the membrane receptors on the host. Davis et al., (2010) reported that the pre treatment of lymphocytes with micromolar concentrations of OdDHL causes significant reduction in the binding capacity of saquinavir (antiretroviral drug), which is a HIV-1 protease inhibitor to membrane protein P group and increase in dissociation constant by preventing the cleavage of viral polyprotein, preventing maturation of virus.

For adherence and colonization, cell surface hydrophobicity (CSH) is also considered as an important parameter (Absolom et al. 1983; Costa et al. 2006). Studies have shown that hydrophobic cells are more invasive and can result in infections which are difficult to treat (Doyle 2000). On the other hand the positive aspect of hydrophobic organisms can be the bioremediation i.e. cleaning up the environment employing the use of microbes. In the present study, the CSH of *P.aeruginosa* in presence of synthetic as well as conjugated OdDHL as compared to the control(in absence of OdDHL) was checked. Maximum and significant level of CSH (63% and 64%) was observed in presence of KLH conjugated and BSA Conjugated OdDHL respectively. Despite of increased hydrophobicity of conjugated OdDHL, it was able to reduce the adhesive ability of *P.aeruginosa* to UECs. Despite development of various methods of recogonition of hydrophobic properties of microorganism, knowledge about the process of adhesion of microorganism is still insufficient. Reports have indicated that the ability to regulate CSH (cell surfave hydrophobicity) allow microorganism either to promote or hinder attachment (Rosenberg et al. 1981).

Phagocytosis of macrophages after treatment with different concentration of conjugated OdDHL's (1μM, 10μM, 100μM) and 50μM synthetic OdDHL was studied in the present study. Pinocytosis of neutral red, which is weak cationic dye that readily penetrates cell membrane by non ionic diffusion, is indicative of phagocytosis of *P.aeruginosa*. When cell is intact, it binds and accumulates with anionic sites in lysosome, becomes charged and doesn't freely pass out into the cytoplasm. Phagocytosis was decreased in case of synthetic OdDHL but increased in case of conjugated OdDHL,s as compared to the control. The decline in phagocytosis after the pre-treatment of macrophages with synthetic OdDHL may be due to the decline in cell viability as shown by MTT and SRB assays. It was shown in the previous findings that effect of synthetic OdDHL on phagocytic capacity of macrophages depends on the concentration (Vikstrom et al. 2005). The higher concentration of synthetic OdDHL significantly reduced phagocytosis. Yang *et al* (2013), also showed decline in pinocytosis of neutral red and phagocytosis of *P.aeruginosa* after treatment with different concentration of OdDHL as compared with the control group (Yang et al. 2013). Since macrophages are the main cells responsible for clearing the dead bacteria, necrosed tissue and cell debris at body injury sites, alteration in their main function i.e. phagocytosis is indicative of immunoinhibited property of OdDHL. This may hamper their role in healing process and in providing anti-infective immunity. The improved phagocytosis in case of conjugated OdDHL might be attributed to increased molecular size of the conjugated OdDHL which led to engulfment by macrophages followed by secretion of cytokines. Results of the present study indicate the immunomodulatory role of conjugated OdDHL.

5. Conclusion

Recent progress in QS research as well as the results of the present study demonstrated that Pseudomonas AHLs can affect immune responses hence acting as immunomodulator. Based on the results of the present study, OdDHL molecule can be considered as the probable candidate for vaccine development. OdDHL molecule when conjugated with KLH may offer a better advantage. However, further investigation regarding maximum induction of protective efficacy *in vivo*, safety and possible adverse reactions, if any, are required to certify its use as a noval vaccine strategy against *P.aeruginosa* infections.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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