

**EFFECT OF ATORVASTATIN AND ROSUVASTATIN ON QUORUM SENSING,  
BIOFILM FORMATION AND BACTERIAL MOTILITIES OF *PSEUDOMONAS  
AERUGINOSA*****SAJAL SARABHAI<sup>1</sup>, LOVEDEEP KAUR DHALIWAL<sup>1</sup>,  
NEENA CAPALASH<sup>2</sup> AND PRINCE SHARMA<sup>1\*</sup>**<sup>1</sup>*Department of Microbiology, Panjab University, Chandigarh*<sup>2</sup>*Department of Biotechnology, Panjab University, Chandigarh***ABSTRACT**

Statins have been widely studied as antibacterial compounds, but the mechanism of their bacterial inhibition is still not known. In the present investigation two statins, atorvastatin and rosuvastatin, were tested for their antimicrobial activity against *Pseudomonas aeruginosa* and both showed MIC of 625µg/ml. Both the statins shared structural similarity with quorum sensing signal molecule of Gram negative bacteria i.e. N-acylhomoserine lactone (AHL) but showed no functional agonist or antagonist activity, as indicated by QS-deficient *Agrobacterium tumefaciens* A136 biosensor strain. Atorvastatin increased the flagella-mediated swarming, swimming and type IV pili-mediated twitching motilities and biofilm formation in *P. aeruginosa* PAO1 while rosuvastatin decreased all three motilities slightly but had no effect on biofilm formation.

**KEYWORDS:** Atorvastatin /Rosuvastatin/ Biofilm/ Motilities/ *Pseudomonas aeruginosa*/ Quorum sensing

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## INTRODUCTION

In recent years, several studies have supported that statin treatment is associated with a better prognosis in severe bacterial and viral infections<sup>1,2</sup>. Statins therapy effectively attenuated the clinical course of experimental sepsis in animal model<sup>3,4</sup>. Experimental studies have also shown that statins affect the modulation of cytokine cascade and the organization of immunological response to respiratory infections. Most observational studies published to date support the idea that the use of statins may improve the prognosis of Community Associated Pneumonia (CAP), mediated by bacteria, viruses, fungi and different parasites. Statins have been reported to be antimicrobial in nature. Growth of methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), vancomycin-susceptible enterococci (VSE), vancomycin-resistant enterococci (VRE), *Acinetobacter baumannii*, *Streptococcus epidermidis*, *Enterobacter aerogenes* and *Proteus mirabilis* (2,5) is inhibited by statins. HMG-CoA-reductase of prokaryotes showed 10,000 times lower affinity for statins as compared to eukaryotic or human HMG-CoA-reductase<sup>1</sup>. Therefore, it is unlikely that antimicrobial effect works through this mechanism. Statins are cytotoxic in nature and lead to apoptosis of eukaryotic cells and this could be a plausible mechanism behind antibacterial activity<sup>6</sup>. In fungi, statins reduce the membrane fluidity by interfering with ergosterol biosynthesis. However, the exact mechanism behind the anti-microbial activity of statins is still unexplored. Quorum sensing (QS) is an important intercellular communication system in bacteria and plays an important role in their pathogenesis. Interference with the QS affects the severity of bacterial infections<sup>7</sup>. As statins showed structural similarity with quorum sensing signal molecules (N-acylhomoserine lactone) of Gram negative bacteria, therefore, this study hypothesized that reduction in the severity of bacterial infections by statins could be due to QS interference. Atorvastatin and rosuvastatin are widely used anti-cholesterol drugs which are reported to be antimicrobial in nature<sup>5</sup>. The present study investigates the effect of atorvastatin and rosuvastatin on the quorum sensing and virulence factors viz.

biofilm formation and motility of *Pseudomonas aeruginosa* PAO1.

## MATERIALS AND METHODS

### Chemicals

Atorvastatin(3*R*,5*R*)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid) and Rosuvastatin(3*R*,5*S*,6*E*)-7-[4-(4-fluorophenyl)-2-(*N*-methylmethanesulfonamido)-6-(propan-2-yl)pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoic acid) calcium salts were obtained from InnovaCapTab, India (98.8% purity) and dissolved in DMSO to a stock concentration of 20 mg/ml.

### Bacterial strains and culture media

*Pseudomonas aeruginosa* PAO1<sup>8</sup> was grown in Luria Bertani broth at 37°C unless otherwise stated. Biosensor strain *Agrobacterium tumefaciens* A136<sup>9</sup> was grown in LB at 30°C, supplemented with 50 µg/ml of spectinomycin and 5 µg/ml tetracycline.

### Minimum inhibitory concentration

MIC of statins (atorvastatin and rosuvastatin) for PAO1 was determined by broth microdilution assay as recommended by National Committee for Clinical Laboratory Standards (NCCLS), Wayne county, USA, using 96 well microtiter plate

### Quorum Sensing activity of statins

#### (i) Disc diffusion assay

100 µl of overnight grown culture of *A. tumefaciens* A136 was spread on the LB agar plate. For agonist activity, each statin at 50 and 100 µg/ml was loaded onto sterile whatman filter paper disks (6 mm diameter) along with X-gal (20mg/ml) while for antagonist activity, 10 µM 3-oxoC<sub>12</sub> homoserine lactone was also supplemented to the mixture. Curcumin (3 µg/ml) and 3-oxo-C<sub>12</sub> HSL served as positive controls for antagonist and agonist activities, respectively. Plates were incubated at 30°C overnight and checked for the zone of colour development/ inhibition to determine agonist or antagonist activities, respectively<sup>10</sup>.

**(ii) Liquid bioassay**

Overnight grown culture of *A. tumefaciens* A136 was diluted with LB to obtained OD<sub>600nm</sub> 0.5-0.6. For checking the agonist activity, atorvastatin and rosuvastatin were added at concentration of 50 and 100 µg/ml while for antagonist activity, 3-oxo-C<sub>12</sub> HSL were added at 10µM concentration with statins. The culture was then incubated at 30°C, 180 rpm for 2h and β-galactosidase activity was determined by ONPG method<sup>11</sup>.

**Effect of statins on PAO1 virulence****(i) Surface motilities**

*P. aeruginosa* PAO1 was grown in LB broth at 37°C and 180 rpm for 16 h. For swimming, LB with 0.3% agar was used. Swarming was observed on M9 minimal medium with 0.5% each of glucose, peptone and agar supplemented with 50 and 100µg/ml of statin. 5 µl of bacterial inoculum (OD<sub>600nm</sub>~ 1.5) was spotted on the medium and incubated at 37°C for 48h. The diameter of swimming or swarming zone was measured<sup>12</sup>. For twitching, King's B medium (0.5%peptone, 0.15% of Mg<sub>2</sub>SO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub> and 1% each of glycerol and agar) was used. The medium was supplemented with 50 and 100 µg/ml of statin. Fresh *P. aeruginosa* culture, grown in LB agar plate, was used for stab inoculation of twitching plate. The plates were incubated for 16 h at 37°C with prolonged incubation at room temperature for two days. The agar was removed and plate was stained with 1% crystal violet for 1min and washed with sterile distilled water. The diameter of twitching zone was measured<sup>13</sup>.

**(ii) Biofilm formation**

200 µl of *P. aeruginosa* (OD<sub>600nm</sub>~1.0) culture in LB broth containing 1% glycerol and 50 or 100 µg/ml of statin was incubated for 24 h at 37°C under static conditions in 96 well polystyrene microtiter plate. Thereafter, biofilm was washed three times with phosphate buffer (50mM, pH7.0) to wash off loosely adhered planktonic bacterial cells. Subsequently, the biofilm was fixed with 200µl of methanol for 15 min. Methanol was poured off and plate was air dried. Biofilm was then stained with 200 µl of 0.5% (w/v) crystal violet for 15 min and washed with phosphate buffer (50mM, pH7.0) three times to remove excessive stain. 200µl of 95% (v/v) ethanol was added to extract bound crystal violet and biofilm index was calculated as OD 570 nm/600nm<sup>14</sup>.

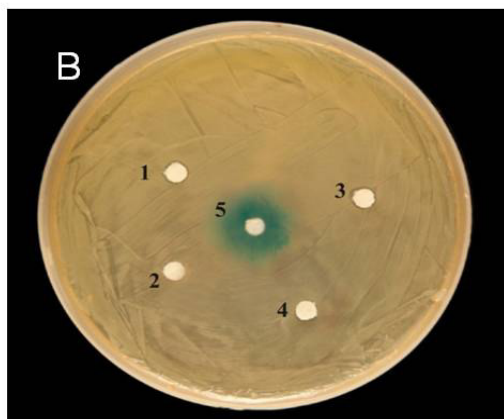
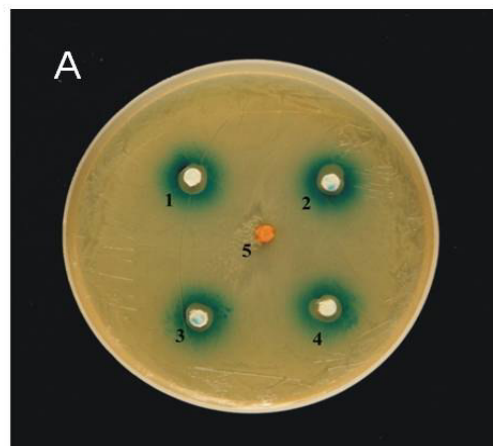
**Statistical analysis**

Statistical analyses were performed by student t tests and p≤0.05 was considered as significant. All experiments were performed in triplicates with two biological replicates

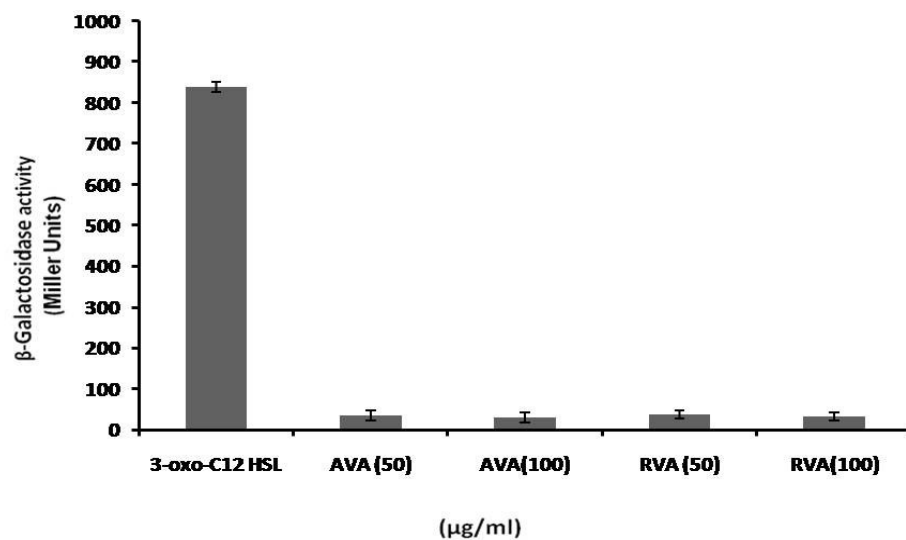
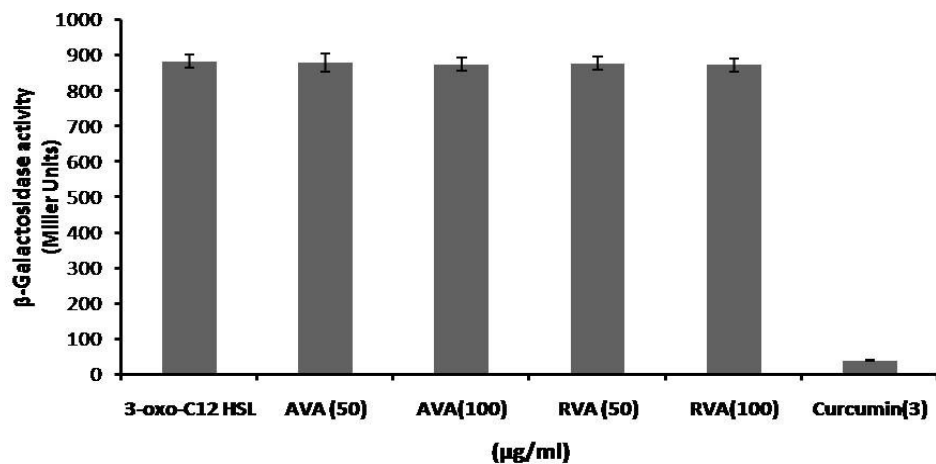
**RESULTS****QS activity of statins**

The MIC of both, atorvastatin and rosuvastatin, was 625 µg/ml for *P. aeruginosa* PAO1. Antagonist and agonist activities of statins were determined at sub-inhibitory concentration of 50 and 100 µg/ml using *A.tumefaciens* A136 biosensor strains. Both rosuvastatin and atorvastatin lacked agonist and antagonist activities as determined by both liquid bioassay and disc diffusion assay (Fig 1).

## DISC DIFFUSION ASSAY



## LIQUID BIOASSAY



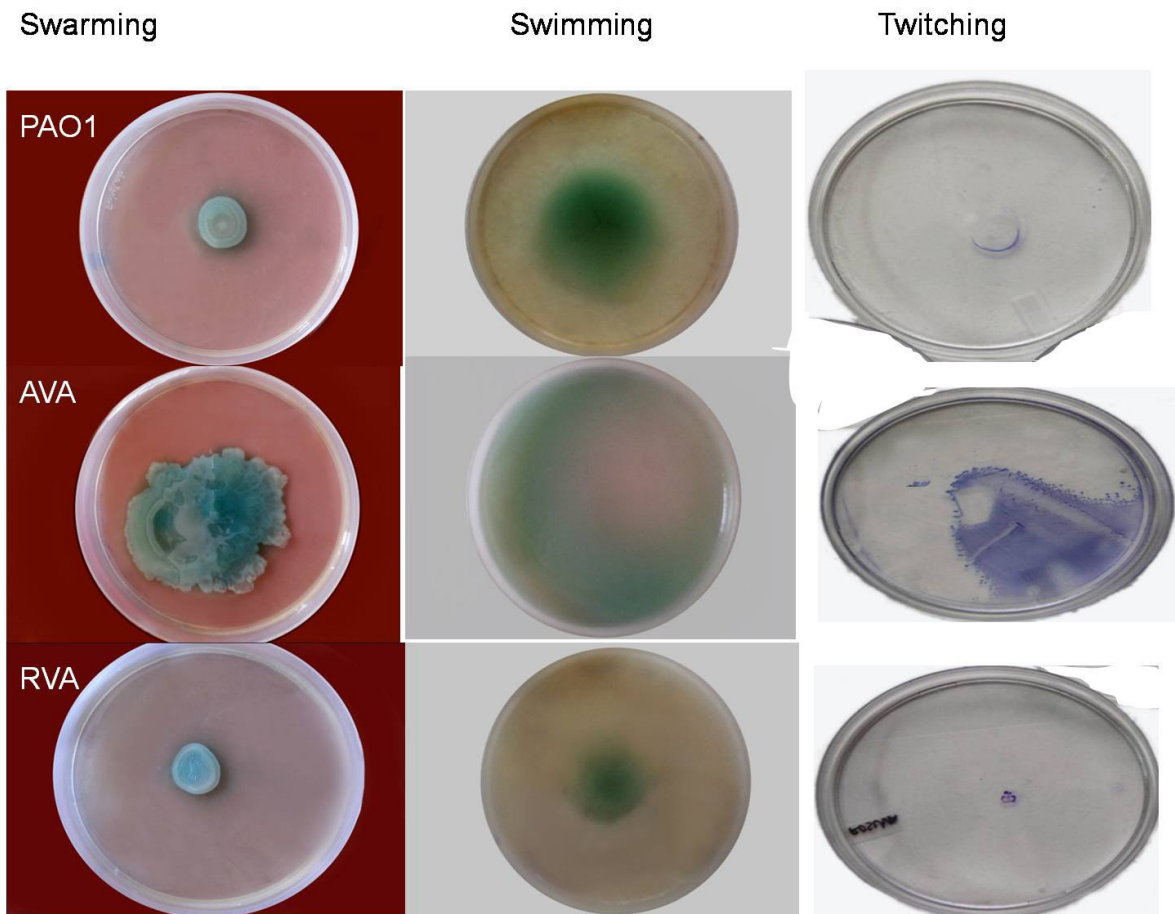
**Figure 1**

A) Antagonist and B) agonist activities of statins by disc diffusion assay and liquid bioassay using *A.tumefacines* A136. Atorvastatin (AVA)(1,2) and Rosuvastatin(RSV) (3,4) used at 50 $\mu\text{g/ml}$  and 100  $\mu\text{g/ml}$  respectively ; 5) Positive control: 3  $\mu\text{M}$  curcumin (A) and 10  $\mu\text{M}$  3-oxo-C<sub>12</sub>HSL (B).

**Surface motilities**

Atorvastatin enhanced motilities of *P. aeruginosa* PAO1. There was a significant ( $p < 0.05$ ) increase of 179, 150, 165 % in spread diameter of swarming, swimming and twitching motilities, respectively in the

presence of 200  $\mu\text{g/ml}$  atorvastatin (Fig 2A). In contrast, rosuvastatin at same concentration showed reduction in the surface motilities by 20, 25, 30% ( $p < 0.05$ ) in spread diameter of swarming, swimming and twitching motilities, respectively.



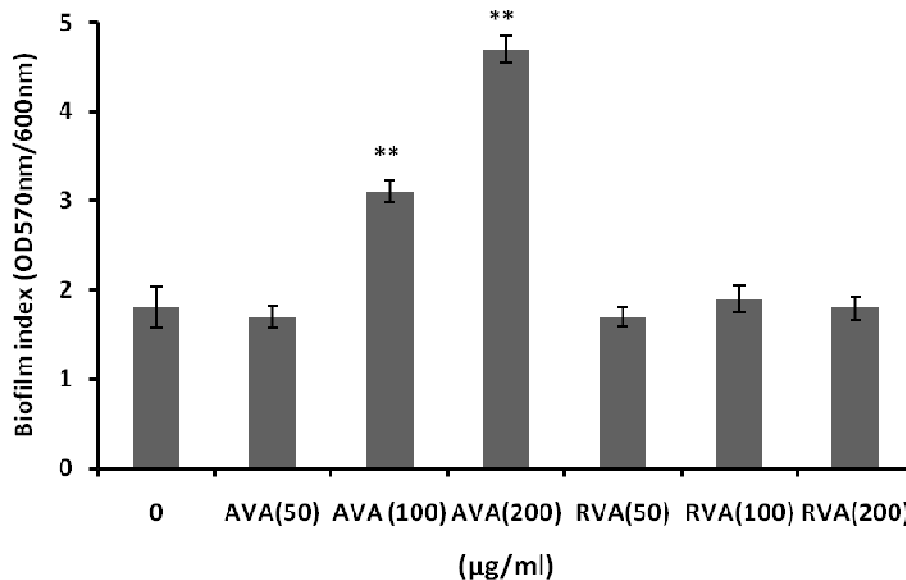
**Figure 2**

**Effect of atorvastatin (AVA) and rosuvastatin (RVA) on swarming, swimming and twitching motilities of *P. aeruginosa* PAO1 at 200  $\mu\text{g/ml}$ .**

**Biofilm formation**

There was concentration dependent enhancement of biofilm formation by *P. aeruginosa* PAO1 grown in the presence of atorvastatin. An increase of 125 and 152%

( $p < 0.05$ ) in biofilm index was observed at 100 and 200  $\mu\text{g/ml}$  of AVA. However, rosuvastatin showed no significant change in the formation of *P. aeruginosa* biofilm at same concentrations (Fig 3).



**Figure 3**  
**Biofilm index of *P.aeruginosa* PAO1 treated with increasing concentration (50-200µg/ml) of atorvastatin (AVA) and rosuvastatin (RVA) \*\*p<0.05.**

## DISCUSSION

Statins are antimicrobial in nature and enhance the prognosis of antimicrobial treatment in hospitalized patients due to their pleiotropic effect. The present study investigated the effect of two statins viz. atorvastatin (AVA) and rosuvastatin(RVA) on the pathogenesis of opportunistic pathogen *P. aeruginosa*. Both statins showed antimicrobial activity against *P. aeruginosa* PAO1 at MIC of 625µg/ml. Masadeh et al. (2012) also reported antimicrobial activity of AVA and RVA but the MICs obtained were lower than reported in the present work which was probably due to variability in susceptibility of strains. Statins such as simvastatin and fluvastatin have been reported to be effective in inhibiting the growth of Gram positive *S. aureus*. Interference with quorum sensing system during bacterial infections has improved the clinical outcome of antimicrobial treatments<sup>15</sup>. Signal molecules (AHLs) are the inducer of QS and molecules that act as agonists or antagonists of AHLs could affect bacterial persistence in host system. Statins are substituted hexahydro naphthaline lactones and share structural similarity with AHLs. Therefore, it was speculated that statins might act through interference with QS system. But in the present study, both atorvastatin and rosuvastatin lacked agonist or antagonist activities.

Lumichrome (a derivative of Riboflavin) was the first metabolite from a eukaryote identified as a bacterial QS agonist<sup>16</sup>. Antibiotics like azithromycin and erythromycin at sub-inhibitory concentration act as antagonist to AHLs and attenuate *P. aeruginosa* virulence<sup>17,18</sup>. Motility is strongly associated with the pathogenesis of *P. aeruginosa* as it enables the bacteria to colonize different environments, attach to surfaces, and form biofilms<sup>19</sup>. *P. aeruginosa* is capable of flagella-mediated swimming, swarming motilities and exhibits twitching motility on solid surfaces or interfaces, mediated by type IV pili<sup>20</sup>. Atorvastatin enhanced all the three motilities of *P. aeruginosa* as compared to rosuvastatin. *P. aeruginosa* utilizes rhamnolipids biosurfactant to minimize the surface tension but statins didn't affect the production of surfactant molecules (data not shown). Also atorvastatin itself lacked any biosurfactant property as tested by drop collapse and oil spread method (data not shown). Therefore the mechanism through which the swarming and swimming motilities are increased is unexplained. Bacterial motilities form an important factor in dispersal of bacterial cells present within the biofilm matrix<sup>21</sup>. At this stage, bacterial cells show similar phenotype as that of planktonic cell. An increase in bacterial motilities by

atorvastatin might be enhancing the dispersal of biofilm cell resulting in effective antimicrobial treatment. In contrast, rosuvastatin showed inhibition of bacterial motilities which might affect *P. aeruginosa* adherence and colonization on biotic surfaces. Biofilm is another virulence factor that influences *P. aeruginosa* persistence in host<sup>22</sup>. Biofilm formation was increased by atorvastatin while rosuvastatin showed no effect. As swarming motility is a prototype of intercellular communication system in biofilm, therefore

increase in biofilm mass by atorvastatin was anticipated but its implication *in vivo* is unexplained. Both atorvastatin and rosuvastatin showed opposite effects on virulence determinants of *P. aeruginosa* probably due to their structural difference. But they do exhibit antimicrobial activity against *P. aeruginosa*. Further studies are warranted to elucidate the antibacterial mechanism of statins and to study adjuvant activity of statins with antibiotics for the treatment of infections by opportunistic pathogens.

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