

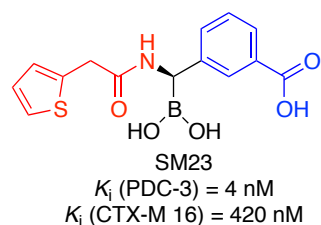
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**Introduction.** *Pseudomonas aeruginosa* is a Gram-negative nosocomial pathogen, often causative agent of severe device-related infections, given its great ability to form biofilm. *Pseudomonas* regulates the expression of numerous virulence factors, including biofilm production, by Quorum Sensing (QS), a cell-to-cell communication mechanism used by many bacteria. Inhibition of QS-controlled pathogenicity without affecting the *P. aeruginosa* growth, may thus represent a promising strategy to overcome its widespread and constantly increasing drug-resistance.

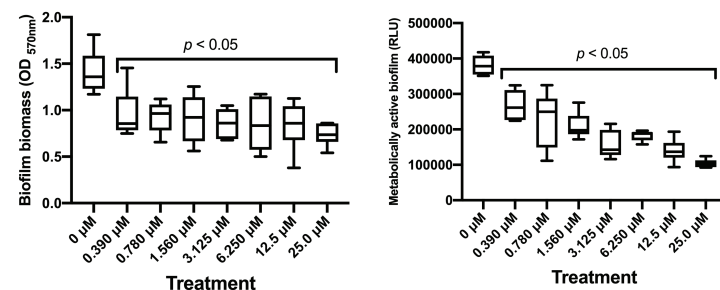
**Aim.** We investigated the effects of SM23, a boronic acid derivative, specifically designed as beta-lactamase inhibitor, on biofilm formation and virulence factors production by *P. aeruginosa*.

**Methods.** The *P. aeruginosa* bioluminescent strain P1242 was used employing bioluminescence, colorimetric and fluorescence assays (microbial growth, biofilm, elastase) as well as mass spectrometry (pyocyanin, pyoverdine and quorum sensing molecules)

## Results: 1. Inhibitory Effects of SM23 on *P. aeruginosa* Biofilm Formation



SM23 is a boronic acids transition state analog inhibitor (BATS) of  $\beta$ -lactamases from class C (PDC-3) and class A (CTXM-16)



**Dose-dependent effect of SM23 on *P. aeruginosa* biofilm formation.** Both box-plot graphs show the mean  $\pm$  SEM of microbial biofilm produced after 24 h of incubation in medium or in the presence of scalar doses of SM23. Left panel: OD<sub>570nm</sub> (biofilm biomass). Right panel: Relative Luminescence Units (RLU).

## 2. Inhibition of *P. aeruginosa* Quorum Sensing-Related Virulence Factors by SM23

| Groups               | Elastase activity (OD <sub>495</sub> ) |                   |                    |
|----------------------|--|-------------------|--------------------|
|                      | 6h                                     | 12h               | 24h                |
| Untreated            | 0.142 $\pm$ 0.030                      | 0.114 $\pm$ 0.017 | 0.155 $\pm$ 0.013  |
| SM23 (0.780 $\mu$ M) | 0.114 $\pm$ 0.011                      | 0.128 $\pm$ 0.018 | 0.092 $\pm$ 0.004* |
| SM23 (1.560 $\mu$ M) | 0.103 $\pm$ 0.011                      | 0.134 $\pm$ 0.018 | 0.088 $\pm$ 0.003* |
| SM23 (3.125 $\mu$ M) | 0.106 $\pm$ 0.013                      | 0.109 $\pm$ 0.015 | 0.087 $\pm$ 0.006* |

| Groups               | Py D |      |                                  | Py E |      |                                   |
|----------------------|------|------|----------------------------------|------|------|-----------------------------------|
|                      | 6h   | 12h  | 24h                              | 6h   | 12h  | 24h                               |
| Untreated            | n.f. | n.f. | 21.636.937,83 $\pm$ 2.064.621,26 | n.f. | n.f. | 107.118.272,50 $\pm$ 6.827.575,27 |
| SM23 (0.780 $\mu$ M) | n.f. | n.f. | 12.791.348,09 $\pm$ 3.054.805,04 | n.f. | n.f. | 89.272.480,54 $\pm$ 9.299.967,42  |
| SM23 (1.560 $\mu$ M) | n.f. | n.f. | 13.394.820,07 $\pm$ 3.644.560,57 | n.f. | n.f. | 78.809.117,60 $\pm$ 15.530.420,87 |
| SM23 (3.125 $\mu$ M) | n.f. | n.f. | 11.863.503,70 $\pm$ 2.030.689,62 | n.f. | n.f. | 68.465.442,68 $\pm$ 8.162.020,46  |

| Groups               | Py Succ-P-Ser-Y <sub>isom1</sub> |      |                                  | Py Succ-P-Ser-Y <sub>isom2</sub> |      |                                  |
|----------------------|----------------------------------|------|----------------------------------|----------------------------------|------|----------------------------------|
|                      | 6h                               | 12h  | 24h                              | 6h                               | 12h  | 24h                              |
| Untreated            | n.f.                             | n.f. | 12.195.698,41 $\pm$ 694.005,68   | n.f.                             | n.f. | 13.864.633,08 $\pm$ 890.328,25   |
| SM23 (0.780 $\mu$ M) | n.f.                             | n.f. | 17.853.144,48 $\pm$ 1.894.407,84 | n.f.                             | n.f. | 20.815.367,39 $\pm$ 1.882.888,28 |
| SM23 (1.560 $\mu$ M) | n.f.                             | n.f. | 17.177.294,38 $\pm$ 1.481.504,17 | n.f.                             | n.f. | 17.219.927,21 $\pm$ 2.725.080,39 |
| SM23 (3.125 $\mu$ M) | n.f.                             | n.f. | 15.410.576,46 $\pm$ 33.533,32    | n.f.                             | n.f. | 15.854.231,57 $\pm$ 1.705.318,16 |

| Groups               | Py Succa-P-Ser-Y <sub>isom1</sub> |      |                               | Py Succa-P-Ser-Y <sub>isom2</sub> |      |                               |
|----------------------|-----------------------------------|------|-------------------------------|-----------------------------------|------|-------------------------------|
|                      | 6h                                | 12h  | 24h                           | 6h                                | 12h  | 24h                           |
| Untreated            | n.f.                              | n.f. | 1.443.163,09 $\pm$ 118.385,60 | n.f.                              | n.f. | 2.740.841,46 $\pm$ 256.622,87 |
| SM23 (0.780 $\mu$ M) | n.f.                              | n.f. | 1.745.369,67 $\pm$ 327.147,82 | n.f.                              | n.f. | 2.791.882,23 $\pm$ 154.538,85 |
| SM23 (1.560 $\mu$ M) | n.f.                              | n.f. | 846.415,5 $\pm$ 400.566,41    | n.f.                              | n.f. | 2.853.552,30 $\pm$ 557.608,16 |
| SM23 (3.125 $\mu$ M) | n.f.                              | n.f. | 1.133.441,13 $\pm$ 30.587,47  | n.f.                              | n.f. | 2.205.285,65 $\pm$ 499.846,96 |

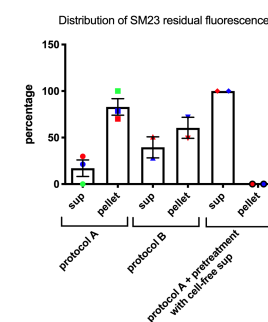
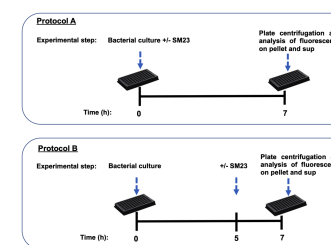
| Groups               | Pyocyanin |      |                                      |
|----------------------|-----------|------|--------------------------------------|
|                      | 6h        | 12h  | 24h                                  |
| Untreated            | n.f.      | n.f. | 1.604.817.246,57 $\pm$ 56.645.573,69 |
| SM23 (0.780 $\mu$ M) | n.f.      | n.f. | 804.321.499,25 $\pm$ 71.053.820,19*  |
| SM23 (1.560 $\mu$ M) | n.f.      | n.f. | 945.605.723,51 $\pm$ 50.626.110,98*  |
| SM23 (3.125 $\mu$ M) | n.f.      | n.f. | 696.891.913,37 $\pm$ 34.697.351,25*  |

Py= Pyoverdine  
3-oxoC<sub>12</sub>-HSL= N-(3-Oxododecanoyl)-L-homoserine lactone  
C<sub>4</sub>-HSL= N-(butanoyl)-homoserine lactone

## 3. Inhibition of *P. aeruginosa* Quorum Sensing Molecules by SM23

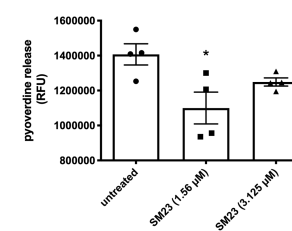
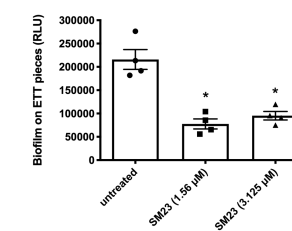
| Groups               | 6h   | 3-oxo-C <sub>12</sub> -HSL        |                                    | C <sub>4</sub> -HSL |      |                                   |
|----------------------|------|-----------------------------------|------------------------------------|---------------------|------|-----------------------------------|
|                      |      | 12h                               | 24h                                | 6h                  | 12h  | 24h                               |
| Untreated            | n.f. | 37.575.520,70 $\pm$ 2.315.838,91  | 201.150.062,20 $\pm$ 23.359.699,90 | n.f.                | n.f. | 69.707.031,01 $\pm$ 1.143.025,45  |
| SM23 (0.780 $\mu$ M) | n.f. | 23.645.421,84 $\pm$ 980.613,63*   | 55.787.081,98 $\pm$ 5.098.512,17   | n.f.                | n.f. | 56.319.203,07 $\pm$ 2.121.398,89  |
| SM23 (1.560 $\mu$ M) | n.f. | 24.783.514,13 $\pm$ 339.890,28*   | 51.160.160,28 $\pm$ 1.534.757,47   | n.f.                | n.f. | 57.213.308,48 $\pm$ 1.431.287,96  |
| SM23 (3.125 $\mu$ M) | n.f. | 26.816.303,02 $\pm$ 1.469.095,35* | 45.531.599,65 $\pm$ 4.653.861,29*  | n.f.                | n.f. | 48.215.432,52 $\pm$ 3.107.395,93* |

## 4. SM23 Interaction With *P. aeruginosa*



**SM23 interaction with *P. aeruginosa*.** Mean percent  $\pm$  SEM of the fluorescence signal detected both in the cell-free supernatant (sup) and in the pellet as specified in the experimental time-line (see above).

## 5. SM23 Inhibitory Effects on Endotracheal Tubes-Associated Biofilm



**SM23 effects on medical device-associated biofilm by *P. aeruginosa*: impairment of pyoverdine release and biofilm formation.** Biofilm mass (upper panel), expressed as mean RLU  $\pm$  SEM, and pyoverdine release (lower panel), expressed as mean RFU  $\pm$  SEM, by a 24 h-old *Pseudomonas* biofilm produced on ETT pieces, in the absence and in the presence of SM23.

**Conclusions.** Our results indicate that, besides inhibiting  $\beta$ -lactamase, SM23 can also act as powerful inhibitor of *P. aeruginosa* biofilm, suggesting that it may have a potential application in the prevention and treatment of biofilm-associated *P. aeruginosa* infections.