DEVELOPMENT OF AN IN-HOUSE *IN VITRO* QUORUM SENSING INHIBITOR DETECTION ASSAY AND CONFIRMATION OF THE QUORUM SENSING INHIBITORY ACTIVITY OF NX-AS-401

WILLIAMS, D.M., OLDHAM, H. AND GRAZ, M.

NEEM BIOTECH, WALES, UK.

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BACKGROUND

Opportunistic *Pseudomonas aeruginosa* bacteria colonise the lungs of many individuals with cystic fibrosis (CF) from a young age, leading to significant loss of lung function and quality of life. Quorum Sensing Inhibitors (QSIs) are a novel approach in the treatment of chronic *P. aeruginosa* infections. They restrict the capability of *P. aeruginosa* to form biofilms that, in turn, limit the effectiveness of conventional antibiotics used against them. Neem Biotech has been granted Orphan Drug Designation by the FDA for treatment of *P. aeruginosa* lung infections in CF patients using NX-AS-401, as an adjunct to conventional antibiotics.

AIMS

- To develop an *in vitro* assay to identify QSIs of *P. aeruginosa* using a known QSI, 4-Nitropyridine-*N*-Oxide (4-NPO) as a control.

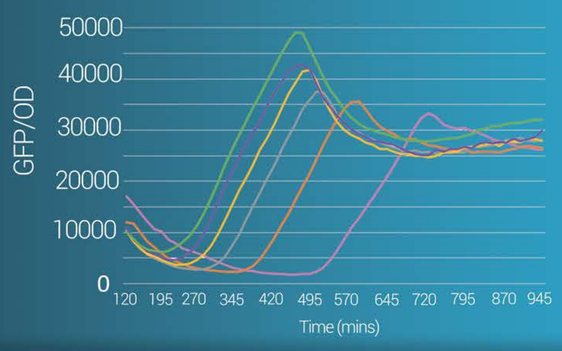
- Test batches of NX-AS-401 to demonstrate QSI using the developed method.

METHODS

A reporter gene assay was developed centred around key genes in the quorum sensing system of *P. aeruginosa*, *lasB* and *rhlA*. *LasB-gfp* and *rhlA-gfp P. aeruginosa* were grown overnight in minimal media, adjusted to OD_{600} 0.08 and incubated in the presence and absence of 4-NPO/NX-AS-401. GFP fluorescence and absorbance were measured over time; GFP data were normalised to OD.

SECTION 1: 4-NPO

Figure 1: Fluorescence output of *P. aeruginosa* containing a *lasB-gfp* construct; fluorescence output has been normalised to the absorbance OD. An untreated control of *lasB-gfp P. aeruginosa* is compared to six concentrations of treatment with 4-NPO.



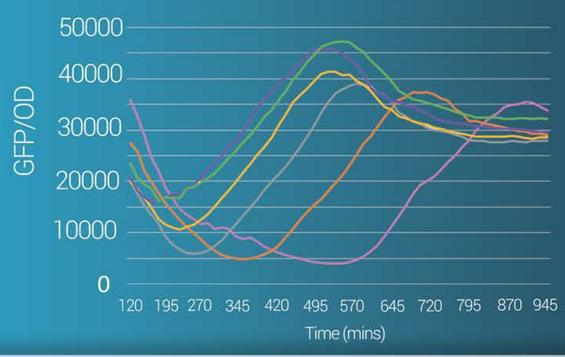
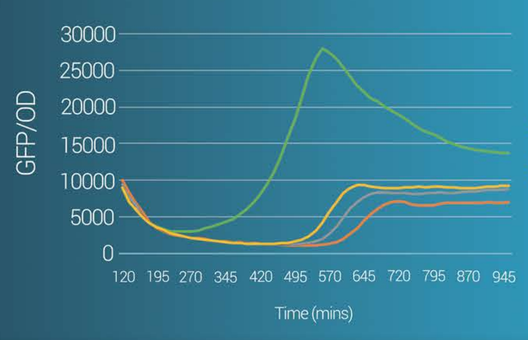


Figure 2: Fluorescence output of *P. aeruginosa* containing a *rhlA-gfp* construct; fluorescence output has been normalised to the absorbance OD. An untreated control of *rhlA-gfp P. aeruginosa* is compared to six concentrations of treatment with 4-NPO.

SECTION 2: NX-AS-401

Figure 3: Fluorescence output of *P. aeruginosa* containing a *lasB-gfp* construct; fluorescence output has been normalised to the absorbance OD. An untreated control of *lasB-gfp P. aeruginosa* is compared to three concentrations of treatment with NX-AS-401. Results represent an average of ten separate batches of NX-AS-401.



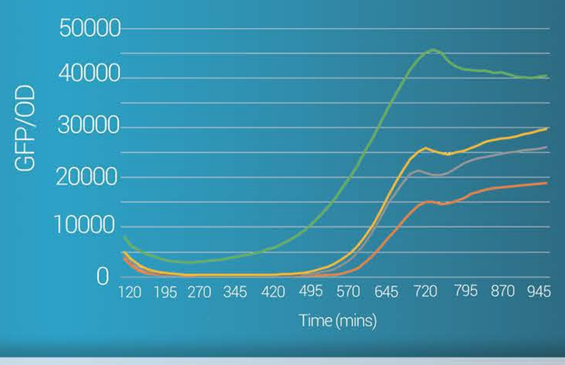


Figure 4: Fluorescence output of *P. aeruginosa* containing a *rhlA-gfp* construct; fluorescence output has been normalised to the absorbance OD. An untreated control of *rhlA-gfp P. aeruginosa* is compared to three concentrations of treatment with NX-AS-401. Results represent an average of ten separate batches of NX-AS-401.

RESULTS SECTION 1: 4-NPO

Monitoring the fluorescence and absorbance of cultures of *P. aeruginosa* with a l*asB-gfp/rhlA-gfp* construct with and without treatment of known QSI compound 4-NPO has confirmed the viability of the method as a screen for QSI compounds. 4-NPO produced a dose dependent response against both *lasB* (Figure 1) and *rhlA* (Figure 2) strains of *P. aeruginosa* consistently between multiple experiments.

RESULTS SECTION 2: NX-AS-401

A dose dependent QSI response was observed for the three tested concentrations of NX-AS-401 against both *lasB-gfp* (Figure 3) and *rhlA-gfp* (Figure 4) strains. The results

Table 1: Average fluorescence over time (120-960 min) of *P. aeruginosa* treated with NX-AS-401 reported as a percentage of the untreated control. Measurements are included from two gene reporter strains, *lasB-gfp* and *rhlA-gfp*. A mean of the results of ten batches of NX-AS-401 was determined and the standard deviation was calculated.

	lasB		*rhlA*	
Conc. NX-AS-401 (µg/mL)	Average Fluorescence	St. Dev	Average Fluorescence	St. Dev
64	43%	0.04	21%	0.02
32	47%	0.03	30%	0.02
16	50%	0.02	36%	0.03

CONCLUSIONS

- The development of an *in vitro* reporter gene assay for the purpose of screening for QSI compounds in house was successful. The assay can be utilised to screen for further putative QSI compounds active against *P. aeruginosa*.

- The QSI activity of multiple batches of Neem Biotech's lead compound, NX-AS-401, was demonstrated against *lasB* and *rhlA* quorum sensing systems in *P. aeruginosa*.

- This assay allows verification of the biological activity of every NX-AS-401 batch produced.