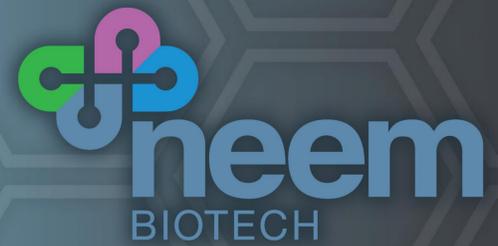


NX-AS-401: A QUORUM SENSING INHIBITOR THAT ENHANCES THE EFFECT OF TOBRAMYCIN ON *P. AERUGINOSA* IN VITRO AND IN VIVO



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BACKGROUND

Pseudomonas aeruginosa bacteria colonise the lungs and upper respiratory tract of many people with cystic fibrosis (CF). The nasopharynx and paranasal sinuses have been identified as reservoirs for *P. aeruginosa*, having a key role in repeat and chronic infections. Bacteria are able to adapt to an anoxic environment, then migrate along the respiratory tract to descend into the lower lung [1]. Adaptations can lead to mutations of quorum sensing (QS) regulator genes, as well as increased secretion of virulence factors such as pyocyanin (PCN). QS systems and virulence factors such as *algD*, *flgD*, and *pslD* play a key role in the formation of biofilms. It is well established that biofilms limit the effectiveness of conventional antibiotics, leading to chronic infections that are difficult to eradicate. This results in significant loss of lung function and quality of life for people with CF. Inhibiting QS and production of virulence factors in *P. aeruginosa* provides a novel treatment approach for chronic infections. Neem Biotech's lead compound, NX-AS-401, is a QS inhibitor (QSI) with efficacy as an adjunct to antibiotics currently used to treat chronic CF infections.

AIMS & METHODS

1. Demonstrate QSI activity and inhibition of virulence factors by NX-AS-401 in *P. aeruginosa*.
 2. Determine effects of NX-AS-401 and/or tobramycin on clinical isolates and chronic biofilms, *in vitro* and *in vivo*.
- A gene reporter assay was used with *rhIA-gfp* and *lasB-gfp* modified PAO1 *P. aeruginosa*. Fluorescence and optical density were monitored over time for treated cultures (NX-AS-401 4µg/ml-64µg/ml) and untreated controls.
 - Expression of genes involved in QS (*lasA*, *lasR*, *rhIR*), exopolysaccharide production (*phzF*), biofilm formation (*algD*, *flgD*, *pslD*) and CFTR inhibition (*cif*), was quantified at 1, 3 and 7 days post treatment using RT-qPCR [2].
 - Glass coupons in a 12-well plate were inoculated with 0.9ml *P. aeruginosa* or mixed species (OD600 of 0.1). Bacteria were grown statically in the absence of treatment for 6 hrs then incubated for 24 hrs with gentle agitation (37°C, 60rpm) with NX-AS-401 (8-32µg/ml) to determine effects on established biofilms.
 - C57Bl6 mice were inoculated intra-nasal installation with *P. aeruginosa* (LESB58). NX-AS-401 and/or tobramycin were dosed 24 hours later. CFU burden was assessed at days 3, 5 and 7 [1].

INHIBITION OF *RHLA* AND *LASB* QUORUM SENSING MECHANISMS

Addition of NX-AS-401 to cultures of modified PAO1 resulted in dose-dependent inhibition of *rhIA* (Figure 1a) and *lasB* (Figure 1b). GFP expression was significantly reduced at 16 hrs ($F(5,37) = 2.79, p = 0.031$ and $F(5,37) = 52.26, p = 9.41E-16$ respectively).

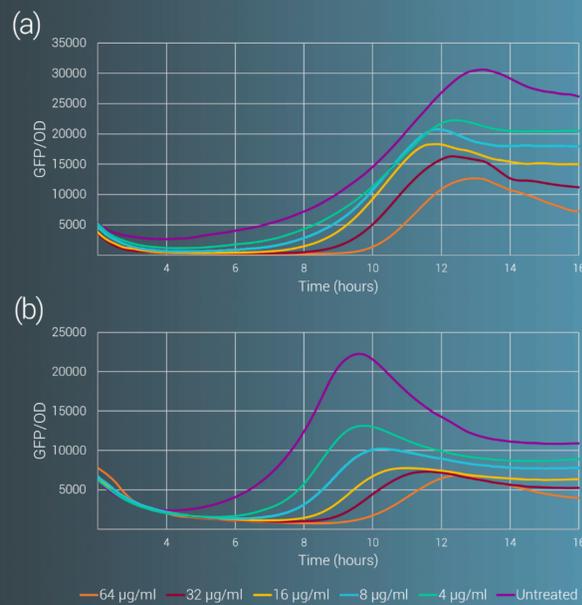


Figure 1 Dose-dependent effects of NX-AS-401 over time on the fluorescent signal of GFP for modified strains of PAO1: *rhIA-gfp* (a) and *lasB-gfp* (b). The y-axis shows GFP signal normalised to growth (GFP/OD). Measurements were taken every 15 mins for 16 hrs (data not shown for 0-2hrs).

REDUCED EXPRESSION OF VIRULENCE GENES

NX-AS-401 treatment reduced expression of all studied genes 3 days following treatment. Tobramycin + NX-AS-401 led to a long-term decrease in expression of *lasA*, *rhIR*, *phzF* and *cif*, evident at day 7, compared to Tobramycin alone.

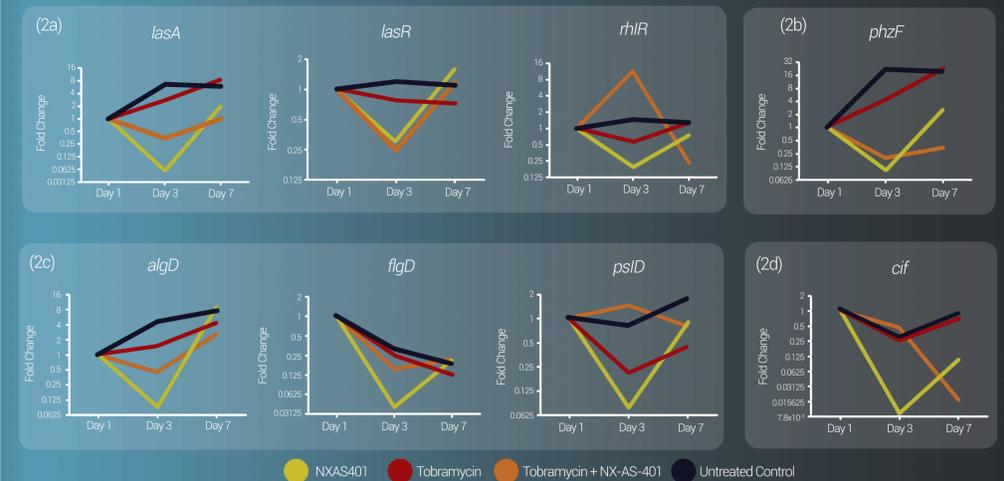
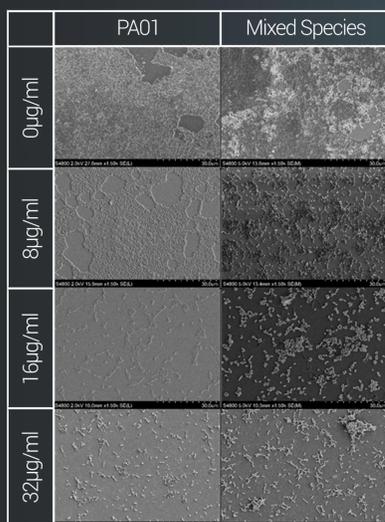


Figure 2 Fold change in gene expression of *P. aeruginosa* at day 3 & 7 following treatment, normalised to day 1 for genes related to (2a) QS; (2b) pyocyanin production; (2c) biofilm formation; (2d) CFTR inhibition.

DISRUPTION OF BIOFILMS IN VITRO



Overnight treatment with 16 µg/ml or 32µg/ml NX-AS-401 led to full retardation of pre-formed *P. aeruginosa* and mixed species biofilms (Figure 3); although single treatment with pure compound had no effect on cell viability.

Combined treatment of *P. aeruginosa* biofilms with NX-AS-401 and 10 µg/ml tobramycin has been shown to have a synergistic effect; resulting in more than 90% killing of cells, with full penetration through the biofilms of PAO1 and clinical isolate CF438 (Figure 4).

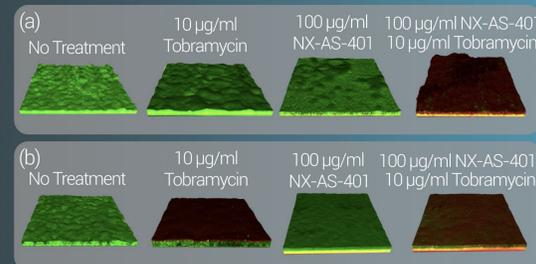


Figure 4 Confocal scanning laser microscopy images of PAO1 (a) and CF438 (b) biofilms stained with Syto 9 at day 4 following treatment with NX-AS-401 and tobramycin alone or in combination. Dead cells are stained with DNA stain PI (red). Adapted figure from Jakobsen et al., (2012) [2].

BACTERIAL CLEARANCE IN VIVO

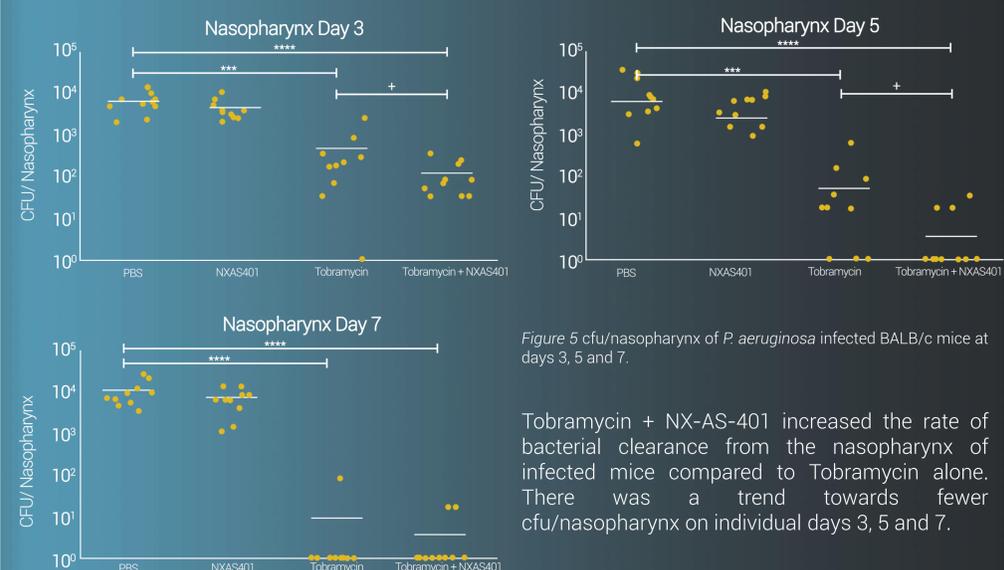


Figure 5 cfu/nasopharynx of *P. aeruginosa* infected BALB/c mice at days 3, 5 and 7.

Tobramycin + NX-AS-401 increased the rate of bacterial clearance from the nasopharynx of infected mice compared to Tobramycin alone. There was a trend towards fewer cfu/nasopharynx on individual days 3, 5 and 7.

(+ $p < 0.1$, *** $p < 0.001$, **** $p < 0.0001$.)

CONCLUSIONS

NX-AS-401 has QSI activity against *P. aeruginosa* and inhibits the expression of virulence genes known to have a role in biofilm formation and the inhibition of CFTR.

NX-AS-401 disrupts biofilms of single and mixed bacteria colonies *in vitro* and enhances the activity of tobramycin against clinically relevant strains of *P. aeruginosa* *in vivo*.

Neem Biotech has been granted Orphan Drug Designation by the FDA for the treatment of *P. aeruginosa* lung infections in CF patients, using NX-AS-401 as an adjunct to conventional antibiotics.

