

# Efficacy of MAR Inhibitors in a *Pseudomonas aeruginosa* Pneumonia Model

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## ABSTRACT

**Background:** Multiple Adaptation Response (MAR) proteins are a family of bacterial transcription factors that regulate the expression of antibiotic resistance and virulence determinants. In *P. aeruginosa* (PA), the MAR protein ExsA is an important virulence regulator of the type III secretion (TTS) system. Treatment with small molecule inhibitors of ExsA reduces infection and mortality in a PA pneumonia model.

**Methods:** An *in vitro* DNA-protein binding assay was used to identify compounds with potency against the ExsA protein as well as MAR proteins from other bacteria. Compounds were further screened for inhibition of TTS dependent cytotoxicity toward infected J774.1 cells. A murine model of PA pneumonia was used to assess the efficacy of the compounds.

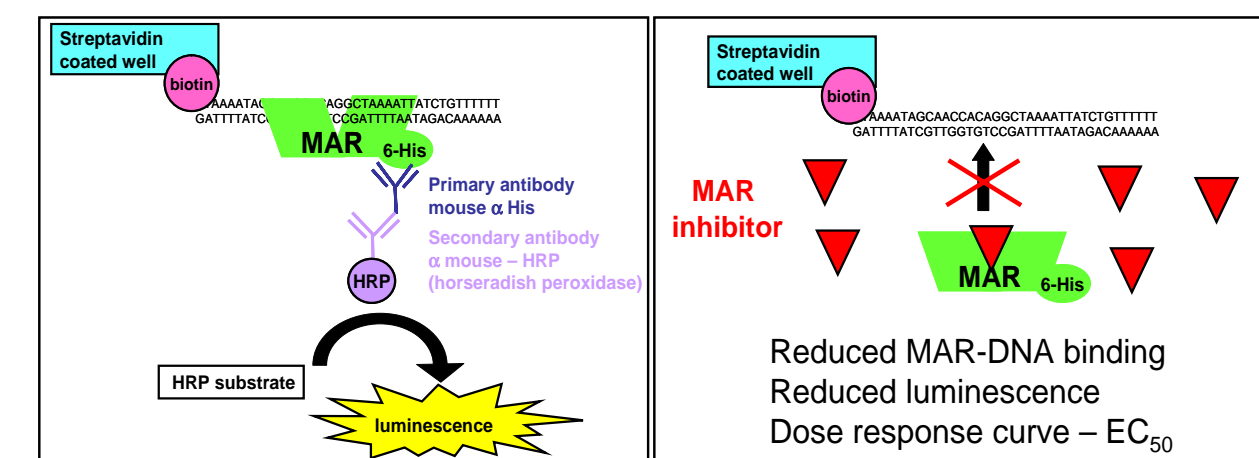
**Results:** Selected benzimidazole derivatives exhibited good *in vitro* potency and activity in cytotoxicity assays. Several ExsA inhibitory compounds also showed low micromolar potency against MAR proteins from *E. coli* (SoxS) and *Y. pseudotuberculosis* (LcrF) in DNA-protein binding assays. In the PA pneumonia model, survival increased (4-fold,  $p < 0.05$ ) in mice treated daily IP for four days post infection with compound P005784 or P005707 compared to vehicle alone. A significant reduction in the bacterial load in the lungs (63%) and liver (75%) was observed in mice treated with P005784 compared to vehicle alone ( $p < 0.05$ ).

**Conclusion:** Targeting MAR proteins with small molecule drugs represents a new paradigm for anti-infective chemotherapy, namely disease prevention. These data demonstrate the conceptual validity of this approach including the potential for the development of broad spectrum agents.

### Identification of MAR Inhibitors

- MAR proteins are members of the AraC family of transcription regulators - defined by 2 conserved helix-turn-helix DNA binding domains
- MAR proteins regulate virulence factor expression in bacterial pathogens, but are not found in eukaryotes
- A cell free DNA binding assay was used to identify small molecule inhibitors of the MAR proteins ExsA (*P. aeruginosa*), SoxS (*E. coli*), and LcrF (*Y. pseudotuberculosis*)

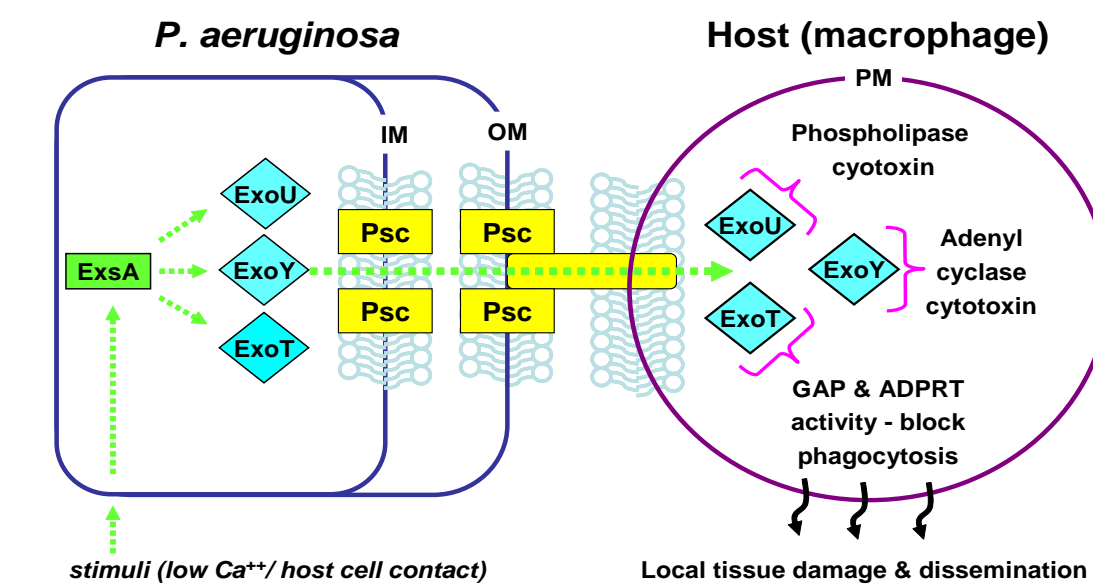
### Cell Free DNA Binding Assay



### MAR Inhibitor Activity *in vitro* – Potential for a Broad Spectrum Inhibitor

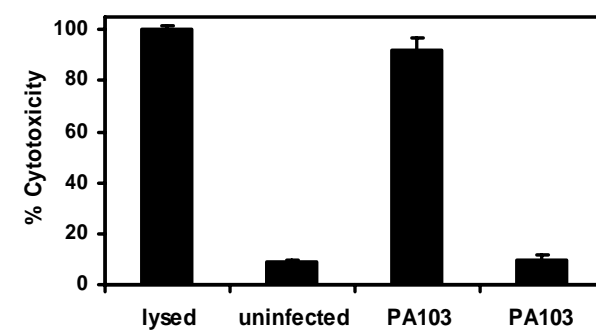
Compound	X / Y	<i>P. aeruginosa</i>			
		ExsA EC <sub>50</sub> (μM)	SoxS EC <sub>50</sub> (μM)	LcrF EC <sub>50</sub> (μM)	SlyA EC <sub>50</sub> (μM)
P005375	4-CH <sub>3</sub>	2.5	3.6	1.6	>57
P005389	2,4-F,F	3.1	1.8	5.0	>48
P005784	4-COCH <sub>3</sub>	3.0	18.3	5.8	>38

### ExsA Regulates Expression of the Type III Secretion System

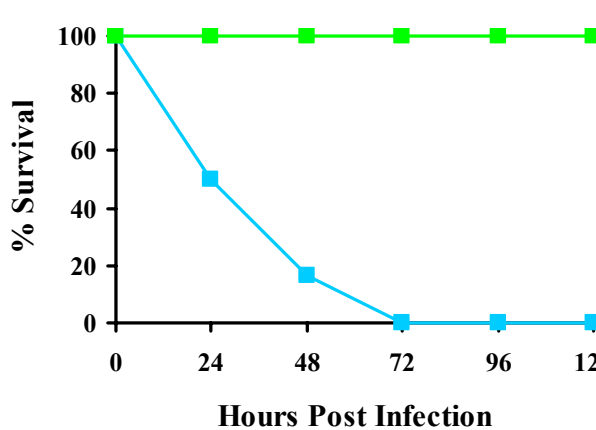


### ExsA Mutants are Avirulent in a Whole Cell Assay and Animal Model of Infection

#### Whole Cell Cytotoxicity Assay



Whole Cell Cytotoxicity Assay: J774.1 cells were infected for 1.75 hours with PA103 or PA103ΔExsA bacteria at an MOI of ~10. Lactate dehydrogenase (LDH) released into the culture media was measured using a standard kit. Cytotoxicity results are expressed as a percentage of the total LDH released from uninfected wells lysed with detergent.



Murine Pneumonia Model: Groups of 6 Swiss Webster mice were infected intranasally with ~1 x 10<sup>6</sup> cfu of either PA103 or PA103ΔExsA in 50μL of sterile PBS.

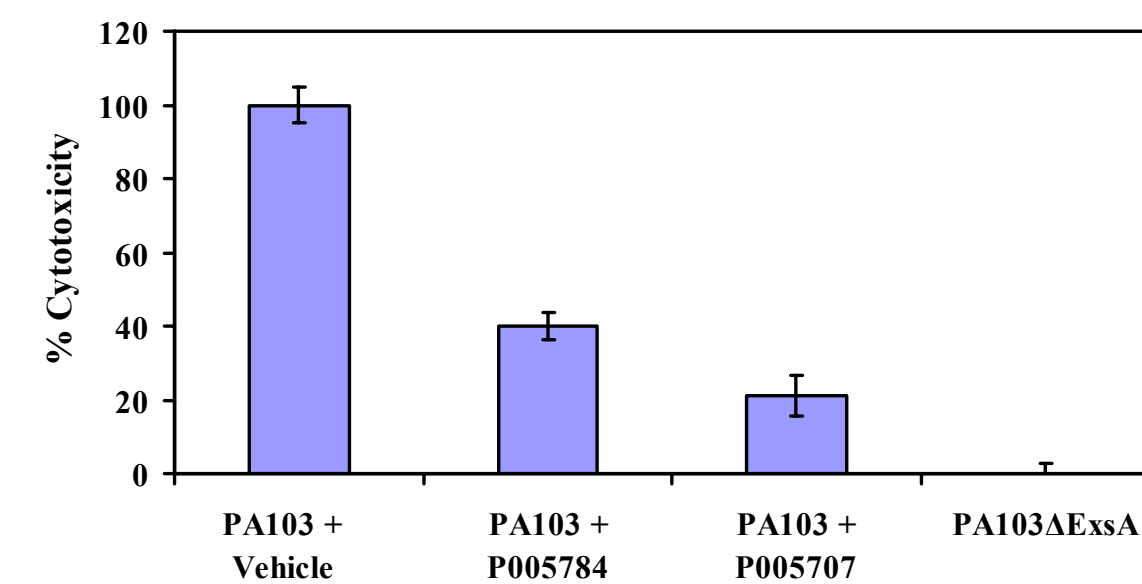
### Lead ExsA Inhibitors

Compound	Structure	ExsA EC <sub>50</sub> (μM)
P005784		3.0
P005707		6.6

### ExsA Inhibitors are Non-Antibacterial

Compound	MIC Values (μg/mL)			
	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> K201 PAO6609	<i>S. aureus</i> RN450	<i>S. pneumoniae</i> 157E
P005784	>64	>64	>64	>64
P005707	>64	>64	>64	>64

### ExsA Inhibitors Reduce *P. aeruginosa* Cytotoxicity Toward J774.1 Cells

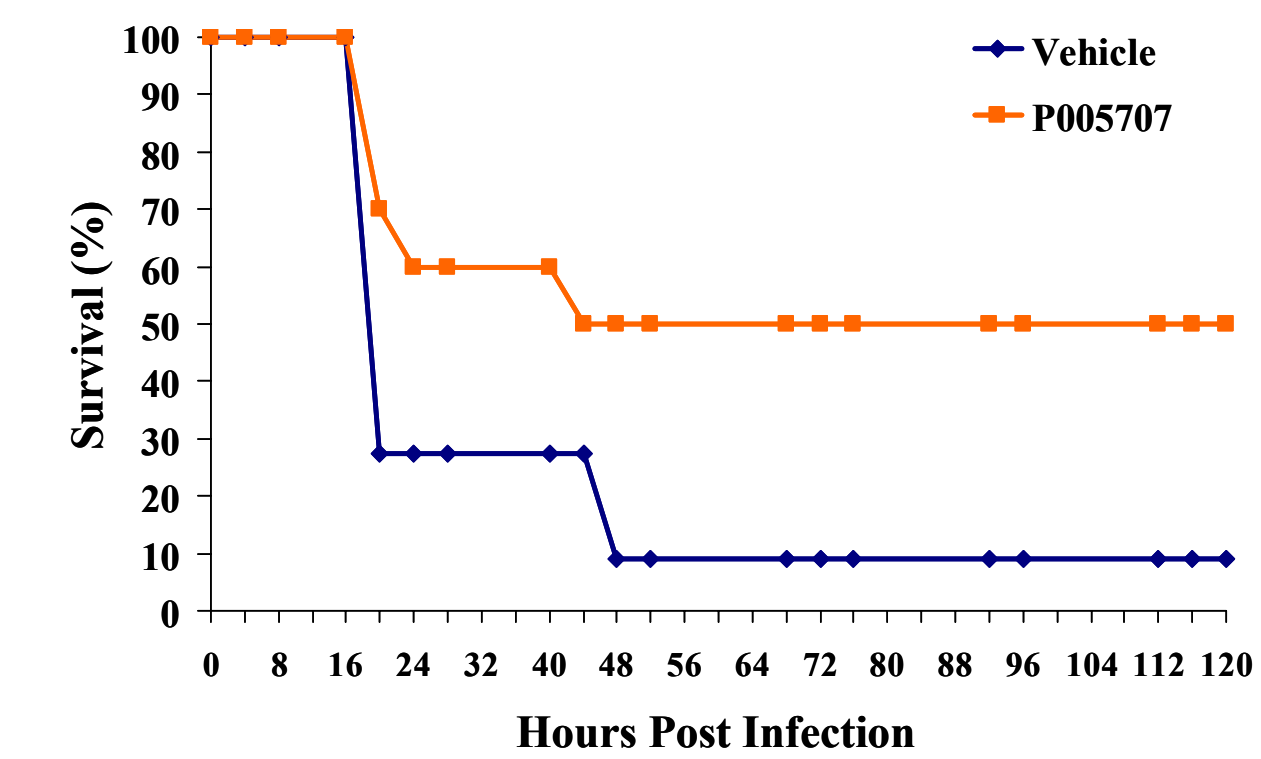
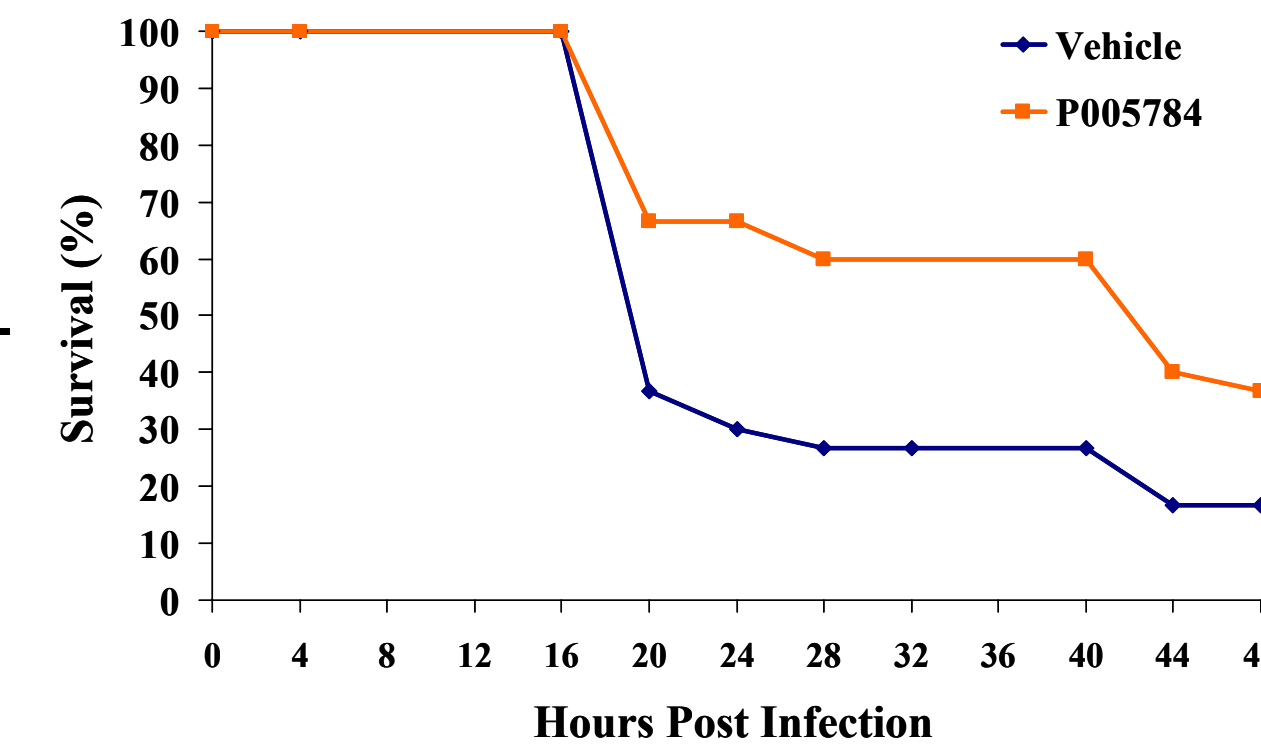


J774.1 cells were infected with vehicle or ExsA inhibitor-treated PA103 (wild type) or vehicle-treated PA103ΔExsA *P. aeruginosa*. Bacteria were cultured under Type III Secretion inducing conditions with ExsA inhibitor or equal volume of vehicle. Lactate dehydrogenase (LDH) release was measured. Results are expressed as a % of the LDH released by infection with the vehicle-treated PA103 control. Infection with untreated wild type PA103 bacteria typically resulted in the release of 75-90% of the total LDH activity.

### ExsA Inhibitors Reduce Mortality in a Lethal *P. aeruginosa* Pneumonia Model

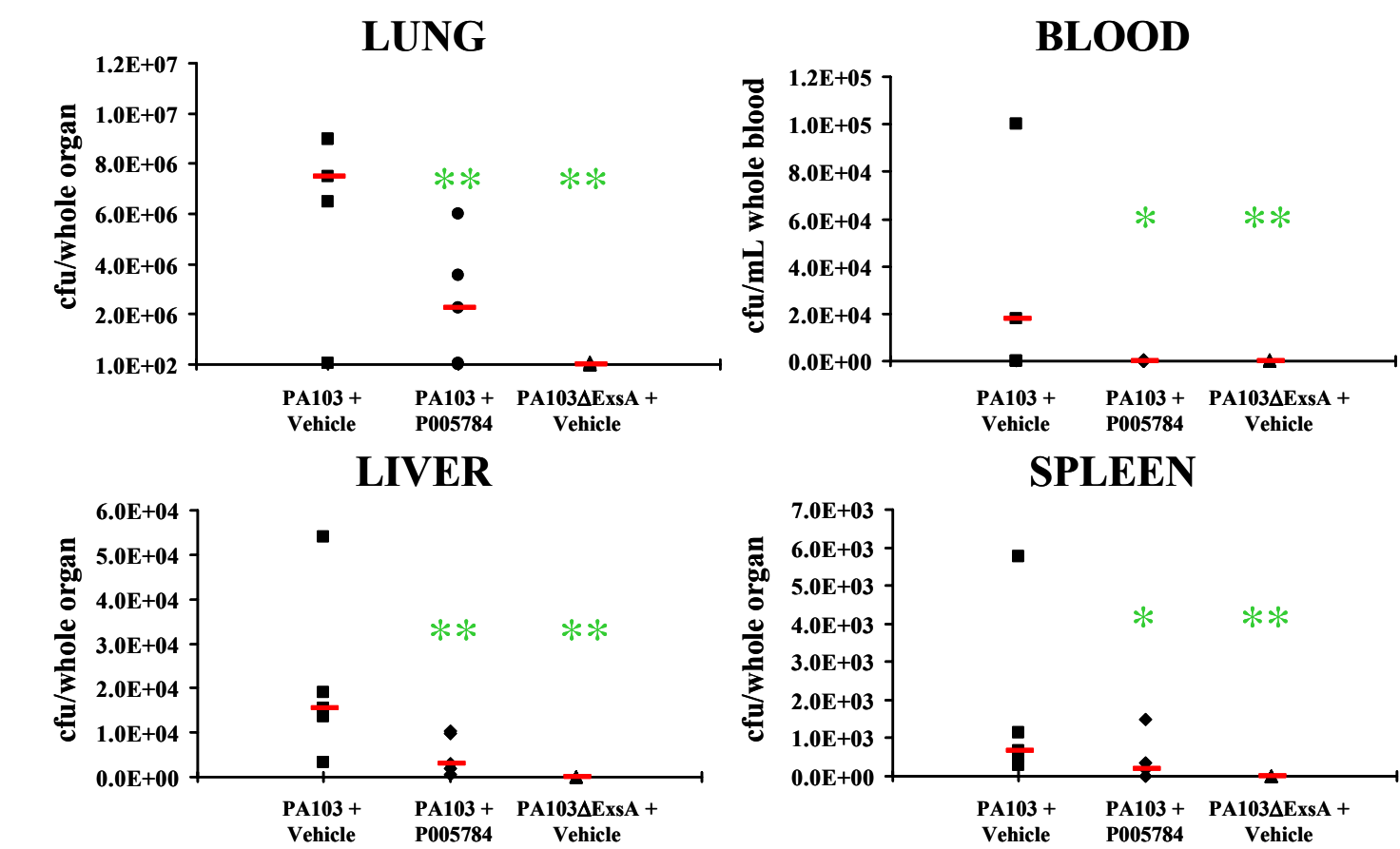
#### METHODS AND RESULTS

- Female Swiss Webster mice (18-22 grams)
- Mice were infected intranasally (i.n.) with *P. aeruginosa* at ~1 x 10<sup>6</sup> cfu in 50μl sterile PBS
- Compounds were administered intraperitoneally (i.p.) at 25 mg/kg at -18, -0.5, 2, 5, 20, 26, 44 and 48 hours post-infection
- Mice were observed for up to 5 days post infection



Kaplan Meier analysis revealed a significant difference in survival between vehicle-treated and P005784 or P005707-treated groups ( $P < 0.05$  by a Wilcoxon Test),  $n = 30$  or 10-11 mice/group, respectively.

### P005784 Prevents *P. aeruginosa* Dissemination in Mice Following Intranasal Infection



*P. aeruginosa* PA103 or PA103ΔExsA was inoculated i.n. (~5 x 10<sup>5</sup> cfu in 50μl sterile PBS). Drugs were administered i.p. at 25 mg/kg at -18, -1, 2, 5 hours post-infection. Mice were sacrificed at 18 hours post infection and the bacterial loads in the blood and whole organs were determined. — Group median. \*\*  $P < 0.05$ , \*  $P < 0.1$  by a Kruskal-Wallis Test with a ChiSquare Approximation,  $n = 5$  mice/group.

## CONCLUSIONS

- The MAR family transcription factor ExsA is required for full virulence in tissue culture and animal models of *P. aeruginosa* infection.
- ExsA inhibitors are non-antibacterial and thus not under the same selection pressure for resistance as traditional antibiotics.
- Treatment with the ExsA inhibitors P005784 and P005707, attenuated virulence and protected animals from mortality due to *P. aeruginosa* pneumonia.
- P005784 was effective in significantly reducing the bacterial load of *P. aeruginosa* in the lungs of infected mice and significantly reducing the dissemination of bacteria to the blood, liver and spleen.
- Inhibitors that act against multiple members of the MAR family of transcription factors may further inhibit *P. aeruginosa* virulence and could be used as broad spectrum agents against multiple bacterial pathogens.