The 8-hydroxyquinoline INP1855 reduces Type Three secretion system (T3SS)-dependent virulence of *Pseudomonas aeruginosa in vitro* and *in vivo*

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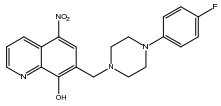
INTRODUCTION AND OBJECTIVES

Because of the alarming increase of resistance to current antibiotics and of the lack of novel drugs acting on multiresistant strains, new therapeutic approaches need to be explored, among which the inhibition of virulence mechanisms. T3SS is a major virulence factor in *Pseudomonas aeruginosa* (*P.a*) that allows the translocation of effector proteins from the bacterial cytoplasm directly into the eukaryotic host cells, contributing to the establishment and dissemination of acute infections [1]. The 8-hydroxyquinolines have shown inhibitory activity against T3SS of *Yersinia* and *Chlamydia* spp [2]. In this study, we evaluated the activity of INP1855 against *P.a in vitro* and *in vivo*.

INP1855
(8-hydroxyquinoline)



MW = 382.4
pK_a = 5.75 and 8.02 (zwitterionic at pH 7)
log P = 3.59
log $D_{pH\ 7}$ = 2.23
} calculated with Reaxys

MATERIAL AND METHODS

- Bacterial strains: CHA (clinical isolate with high expression of T3SS) and CHAΔExsA (deletion of T3SS regulon).
- Eukaryotic cells: human alveolar epithelial A549 cells and THP-1 monocytes. Mice: C57BL6. Chemical: INP1855
- Growth inhibition assay: Optical density (620 nm) of the bacterial suspension
- Cell viability (eucaryotic cells): lactate dehydrogenase (LDH) release
- Motility assay (bacteria): swarming in LB agar 0.5% plate.
- Internalization assay: Bacteria pre-treated with INP1855 (3 h) and opsonised with human serum (1 h). Phagocytosis initiated with 10 CFU/ cell for 2 h (THP-1 cells) in medium containing 60 µM of INP1855 (elimination of non-phagocytised bacteria by incubation with 100 µg/mL gentamicin (1 h). After washing, cells were lysed in water and aliquots plated for CFU counting [3].
- T3SS transcription assay: (1) the bioluminescence of the reporter strain CHA pC lux (constructed by fusion of *luxCDABE* to the promoter of the regulator operon *exsCEBA* [4]) in the presence of THP-1 cells or A549 cells over time of incubation with 60 µM of INP1855, (2) the levels of T3SS genes expression by RT-PCR in CHA strain in the presence of INP1855 60 µM.
- T3SS functionality : Detection of ExoS by Western Blot
- In vivo assay. Mouse model of acute lung aggression induced by CHA with co-instillation of bacteria and INP1855 200 µM. After 16 h, mice were sacrificed and different parameters were evaluated (weight gain, bacterial burden, lung lesion, bacterial dissemination and bronchoalveolar lavage cellularity).

RESULTS

INP1855 is not toxic for eukaryotic cells up to 60 µM, neither for bacteria at 200 µM

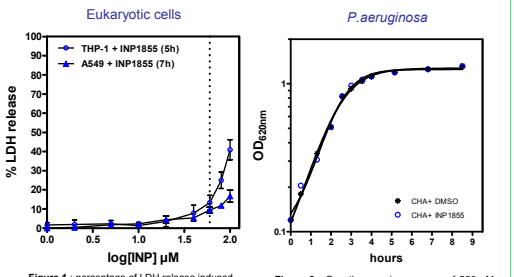


Figure 1 : percentage of LDH release induced by INP1855 after 5h (THP-1 monocytes) or 7h (A549 cells) of incubation. Mean ± SD (n=3). Dotted line : 60 µM

Figure 2 : Growth curve in presence of 200 µM of INP1855

INP1855 decreases T3SS-mediated virulence

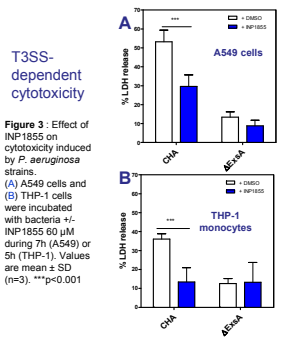


Figure 3 : Effect of INP1855 on cytotoxicity induced by *P. aeruginosa* strains. (A) A549 cells and (B) THP-1 cells were incubated with bacteria +/- INP1855 60 µM during 7h (A549) or 5h (THP-1). Values are mean ± SD (n=3). ***p<0.001

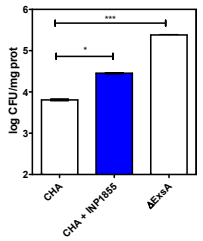


Figure 4: Effect of INP1855 on THP-1 monocytes internalization of CHA. Values are mean ± SD (*n*=3). * p<0.05, ***<0,001

INP1855 reduces flagellar motility

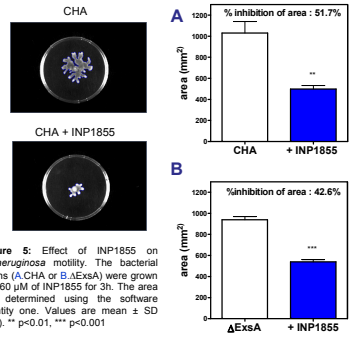


Figure 5: Effect of INP1855 on *P. aeruginosa* motility. The bacterial strains (A.CHA or B.ΔExsA) were grown with 60 µM of INP1855 for 3h. The area was determined using the software quantity one. Values are mean ± SD (*n*=3). ** p<0.01, *** p<0.001

INP1855 decreases T3SS transcription without affecting QS system

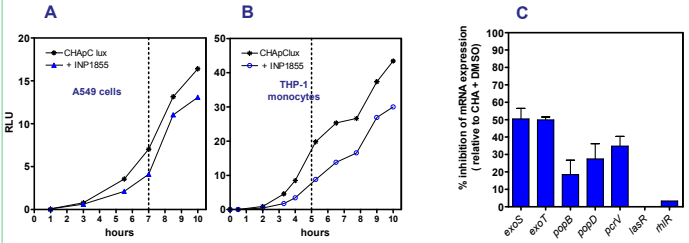


Figure 6 : (A et B) Effect of INP1855 on T3SS transcriptional activation of *exsCEBA* operon. Reporter strain CHA pC lux was incubated with 60 µM of INP1855 for 10h in A549 cells (A) and in THP1- cells (B). Luminescence was measured over time. Dotted line: time selected for further experiments. (C) Gene expression evaluated by RT-PCR. CHA strain was incubated with 60 µM of INP1855 or DMSO in LB supplemented with EGTA 5 mM and $MgCl_2$ 20 mM. Mean ± SD (n=2)

INP1855 impairs ExoS secretion

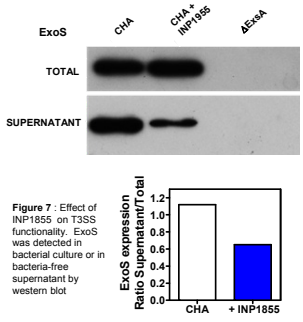


Figure 7 : Effect of INP1855 on T3SS functionality. ExoS was detected in bacterial culture or in bacteria-free supernatant by western blot

INP1855 increases mice survival and bacterial clearance

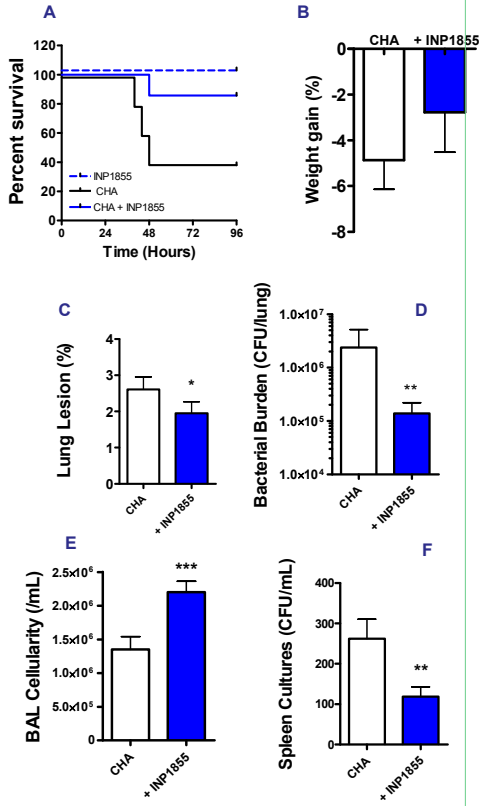
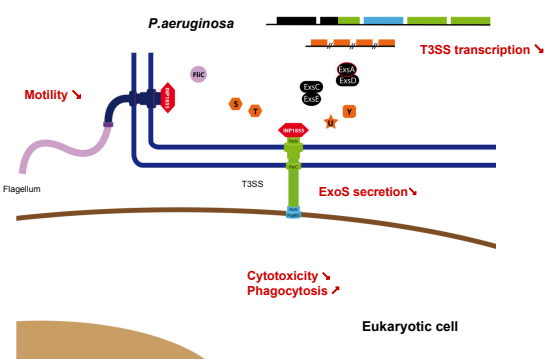


Figure 8: Survival assay and in vivo assay at 16 h. Mice infected by CHA were treated with DMSO or INP1855 200 µM. **A**. Survival assay. **B**. Weigh gain **C**. Lung injury assessed by alveolar capillary barrier permeability **D**. Bacterial burden in CFU/lung. **E.** Cell count from bronchoalveolar lavage liquid **F.** Bacterial dissemination assessed by spleen cultures homogenates. Values are mean ± SD (n=5). *p<0.05, **p<0.01, ***p<0.001

CONCLUSION

- INP1855 protects epithelial and phagocytic cells from T3SS-induced toxicity and is also effective *in vivo*.
- It inhibits both secretion of T3SS toxins and flagellar motility, suggesting a molecular target common to these two secretion systems.
- Although further studies are needed to better characterize its mode of action, our data highlights a potential interest for IN1855 as anti-pseudomonal agent.



Decreased functionality of T3SS as potential mechanism of action of INP1855:

- direct or indirect inhibition of the T3SS export apparatus and of flagellar basal body (which show a high degree of similarity) causing
  - a concomitant decrease in motility, toxin secretion and T3SS transcription (ExsE, the repressor of the transcriptional factor ExsA, being translocated via the T3SS)
  - a reduction in toxicity of *P. aeruginosa* for the host cells and an increased phagocytosis (demonstrated *in vitro*).
  - a reduction in pulmonary lesions and infection dissemination observed in *vivo*

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