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Inhibitors of type three secretion system [TTSS] protect against *Pseudomonas aeruginosa* cellular toxicity by inhibiting the transcription of TTSS

Guillaume de Laminne de Bex, Julien Buyck, Paul M. Tulkens and Françoise Van Bambeke

Pharmacologie cellulaire et moléculaire & Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium.

Mailing address:
F. Van Bambeke
av. Mounier 73, B1.73.05
1200 Brussels – Belgium
francoise.vanbambeke@uclouvain.be

Abstract (revised)

Objectives: TTSS is a major virulence factor in *P. aeruginosa* (Pa), inducing destructuration of cytoskeleton and direct necrosis of host cells. Inhibitors of this system may therefore constitute a complementary therapeutic approach to antibiotics. Substances INP0010, INP0341, and INP0406 are new TTSS inhibitors that have not yet been evaluated against Pa (1). The aim of the study was to determine their effects on Pa virulence and their mode of action.
Methods: Strains: PAO1; CHA (clinical isolate expressing an active TTSS); CHA-D1 (isogenic *exsA*::Gm mutant; no TTSS expression (2)). Toxicity: (i) towards bacteria: kinetics of growth in broth over 24 h (OD_{620} nm); (ii) towards THP-1 cells: trypan blue exclusion assay after 5 h incubation. TTSS transcription: bioluminescence assay at 5 h using CHA with lux operon inserted downstream of the TTSS regulator operon ExsCEBA or of the ExoS effector operon (3).
Results: As shown in the Table, none of the INP markedly altered bacterial growth and viability of THP-1 cells. CHA induced a marked cytoxicity towards THP-1 compared to PAO1 or CHA-D1. INP10 and INP341 protected against cytotoxicity induced by CHA and reduced the transcriptional activation of the promoter ExsCEBA. INP10 also inhibited the transcriptional activation of the ExoS promoter.

TTSS inhibitor (10 µM)	Cytotoxicity of TTSS inhibitors		Pa virulence (% cell death of THP-1 induced by PA)			Transcriptional activation of TTSS in CHA (10^6 cpm/cm^2)	
	PAO1 growth (OD620; % control)	% cell death (THP-1)	PAO1	CHA	CHA-D1	ExsCEBA Regulator	ExoS effector
CTL	100	6.7 ± 1.8	29.9 ± 5.2	41.4 ± 8.7	16.4 ± 3.6	15.2 ± 2.6	15.7 ± 2.6
INP10	104.5	18.5 ± 3.1	36.8 ± 5.0	4.7± 0.7	16.7 ± 1.6	5.2 ± 1.4	5.2 ± 3.4
INP 341	105.7	12.5 ± 1.0	27.5 ± 1.7	7.5 ± 2.1	18.1 ± 1.4	7.7 ± 1.1	17.7 ± 3.2
INP 406	111.3	9.9 ± 3.1	30.3 ± 13	7.0 ± 1.3	19.6 ± 3.1	13.5 ± 3.9	18.0 ± 1.9

Conclusion: These new TTSS inhibitors are able to inhibit cell death induced by TTSS of Pa probably by interaction with the main regulator of TTSS transcription. These results suggest a potential interest for these molecules as adjuvant therapy, which needs to be further explored *in vivo*.

Acknowledgments

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References

(1) Keyser *et al.*, J Intern Med 2008; **264:** 17–29.
(2) Toussaint *et al.*, Infect Immun 1999, **67:**6164-7
(3) Shen *et al.*, Infect Immun 2006, **74:**1121-9
(4) Roy-Burman, J Infect Dis 2001, **183 (12):** 1767-74

Background and aim

P. aeruginosa is an opportunistic pathogen causing life-threatening infections in immunocompromised patients. TTSS plays a major role in pathogenicity, since the relative risk of mortality is 6-fold greater when patients are infected by strains expressing TTSS (4). TTSS is capable of inducing destructuration of cytoskeleton and direct necrosis of host cells *in vitro*. In a view of the increasing resistance of *P. aeruginosa* to antibiotics, inhibitors of this system may therefore constitute a complementary therapeutic approach. INP are new TTSS inhibitors that have not yet been evaluated against *P. aeruginosa*.
The aim of the study was to determine their effects on *P. aeruginosa* virulence and their mode of action.

Materials & Methods

Bacterial strains:
ATCC PAO1 as a reference; CHA (clinical isolate expressing an active TTSS); CHA-D1 (isogenic mutant with deleted for TTSS [3]).
INPs Toxicity:
for bacteria: measure of the kinetics of growth in broth or RPMI over 24 hours (OD620nm) in the presence of INPs (20 µM);
for THP-1 cells: trypan blue exclusion assay after 5 h of incubation with increasing concentration of INPs.
TTSS inhibition.
Effect on cytotoxicity: trypan blue exclusion or LDH release assays in RPMI medium (or RPMI supplemented by EGTA (5mM) to activate TTSS) after 5 h of incubation with INPs (20 µM).
TTSS transcription: bioluminescence assay for 5 h using CHA with lux operon inserted downstream of the TTSS regulator operon ExsCEBA or of the ExoS effector operon.

Results

Inocuity of INPs for THP-1 cells

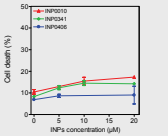


Figure 1 Percentage of cell death (evaluated by trypan blue exclusion assay) induced by INPs after 5 h of incubation. N=3. Mean ± sem

✓ INPs are not toxic for THP-1 cells at 20µM

✓ INPs do not alter the growth rate of *P. aeruginosa*

Inocuity of INPs for *Pseudomonas* strains

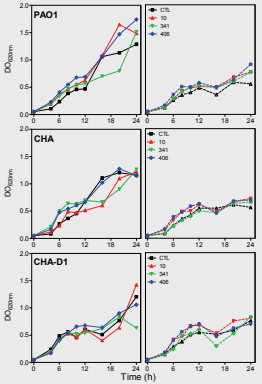


Figure 2: Influence of INP (20 µM) on growth rates of *P. aeruginosa* strains in MHB (left) and in RPMI (right)

Effect of INPs on *Pseudomonas* toxicity for THP-1 cells

Trypan blue exclusion assay

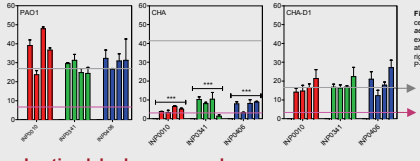


Figure 3: Effect of INP at 5 h on cell death induced by *P. aeruginosa* strains (trypan blue exclusion assay). INPs were used at 1, 5, 10 and 20 µM (from left to right). N=3. Mean ± sem. ***: P<0.001. Difference from the CTL.

Lacticodehydrogenase release

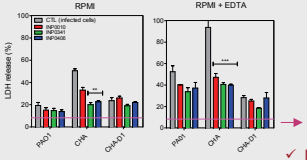


Figure 4: Effect of INP (20 µM) at 5 h on cell death induced by *P. aeruginosa* strains (LDH release). Cells were incubated in RPMI medium (left) and in RPMI supplemented with 5 mM EDTA to activate the TTSS (Right). N=3. Mean ± sem. **: P<0.01, ***: P<0.001 . Different from CTL)

✓ INPs specifically reduce cell toxicity of CHA mediated by TTSS

Effect of INPs on TTSS transcription

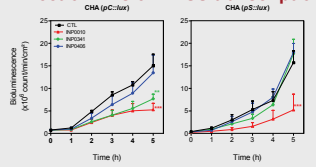
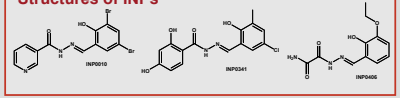


Figure 5: Activation of ExsA promoter (CHA (pC ::lux), left) and of ExoS promoter (CHA (pS ::lux), right) incubated for 5 h with INPs (20 µM). (N = 3, Mean ± sem. **: P<0.01, ***: P<0.001, difference from CTL).

✓ INP0010 inhibits the transcriptional activation of ExsA and ExoS
✓ INP0341 inhibits the transcriptional activation of ExsA

Structures of INPs



Conclusions

✓ INPs prove useful for reducing cytotoxicity of *P. aeruginosa*. They seem to specifically exert their action on the TTSS, because (I) they do not modify bacterial growth in broth, (ii) they protect against the toxicity induced by a strain expressing TTSS and their effect is maintained when TTSS is further activated by EDTA, and (iii) some of them inhibit the transcription of the TTSS.
✓ Further studies are needed however to explain the differences in the effects of the three molecules investigated on TTSS transcription and to evidence potential additional effects that may contribute to their pharmacological activity.
✓ Yet, our data underline the potential interest of these molecules as adjuvant therapy in the treatment of *P. aeruginosa* infection, which will need to be investigated also in vivo.

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