Salicylidene acylhydrazides and hydroxyquinolines decrease type three secretion system (T3SS)-dependent cytotoxicity induced by *Pseudomonas aeruginosa* (*P.a*) clinical isolates

A. Anantharajah[1#], C. Sundin[2$], M.P. Mingeot-Leclercq[1], P.M. Tulkens[1], F. Van Bambeke[1]

[1]Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium;
[2]Creative Antibiotics, Umeå, Sweden ; Current affiliations: # Cliniques universitaires Saint Luc, Université catholique de Louvain;
[$]Department of Chemistry, Umeå University, Sweden

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

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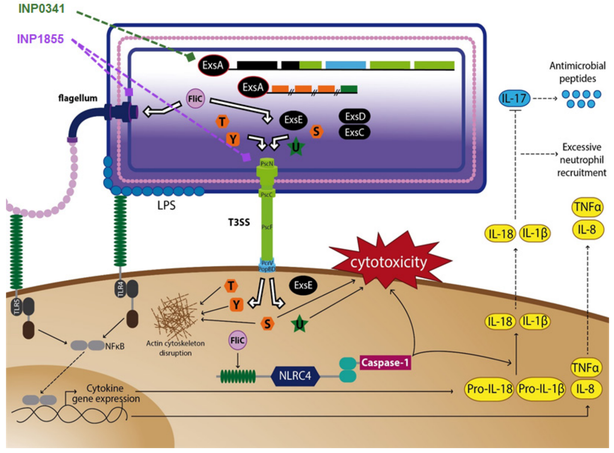
INTRODUCTION and OBJECTIVE

Expression of T3SS in *P.a* is associated with poor clinical outcome and high morbidity in acute infections. T3SS allows bacteria to inject exotoxins (e.g. ExoU or ExoS) into the host cell cytoplasm, causing cytotoxicity and preventing *P.a.* internalization (1). T3SS can also deliver proteins like flagellin into the cytosol of phagocytic cells, inducing caspase-1 proteolysis via NLCR4 inflammasome activation. Active caspase-1 causes not only cytotoxicity but also the secretion of IL-1β and IL-18, thereby impairing *P.a.* clearance (2;3). Using THP-1 monocytes, we previously distinguished T3SS+ strains expressing ExoU (high cytotoxicity, causing cell death without inflammasome activation [T3SS+ExoU+]) from those expressing ExoS or no toxins (moderate cytotoxicity, decreased by caspase-1 inhibitor and thus related to inflammasome activation [T3SS+ExoU-]) (4). However, in epithelial cells (not expressing NLRC4 inflammasome), *P.a* cytotoxicity was only T3SS toxin-dependent.

Here, we compare the protective effects of two T3SS inhibitors (INP0341 [salicydene acylhydrazide (5)] and INP1855 [hydroxyquinoline (6)]) to caspase-1 inhibitor on inflammasome activation and cytotoxicity caused by *P.a.* clinical isolates differing in their expression of T3SS and antimicrobial susceptibility.



Schema: Pictorial view of the physiopathology of cytotoxicity mediated by T3SS and of the putative targets of INP0341 and INP1855

MATERIALS & METHODS

Strains: CHA (clinical isolate expressing T3SS) and PA103 (cytotoxic strain expressing ExoU); 20 clinical T3SS+ isolates from patients suffering from acute infections

Antimicrobial susceptibility testing: MICs determined by microdilution in cation-adjusted Mueller–Hinton broth.

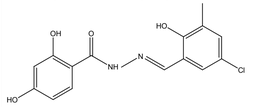
Caspase-1 inhibitor : Ac-YVAD-cmk (N-acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethyl ketone) 40 µM

Cells: THP-1 monocytes and A549 lung epithelial cells

Inflammasome activation: IL-1β secretion (ELISA).

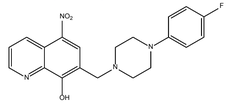
Cytotoxicity: release of the cytosolic enzyme lactate dehydrogenase (LDH) in culture medium after incubation with bacteria (10 bact/cell).

INP0341
Salicylidene acylhydrazides



60 µM

INP1855
Hydroxyquinolines



60 µM

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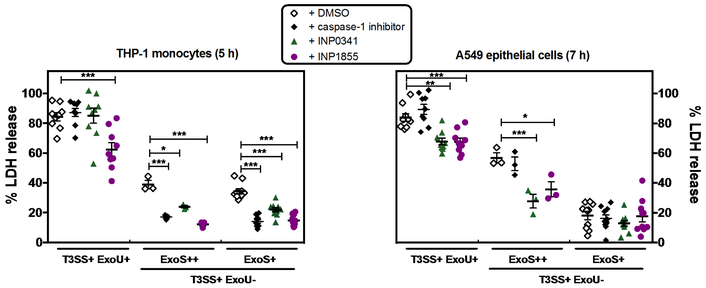
RESULTS

Cytotoxicity induced by *P.a* clinical isolates



T3SS+ ExoU+ :
- High cytotoxicity
- caspase-1 inhibitor : no effect on LDH release

⇨ **Cytotoxicity unrelated to inflammasome activation**

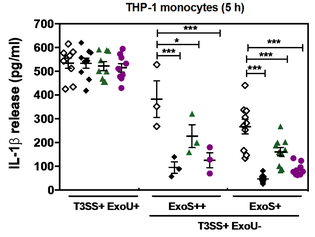
- INP0341 ↓ LDH release in A549 cells
- INP1855 ↓ LDH release in both cell types

T3SS+ ExoU- :
- Cytotoxicity ExoS++[b] > ExoS+[a] in epithelial cells ≠ in THP-1 cells
- caspase-1 inhibitor : ↓ LDH release only in THP-1 monocytes. No effect in A549 epithelial cells.

⇨ **Cytotoxicity related to inflammasome activation in THP-1 cells only**

- INP0341 and INP1855 : ↓ LDH release for ExoS+[a] and ExoS++[b] in THP-1 cells and only for ExoS++ in A549 epithelial cells

Inflammasome activation induced by *P.a* clinical isolates



T3SS+ ExoU+ :
- caspase-1 inhibitor : no effect on IL-1β release

⇨**IL-1β release not releated to inflammasome activation**

- INP0341 and INP1855: no effect on IL-1β release

T3SS+ ExoU- :
- caspase-1 inhibitor : ↓ IL-1β release

⇨**IL-1β release releated to inflammasome activation**

- INP0341 and INP1855: ↓ IL-1β release

[a]ExoS+: level of expression lower than that detected in reference strain CHA; [b]ExoS++ : level of expression higher than that detected in CHA;

Resistance profile

Table : MICs values and susceptibility patterns[a] of the studied clinical isolates of *Pseudomonas aeruginosa*

		Strains	Antibiotics[a]							
			AMK	TOB	MEM	TIC	CAZ	FEP	CIP	CST
clinical isolates	T3SS+ ExoU+	13846184	16	0.5	**16**	**128**	**32**	**16**	0.125	1
		9101/2	**128**	**256**	**32**	**256**	8	**16**	**64**	1
		14081972	4	0.5	**16**	32	2	4	0.25	1
		14241108	**128**	**128**	**32**	**>512**	**256**	**256**	**8**	1
		2504/6	1	**32**	2	**128**	**16**	**16**	1	1
		24138438	4	1	**8**	**64**	8	4	0.5	2
		24139146	4	0.5	1	**32**	2	4	0.125	4
		24138943	1	**32**	2	**128**	**16**	**16**	**8**	2
	T3SS+ ExoU-	NSIH 4603	4	1	1	**32**	4	8	0.125	2
		9101/1	1	0.125	**64**	**512**	8	**64**	0.5	1
		ZIV889	4	0.5	4	**64**	2	8	0.5	1
		24134699	4	1	4	**32**	2	4	0.125	4
		24140250	4	1	0.25	**32**	2	4	0.125	1
		05/1592	8	0.5	**32**	**128**	8	**16**	**4**	0.25
		15031978	4	1	0.5	**128**	8	8	0.25	1
		24128193	1	0.25	1	**64**	8	8	0.5	2
		BG0501/9344	2	1	0.5	**32**	2	2	0.25	0.25
		24138431	4	0.5	**8**	**64**	4	4	0.5	1
		24137296	4	1	0.5	**32**	2	4	0.5	1
		ZKT097	4	0.5	0.5	16	2	4	0.125	4

[a] v%values in bold characters are above the susceptibility breakpoints of CLSI (black), EUCAST (blue), or both CLSI and EUCAST (red), highlighting resistance
[b] AMK: amikacin; TOB: tobramycin; MEM: meropenem; ATM: aztreonam; TIC: ticarcillin; TZP: piperacillin-tazobactam; FEP : cefepime ; CIP : ciprofloxacin ; CST : colistin

Conclusions

- **T3SS+ ExoU- isolates: cytotoxicity, causing cell death without inflammasome activation**
- **T3SS+ ExoU- isolates: moderate cytotoxicity, related to inflammasome activation, which induces IL-1β secretion.**
- **Both INP341 and INP1855 protect eukaryotic cells from the toxic effects of *P.a.* clinical isolates mediated by the T3SS toxins (ExoU and ExoS) or by inflammasome activation.**
- **INP1855 is more potent than INP0341.**
- **Protective effects are independent of the antibiotic resistance profile of the isolates.**

⇨ **Inhibiting T3SS is thus a promising strategy deserving further evaluation in models of acute infections.**

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[5] Muschiol S *et al.*, BMC Microbiol. 2009;9:75
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