

## Review

# Targeting the Type Three Secretion System in *Pseudomonas aeruginosa*

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**The injectisome type three secretion system (T3SS) is a major virulence factor in *Pseudomonas aeruginosa*. This bacterium is responsible for severe infections in immunosuppressed or cystic fibrosis patients and has become resistant to many antibiotics. Inhibitors of T3SS may therefore constitute an innovative therapeutic target. After a brief description of the T3SS and its regulation, this review presents strategies to inhibit T3SS-mediated toxicity and describes the main families of existing inhibitors. Over the past few years, 12 classes of small-molecule inhibitors and two types of antibody have been discovered and evaluated *in vitro* for their capacity to inhibit T3SS expression or function, and to protect host cells from T3SS-mediated cytotoxicity. While only one small molecule has been tested *in vivo*, a bifunctional antibody targeting both the translocation apparatus of the T3SS and a surface polysaccharide is currently in Phase II clinical trials.**

## Targeting the Injectisome in *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is an opportunistic Gram-negative bacterium chronically colonizing the respiratory tract of cystic fibrosis patients and causing severe healthcare-associated infections in immunocompromised hosts. Despite appropriate antibiotic treatment, mortality rates can reach more than 60% in specific settings, such as ventilator-associated pneumonia. Therapeutic options are limited because *P. aeruginosa* has become resistant to almost all antibiotic classes available in the clinic.

In this context, innovative therapeutic strategies are needed, among which targeting virulence is particularly appealing [1], because it may reduce morbidity, improve immune response, and preserve commensal flora. This strategy also remains compatible with antibiotic usage and is expected to have a lower risk of selecting resistance, because it only disarms bacteria, leaving the task of killing them to the immune system [2]. In this context, the injectisome T3SS is one of the main virulence factors of *P. aeruginosa*. In acute respiratory infections, strains expressing a functional T3SS and secreting toxins persist in the lungs and are associated with higher relapse rates and bacterial burden, causing sixfold higher mortality [3]. By contrast, T3SS expression regresses over time in chronically colonized cystic fibrosis patients [4]. Thus, T3SS appears to be a highly attractive target for innovative therapies against acute infections. After a brief description of the architecture of the system, the regulation of its expression, and its toxic effects for the host, this work presents the possible ways to target T3SS and reviews the inhibitors described in the current literature (Figure 1, Key Figure).

## Structure and Regulation of the T3SS

T3SS is highly conserved among Gram-negative bacteria and evolved from the flagellum [5]. It consists of a syringe-like export machine injecting toxins (called effectors) from the bacterial

### Trends

Disarming bacteria by reducing their virulence is a promising therapeutic strategy in a world of increasing antibiotic resistance. The injectisome T3SS of *P. aeruginosa* causes direct cytotoxicity and also activates NLR4 inflammasome cascade in the host.

A series of small-molecules inhibitors have been identified acting on gene transcription, ATPase or basal body export activity, effector secretion or translocation, and blocking effector enzymatic activity. Most of them have proved effective *in vitro*, but *in vivo* demonstration of their efficacy remains scarce.

Antibodies targeting the translocon and bispecific antibodies targeting both the translocon and a surface polysaccharide have reached Phase II trials, but the first antibodies were abandoned for insufficient efficacy.

Immunomodulation can counteract cytokine imbalance related to inflammasome activation.

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cytosol directly into the host cell cytosol [5] by the concerted action of a secretion apparatus (transporting effectors through the bacterial membranes) and a translocon (translocating them through the host cell membrane). The *P. aeruginosa* T3SS and its regulators are encoded by 43 coordinately regulated genes [6], with genes encoding proteins associated with secretion, translocation, and regulation functions located on five operons, and those encoding effectors and associated chaperones located elsewhere on the chromosome (*exoS*, *exoT*, *exoY*) or on a pathogenicity island (*exoU*) [7].

#### The Secretion Apparatus or Needle Complex

The needle complex consists of a multi-ring basal body and a needle, made up of approximately 25 different proteins. The basal body spans the inner membrane, the peptidoglycan layer, and the outer membrane, and anchors the needle-like structure into the bacterial membrane [5]. The basal body comprises an inner membrane ring made of the lipoprotein PscJ [8] and an outer membrane ring made of the oligomerized secretin PscC [9] interconnected by a short rod in the bacterial cytoplasm. Of note, secretins are also found in other microbial nanomachines, like T2SS and type IV pili (see Glossary). The cytoplasmic ATPase PscN facilitates recognition of chaperone/effector complexes and renders secreted proteins export-competent by unfolding them; its role as an energy source for T3SS is not well established [10]. The needle is hollow, or barrel-shaped, and assembled by helical polymerization of PscF proteins. It brings effectors from the basal body to the translocation apparatus and probably serves as a sensor for host–cell contact [11], which is thought to trigger effector delivery *in vivo*.

#### The Translocation Apparatus

The translocation apparatus is made of two hydrophobic proteins (PopB, PopD) and one hydrophilic protein (PcrV) secreted by the T3SS itself. PopB and PopD interact with each other and the host cell membrane to form the translocation pore [12], transporting effectors from the needle across the host cell membrane and delivering them into the host cytosol. PcrV is required for the functional assembly of the PopB/D translocon complex [13].

#### Effectors and Other Translocated Proteins

Once the pore is formed, effectors are translocated through the needle after removal of their chaperone by the ATPase of the basal body [14]. At least four effectors are secreted via this system (Box 1). For reasons still unclear, coexpression of *exoS* and *exoU* is rare in a single strain. *ExoS*-secreting strains cause apoptotic delayed cell death, whereas *ExoU*-producing strains induce rapid cell lysis.

Proteins other than effectors are also delivered by the injectisome, among which are pilin (PilA; main constituent of type IV pili [15]) or PscI (basal body rod component of the T3SS apparatus [16]), and different proteins from the flagellum, including flagellin (FliC) [17]. The recognition of FliC is explained by the fact that the flagellum also uses a T3SS to export its substrates.

#### Regulation of T3SS Expression

T3SS transcription is intimately coupled to its secretory activity. In the absence of a signal, proteins are expressed at basal level. Under inducing conditions ( $\text{Ca}^{2+}$ -depleted medium, serum, contact with eukaryotic cells [18]), the negative regulator *ExsE* is secreted from the bacteria, which activates *ExsA*, the transcriptional activator of the operons encoding T3SS. *ExsA* itself is under the control of a complex regulatory cascade (Box 2) [19].

#### Chaperones

Some type III-secreted proteins have cognate chaperones to which they bind before being secreted. Chaperones have been identified for effectors (*SpcS* for *ExoS* and *ExoT* [20], *SpcU* for

#### Glossary

**Bleb niche:** membrane-bound vacuoles and cell membrane protrusions in which internalised bacteria can survive, multiply, and swim, while remaining physically separated from the rest of the cytoplasm. The mechanism of their formation is unknown, but requires the expression of *ExoS*.

**Neuronal apoptosis inhibitory proteins (NAIP):** a subfamily of the nucleotide oligomerisation domain receptors (NLRs), containing a Baculovirus inhibitor of apoptosis repeat (BIR) domain, are direct sensors of flagellin or PrgJ; flagellin binds to NAIP5, and PrgJ to NAIP2. These ligand–NAIP complexes can subsequently interact with NLRC4 and trigger inflammasome activation.

#### NLR family, CARD domain containing 4 (NLRC4)

**inflammasome:** inflammasome complexes generally have three main components: a cytosolic pattern-recognition receptor, the enzyme caspase-1, and an adaptor protein that facilitates the interaction between the two other proteins. The complexes mediate the activation of caspase-1 to promote the maturation of IL-1 $\beta$  and IL-18. The receptor is either a member of the NLR family of proteins or a member of the pyrin and HIN domain-containing (PYHIN) family of proteins. The NLRs contain a carboxy-terminal leucine-rich repeat (LRR) domain, a conserved central NACHT domain (which is essential for the nucleotide binding and protein oligomerisation that are required to form multiprotein inflammasome complexes) and a variable amino-terminal domain that defines several NLR subfamilies. NLRC4 contains an N-terminal caspase activation and recruitment domain (CARD).

**Pyroptosis:** a rapid form of cell death following caspase-1 activation, which shares characteristics with both apoptosis (such as DNA fragmentation) and necrosis (such as cell swelling and rupture).

**Type II secretion system (T2SS):** a membrane-bound protein complex found in Gram-negative bacteria. While T3SS secretes proteins and translocates them in the cytosol of the host cells, T2SS secretes cytoplasmic proteins to the extracellular medium. Secretion substrates for this system include bacterial exotoxins and degradative

ExoU [21]; none so far for ExoY), translocation proteins (PcrH for PopB and PopD [22]), structural subunits of the needle complex (PscE and PscG for PscF [23]), and the regulatory protein ExsE (ExsC [24]). They facilitate the storage of secreted proteins in the bacterial cytosol and their appropriate delivery via the needle complex [14].

### Pathogenicity Related to the T3SS

The T3SS is generally associated with acute invasive infections. Once activated by contact with eukaryotic cells via pili or flagella, T3SS interferes with signal transduction, causing cytotoxicity directly mediated by effectors, as well as alterations in immune responses.

#### Effector-Dependent Pathogenicity

ExoS and ExoT have GTPase (GAP) and ADP ribosyltransferase (ADPRT) domains (Box 1). The GAP activity of both effectors targets small GTPases that maintain organization of the host cell actin cytoskeleton [25], thus inducing cell rounding and detachment, and inhibition of cell migration and phagocytosis. The ADPRT activities of the two effectors are different. The ADPRT domain of ExoS causes cytotoxicity and apoptosis, inhibits DNA synthesis, alters endocytosis, vesicular trafficking, and bleb-niche formation, and favors intracellular multiplication [26]. ExoS also disrupts the pulmonary–vascular barrier *in vivo*, leading to bacterial dissemination [27] and pulmonary damage [28]. The ADPRT and the GAP domains of ExoT cooperate to disrupt actin cytoskeleton and impair phagocytic activity [29]. *In vivo*, ExoT delays wound healing, favoring bacterial invasion [30].

By its phospholipase A<sub>2</sub> activity, ExoU causes irreversible damage to cellular membranes as well as rapid necrotic death of epithelial cells, macrophages, and neutrophils. ExoU also increases the production of proinflammatory eicosanoids. It thereby triggers an arachidonic acid-dependent inflammatory response [31] and induces the expression of inflammatory genes [32]. ExoU may also cleave lipids in pulmonary surfactant [33]. ExoU is considered the major cytotoxin secreted via the T3SS because it is associated with severe acute lung injury, sepsis, and mortality [34].

ExoY was first described as an adenylate cyclase increasing intracellular cAMP, but has also been reported to increase the intracellular levels of other cyclic nucleotides (cCMP, cGMP, cUMP) [35]. As a consequence, ExoY causes actin cytoskeleton disorganization, cell necrosis, and alteration of endothelial cell barrier integrity following lung injury [36].

#### Effector-Independent Pathogenicity

T3SS can induce cell death by **pyroptosis** independently of effector injection, by activating the cytosolic sensor **NLR family, CARD domain containing 4 (NLRC4) inflammasome** [37]. Several bacterial proteins can trigger NLRC4-inflammasome activation (PilA [15], RhsT [38], FliC [17], PscI [16]), but only FliC is secreted by the T3SS. When inside the host cell, these proteins bind to **NLR family, apoptosis inhibitory proteins (NAIPs)**, which, once activated, cause oligomerization and activation of NLRC4 and subsequent recruitment and activation of caspase-1. Caspase-1 induces cell death by pyroptosis and proteolytic cleavage of pro-IL-1 $\beta$  and pro-IL-18 in mature cytokines [39]. Mitochondrial DNA release triggered by T3SS can also activate NLRC4, while autophagy downregulates this effect [40].

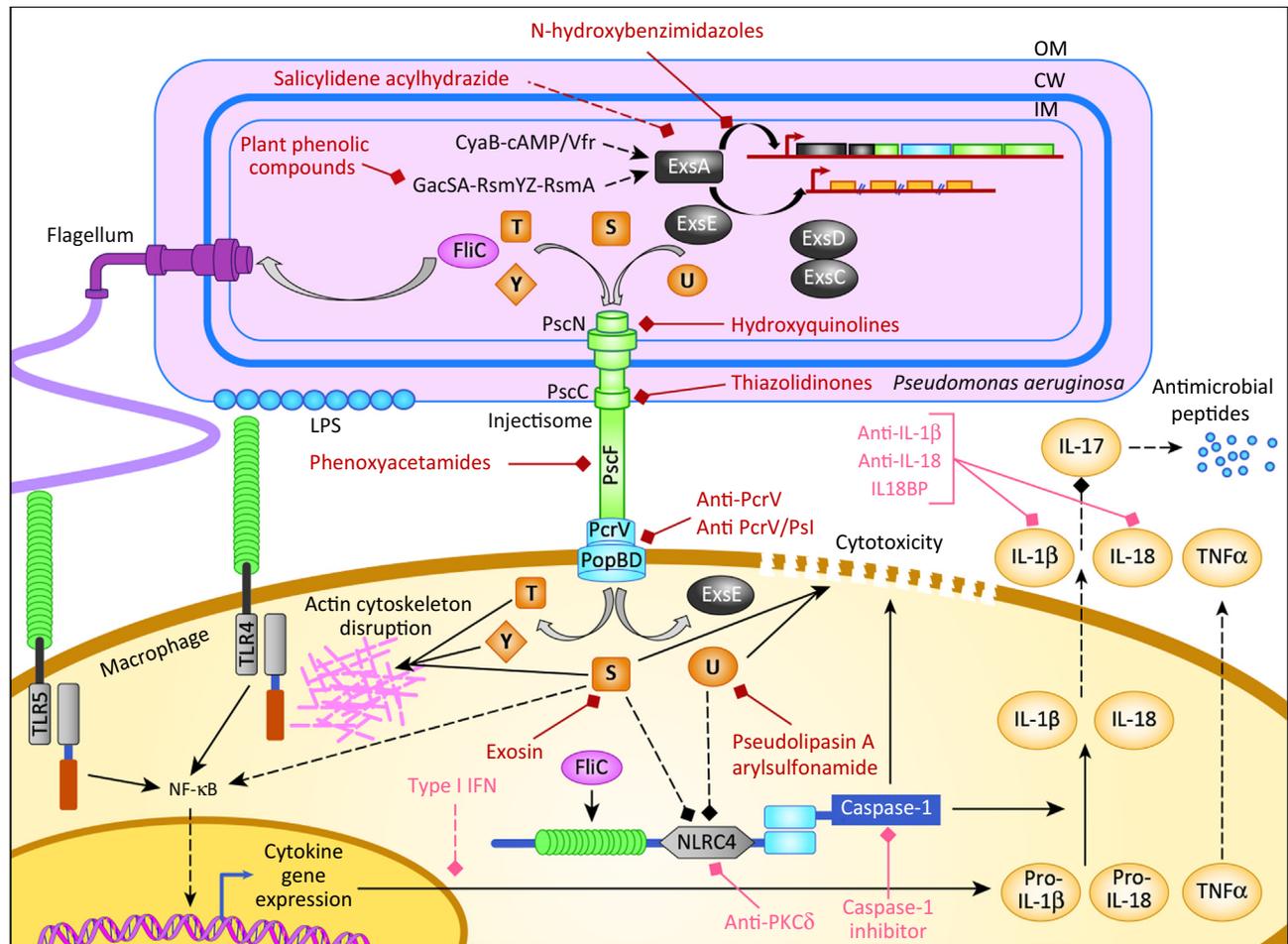
Although still controversial, a deleterious role of NLRC4 inflammasome activation has been demonstrated for *P. aeruginosa* in animal models of acute pulmonary infections [41,42]. Of note, ExoU and ExoS can inhibit NLRC4 inflammasome activation [43], an effect suppressed by FliC overexpression [17]. Conversely, or flagellar motility stimulates NLRC4 inflammasome activation [44].

enzymes, such as proteases and lipases.

**Type IV secretion system (Type IV pili or T4SS):** another type of membrane-bound protein complex in Gram-negative bacteria. Its subunits form a channel in the bacterial cell membrane, and often a pilus or pilus-like structure involved in twitching motility. In contrast to T3SS, T4SS delivers not only proteins, but also nucleoprotein complexes from a donor bacterium to recipient bacteria. Thus, it also plays a role in horizontal gene transfer.

## Key Figure

Type 3 Secretion System (T3SS)-Mediated Interactions between *Pseudomonas aeruginosa* and the Host Cells as a Target for Antivirulence Therapeutic Strategies



Trends in Pharmacological Sciences

**Figure 1.** T3SS (injectisome) can inject several proteins into the host cell cytosol, among which are the exoenzymes S, T, Y, or U and the FliC protein (monomer of flagellin), which can induce cytotoxicity, disrupt the actin cytoskeleton, and activate the NLRC4 inflammasome, causing caspase-1 activation. The active enzyme cleaves pro-IL-1 $\beta$  to IL-1 $\beta$  and pro-IL-18 to IL-18, which are released from the cell and inhibit IL-17 signaling. Lipopolysaccharide (LPS) or T3SS (flagellum) can activate the NF- $\kappa$ B pathway via TLR4 or TLR5 signaling and induce production of inflammatory cytokines. This system is the target of a series of molecules acting on T3SS constituents, effectors, or gene regulation (shown in dark red) or on the host cell response (shown in light red). Stimulating effects are represented by arrows (plain lines for direct effects, broken lines for indirect effects) and inhibiting effects by lines ending with a lozenge. Abbreviations: CW, cell wall; IM, inner membrane; OM, outer membrane.

Together, T3SS effectors and NLRC4 inflammasome activation enhance the proinflammatory response by triggering the release of eicosanoids, TNF- $\alpha$ , IL-1 $\beta$ , and IL-18. The ensuing recruitment of neutrophils and macrophages causes collateral damage to host tissues while failing to eradicate bacteria, perhaps because recruited cells are killed or otherwise impaired by effectors or by NLRC4 inflammasome activation [45]. Moreover, the release of IL-1 $\beta$  and IL-18 reduces the production of IL-17 and the expression of lung epithelial antimicrobial peptides (AMPs), thereby preventing bacterial clearance [41]. At the end, disruption of epithelial barriers

## Box 1. Properties of T3SS Effectors

**ExoS and ExoT (76% Similarity)**

- **Activity:** they are bifunctional toxins with Rho GTPase activating protein (RhoGAP) activity and ADP-ribosyltransferase (ADPRT) activity. These activities work in cohesion to disrupt the host cell actin cytoskeleton, block phagocytosis, and cause cell death [72].
- **Primary structure:** they consist of 453 (ExoS) and 457 (ExoT) amino acids. Both have an N-terminal secretion domain, a chaperone binding domain, a membrane localization domain, enzymatic domains (GAP and ADPRT) and a cofactor binding site. The secretion signal directs effectors to the T3SS apparatus. The chaperone binding domain binds to SpcS, which is important for effector secretion, probably by maintaining them in a secretion-competent conformation [20].
- **Intracellular fate:** once injected inside host cells, ExoS localizes transiently to the plasma membrane and then traffics to the membranes of internal organelles, such as endosomes and the Golgi/endoplasmic reticulum. The localization of ExoS at the plasma membrane is essential for its RhoGAP activity and at the membrane of subcellular compartments for its ADPRT activity [73]. Interestingly, the ADPRT portion of ExoS (and presumably also of ExoT) only becomes activated upon interaction with a host-derived cofactor identified as 14-3-3 proteins (also termed FAS for 'Factor Activating ExoS') [74]. ExoS thus illustrates the propensity of type III effector proteins to hijack host processes and factors, using them to subvert the injected cell.

**ExoU**

- **Activity:** ExoU is the most virulent among the injected effectors, having phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity, but only after interaction with the host cell superoxide dismutase 1 (SOD1) acting as a cofactor.
- **Primary structure:** it has 687 amino acids. ExoU includes a secretion signal directing it to the type III secretion apparatus, a binding domain for SpcU (chaperone of ExoU), immediately adjacent to the patatin-like domain responsible for the PLA<sub>2</sub> activity, and a membrane localization domain.
- **Intracellular fate:** like ExoS, ExoU localizes to the plasma membrane [75].

**ExoY**

- **Activity:** the two domains of ExoY are similar to the corresponding domains of the extracellular adenylate cyclases of *Bordetella pertussis* (CyaA) and *Bacillus anthracis*. However, it has recently been shown that ExoY is a nucleotidyl cyclase with preference for cGMP and cUMP production; its effect is enhanced by an unknown eukaryotic cofactor [35].
- **Primary structure:** it consists of 378 amino acids and contains two adenylate cyclase domains that act together to bind ATP.

allows for the dissemination of bacteria and inflammatory mediators, which eventually results in bacteremia.

### Pharmacological Inhibitors of *P. aeruginosa* T3SS

Taking advantage of the knowledge of the structure of T3SS, the regulation of its expression, and its deleterious effects for the host, different strategies can be envisioned to impair T3SS activity or prevent the consequences of its activation. A series of pharmacological inhibitors have been discovered (Table 1), reflecting these approaches. In most of the cases, however, these inhibitors have been identified by screening and not by rational design towards a specific target. We focus in the text on those for which mechanistic studies led to the identification of a possible molecular target.

#### Targeting the Regulation of T3SS Expression

Many T3SS inhibitors were initially screened for their capacity to inhibit gene transcription, making use of reporter strains. Yet, repression of the T3SS expression was eventually identified as the primary pharmacological effect of inhibitors for three families only.

TS027 and TS103 [46] were identified out of a library of plant phenolic compounds inhibiting exoS transcription by targeting the GacSA-RsmYZ-RsmA-ExsA regulatory pathway. They induce the expression levels of the regulatory small RNAs *rsmY* and *rsmZ*, which repress *exsA* expression by sequestering RsmA. At this stage, their effect on T3SS-mediated cytotoxicity was not investigated.

*N*-hydroxybenzimidazoles emerged from an *in silico* screening for small molecules that interact with the DNA-binding domains of regulators of gene expression in *Escherichia coli* belonging to the AraC family, such as ExsA [47]. *N*-hydroxybenzimidazoles interact with the carboxy-terminal domain of ExsA, preventing its binding to promoter sites on DNA [47,48]. The putative

*N*-hydroxybenzimidazole-binding pocket is located in the ExsA DNA-binding domain. Specific amino acid substitutions in this pocket resulted in altered sensitivity to *N*-hydroxybenzimidazoles, which enabled researchers to determine putative ligand-binding sites and helped designing higher-affinity inhibitors [48]. The interaction between *N*-hydroxybenzimidazoles and ExsA seems to be specific, because no effect has been observed on the binding to DNA of other virulence regulators, such as Vfr. *N*-hydroxybenzimidazole biological effects include a decrease in T3SS gene expression and in T3SS-mediated cytotoxicity *in vitro*.

### Box 2. Regulation of T3SS Expression by ExsA

#### ExsA Activation by T3SS Itself (Figure I)

- ExsD is a negative regulator of ExsA;
- ExsC is an anti-anti-activator; binding to and inhibiting the negative regulatory activity of; ExsD;
- ExsE is a negative regulator of ExsC

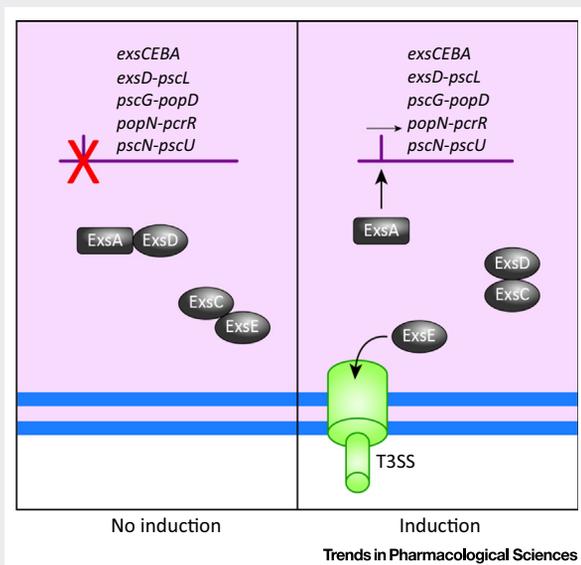
#### ExsA Activation by T3SS-Independent Pathways

Three other global regulatory pathways control ExsA expression (Figure II):

- CyaB-cAMP/Vfr: under inducing conditions, the adenylate cyclase (CyaB) is activated. Together with cAMP (produced by CyaB), Vfr (cAMP-dependent DNA-binding protein) increases *exsA* transcription from a newly identified promoter located immediately upstream of *exsA* [76].
- GacSA-RsmYZ-RsmA: in the presence of environmental stimuli, the tripartite sensor histidine kinase GacS activates its cognate response regulator GacA by phosphorylation, which in turn induces the expression of regulatory small RNAs *RsmY* and *RsmZ*. *RsmY* and *RsmZ* transcripts bind to and sequester the carbon storage regulator RsmA, leading to a repression of *exsA* [19].
- PsrA-RpoS: PsrA, a long-chain fatty acid sensory regulator, directly binds to the promoter region of the *exsCEBA* operon and positively regulates the expression of these genes [18]. It also binds to the promoter region of *rpoS* and positively regulates its transcription, which in turn represses *exsA* expression.

#### Post-Transcriptional Control of ExsA Synthesis

The DeaD RNA helicase increases ExsA expression at the post-transcriptional level, possibly by relieving an inhibitory structure within the *exsA* mRNA that normally prevents its translation [77].



**Figure I. Regulation of T3SS by ExsA.** In the absence of environmental signals, ExsC preferentially binds to ExsE, and ExsD to ExsA, leading to an inhibition of ExsA-dependent transcription. Under inducing conditions, ExsE is secreted by the type 3 secretion system (T3SS), releasing ExsC, which binds to ExsD, thereby liberating ExsA and activating T3SS gene expression [19].

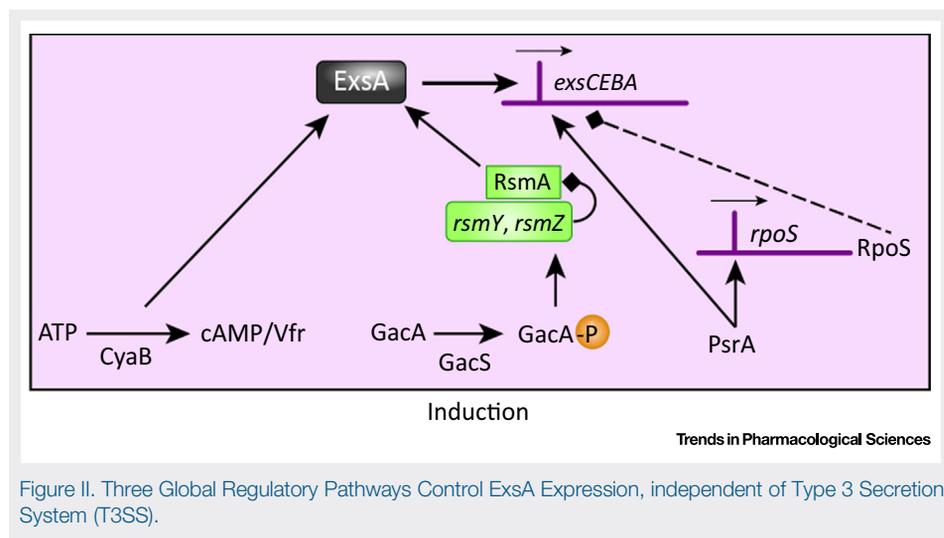


Figure II. Three Global Regulatory Pathways Control ExsA Expression, independent of Type 3 Secretion System (T3SS).

Salicylidene acylhydrazides have been screened as inhibitors of T3SS transcription in different bacterial species, such as *Chlamydia trachomatis* or *Yersinia pseudotuberculosis* [49]. They have been suggested to target three enzymes in *Y. pseudotuberculosis* and *E. coli* (WrbA, Tpx, FolX), which probably indirectly interfere with T3SS regulation by altering cellular metabolism [50,51]. Some of these compounds were also screened as inhibitors of T3SS transcription against *P. aeruginosa*, with INP0341 being the more potent<sup>i</sup>. Although homologs of the target enzymes in *Y. pseudotuberculosis* and *E. coli* are present in *P. aeruginosa*, the antipseudomonal mode of action of INP0341 has not yet been elucidated.

#### Targeting the T3SS Apparatus Functionality

The complex structure of the secretion and translocation apparatus is an opportunity for multiple pharmacological interventions, because a disruption of the function of one of these proteins would compromise the activity of the whole system.

#### Targeting the ATPase Activity

Hydroxyquinolines were first described as inhibitors of T3SS gene expression in *Y. pseudotuberculosis* [52] and then in *P. aeruginosa*, with INP1855 being more potent than IN1750<sup>i</sup>. Further mechanistic studies demonstrated that inhibition of transcription is a consequence of the inhibition of the secretion of the negative regulator ExsE via the T3SS. INP1855 also inhibits the secretion of ExoS and FliC as well as the flagellar motility, suggesting that hydroxyquinolines act upon a target common to both flagellar and injectisome T3SS. This target could be the ATPase, because INP1855 increases ATP levels specifically in strains expressing T3SS and/or flagellum and inhibits the activity of the *Yersinia* T3SS ATPase (Anantharajah et al., unpublished data, 2016). In addition, hydroxyquinolines protect eukaryotic cells from T3SS-mediated cytotoxicity and impair caspase-1 activation and subsequent IL-1 $\beta$  release in phagocytic cells. In a model of acute pulmonary infection, INP1855 improves survival of mice infected with a T3SS-positive strain, reducing lung injury, bacterial burden, and dissemination. It also impairs IL-1 $\beta$  secretion and increases IL-17 secretion in mice infected by a T3SS-positive strain or by a T3SS-negative but flagellated strain, suggesting its usefulness against both types of strain.

#### Targeting the Basal Body

Among thiazolidinones, 2-imino-5-arylidene thiazolidinone was identified as a promising T3SS inhibitor with broad-spectrum activity against Gram-negative pathogens (Anantharajah et al., unpublished data, 2016; [53]). It was discovered by high-throughput screening for inhibitors of

Table 1. Inhibitors of *Pseudomonas aeruginosa* T3SS Described in the Literature and their Main Characteristics<sup>a</sup>

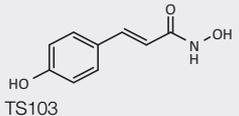
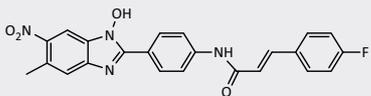
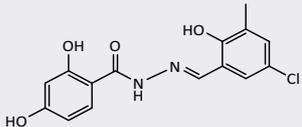
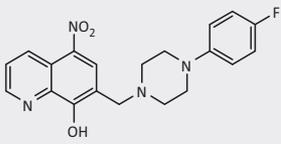
Inhibitors	Chemical Structure	Spectrum of Activity (beside <i>P. aeruginosa</i> )	Possible Molecular Target	Effects on Host Cells	<i>In vivo</i> Activity	Refs
<b>T3SS Genetic Regulation</b>						
Plant phenolic compounds (TS027 and TS103)	 TS103	ND	GacSA-RsmYZ-RsmA-ExsA regulatory pathway	ND	ND	[46]
<i>N</i> -hydroxy-benzimidazoles		<i>Yersinia pseudotuberculosis</i>	Carboxy-terminal domain of ExsA, preventing its binding to promoter sites on bacterial DNA	↘ T3SS-mediated cytotoxicity	ND	[47,48]
Salicylidene acylhydrazides (INP0341)		<i>Chlamydia trachomatis</i> , <i>Y. pseudotuberculosis</i>	WrbA, Tpx, and FolX enzymes, thereby indirectly interfering with T3SS regulation by altering cellular metabolism	↗ Bacterial internalization; ↘ T3SS-mediated cytotoxicity	ND	[49]
<b>T3SS Apparatus Functionality</b>						
Hydroxyquinolines (INP1855)		<i>C. trachomatis</i> , <i>Y. pseudotuberculosis</i>	ATPase of T3SS (PscN) and flagellum	↗ Bacterial internalization; ↘ translocation of ExsE, ExoS, FlhC; ↘ ExoS, ExoU, and NLRC4-mediated cytotoxicity; ↘ inflammatory response	Animal models of acute pulmonary infection (prophylactic and therapeutic)	[52]

Table 1. (continued)

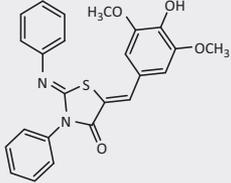
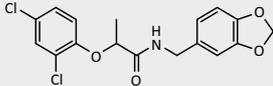
Inhibitors	Chemical Structure	Spectrum of Activity (beside <i>P. aeruginosa</i> )	Possible Molecular Target	Effects on Host Cells	<i>In vivo</i> Activity	Refs
Thiazolidinones 2-imino-5-arylidene thiazolidinone (TTS29)		<i>Yersinia enterocolitica</i> , <i>Salmonella enterica</i> ,	PscC secretins (components of basal body shared by type II, III, and IV secretion systems)	ND	ND	[53,54]
Phenoxy-acetamide (MBX1641)		<i>C. trachomatis</i> , <i>Yersinia pestis</i>	PscF (component of T3SS needle protein)	↗ Bacterial internalization; ↘ T3SS-mediated cytotoxicity	ND	[55–57]
Anti-PcrV antibody (KB001; V2L2MD)	Antibody	ND	PcrV (component of T3SS translocon apparatus)	↘ T3SS effector translocation; ↗ phagocytic function of macrophages	Animal models of acute and chronic pulmonary infection, bacteremia, or septic shock; clinical trial for (i) ventilator-associated pneumonia in intensive care units and (ii) chronic pneumonia in cystic fibrosis patients	[63,64]
Anti-PcrV/PsI (MEDI3902)	Antibody	ND	PcrV (component of T3SS translocon apparatus) and PsI (exopolysaccharide)	↘ Cytotoxicity and attachment to epithelial cells	Animal models of acute pulmonary infection (prophylactic and therapeutic), immunocompromised pneumonia, thermal injury and bacteremia; clinical trials for prevention of hospital-acquired pneumonia in adults and the elderly	[66]

Table 1. (continued)

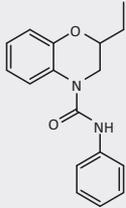
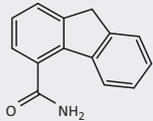
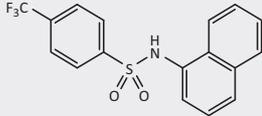
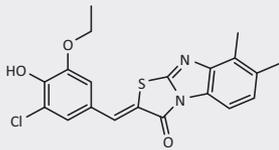
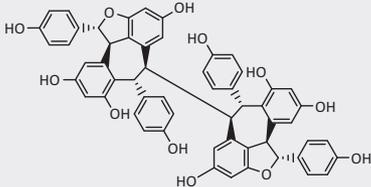
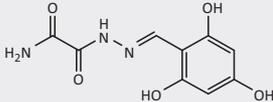
Inhibitors	Chemical Structure	Spectrum of Activity (beside <i>P. aeruginosa</i> )	Possible Molecular Target	Effects on Host Cells	<i>In vivo</i> Activity	Refs
<b>T3SS Effector Proteins</b>						
Exosin		ND	ADP-ribosyltransferase enzymatic activity of ExoS	↘ ExoS cytotoxicity (apoptosis and necrosis) against mammalian cells	ND	[67]
Pseudolipasin A		ND	Phospholipase A2 activity of ExoU	↘ ExoU-dependent cell death	ND	[68]
Arylsulfonamides		ND	Phospholipase A2 activity of ExoU	↘ ExoU-dependent cell death	ND	[69]
<b>Not Well Defined</b>						
C20, C22, C24, C34, and C38	 C22	<i>Y. pseudotuberculosis</i>	T3SS secretion/translocation?	↘ T3SS-dependent cell rounding	ND	[78]

Table 1. (continued)

Inhibitors	Chemical Structure	Spectrum of Activity (beside <i>P. aeruginosa</i> )	Possible Molecular Target	Effects on Host Cells	<i>In vivo</i> Activity	Refs
Resveratrol tetramer (-)-hopeaphenol		<i>C. trachomatis</i> , <i>Y. pseudotuberculosis</i>	T3SS secretion?	∖ T3SS-dependent cytotoxicity	ND	[79]
Gallium(III)-salicylidene acylhydrazides	 ME0163	ND	T3SS expression/secretion?	Antibiofilm properties	ND	[80]

<sup>a</sup>ND, not determined.

the secretion of effectors with phospholipase A2 activity. In *P. aeruginosa*, this compound inhibits the secretory activity of both T3SS and T2SS, as well as the twitching motility depending on Type IV pili, without affecting flagellar motility. It was therefore hypothesized that thiazolidinones could target secretin, the only protein shared by type II, III, and IV secretion systems [9].

This pharmacophore has then been used to explore structure–activity relationships, leading to the design of more potent inhibitors constructed on a *N*-3-dipeptide scaffold, and of thiazolidinone dimers, which showed improved physicochemical properties and activity when tested as inhibitors of secretion in *Salmonella enterica* [54]. These studies should be expanded to *P. aeruginosa*.

#### Targeting the Needle Complex

Phenoxyacetamides represent another class of inhibitors discovered by screening as acting on transcription [55], among which MBX1641 was selected for further studies. MBX1641 decreases the secretion and translocation activities of T3SS without affecting T2SS activity and general protein expression. Phenoxyacetamides were suggested to specifically bind to the needle protein PscF, based on the observation that mutations in this protein make them inactive [56]. Phenoxyacetamides reduce T3SS-mediated cytotoxicity and facilitate internalization of bacteria in HeLa cells. Recent structure–activity relationships have allowed developers to optimize the potency of these compounds, their specificity of action as inhibitors of secretion, and their innocuity for eukaryotic cells [57].

#### Targeting the Translocon Apparatus

Given that translocon is the most accessible part of the T3SS, it has been selected for targeting by antibodies. A rabbit polyclonal anti-PcrV antibody and a murine monoclonal anti-PcrV antibody (mAb166.2a) were shown to inhibit effector translocation and to restore phagocytic function of macrophages [58,59]. Despite sequence variability of PcrV among strains, anti-PcrV antibodies are effective in reducing cytotoxicity of a wide range of clinical isolates, suggesting their applicability in the clinics [60]. Anti-PcrV vaccination has also proved effective in mice with acute and chronic pulmonary infection, bacteremia, or septic shock, decreasing tissue inflammation and injury [59,61].

On these bases, KB001 (Kalobios Pharmaceuticals; San Francisco, CA, USA), a molecular-engineered humanized anti-PcrV IgG antigen-binding fragment, was prepared starting from mAb166.2a IgG and developed for clinical use [62]. KB001 has completed Phase II clinical trials for ventilator-associated pneumonia [63] and chronic pneumonia in cystic fibrosis. While KB001 demonstrated a favorable pharmacokinetic profile, innocuity, and promising effects in acute infections, it failed to improve symptoms in cystic fibrosis patients and was, therefore, abandoned<sup>ii</sup>. It could be argued that cystic fibrosis patients were probably not the most appropriate target population, because T3SS expression is low in chronic infections [4].

Research is still active in this field, as witnessed by the demonstration of the superiority of the novel anti-PcrV MAb V2L2MD over MAb166.2a, in multiple animal models [64].

Bis4 $\alpha$ Pa (MEDI3902; MedImmune LLC; Nijmegen, The Netherlands) is a bispecific monoclonal antibody targeting both PcrV and Psl (an exopolysaccharide involved in the attachment of *P. aeruginosa* to host cells, immune evasion, and biofilm formation). It reduces cytotoxicity of clinical isolates expressing PcrV (100%) and Psl (98%), underlining the pertinence of the selected targets [65]. It has proved protective against diverse clinical strains, including multidrug-resistant ones, in animal models of infection, when used both as a prophylaxis or a treatment [66]. It shows synergy with antibiotics, even against drug-resistant clinical isolates. MEDI3902 has completed a Phase I clinical trial to evaluate its safety and pharmacokinetics in healthy adults and is currently in Phase II evaluation for the prevention of hospital-acquired pneumonia in adults and the elderly.

### Targeting T3SS Effector Proteins

Besides inhibiting the T3SS itself, an alternative strategy has been to find molecules acting downstream to counteract effector toxic effects on the host cells. This approach has proved successful, at least *in vitro*. However, a limitation is that such inhibitors could have a narrower spectrum of effectiveness because all effectors are not expressed in each single strain.

The ExoS inhibitor exosin impairs ExoS ADP-ribosyltransferase enzymatic activity by acting as a competitive inhibitor against NAD<sup>+</sup> substrate [67], which is accompanied by a reduction in ExoS cytotoxicity for mammalian cells. No effect was observed on strains expressing other effectors.

Pseudolipasin A specifically inhibits the phospholipase A<sub>2</sub> activity of ExoU, without affecting any of other eukaryotic phospholipases tested [68], or modifying ExoU secretion or translocation in the host cells. Again, this effect protects cells from cytotoxicity of strains expressing ExoU. A series of arylsulfonamides was compared with pseudolipasin A, but proved less potent to inhibit ExoU-mediated cytotoxicity [69].

### Targeting the Immune Response

Besides effector-related effects, T3SS also exerts deleterious effects by modulating host immune response. Instead of targeting bacteria or virulence factors, another approach could consist of acting upon the host, with the objective of initiating or enhancing protective antimicrobial immunity while limiting inflammatory tissue injury.

In the specific case of *P. aeruginosa*, counteracting the deleterious effects of NLRC4 inflammasome activation could be useful. Although this strategy has not yet been exploited in therapeutics, its relevance has been demonstrated in experimental models.

Pharmacological inhibition of IL-1 $\beta$  and IL-18 production can be achieved by stimulating type I IFN signaling using polyinosinic:polycytidylic acid or by inhibiting caspase-1. In mice, the latter strategy not only reduces IL-1 $\beta$  production, but also improves bacterial clearance and lung pathology without altering the nature of immune cell recruitment into the airways and the levels of other inflammatory cytokines [42]. Likewise, anti-PKC $\delta$  antibodies reduce inflammasome activation (demonstrated so far only for macrophages infected by *Salmonella enterica*) [70], but at the same time, they downregulate antimicrobial peptide expression. The latter effect is deleterious because antimicrobial peptides are needed to ensure bacterial clearance, even when IL-1 $\beta$  or IL-18 are reduced [41]. By contrast, specific inhibition of IL-18 by recombinant IL-18 binding proteins (IL-18BP; soluble receptor for IL-18) or anti-IL18 antibodies decreases airway inflammation and restores IL-17-mediated antimicrobial peptides expression, leading to enhanced bacterial clearance [41].

Vaccination is another way to modulate the immune response. In this context, immunization with purified PopB stimulates IL-17 production, conferring protection against *P. aeruginosa* pneumonia [71].

### Concluding Remarks and Future Perspectives

Over the past few years, we have witnessed remarkable progress in the understanding of the structure, function, and host cell response to the T3SS of *P. aeruginosa*. This knowledge has set the scene for the research of effective inhibitors, and we have now in our hands a few families of small-molecule inhibitors and antibodies having demonstrated their relevance as inhibitors of *P. aeruginosa* virulence. Although the results collected so far with these molecules are encouraging, they remain nevertheless preliminary, paving the way for future research in this area (see Outstanding Questions). First, in most of the cases, the molecular target of the inhibitors remains to be identified, because the majority of them have been identified by simple screening based on

### Outstanding Questions

#### At the Molecular Level

What is the molecular target of the existing T3SS inhibitors and their mode interaction with this target?

Can more specific, potent, and effective inhibitors be developed, taking advantage of the current knowledge of T3SS structure and function, based on structure-activity relationships, computer-assisted drug design, and the crystallization of inhibitor-target complexes?

How fast will resistance to T3SS inhibitors emerge? Is it avoidable by dose optimization (as for antibiotics), combination therapy, or multi-target molecules?

#### At the Cellular Level

What are the consequences for bacterial cells of T3SS inhibition: fitness cost, dysregulation of the expression of other virulence factors, induction of stress responses, cross-talk with quorum sensing, or the expression of resistance mechanisms?

How do eukaryotic cells react to the deleterious effects of effectors and T3SS: mechanisms of cell death, modulation of inflammation and cytokine response, or modulation of gene expression and signal transduction? On this basis, is there any relevance to combining T3SS inhibitors with other immunomodulatory therapies?

#### At the Therapeutic Level

Are these inhibitors effective *in vivo*? Do they modulate inflammasome activation *in vivo* and is it deleterious or beneficial for the host (still controversial)?

Do we need to develop diagnostic tests to demonstrate the expression of T3SS or of specific effectors before initiating therapy?

Which clinical indications need to be explored first: acute or chronic infection, in specific tissues or organs? Prophylaxis or treatment? Monotherapy or combination? What is the most appropriate route of administration?

inhibition of gene transcription. Yet, because of the complex regulation of T3SS, this effect can be indirect as observed for hydroxyquinolines. Second, improving inhibitor efficacy, based on experimentally determined structure–activity relationships or on rational drug design, should be more feasible in the future if progress can be made in the elucidation of the crystal structures of the T3SS components. Third, the biological evaluation of the pharmacological effect of these compounds is often limited to the demonstration of a reduction in cytotoxicity and the majority of these compounds have not yet been tested *in vivo*. In-depth study on how they modulate the host response would not only help to better define their potential interest, but also to further characterize the host–pathogen interaction. *In vitro* studies should examine the influence of inhibitors on phagocytosis, host cell death (including its mechanisms), inflammatory response, and their interference with signal transduction. *In vivo* studies should demonstrate the protection in acute models of infection as well as innocuity for the host and establish their pharmacokinetic profile. Fourth, due to the multiplicity of possible targets within the T3SS and among other virulence factors, multi-target approaches (as the bifunctional anti-PcrV/Psi antibodies) are worth further investigation, because they can broaden the spectrum to strains expressing different profiles of virulence factors. If successful, all these steps could help to put forward some inhibitors deserving clinical evaluation.

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### Resources

<sup>i</sup> [www.facm.ucl.ac.be/posters/2012/2-ICAR/Anantharajah-et-al-ICAR-2012.pdf](http://www.facm.ucl.ac.be/posters/2012/2-ICAR/Anantharajah-et-al-ICAR-2012.pdf).

<sup>ii</sup> <http://ir.kalobios.com/releasedetail.cfm?ReleaseID=889910>.

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