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REVIEWS

The Type Three Secretion System of *Pseudomonas aeruginosa* as a Target for Development of Antivirulence Drugs

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Abstract—*Pseudomonas aeruginosa* is one of the leading antibiotic-resistant gram-negative organisms responsible for nosocomial infections. Multidrug pathogen resistance leads to the low antibiotic therapy efficiency. The solution for this problem involves developing new therapeutic agents that operate under different principles to the currently available antibiotics. The Type Three Secretion System (T3SS) is a major virulence factor in *Pseudomonas aeruginosa*. This review presents a brief description of structure and regulation of T3SS, which has been shown to contribute to the virulence of Gram-negative bacteria with different types of parasitism and is extremely necessary for the manifestation of the pathogenesis of diseases caused by them. The secretion apparatus is formed after bacteria contact with eukaryotic cell and allows the bacterium to inject toxins directly into the host cell cytoplasm. The T3SS regulation is a strictly hierarchically organized process that occurs at least at two levels, transcriptional and secretory. Thus, T3SS appears to be a highly attractive target for innovative therapies as it possesses a number of advantages over antibiotics: T3SS inhibitors are expected to have a lower risk of selecting resistance because they do not suppress the viability of pathogens, but only reduce bacterial virulence; inhibitors will be effective regardless of acquired antibiotic resistance; inhibitors will not to exert negligible effects on commensal bacteria. To date, a number of T3SS inhibitors with various nature and different mechanism of action have been identified. The discovered inhibitors suppress the transcription of the T3SS genes, toxins translocation and inhibit the effector molecules. For many of the developed inhibitors, their specific activity was shown in in vitro experiments, for few of them the antibacterial effect was shown in animal models and only two inhibitors are ongoing to test in clinical trials now: the Ftortiazinon and the antibodies MEDI3902.

Keywords: Pseudomonas aeruginosa, antibiotic resistance, Type III Secretion System, virulence, inhibitors, infectious process, review

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INTRODUCTION

Pseudomonas aeruginosa is a opportunistic gram-negative pathogen that causes up to 30% (in some departments up to 50%) of all opportunistic nosocomial infections, which are accompanied by a high level (34–48%) mortality. In a high percentage of cases, *Pseudomonas* is a cause of nosocomial pneumonia, primary bacteremia, urinary tract infections, and purulent surgical and burn infections. Despite timely treatment with antibiotics, rates of mortality from *Pseudomonas* infections remain high, with mortality reaching 60% in the case of patients undergoing mechanical ventilation (MV) [1].

Difficulties in the treatment of *Pseudomonas* infection are caused by an extremely high prevalence of multiple antibiotic resistance in this pathogen. *Pseudomonas* strains circulating in hospitals are frequently resistant to almost all classes of antibiotics available in hospitals [2].

In the current situation, a need to develop new strategies for solving this problem is becoming apparent. Changing the paradigm of infection control is an innovative solution: the drug should suppress virulence, but do not kill the pathogen, thus preventing the selection of emerging mutations of resistance to the drug used. Destruction of a pathogen that has lost its virulent properties will occur as a result of the effect of the host organism's immune defenses. The suppression of virulence will lead to a limitation in manifestation of symptom complex of the disease, as well as allowing the death of normal (nonpathogenic) human microflora to be avoided [3].

Pseudomonas has a variety of pathogenicity factors, but the leading role among them belongs to the type-

three secretion system (T3SS). Mutations leading to violation of T3SS functions cause a decrease or loss of the virulence [4]. Acute *Pseudomonas* infections caused by the strains secreting T3SS effector toxins are characterized by a high concentration of the pathogen in affected organs and tissues and frequent relapses after recovery, as well as leading to a significant increase in mortality [5].

Thus, T3SS of *Pseudomonas* is a promising target for the development of innovative antivirulence drugs based on T3SS inhibitors. In this review, modern ideas about the structure, function, and regulation of *Pseudomonas* T3SS are considered, promising targets for the effect of inhibitors as part of the secretory apparatus are characterized, and data on the most efficient *P. aeruginosa* T3SS inhibitors are presented.

STRUCTURE OF *P. aeruginosa* T3SS

T3SS is a highly conservative structure, which has a significant similarity to unrelated species of gram-negative microorganisms [6, 7]. Structurally, T3SS is a transmembrane complex (or injectisome), which allows the bacterium to introduce its toxins (called "effectors") directly into the cytosol of eukaryotic target cell. The process of the formation of T3SS structural apparatus and the transport of effectors is activated as a result of the contact between bacterial and eukaryotic cells [8]. T3SS passes through the inner and outer membrane of the bacterium and the host cell membrane. Three structural parts of T3SS can be allocated: a cytoplasmic part, the basal body, and an extracellular part.

The cytoplasmic part of T3SS includes the export apparatus (EA), cytoplasmic ring, and ATPase complex. EA is located in the inner membrane of the cell wall and is assembled from five membrane proteins: PscR, PscS, PscT, PscU, and PcrD [8]. The PcrD protein forms export gates that have the form of a ring consisting of seven subunits; in a complex with other proteins, EA performs the function of the entrance portal of T3SS channel for the passage of substrates [9]. The cytoplasmic ring (C-ring), formed by the PscQ protein and located around the ATPase complex, is located directly under the export apparatus. The ATPase complex consists of PscN ATPase protein, PscO stalk protein, and PscL protein, which connects the C-ring and ATPase and is a negative regulator, as well as PscK cofactor protein [10]. These proteins form a sorting platform.

The basal body consists of ring structures integrated into the bacterium cell wall. The proteins of the inner membrane (PscJ and PscD) form two membrane rings built into each other, EA is located inside these rings [11]. The ring of the outer membrane formed by N-terminal domains of the PrsC protein (or secretin) penetrates deep into the periplasm and interacts directly with the ring of the inner membrane OscD, thus connecting inner and outer membrane rings.

The extracellular part of T3SS consists of a needle and translocation apparatus. The needle consists of helical hundreds of copies of the PscF protein and PscI inner core protein conditioning the association with the basal body [12]. The needle tip PcrV protein is located at the end of the needle. The needle length is controlled by the PscP protein, and the needle length can differ in different species of bacteria [13].

Upon coming into contact with the host cell, T3SS forms a translocation apparatus in the membrane of eukaryotic cell; it consists of three structural proteins, two secreted PopB and PopD proteins (that interact with each other and form a PopB/D complex required for the formation of a pore in the host cell membrane), and one PcrV protein. The *Pseudomonas* PcrV protein is a highly specific protein. The transport of effectors from bacterial cell to the target cell cytosol is performed through the translocation pore [14].

REGULATION OF *P. aeruginosa* T3SS SECRETORY ACTIVITY

It is known that, first of all, direct contact of injectisome with the host cell is a signal that triggers the expression of T3SS genes, but the exact mechanism of signal transduction is still poorly studied. The secretion process is regulated at two levels: transcriptional and secretory. In the absence of secreting conditions, the secretion of T3SS proteins is at a basic level.

Regulation of T3SS Gene Expression by ExsA Transcription Activator

The *P. aeruginosa* T3SS regulon consists of approximately 40 genes organized inside ten transcriptional units. They encode structural, regulatory, and effector proteins, as well as cytoplasmic chaperons [15].

All T3SS genes, including the genes encoding the effectors, are under the control of ExsA protein (AraC-type transcriptional activator, which is a central regulator of the *P. aeruginosa* T3SS regulon. Blocking its functions leads to a decrease in the expression of T3SS genes and inhibition of the *P. aeruginosa* virulence [15].

The regulation of T3SS gene expression is controlled by an *ExsADCE* cascade out of regulatory ExsE, ExsC, ExsD, and ExsA proteins. In the absence of T3SS-inducing signals, ExsC is linked to ExsE, and ExsD to ExsA, which leads to the inhibition of ExsA-dependent transcription. In conditions of induction (contact with eukaryotic cell, reduced amount of calcium), there is an allocation of ExsE negative regulator, release of ExsC, and its binding to ExsD, and finally release of ExsA, which binds to its promoter and activates the transcription of T3SS genes. The

ExsADCE cascade allows the expression of T3SS genes to be induced rapidly [16].

Activation of ExsA by T3SS-Independent Pathways

In addition to the activation of ExsA with the involvement of the above cascade, the *exsA* gene expression is also regulated by three regulatory pathways: CyaB-cAMP/Vfr, GacSA-RsmYZ-RsmA, and PsrA-RpoS.

CyaB-cAMP/Vfr pathway. Vfr is cAMP-dependent regulator of transcription known as a global regulator of virulence gene expression. Vfr-regulon consists of approximately 200 genes involved in the regulation of expression of type-three and -two secretion systems, type-IV pili, and quorum sensing system genes. In conditions of induction of secretion, adenylate cyclase (CyaB) is activated and produces cyclic AMP interacting with the Vfr protein. Together with cAMP, Vfr regulator increases the *exsA* gene transcription, interacting with the promoter located directly before the *exsA* [17].

GacSA–RsmYZ–RsmA pathway. The expression of *P. aeruginosa exsA* gene is also regulated by a carbon storage regulator (RsmA). GacS is a trilateral sensory histidine kinase, which perceives secretion induction signals from the environment that activate the GacA response regulator by phosphorylation; in turn, this indices the expression of RsmY and RsmZ small regulatory RNAs. The RsmY and RsmZ transcripts bind the RsmA carbon storage regulator, which leads to a decrease in the *exsA* expression [15].

PsrA–RpoS pathway. PsrA, a sensor regulator of long chain fatty acids, binds directly to the exsCEBA operon promoter region and positively regulates the expression of these genes. Along with this, PsrA also binds to the rpoS gene promoter region and positively regulates its transcription, which in turn represses the expression of *exsA* and other T3SS genes [18].

Secretory Pathway of T3SS Regulation

The control of T3SS assembly and functioning is also performed at the level of secretion of the proteins that are divided into the following groups: early (needle and core proteins), medium (translocators), and late (effector) substrates. The secretion process has a strict hierarchy and happens step by step [8].

At the first stage, PscU autoprotease and PscP protein, which controls the needle length, are involved in the regulation of secretion of early substrates that directly form the T3SS needle. At the first stage, the PscU is involved in switching the secretion to medium substrates, which creates a translocation pore in the host cell membrane. Such switching is controlled by changing the PscU protein conformation and its interaction with the export apparatus proteins [19].

At the following stage, a whole complex of cytoplasmic proteins works: PcrG regulator, PopN gatekeeper, T3SS ATPase (PscN), and late substrate-specific chaperons. These proteins switch the secretion from medium to late substrates, which allows T3SS effectors to be delivered through the needle and translocation pore to the host cell [8, 20].

The PopN protein is linked to three proteins: Pcr1, Pcr2, and PscB. The Pcr2 and PscB proteins form a heterodimeric chaperon. This complex of four proteins is connected to T3SS through the interaction between Pcr1 and PcrD. The PopN protein and related regulators control the secretion of effectors due to the fact that they partially block the secretion channel, being attached to T3SS. In inducing conditions, the PopN releases the secretion channel, while the effectors obtain access to the secretory apparatus. The access occurs through the sorting platform [20].

For subsequent secretion from the bacterial cell, the effectors and their related chaperones interact with the ATPase complex. Here, ATP hydrolysis using ATPase helps to remove chaperones from the effector–chaperon complex and simultaneously unfold them, and them secrete the effectors though the T3SS needle [10].

Thus, attaching the needle to the host cell membrane and the formation of the translocation pore starts the delivery of unfolded effectors through a hollow channel, formed inside the needle, directly to the cytosol of eukaryotic cells.

It has become known recently that another cytoplasmic protein (PscO) energetically provides the functioning of the secretory apparatus with the involvement of a proton-motive force. Thus, P. Halder et al. [10] demonstrated on a model of rotational secretion that the proton-motive force (along with ATP hydrolysis) is responsible for the injectisome base rotation and, thus, secretes unfolded molecules of effector proteins through revolution through the cytoplasmic complex, basal body, needle, and, finally, to the eukaryotic cell.

EFFECTORS AND OTHER SECRETED T3SS PROTEINS

P. aeruginosa has a variety of pathogenicity factors, but a leading role in the pathogenesis belongs to effector T3SS proteins. At present, four effector exotoxin proteins have been identified in *P. aeruginosa*: ExoU, ExoS, ExoT, and ExoY [6].

The most virulent *P. aeruginosa* strains produce ExoU exotoxin. N-terminal fragment of ExoU exotoxin has a phospholipase activity. The cell death, which is characterized by a sudden (within 1–2 h) violation of the cytoplasmic membrane integrity (as during necrosis), is a final result of the toxic effect of ExoU on the cell. The toxic effect of ExoU is targeted at phagocytes, as well as at overcoming the epithelial

barrier, which contributes to bacterial dissemination and persistence [21].

The ExoS and ExoT exotoxins are bifunctional proteins activating GTPases and having ADP-ribosyltransferase activity. Despite the fact that the ExoT and ExoS effectors are 76% identical in terms of amino acids and structural similarity, they are functionally different.

The activity of ExoS works toward breakage of the cytoskeleton, which leads to cell rounding and a decrease in the Pseudomonas capture by certain types of cells (that is, causes the inhibition of phagocytosis). Irreversible destruction of the cytoskeleton can lead to a violation of the cell contacts and contribute to the penetration of *Pseudomonas* through the epithelial barrier. The death of immune cells under the influence of *P. aeruginosa* ExoS allows the pathogen to persist in the organism [22].

The activity of ExoT is aimed at the suppression of migration, adhesion, and proliferation of cells, as well as blocking phagocytosis and violation of the epithelial barrier integrity, which contributes to bacterial dissemination [23].

The significance of the fourth effector protein (ExoY, which is adenylate cyclase) is still not fully studied. Its activity leads to violation of the cytoskeleton, inhibition of *Pseudomonas* capture by host cells, and an increase in endothelium permeability [24].

Other proteins, such as pilin (PilA, the main component of type IV pili) or PcsI (the component of T3SS basal body core), as well as different flagellum proteins, including flagellin (FliC), are also secreted by means of T3SS. The recognition and transport of FliC is caused by the homologous and structural similarity of the flagellum basal body and T3SS [8].

TARGETS AND MECHANISM OF ACTION OF *P. aeruginosa* T3SS INHIBITORS

To date, several classes of small-molecule substances that specifically inhibit T3SS of gram-negative bacteria have been identified. In addition to small-molecule compounds, T3SS inhibitors are also represented by polymers, proteins, mimetic polypeptides, and polysaccharides. Specific targets in T3SS have been detected for several inhibitors, but most targets for T3SS inhibitors have yet to be identified and characterized. Summary information about currently known T3SS inhibitors, their molecular targets, and members of bacteria genera for which the effect of inhibitors was demonstrated, as well as on chemical classes of compounds, is presented in Table 1.

According to the targets of the effect on T3SS, the inhibitors can be divided into the following groups.

(1) Ones that act on the genetic regulation of T3SS (hydrazones of salicylic aldehyde, N-hydroxy-benzimidazoles, plant phenolic compounds).

(2) Ones that act on the functioning of T3SS apparatus (hydroxyquinolines, thiazolidinones, phenylacetamides, PcrV antibodies (KB001, V2L2MD), PcrV/PsI antibodies MEDI3902).

(3) Ones that act on the effector T3SS proteins (exocin, aryl sulfonamides, pseudolipase A, (-)-hopeaphenol).

INHIBITORS OF T3SS GENETIC REGULATION

Compounds of the class of salicylidene acylhydrazides, which are active relative to a wide range of bacteria, such as *Y. pseudotuberculosis*, *S. enterica* serovar Typhimurium, *Shigella* spp., *Chlamydia* spp., *E. coli* O157:H7, *P. aeruginosa*, and *Erwinia amylovora* plant pathogen, were some of the first well-characterized T3SS inhibitors [25, 26].

In 2003, as a result of screening a chemical library consisting of 9400 compounds, A. Kauppi et al. [25] identified several salicylidene acylhydrazides , such as INP0007 and INP0010, efficiently inhibiting the secretion of *Y. pseudotuberculosis* YopE effector in vitro.

Another compound of this class (INP0341) suppressed the transcription of T3SS genes, including in strains with their constitutive expression, and reduced the level of expression of exotoxins. It was suggested that the suppression of transcription of an operon encoding the *P. aeuginosa* T3SS is a possible mechanism of action of the inhibitor [26].

INP0341 has been shown to inhibit T3SS-dependent intracellular replication of *C. trachomatis* in HeLa cells, suppressing the secretion of chlamydial effector protein IncA. Compounds of this class also suppress the secretion of effector proteins in *Salmonella enterica* in vitro [27].

The results of studies on models in animals have demonstrated a decrease in clinical symptoms of infections caused by *S. enterica* serovar Typhimurium and *Citrobacter rodentium* after therapy with salicylidene acylhydrazides [28]. In another work [29], the efficiency of therapy with INP0341 compound for vaginal chlamydial infection in mice was demonstrated.

The effect of INP0341 inhibitor on the *P. aeruginosa* was demonstrated on a model of burn infection in mice. Therapy with INP0341 significantly increased the lifespan in animals in the treatment group; however, it did not prevent the systemic spread of infection and death of mice [30].

Unsatisfactory pharmacokinetic parameters associated with inability to reach high concentrations in blood plasma, as well as the short half-life period of this substance, can be attributed to disadvantages of INP0341 salicylidene acyl hydrazide compound. Thus, hydrazones of salicylic aldehyde can be considered promising drugs based on specific T3SS inhibitors of a

Table 1. Summary information on *Pseudomonas aeruginosa* T3SS inhibitors and their main characteristics

Inhibitors	Proposed mechanism of action	Effect on eukaryotic cell	Action in vivo	Reference
Salicylidene acylhydrazides (INP0341)	Suppression of expression of the operon encoding T3SS is a possible mechanism of action	↓T3SS-mediated cytotoxicity, expression and secretion of ExoS; formation of biofilms; mobility due to the action on the flagellum ↑Internalization of bacteria	Model of burn infection against a background of immunodeficiency	[26—30]
N-hydroxy-benzimidazoles	Carboxy-terminal domain of ExsA preventing binding to promoter sites on bacterial DNA	↑T3SS-mediated cytotoxicity	ND	[31—33]
Plant phenolic compounds (TS027 and TS103)	Effect on ecoS expression though Gac–SA–RsmYZ–RsmA–ExsA regulatory pathway	ND	ND	[34]
Hydroxyquinolines (INP1855)	Violation of ExsE secretion; inhibition of ATPase activity of T3SS and flagellum	↑Internalization of bacteria ↓Inflammatory response; ExsE, ExoS, FliC translocation; ExoS, ExoU, and NL-RC4-mediated cytotoxicity	Model of acute pneumonia in animals (prevention and treatment)	[35—38]
Thiazolidinones (TTS29)	Presumably affects PscC secretin	ND	ND	[39—41]
Phenoxy-acetamides	PscF (needle protein component)	↓ExoS secretion and translocation ↑Internalization of bacteria	↓Abscess area	[42—44]
Thiadiazinones (fluorothiazinone)	Presumably acts at the stage of translocation of effector proteins	↑Internalization of bacteria by HeLa cells; phagocytic activity; ↓Translocation of effector proteins; inflammation response; dose-dependent decrease in cytotoxicity, apoptosis, necrosis	Model of acute pneumonia in mice, prevention and treatment; successful completion of phase 1 clinical trials in healthy volunteers; phase II clinical trials	[45—50]
Anti-PcrV/PsI antibodies (MEDI3902)	PcrV (component of T3SS translocation apparatus) and PscI (exopolysaccharide)	↓Cytotoxicity and ability of bacteria to attach to epithelial cells	Model of acute pneumonia in animals (prevention and treatment), pneumonia in immunodeficient animals, burn infection, and bacteremia; Successful completion of phase I clinical trials; stage IIb clinical trials	[51]
Anti-PcrV antibodies (KB001, V2L2-MD)	PcrV (component of T3SS translocation apparatus)	↓Translocation of effector proteins; ↑Phagocytic function of macrophages	Acute and chronic infection pneumonia, bacteremia, septic shock; successful completion of phase I clinical trials; stage II clinical trials	[52, 53]

Table 1. (Contd.)

Inhibitors	Proposed mechanism of action	Effect on eukaryotic cell	Action in vivo	Reference
Pseudolipasin A	Presumably affects secreted enzyme phospholipase, possibly on the allosteric site	↓ExpU-mediated cell death	ND	[55]
Arylsulfonamides	Phospholipase A2 activity of ExoU	↓ExpU-mediated cell death	ND	[56]
Cyclic peptomeres	T3SS secretion	↓ExoU secretion	ND	[57]
Exocin	ADP-ribosyltransferase enzymatic activity of ExoS	↓ExoS-mediated cytotoxicity on mammalian cells	ND	[58]
Thienopyrimidinones	ADP-ribosyltransferase enzymatic activity of ExoS	ND	ND	[59]
(–)-Hopeaphenol	T3SS secretion?	↓T3SS-mediated cytotoxicity, secretion, and expression of ExoS	ND	[60]

↑, increase; ↓, decrease; ND, no data.

wide range of gram-negative bacteria, which require, however, further pharmacological optimization.

N-hydroxy-benzimidazoles compounds were obtained by screening of small-molecule compounds and represent a class of antivirulence molecules that inhibit DNA-binding activity of some AraC proteins [31]. In *Pseudomonas*, the ExsA protein is a key regulator of AraC-type regulon T3SS transcription. It was demonstrated that N-hydroxy-benzimidazoles interact with the DNA-binding domain of ExsA, thus inhibiting the DNA-binding activity of ExsA protein and suppressing ExsA-dependent activation of transcription. Such interaction is apparently specific, since the inhibitors have been seen to not suppress the activation of transcription associated with a global virulence regulator (Vfr protein) [32].

The biological effects of N-hydroxy-benzimidazoles have resulted in a decrease in the expression of T3SS genes and T3SS-mediated cytotoxicity in vitro. The effect of N-hydroxy-benzimidazoles in vivo has not yet been demonstrated [33].

The compounds TS027 and TS103 were identified as a result of screening of plant phenolic compound library. They suppressed the *exoS* gene transcription through a GacSA–RsmYZ–RsmA–ExsA regulatory pathway. At this stage of development, the effect of TS027 and RS103 compounds on T3SS-mediated cytotoxicity has not been studied [34].

INHIBITION OF THE FUNCTIONAL APPARATUS OF T3SS

Hydroxyquinolines were, first, described as inhibitors of T3SS gene expression in *Y. pseudotuberculosis* [35] and, then, in *P. aeruginosa*. INP1855 (an inhibitor from the class of hydroxyquinolines) was obtained by screening a library consisting of 17500 synthetic small-molecule organic molecules. It was demonstrated that INP1855 hydroxyquinoline suppressed the secretion of ExsE negative regulator of transcription, ExoS effector protein, and FliC flagellum protein, which gave reason to hypothesize that the common target can may be in hydroxyquinolines in T3SS and in the flagellum [36]. Studies of the mechanism of action indicate that ATPase may be such a target of INP1855, since the effect of INP1855 on bacterial cells inhibits the level of ATP only in strains expressing T3SS and/or flagellum. This study was carried out on YscN ATPase complex protein in *Y. pseudotuberculosis*. At the moment, there are no clear data that show that there is a direct effect of INP1855 inhibitor on PscN ATPase complex protein in *P. aeruginosa*. However, since YscN protein has 57% homology with PscN protein (according to BLAST alignment data), it can be supposed that INP1855 will also inhibit the activity of PscN protein [37].

INP1855 protected in vitro eukaryotic cells from T3SS-mediated cytotoxicity and suppressed the activation of caspase-1 with subsequent release of IL-1β in phagocytic cells, which indicated suppression of the process of activation of NLRC4 inflamosomal signal-

ing pathway [38]. Treatment with INP1855 inhibitor in vivo on a model of acute *Pseudomonas pneumonia* in mice led to a decrease in the damage to, and amount of *Pseudomonas* in, the lungs, as well as to a restriction of the dissemination of bacteria to the spleen.

At the same time, there were no differences in seeding of lung tissue from animals of the treatment group and the control group, since the INP1855 inhibitor did not affect the viability of bacteria. However, at the same time, a decrease in the influx of neutrophils and macrophages into the focus of infection, as well as a significant decrease in the level of IL-1β and an increase in the level of IL-17 in the bronchoalveolar lavage (BAL), was demonstrated, indicating the cessation of acute inflammation. Work with hydroxyquinolines is continuing; however, data on the transition to the stage of preclinical studies have not yet been described in the literature [37].

Thiazolidinones. The compound of a class of thiazolidinones (2-imino-5-arylidentiazolidinone) was selected as a promising T3SS inhibitor with a wide spectrum of action against gram-negative pathogens [39]. This inhibitor was obtained as a result of screening of a library consisting of 92000 small-molecule compounds using a high-performance experimental testing of compounds in relation to the suppression of T3SS function in *S. enteric* serovar Typhimurium. For this purpose, a strain of *Salmonella* in which the effector protein SipA was "fused" with the *Y. enterocolitica* YplA protein with phospholipase activity was secreted in a T3SS-dependent manner was constructed. It was demonstrated that 2-imino-5-arylidentiazolidinone dose-dependently inhibits the secretion of SipA and SspH1 *Salmonella* effector proteins without affecting the growth of bacteria [39]. When studying the mechanism of action of the obtained inhibitor, it was detected that it causes a decrease in the number of structural T3SS proteins (InvG, PrgH, and PrgK) that form a secreting complex in the inner and outer membrane of *S. enteric* serovar Typhimurium cell wall, which suggests the inhibition of the assembly and destabilization of the secretory apparatus [40].

Taking into account the structural similarity of T3SS and the flagellum apparatus in the area of the basal structure located in the cytoplasmic membrane, the effect of 2-imino-5-arylidentiazolidinone inhibitor on the mobility of *S. enteric* serovar Typhimurium bacteria was studied. It was demonstrated that 2-imino-5-arylidentiazolidinone does not suppress the flagellar system, indicating the localization of a possible target of the inhibitor in the outer membrane of the cell wall. It was supposed that a conservative protein secretin, which is present not only as a part of T3SS, but also as a part of type-two (T2SS) and -four (T4SS) secretion systems, may be a such target. Using the *P. aeruginosa* model, it was established that 2-imino-5-arylidentiazolidinone suppressed the translocation of T2SS effector protein elastase (a significant virulence factor of *Pseudomonas*), as well as inhibiting the mobility dependent on T4SS pili. Thus, the secretin (the only protein common for type-two, -three, and -four secretion systems) is a possible target of the obtained inhibitor [40].

It was demonstrated that 2-imino-5-arylidentiazolidinone suppresses T3SS of other gram-negative pathogens, such as *Yersinia*. spp., *Pseudomonas* spp., and *Francisella novicida* [39]. That is, the obtained inhibitor suppressed the virulence of mammalian and plant pathogens, reducing *Salmonella*-induced death of macrophages and suppressing hypersensitivity reactions in tobacco plants induced by *P. syringae* bacteria. Thus, 2-imino-5-arylidentiazolidinone inhibitor is a promising broad-spectrum antivirulence drug; however, we found no data on further development of this class of inhibitors in the available literature [41].

Phenoxyacetamides are another class of T3SS inhibitors. They were determined as *P. aeruginosa* T3SS inhibitors by screening in vitro [42]. It was suggested that phenoxyacetamides specifically bind to the PscF needle protein. This hypothesis was based on the observation that mutations in this protein deactivated the inhibiting properties of MBX1641 phenoxyacetamide [43]. It was demonstrated in vitro that phenoxyacetamides reduced T3SS-mediated cytotoxicity and facilitated internalization of bacteria in HeLa cells. Phenoxyacetamide therapy demonstrated positive results during the suppression of *Pseudomonas* infection on an abscess model in mice [44].

Thiadiazinones. A new small-molecule T3SS inhibitor, called CL-55 and belonging to the class of 2,4-disubstituted-4H-[1,3,4]-thiadiazine-5 ones, was developed at the Gamaleya National Research Center for Epidemiology and Microbiology, Ministry of Health of the Russian Federation. The compound was obtained as a result of experimental screening of more than 400 small-molecule compounds of different classes and modified in order to improve the physicochemical properties (such as solubility and stability) and to decrease the toxicity for eukaryotic cells [45, 46].

The mechanism of action was demonstrated on *Pseudomonas*, *Chlamydia*, and *Salmonella* when using specific sera to the effector proteins, which manifested suppression of translocation of these proteins [47–50].

On models in vitro, inhibition of the activity of T3SS led to a dose-dependent suppression of cytotoxicity regarding eukaryotic cells associated with the activity of *P. aeruginosa* ExoU and ExoS effectors. The inhibitor led to the restoration of phagocytic activity of unprofessional phagocytes, which is suppressed with the involvement of ExoS protein with ADP-ribosyltransferase activity. For *Pseudomonas*, it was demonstrated that T3SS inhibitor also suppressed the mobility caused by flagellar apparatus [47].

In *Chlamydia*, the suppression of the transport of effector proteins from Inc family led to a blocking of intracellular reproduction with both productive and

persistent infection in cell culture [48, 49]. For *Salmonella*, it was demonstrated that the inhibitor suppressed the translocation of proteins encoded by two pathogenicity islands (SPI-1 and SPI-2), which led to a blocking of the invasion and suppression of intracellular survival [50].

The minimal inhibitory concentration (MIC) of the drug, which was 5–20 μg/mL, was determined in the used tests in vitro. According to the mechanism of action, the selected inhibitor had no antibacterial action in vitro against either the studied pathogens and representatives of normal human microflora.

The studies that have been conducted on the possibility of developing resistance demonstrated that unlike antibiotics, the sensitivity to the drug did not change in conditions of a long passage in the presence of the drug. In a study by L. Nesterenko et al. [50], CL-55 was shown to have an effect on an experimental infection caused by *S. enterica* serovar Typhimurium. It was found that CL-55 suppresses *Salmonella* infection in a model of generalized infection in mice, leading to a complete elimination of the pathogen in the spleen, liver, and blood after the therapy for 5 days.

On the basis of the small-molecular compound CL-55, a dosage form was developed, it was called Fluorothiazinone (FT). The therapeutic effect of FT in vivo was estimated on the model of *P. pneumonia* caused by *P. aeruginosa* clinical isolates with different profiles of multiple antibiotic resistance. A significant decrease of the bacterial load in lung tissues and a high protective effect in the treatment group were shown with the developed model. A blocking of generalization of infection was demonstrated on a model of pneumonia and burn infection caused by *Pseudomonas*; that is, bacteremia typical for such diseases was suppressed. Against a background of treatment, pathomorphological changes in tissues were significantly less pronounced as compared with the control animals.

FT exhibited an equal efficiency with antibiotics regarding the suppression of acute infectious process and elimination of the pathogen from the animal organism in doses comparable with antibiotics [47]. A full cycle of toxicological studies was carried out for FT. No results hindering further development of the drug were detected during a comprehensive preclinical study, which included a study of mutagenicity and carcinogenicity, acute toxicity, chronic toxicity, allergenic properties, immunotoxicity, and reproductive toxicity. The FT drug passed phase I clinical trials, according to the results of which data were obtained indicating the safety of the drug. At present, the drug FT under the name Ftortiazinon is undergoing phase II clinical trials in patients with complicated urinary tract infections.

Inhibitors based on antibodies. Drugs based on specific antibodies can be classified as inhibitors suppressing the functioning of T3SS. Rabbit polyclonal anti-PcrV antibodies and murine monoclonal anti-PcrV antibodies (mAb166.2a) inhibiting the translocation of *Pseudomonas* effectors were developed to inactivate the *P. aeruginosa* T3SS translocon. Such inhibition led to the restoration of macrophage phagocytic activity against the pathogen [51–53]. Despite the variability of PcrV sequence among different *P. aeruginosa* strains, anti-PcrV antibodies were efficient in decreasing the cytotoxicity of a wide range of clinical isolates, which allows it to be suggested that they may be of use in the clinic.

V. Ray et al. [54] conducted studies to evaluate the possibility of antibodies have an effect on biofilms. In this work, these researchers demonstrated that monoclonal antibodies to PsI exopolysaccharide act on the formation and maturation of biofilms, as well as on already-formed biofilms.

MEDI3902 is a drug that based on antibodies to two targets simultaneously. MEDI3902 (MedImmune LLC; Nijmegen, Netherlands) consists of humanized bivalent bispecific monoclonal antibodies (mAb), the effect of which is aimed at inactivation of both the PcrV needle tip protein and PsI exopolysaccharide. MEDI3902 reduced the cytotoxicity of clinical isolates expressing PcrV (100%) and PsI (98%) [51].

Preclinical studies were conducted for MEDI3902. They showed that MEDI3902 had a protective effect on the model of acute lethal pneumonia caused by *P. aeruginosa*, as well as on the model of bacteremia and burn model. It was detected that MEDI3902 maintains the integrity of lung tissue, reduces the bacterial load, and prevents distribution of the pathogen in the spleen and kidneys.

MEDI3902 has passed phase I clinical trials and is currently in phase II clinical trials in the treatment of patients who are on MV against a background of a hospital *Pseudomonas* infection.

Another drug based on antibodies was developed against the PcrV needle tip protein (KB001, Kalobios Pharmaceuticals; San Francisco, California, United States) based on monoclonal antibodies to PcrV, mAb166.2a. For KB001, safety was shown for healthy participants. However, clinical trials in patients with cystic fibrosis did not show sufficient efficacy of the KB001 drug, and its further study was suspended [52].

V2L2MD is another kind of antibody, which has shown to have an effect in both cells and animals. anti-PcrVMAb V2L2MD antibodies in vitro reduced T3SS-mediated cytotoxicity on human bronchoepithelial cells.

Antibodies had a protective effect during infection of animals with a lethal dose of *P. aeruginosa* on the model of acute pneumonia, while a significant decrease in the bacterial load in the lungs, spleen, and kidneys was noted on the model of nonlethal pneumonia.

In the modern literature, there are no data so far on the use of V2L2MD antibodies as a drug for the treatment of *Pseudomonas* infections in human beings [53].

INHIBITORS OF T3SS EFFECTOR PROTEINS

Another strategy to reduce virulence is to act directly on T3SS effector proteins, four of which were have been described in *P. aeruginosa* to date. This approach aims at decreasing the toxic effect on the host cells; however, it has limitations due to the fact that not all effectors are expressed in each individual strain.

Pseudolipasin A was the first inhibitor of ExoU protein. It was obtained as a result of screening in silico. Pseudolipasin acts as a specific inhibitor of the activity of phospholipase A2 of the *P. aeruginosa* ExoU protein, without affecting other eukaryotic phospholipases [55].

Arylsulfamides are also ExoU protein inhibitors. Some compounds of this group of arylsulfamides have significantly inhibited the cytotoxicity mediated by ExoU protein, without causing nonspecific cytotoxicity [56].

When comparing the effect of arylsulfonamides and pseudolipasin A, it was demonstrated that the activity of arylsulfonamides did not exceed the activity of pseudolipasin A during the inhibition of ExoU-mediated cytotoxicity. The effect of these compounds on animals has not been shown to date.

The family of synthetic cyclic peptide–peptoid hybrid molecules (peptomeres) was identified in the experimental screening of T3SS inhibitors. The inhibitors of this class demonstrated a dose-dependent suppression of the secretion of ExoU effector in *P. aeruginosa*, without affecting the growth and mobility of bacteria. When studying the effect of peptomeres on the flagellum, no inhibiting effect was noted. The effect of peptomeres in vivo remains to be studied [57].

The inhibitor exocin was obtained by a screening of small-molecule inhibitors on yeast cells expressing *P. aeruginosa* ExoS. Exocin suppressed the enzymatic activity of ADP-ribosyltransferase of *P. aeruginosa* ExoS protein. Exocin acted as a competitive inhibitor regarding NAD^+ substrate of ExoS protein. The effect in vitro on mammalian cells was demonstrated. Exocin protected Chinese hamster ovary (CHO) cells from lysis caused by *Pseudomonas* bacteria. No data on further studies of this inhibitor could be found [58].

Thienopyrimidinones are a relatively new class of inhibitors of ADP-ribosyltransferase of ExoS protein. The inhibition of ExoS protein was demonstrated in vitro by enzymatic analysis. Several compounds that blocked ADP-ribosyltransferase activity of *P. aeruginosa* ExoS protein were developed. The effect of thienopyrimidinones in vitro and in vivo has not been described in detail [59].

Hopaphenol closes the class of inhibitors acting on effector T3SS proteins. It was obtained as a result of a screening of libraries of natural compounds and refers to the class of polyphenols that are resveratrol tetramers. It was demonstrated that hopaphenol inhibited T3SS in *Y. pseudotuberculosis*, with suppression of the expression and translocation of YopE protein, expression and secretion of YopD translocator, and secretion of YopH protein being noted. In *C. trachomatis*, hopaphenol reduced penetration into the cells and subsequent intracellular growth. When acting on *P. aeruginosa*, hopaphenol blocked the secretion of ExoS protein and reduced T3SS-mediated cytotoxicity. Thus, the effect of hopaphenol in vitro on mammalian cells was demonstrated; however, there are as of yet no data on the effect in vivo [60].

CONCLUSIONS

T3SS, with which the bacterium transports effector proteins into the host cell, is a key factor in *Pseudomonas* virulence. The main action of effector proteins is aimed at the suppression of the immune response: they inhibit the migration and phagocytosis of macrophages and neutrophils, recruited to the focus of infection, cause the death of immune cells. Nosocomial infections caused by *P. aeruginosa* are almost resistant to a standard antibiotic treatment due to multiple antibiotic resistance in clinical strains. It is obvious that it is necessary to select new targets for the development of antibacterial drugs in order to solve the problem of antibiotic resistance and increase the efficiency of treatment. T3SS of gram-negative bacteria is a promising target in virulence factors due to its extremely important role in the development of the infectious process.

Analysis of the literature demonstrates that the studies on the development of inhibitors affecting T3SS of different bacteria have been actively conducted in the last 20 years in laboratories around the world. To date, a number of inhibitors of different nature and with different mechanisms of action have been developed. However, specific molecular targets have been identified only for a small number of described substances, and the mechanisms are still poorly studied. Among the described inhibitors, only an effect in vitro has been demonstrated for the majority of compounds. The effect on the models in vivo was demonstrated for thiadiazinones, hydroxyquinolines and Salicylidene acylhydrazides. The most promising inhibitors at the moment are two compounds: FT, which belongs to the class of thiadiazinones, and a drug based on antibodies MEDI3902, for which clinical trials of phase II are currently completed. It is worth noting that antibody-based drugs have low oral bioavailability and should be injected, in contrast to small-molecular inhibitors, which have a higher oral bioavailability and, therefore, are more attractive as drugs. The described data give hope that new drugs based on T3SS inhibitors will allow one to solve the existing problem of treatment and can be used both as a part of the complex therapy and possibly (with further study) with monotherapy.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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