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Promises and Challenges of the Type Three Secretion System Injectisome as an Antivirulence Target

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ABSTRACT Antibiotic resistance is a major public health threat that has stimulated the scientific community to search for nontraditional therapeutic targets. Because virulence, but not the growth, of many Gram-negative bacterial pathogens depends on the multicomponent type three secretion system injectisome (T3SSi), the T3SSi has been an attractive target for identifying small molecules, peptides, and monoclonal antibodies that inhibit its function to render the pathogen avirulent. While many small-molecule lead compounds have been identified in whole-cell-based high-throughput screens (HTSs), only a few protein targets of these compounds are known; such knowledge is an important step to developing more potent and specific inhibitors. Evaluation of the efficacy of compounds in animal studies is ongoing. Some efforts involving the development of antibodies and vaccines that target the T3SSi are further along and include an antibody that is currently in phase II clinical trials. Continued research into these antivirulence therapies, used alone or in combination with traditional antibiotics, requires combined efforts from both pharmaceutical companies and academic labs.

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INTRODUCTION

Antibiotic resistance is a great and growing threat to public health, motivating scientists to find innovative strategies to cure infections (1–3). An alternative approach to classical antibiotics is to target virulence factors (4): bacterial factors required for infection or damage but not for growth outside the host (2, 5, 6). An antivirulence factor should render the bacteria nonpathogenic by neutralizing a critical virulence element, thereby allowing clearance of the pathogen by the host immune system (5–8).

The type 3 secretion system injectisome (T3SSi) is expressed in a broad spectrum of Gram-negative bacteria and is usually crucial for virulence (4, 9). This needle-and-syringe-like apparatus functions as a conduit for the delivery of effector proteins from the bacterial cytoplasm into host cells (Fig. 1A).

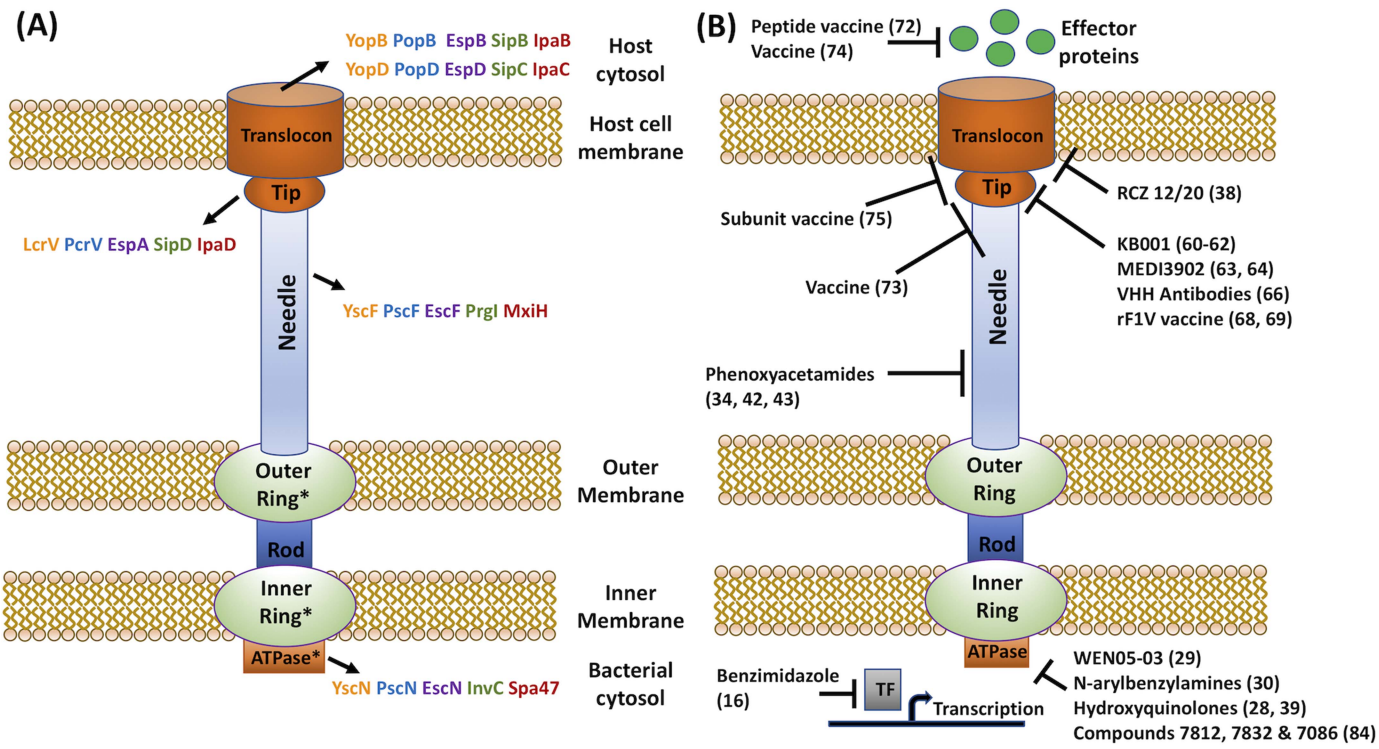

Figure 1 Structure of T3SSi. **(A)** Asterisks indicate regions with conserved components between T3SSi and flagella. Orange, *Yersinia*; blue, *Pseudomonas*; purple, EPEC/EHEC; green, *Salmonella*; red, *Shigella*. **(B)** Potential targets of compounds based on inhibition of T3SSi function, biochemical or binding studies, genetic resistance, or animal studies.

These T3SSi systems share homology with 8 essential core components of flagellar T3SS and contain an additional 20 to 30 proteins involved in expression, secretion, and translocation of effector proteins (9–11). Therapeutic strategies against the T3SSi have been pursued, including interfering with transcriptional regulation, chaperone-effector interaction, assembly of various structures (outer ring, needle, or tip complex), or effector translocation or function (4, 5, 12–18).

Targeting the T3SSi as an effective means of curtailing infection has been rationalized in several ways. Since the injectisome is absent in many resident microbiota, one proposed advantage is that more of the microbiome would be preserved during treatment. Furthermore, the likelihood of developing resistance in resident microbiota that can be transferred by horizontal gene transfer to pathogenic bacteria is minimal. However, due to the homology between some components of the T3SSi and flagella, some inhibitors also affect flagella (13, 19, 20), an observation that may mitigate this advantage. Another potential benefit is that since these antivirulence agents should minimally affect bacterial growth, they may exert low selective pressure in the environment, and therefore, drug resistance may develop infrequently. To our knowledge this has not been experimentally tested in an animal model of infection. On the other hand, disadvantages to be considered include that anti-T3SSi agents may not impede bacterial growth in infected immunocompromised individuals and that some infections require bactericidal agents. Nonetheless, discovering and studying reagents that inhibit the T3SSi remains attractive both for the potential therapeutic benefits and for their use as important tools to elucidate the structure-function relationships of this complex machinery.

This review focuses on advances in T3SSi-targeted therapies in the past 4 years (Tables 1 and 2), including small molecules, antibodies, and vaccines, whose molecular targets are known (Fig. 1B). Excellent in-depth reviews covering progress of the field until 2014–2015 and structure of molecules include references 2, 21, and 22. Some previously well-studied compounds are also summarized in Table 1.

SMALL MOLECULES

Many studies use high-throughput screens (HTSs) to identify small-molecule inhibitors of T3SSi via pheno-

typic readouts of T3SSi functions, including inhibition of T3SSi expression in bacteria (13, 15, 23–25), secretion of effectors into the extracellular supernatant (14, 17, 25–27), or translocation of effector proteins into host cells (14, 18). A benefit of such approaches is that identified molecules are effective in the context of the bacterium. However, complications include the fact that the inhibitors may target more than one protein, may target a host protein, or may alter T3SSi function by generally affecting bacterial cell physiology rather than a specific component of the machinery. Consequently, identification of the specific targets of many small-molecule inhibitors has lagged and structure-activity relationship (SAR) studies are complicated if the molecule targets several proteins.

Recently, several exciting advances have been made in both target identification and identification of lead compounds with sufficiently low 50% inhibitory concentrations for *in vivo* studies. More classical pharmacological approaches that identify compounds that bind to a protein or inhibit its biochemical activity have been fruitfully employed (16, 28–30). Increasingly, the structures of T3SS components are being exploited to elucidate the design of potential inhibitors to these proteins (31–34).

Salicylidene Acylhydrazides

Salicylidene acylhydrazides (SAHs) are the first identified and most widely studied class of synthetic small molecules that target the T3SSi across many bacterial species (13, 14). Several studies suggest that some of these molecules have multiple targets or act indirectly on the T3SSi by impacting bacterial physiology (19, 25, 35–37). Of the derivatives generated, many show promising results. Modifications to improve stability and selectivity of SAH ME0055 resulted in two new synthesized compounds, RCZ12 and RCZ20, that inhibit secretion of EHEC T3SS translocon protein, EspD, as effectively as ME0055 (Fig. 1B). Unlike the parent compound, RCZ12 and RCZ20 have no effect on bacterial growth, suggesting that they are more specific (38). Affinity chromatography experiments revealed coiled-coil domain 1 of EspD as the inhibitors' key domain-binding site (38). These compounds show dual functionality by also downregulating transcription of the locus of enterocyte effacement that encodes the T3SS (38). Recent mechanistic analysis of another SAH, INP0341, showed that it prevents T3SS expression in *Pseudomonas aeruginosa* clinical isolates without affecting growth (39).

A very recent study employed a multiple-assay approach to elucidate the mechanism of action of a group of previously identified T3SS inhibitors (40). Compound SAH INP0007 disrupts YscD punctum formation, suggesting interference with needle assembly, and significantly decreases flagellar motility. Whether inhibition occurs by direct binding to a common core component between the T3SSi and flagella or by interference with other processes that render bacteria less able to build both systems is still unknown (40). Compound 4 (C4), a haloid-containing sulfonamidobenzamide (SAB), which was originally identified along with SAHs as inhibitors of the T3SS (13), is now postulated to have an indirect effect on T3SS transcription by inhibiting the secretion process (40).

Compounds Targeting the T3SS ATPase

Using the known structure of the enteropathogenic *Escherichia coli* (EPEC) EscN ATPase, a computational HTS identified compounds predicted to block the protein's active site (29). One lead compound (WEN05-03) competitively inhibits hydrolysis of ATP by EscN and reduces toxicity to infected HeLa cells (29). Another study using molecular docking and virtual screening identified a series of *N*-arylbenzylamines predicted to target the SctN T3SS ATPase of *Chlamydia trachomatis* (30). Two of these compounds block translocation of the T3SS effector, IncA, into cultured cells and reduce chlamydial survival in these cells (30). Hydroxyquinoline (HQ) derivatives were first described as inhibitors of T3SSi gene expression in *Yersinia pseudotuberculosis* and *C. trachomatis* (41). HQ INP1855 inhibits YscN ATPase activity *in vitro* as well as impairing flagellar motility, providing evidence that it might target conserved ATPases found in the T3SS and flagella (28). In addition, HQ INP1855 reduces *P. aeruginosa* T3SS-mediated cytotoxicity in cultured cells, blocks secretion of ExoS effector protein, and enhances survival and reduces bacterial burden and lung pathology of mice infected intranasally with *P. aeruginosa* (28). HQ INP1750 acts similarly to HQ INP1855 and inhibits both ExoS secretion and flagellar motility (39). However, a direct interaction between these HQ derivatives and T3SS ATPases remains to be shown.

Compounds Targeting Needles or Needle Assembly

Phenoxyacetamide (PXA) was first discovered as an inhibitor of the T3SSi in *P. aeruginosa*, and SAR analysis demonstrated strict stereoselectivity, suggesting an

Table 1 Possible targets and function of small-molecule inhibitors of the T3SS[a]

Compound(s)	Organism(s)	Target	Inhibits bacterial growth?	Toxic to cells?	*In vivo* studies?	Phenotype/readout	Reference(s)
SAH (C1, C2), SAB C4	*Yersinia pseudotuberculosis*		No	NT	No	Inhibit T3SS transcription and Yop secretion; C2 and C4 inhibit flagellar motility	13
SAH (C1–C23), C1-INP0007	*Y. pseudotuberculosis*		No	No	No	Inhibit secretion and translocation	14
SAH C1-INP0007, SAH C11-INP0403	*Salmonella enterica*		No	No	Yes	Inhibit secretion and blocks invasion; first study to validate SAH *in vivo* using bovine intestinal ligated loops	27
SAH C11-INP0403 (ME0053)	*S. enterica*	Suggested indirect effect: iron chelation	No	No	No	Inhibits T3SS transcription and secretion; upregulation of iron acquisition	25
SAH INP0341, SAH INP0400	*Chlamydia trachomatis*	Suggested indirect effect: iron chelation	No	No	Yes	Inhibit T3SS transcription; upregulation of iron acquisition; protect mice against vaginal infection when administered topically	35, 80, 81
SAH INP0341	*C. trachomatis*		No	No	No	Mutations isolated in HemG suggesting indirect effect on T3SS	37
SAH INP0400, SAH INP0402 (C15)	*Shigella flexneri*	Suggested to inhibit T3SS basal needle assembly	No	No	No	Inhibit secretion and blocks invasion; assembly of fewer and shorter needles	17
SAH ME0052 (C8, INP0010), SAH ME0053 (C11, INP0403), SAH ME0054 (C10, INP0401), SAH ME0055 (C17, INP0031)	EHEC	Suggested to inhibit T3SS regulators	No	No	No	Inhibit secretion	15
SAH ME0052 (C8, INP0010), SAH ME0055 (C17, INP0031)	*Y. pseudotuberculosis*, *Escherichia coli*		No	No	No	Inhibit secretion; pulldown assays showed that WrbA, FolX, and Tpx bind to SAH, suggesting indirect effect on T3SS	36
SAH INP0404, SAH INP0405	*S. enterica*		No	No	No	Mutations isolated in FlhA gene suggest targeting of T3SS basal body	19
SAH INP0341	*Pseudomonas aeruginosa*		No	No	No	Inhibits T3SS transcription and ExoS secretion	39
SAH RCZ12 and RCZ20	EHEC	EspD (needle pore protein)	No	No	No	Inhibit EspD secretion; assembly of fewer and shorter needles	38
SAB C4	*Y. pseudotuberculosis*		No	No	No	Inhibits secretion	40
SAH INP0007	*Y. pseudotuberculosis*		No	No	No	Affects YscD punctum formation	40
SAH INP0010	*Y. pseudotuberculosis*		No	Yes	No	Affects YscD punctum formation	40
Salicylideneanilide C3	*Y. pseudotuberculosis*		No	NT	No	Inhibits secretion and transcription	13
Salicylideneanilide	EPEC		No	No	No	Inhibits T3SS transcription and EspB secretion	26

(continued)

Table 1 Possible targets and function of small-molecule inhibitors of the T3SS[a] *(continued)*

Compound(s)	Organism(s)	Target	Inhibits bacterial growth?	Toxic to cells?	*In vivo* studies?	Phenotype/readout	Reference(s)
Benzimidazole	*Y. pseudotuberculosis*	LcrF (T3SS master regulator)	No	No	Yes	Reduces cytotoxicity in infected cells; protective in a murine model	16
C15, C19, C22, C24, and C38	*Y. pseudotuberculosis*, *P. aeruginosa*		No	No	No	Inhibit effector translocation	18
C20	*Y. pseudotuberculosis*, *P. aeruginosa*	Suggested to interfere with adherence	No	No	No	Inhibits effector translocation	18
Compound D	*Y. pseudotuberculosis*, *Yersinia pestis*, *P. aeruginosa*	Suggested to target YopD (translocon)	NT	Yes	No	Inhibits effector secretion	82
Thiazolidinones	*S. enterica*, *P. aeruginosa*, *Yersinia enterocolitica*, *Pseudomonas syringae*	Inhibits T2SS, suggesting common target with T3SS such as secretin	No	No	Yes; tobacco plants	Inhibit transcription and secretion; reduce needle complex formation; reduce hypersensitivity response in plant leaves	83
Phenoxyacetamides	*P. aeruginosa*	Suggested to target PscF (needle protein)	No	No	No	Isolation of *pscF* mutants resistant to phenoxyacetamide inhibitors	34, 42, 43
Phenoxyacetamides	*P. aeruginosa*		NT	NT	Yes	Reduce abscess size in mouse model of *P. aeruginosa* abscess formation	44
Piericidins	*Y. pseudotuberculosis*		No	No	No	Inhibit T3SS-dependent NF-κB activation	45
Piericidin A1	*Y. pseudotuberculosis*	Suggested to target YscF (needle protein)	NT	NT	No	Reduces number of needles present	46
Library of compounds	*Salmonella* spp.	SipD (tip protein), SipB (translocon protein)	NT	NT	No	Surface plasmon resonance screen to find compounds that bind to SipD and SipB	48
Library of compounds	*Shigella* spp.	IpaD (tip protein)	NT	NT	No	Surface plasmon resonance screen to find compounds that bind to IpaD	49
Malic diamide	*Y. pseudotuberculosis*		No	No	No	Inhibits secretion of YopB and YopD	40
Flavonoids	*S. enterica*	Covalent labeling of SPI-1 substrates	No	NT	No	Inhibit bacterial invasion of host cells	47
Compounds 7812, 7832, and 7086	*Y. pestis*	YscN (T3SS ATPase)	No, except for 7086	No	No	Inhibit secretion	84
WEN05-03	EPEC	EscN (T3SS ATPase)	No	No	No	Inhibits ATP hydrolysis; reduces toxicity to infected HeLa cells	29
N-Arylbenzylamines	*C. trachomatis*	Suggested to target SctN (T3SS ATPase)	No	No	No	Reduce secretion and chlamydial inclusions in host cells	30
HQs INP1750, INP1767, and INP1855	*C. trachomatis*, *Y. pseudotuberculosis*		No	No	No	Inhibit cytotoxicity	41

(continued)

Table 1 Possible targets and function of small-molecule inhibitors of the T3SS[a] *(continued)*

Compound(s)	Organism(s)	Target	Inhibits bacterial growth?	Toxic to cells?	*In vivo* studies?	Phenotype/readout	Reference(s)
HQ INP1855	*P. aeruginosa*	Suggested to target T3SS ATPase	No	No	Yes	Reduces cytotoxicity on host cells; reduces bacterial burden and lung pathology in infected mice; reduces activity of homologous T3SS ATPase YscN	28
HQ INP1750	*P. aeruginosa*, *Y. pseudotuberculosis*	Suggested to target T3SS ATPase	No	No	No	Inhibits secretion and flagellar motility; reduces activity of *Yersinia* T3SS ATPase YscN	39
Licoflavonol	*S. enterica*		No	NT	No	Reduces expression of chaperone *sicA* and *invF* (transcriptional regulator for SPI-1 effector proteins)	50
Epigallocatechin gallate	EPEC/EHEC, *S. enterica*, *Y. pseudotuberculosis*		No	NT	No	Reduces adherence of EHEC/EPEC; reduces *Salmonella* invasion into host cells; reduces *Yersinia*-induced cell death	52
Epigallocatechin gallate	*S. enterica*		No	NT	No	Reduces *Salmonella* invasion into host cells	51
Psidium guajava leaf extract	EPEC/EHEC, *S. enterica*, *Y. pseudotuberculosis*		No	NT	No	Reduces adherence of EHEC/EPEC; reduces *Salmonella* invasion into host cells; reduces *Yersinia*-induced cell death	53
Sanguinarine chloride	*S. enterica*		No	Yes at higher concns	No	Inhibits bacterial invasion of host cells	54
Thymol	*S. enterica*		Slightly at higher concns	Slightly at higher concns	Yes	Inhibits bacterial invasion of host cells; protects mice against infection	85
Obovatol	*S. enterica*		No	NT	No	Reduces hemolysis of sheep red blood cells	55
7-Hydroxycoumarin (umbelliferone)	*Ralstonia solanacearum*		Yes (86)	NT	Yes; tobacco plants	Reduces expression of T3SS effector genes; reduces disease progression on tobacco plants	87
SAHs	*R. solanacearum*		Minimal	NT	Yes; tomato plants	Inhibit translocation; reduce bacterial growth on tomato plants	56
SAHs	*Erwinia amylovora*		No	NT	Yes; apple plants	Reduce expression of T3SS genes; reduce disease symptoms on apple plants	57
Phenols	*Xanthomonas oryzae*		No	NT	Yes; rice plants	Reduce expression of *hrpG* and *hrpX* (regulators of *hrp* genes which regulate T3SS effector expression); reduce disease symptoms on rice plants	58
Thiazolidin-2-cyanamide derivatives	*X. oryzae*		No	NT	Yes; rice plants	Reduce expression of *hrpG* and *hrpX* (regulators of *hrp* genes which regulate T3SS effector expression); reduce disease symptoms on rice plants	59

[a]NT, not tested; EHEC, enterohemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; T3SS, type III secretion system.

Table 2 Antibodies, vaccines, and peptomers against T3SS components[a]

Class	Organism(s)	Target(s)	Phenotype/readout	Therapeutic potential	References
Antibody (KB001)	*Pseudomonas aeruginosa*	PcrV (tip)	Protects host cells against T3SS-mediated toxicity and protects mice against acute pulmonary infection (reviewed in reference 88)	Did not meet efficacy endpoints in phase II clinical trials	60–62
Bispecific antibody (MEDI3902)	*P. aeruginosa*	PcrV (tip), Psl (exopolysaccharide)	*In vitro* cytotoxicity protection and *in vivo* protection of acute pneumonia model in mice	Currently in phase II clinical trials	63, 64
Single-VH domain antibodies	*Shigella flexneri*	IpaD (tip)	Reduces hemolysis of sheep red blood cells		66
rF1V vaccine	*Yersinia pestis*	LcrV (tip), F1 protein	Enhances survival of cynomolgus macaques infected with lethal aerosol challenge of *Y. pestis*	Orphan drug designation by FDA	69
Rabbit polyclonal antisera	STEC	$STEC_{O103}$ T3SS proteins	Block adherence of STEC to host cells; immunized mice not protected against fecal shedding		71
Peptide vaccine	*Salmonella enterica*	SseI (effector)	Protects mice against acute infection		72
Vaccine	*S. enterica*	PrgI (needle), SipD (tip)	Protects mice against infection		73
Vaccine	*S. enterica*	SseB (effector), flagellin	Protects mice against infection		74
Subunit vaccine	*S. enterica*	S1 (fusion of SipD and SipB [tip and translocon]), S2 (fusion of SseB and SseC [tip and translocon])	Protects mice against lethal challenge		75
Polypeptide	*S. enterica*, *S. flexneri*	SipB (translocon), IpaB (translocon)	Inhibits bacterial invasion into host cells	Polypeptide too large for therapeutic potential	76
Peptides	EPEC	EspA (tip)	Inhibit EspA polymerization, thereby preventing A/E lesions		77
Peptides	EHEC, *Citrobacter rodentium*	EspA (tip)	Protect mice against colon damage after *C. rodentium* challenge		78
Peptomers (phepropeptin D derivatives)	*Yersinia pseudotuberculosis*, *P. aeruginosa*		Inhibit secretion of T3SS proteins; inhibit *Yersinia* YopM effector translocation and reduce cell rounding		79

[a]STEC, Shiga toxin-producing *Escherichia coli*; A/E, attaching/effacing.

interaction with a specific target or site (42). Isolation of several mutants of PscF resistant to PXA inhibitors provides genetic evidence that PXAs target the needle protein (34, 43). Modeling of PXA inhibitors supports the idea that these molecules intercalate within the needle and interact simultaneously with several assembled PscF subunits; however, biochemical and structural studies are needed to demonstrate a direct interaction. Importantly, injection of PXA (MBX2359) into abscesses formed by *P. aeruginosa* significantly reduces abscess size, providing evidence that these inhibitors are efficacious in infection models in mammals (44).

Piericidins, a class of compounds derived from *Actinomycetales*, inhibit translocation of YopM into cultured cells (45). A follow-up study showed that *Yersinia* treated with piericidin A1 has fewer needles, suggesting that piericidin A1 inhibits a step prior to or during needle assembly (46). The related Psc T3SS of *P. aeruginosa* and the Ysa T3SS of *Y. enterocolitica* are not inhibited, indi-

cating its specificity but potentially limiting its usefulness without additional SAR analysis (46).

Compounds Targeting Translocon and/or Effector Secretion and Activity

Using click chemistry, the flavonoids baicalein and quercetin were found to covalently modify *Salmonella enterica* serovar Typhimurium translocases and effectors, resulting in changes to stability or activity (47). The N-terminal chaperone-binding domain is proposed to be the modified site (47). These flavonoids inhibit invasion of *S.* Typhimurium into cultured cells but have no effect on effector secretion or needle assembly (47). Screening libraries for compounds that bind to *Salmonella* SipD (48) or *Shigella* IpaD tip proteins (49) identified a new class of small molecules based on the indole scaffold as potential inhibitors of the T3SSi. Malic diamide (42), a compound structurally related to PXA, significantly inhibits the secretion of YopB and YopD proteins required for translocation, without disrupting needle YscF punctum formation, indicating that it targets the translocon (40).

In the past few years, several natural compounds have been identified, typically in screens for secretion (50–53) or translocation into target cells (54) or by inhibiting the effects on T3SSi-mediated functions on targeted host cells (55). Potentially promising compounds are listed in Table 1, but to our knowledge, the specificity against T3SSi or protein targets has not been investigated in depth.

Anti-T3SS Compounds Tested against Plant Pathogens

Plants are also susceptible to infection by bacteria harboring T3SSs, and there have been several recent exciting findings. Natural and synthetic compounds were screened for the ability to reduce expression of the *Ralstonia solanacearum* T3SS pilus gene *hrpY* (56). The most potent inhibitors were SAHs, which inhibit secretion of T3SS effector AvrA and limit bacterial growth on tomato plants (56). SAHs also reduce the expression of T3SS genes of *Erwinia amylovora* and reduce disease symptoms on inoculated crab apple pistils (57). Phenolic compounds repress the expression of T3SS transcriptional regulators *hrpG* and *hrpX* of *Xanthomonas oryzae* and reduce disease symptoms on rice leaves (58). Thiazolidine-2-cyanamide compounds also reduce relative expression of *X. oryzae hrpG* and *hrpX* and disease symptoms on rice (59).

ANTIBODIES, VACCINES, AND PEPTIDES

Recent advances in targeting T3SSi using antibodies, vaccines, and polypeptides are summarized below and in Table 2.

Antibodies

A monoclonal antibody, KB001, that binds to the *P. aeruginosa* T3SS tip protein, PcrV, initially showed promise in the treatment of patients with airway-associated *P. aeruginosa* infection or colonization but failed in phase II clinical trials for not meeting efficacy endpoints (60–62). By contrast, a bispecific antibody, MEDI3902, against *P. aeruginosa* PcrV and the Psl exopolysaccharide is effective against a wide range of clinical isolates and is currently in phase II clinical trials for prevention of ventilator nosocomial pneumonia (63, 64).

Single-domain antibodies that consist of the N-terminal variable region of an immunoglobulin heavy chain (VHH) but not the light chain can be isolated from camelid species (65). A panel of VHH single-domain antibodies was raised against the *Shigella flexneri* IpaD tip protein (66). Four such antibodies that bound IpaD significantly inhibit hemolysis of sheep red blood cells, a measure of T3SS translocon functionality (66). Structural binding analysis revealed that these inhibitory VHHs mostly bound to the distal domain of IpaD, suggesting the importance of this region in T3SS function (66).

Vaccines

Work towards a plague vaccine has led to testing a recombinant vaccine consisting of the *Yersinia pestis* F1 protein and the T3SS tip protein LcrV, reviewed in reference 67. The FDA has granted orphan drug status for the development of this rF1V vaccine as a prophylactic for high-risk individuals (68, 69). Efforts to lessen Shiga toxin-producing *Escherichia coli* (STEC) disease burden in cattle to reduce transmission to humans are ongoing. Cohorts of cattle immunized against serotype O157 have reduced shedding of O157 but not of other STEC serotypes due to serotype specificity (70). To develop vaccines against a different prevalent serotype, antisera to five T3SS proteins, EspA, EspB, EspF, NleA, and Tir, of STEC serotype O103 were studied. These antisera block STEC adherence to HEp-2 cells (71). In efficacy studies, mice developed strong serum IgG titers against four of these five proteins but still shed O103 after oral administration, indicating that the bacteria could still be transmitted (71).

Recent attempts to develop T3SS-targeted vaccines against *Salmonella enterica* show some success in mouse studies. A peptide vaccine that elicits a CD4 T cell response against T3SS effector protein SseI protects mice against acute infection, a tantalizing result given that only a single peptide elicits protection (72). Mice were immunized by different routes with *Salmonella* T3SS proteins SipD and PrgI in combination or alone; oral immunization with SipD provides the highest level of protection against lethal challenge (73). Increased protection is observed when flagellin is added to a vaccine against *Salmonella* T3SS protein SseB (74). A subunit vaccine against *Salmonella* consisting of two components, S1 (a genetic fusion of SPI-1 translocon proteins SipB and SipD) and S2 (a genetic fusion of SPI-2 proteins SseB and SseC), elicits strong IgG titers to all four proteins in mice (75). These mice are significantly protected against challenge with *S.* Typhimurium and *S. enterica* serovar Enteritidis and experience reduced cecal inflammation (75). These results warrant studies on long-term protection.

Peptides

Anti-T3SS peptides (Table 2) have been identified against *Salmonella* (76), EPEC (77), enterohemorrhagic *E. coli* (EHEC) (78), and more recently, against *Yersinia* (79). Derivatives of the natural compound phepropeptin D that contained various peptoid substitutions on the cyclic peptide backbone significantly inhibit NF-kB signaling, secretion of the effector protein YopE, and translocation of YopM into HeLa cells by *Yersinia* (79). The peptomers do not affect *Yersinia* growth or flagellar motility, indicating their potential specificity to the T3SSi. Several derivatives also inhibit secretion of the *P. aeruginosa* effector protein ExoU, suggesting that they might target a conserved component of these two injectisome systems (79).

CONCLUSION AND PERSPECTIVE

Discovery of and research into inhibitors of the T3SSi is a highly active area of research, with many candidates from different classes that are effective in blocking the function of T3SS. Although antibodies and vaccines are further along in the pipeline, many small-molecule inhibitors show promise. Some molecules have a narrower spectrum of activity, while others have broader spectra, including those that target components conserved between the T3SSi and flagella. Both have benefits and disadvantages. For instance, an effective but narrow-spectrum molecule against the T3SSi of the multidrug-resistant *P. aeruginosa* could save many lives each year. By contrast, a narrow-spectrum molecule effective towards *Y. pestis* would not save many lives annually unless a major outbreak occurred. Yet importantly, study of such a molecule could help elucidate structure-function relations of the T3SSi and be used as a platform to develop molecules highly effective against homologous components in other T3SSis. Resistance mutants, biochemical assays, structural modeling, and rational designs are helping to identify targets and generate more potent inhibitors. Validation of their efficacy in animal systems is ongoing. Both basic science and clinical translational research from academic and pharmaceutical groups is crucial to the advancement of these molecules to combat the rising threat of antibiotic resistance.

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