A NEW CLASS OF ANTIBIOTICS: TYPE III SECRETION SYSTEM (T3SS) VIRULENCE BLOCKERS

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Abstract

Background: Although antibiotics have saved innumerable lives, due to the disproportionate use of antibiotics for the treatment of humans and farm animals, multidrug resistance (MDR) has emerged. Innovative strategies to tackle antibiotic resistance that allow for a more sustainable therapy to treat bacterial infectious diseases are urgently required.

Objective: This literature review examines the inhibition of conserved growth-dispensable type III secretion system (T3SS) as promising virulence blocking strategy.

Methods: T3SS blockers were categorised according to the inhibitor-screening detection method used to identify them, which were the whole cell-based high-throughput screens or the target-based-inhibition approach. These methods allowed us to perform a comprehensive examination on methodology cross-validation.

Results: Caminoside-A, derived from a marine sponge extract, was the only compound identified via both screening approaches, indicating that this is a very promising candidate.

Conclusion: T3SS has been shown to be an attractive target for the development of anti-bacterial drugs. Further smart and refined inhibitor search designs will improve virulence blocker discovery. Future advances on T3SS inhibition are expected to circumvent the increasing problems derived from MDR.

KEYWORDS: type three secretion system (T3SS), virulence blocker, inhibitor screening

Antibiotic therapy is the most commonly used strategy to overcome bacterial pathogenic infections. Still, the steady misuse of antibiotics has led to multidrug-resistance (MDR), which has recently spread and exacerbated, causing the rise of global resistance. Antibiotics kill bacteria by affecting their key cellular processes, translating in a strong selective pressure to develop resistance against antibiotics [1]. Antibiotics cause a lot of collateral damage as they target not only bacterial pathogens but also resident flora [1]. The decreasing effectiveness of antibiotics is, thus, gradually leading us to a post-antibiotic era.

Although MDR is now one of the top three threats to global public health, the current entry rate of new antibiotics is very low [2]. The lack of novel antibiotic targets, the absence of high throughput screen (HTS)-induced host cell toxicity, and the disinterest of pharmaceuticals to invest in the discovery of long-term solutions are some of the factors causing this novelty shortage. Since, HTS allows us to conduct millions of parallel chemical tests to assess for their inhibition potential of new compounds, this method is auspicious. Conversely, MDR has, unfortunately, given an impulse to shift to more expensive and broad-spectrum antibiotics [3].

Current anti-bacterial therapies

Extension of the post-antibiotic era is highly dependent on the introduction of novel anti-bacterial medicines. Here, narrow-spectrum or even organism-specific antibiotics form a particularly exciting new alternative direction. This includes success stories such as the top-selling narrow-spectrum lipopeptide daptomycin (marketed as Cubicin®) antibiotic [1]. Advances in the field of the so-called "antibiotic stewardship" also promote to stay optimistic with the use of traditional single-agent antibiotics. Furthermore, knowledge on the survival advantage of biofilms has led to promising studies. Interestingly, currently in use, low molecular weight antibiotics were shown to be prone to penetrate and kill species-specific biofilms (e.g., rifampicin) [2]. Other remarkable options include antibiotic adjuvants, which either block resistance or boost host response and monoclonal antibodies or modifiers of the immune system [1].

Disarming bacterial pathogens: virulence blockers

Long term solutions to the challenge of antibiotic resistance are essential. The latest studies on bacterial virulence factors and toxins have allowed us to understand how pathogenic bacteria can manipulate diverse host cellular processes [4]. Upon encountering a human host, they use a variety of mechanisms to either hinder or evade host defences. Bacterial components that interact with the host include capsules, lipopolysaccharides, exo- and endotoxins, invasins, and adhesins [5].

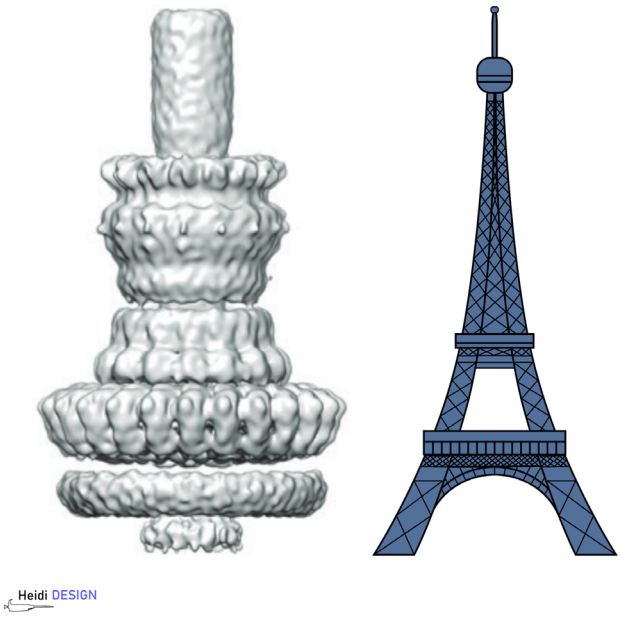

Figure 1: Similarities between type III secretion system and the Eiffel Tower
Left image adapted from Figure 1 of Galan et al. (2014). Surface view of the 3D reconstruction of the cryo-EM map of the Salmonella typhimurium needle complex13. Danger signal represents the hazard for host cells of having an effector protein introduced into their body.

For effective host-microbe interaction, some toxins or effector proteins require microinjection into target cells. Found in many pathogenic gram-negative bacteria, the type III secretion system (T3SS) constitutes one type of sophisticated secretion system. Examples of well-studied T3SS-carrying bacteria encompass *Chlamydia trachomatis*, enteropathogenic or enterohemorrhagic *Escherichia coli* (EPEC/EHEC, respectively), *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Shigella* or *Yersinia* spp. [6]. Back in 1991, preliminary studies in *Yersinia* spp. virulent protein secretion already hinted the existence of T3SS, illustrated in *Salmonella* spp. [7-10]. T3SS specialised effector proteins, thus, allow for many gram-negative bacteria to induce the establishment of infection both in mammals, fish, insects, or plants [11]. Actually, T3SS could form a diversity of weapons that disrupt host homeostasis and immune defences via the disruption of cell cytoskeleton; cell trafficking; epithelium integrity; cell death/survival pathways; inflammatory signalling, or specific adaptive immunity triggering [12].

In this light, small organic molecules targeting T3SS virulence or T3SS-specific virulence blockers could target the virulence factor without affecting bacterial growth. This would translate into a chemical virulence attenuation strategy that would ensure low selection pressure for the emergence of resistant strains. Considering the conservation of T3SS across several bacterial pathogens, the discovery of effective broad-spectrum T3SSs would be ground-breaking. Furthermore, host commensal flora does not use virulence mechanisms and will, therefore, not be targeted [3]. Molecular docking of atomic structures with 3D-cryo EM allows us to observe bacterial injectisome components with remarkable high-resolution [13, 14]. This detailed view aids in virulence blocker search. It could be argued that mutations leading to resistance produce changes on the T3SS *per se*. However, mutations affecting T3SS have shown to lead, with few exceptions, to avirulent phenotypes [15].

T3SS: general architecture and mode of action
T3SS injectisome is a multiprotein complex that transports effector proteins directly from the bacterial cytosol into eukaryotic host cells. The membrane-embedded export apparatus controls the secretion of proteins and anchors the apparatus to the bacterial membrane [13]. The ATPase allows recognition of the chaperone/effector complex as well as provides energy to insert and unfold effector proteins into the apparatus. Partially unfolded effector proteins are secreted through a needle at the extracellular channel (Figure 1) [3].

The needle complex is composed of a base structure embedded in the bacterial envelope and a needle-shaped extension that protrudes from the bacterial surface. On the one hand, the base is formed by two inner (IR1/2) and two outer membrane rings (OR1/2) mediated by a neck [13]. On top, the tip assures regulation of the secretion and forms a scaffold for the pore-forming unit in the host cell (translocon) assembly [3]. The most widely conserved injectisome components have homologues in bacterial flagella, which drive cell motility. In this sense, non-flagellar T3SS (NF-T3SS) structures, like secretin at the outer membrane, form optimal inhibitors (Figure 2) [13].

Ideal requisites for virulence blocker design
Unaffected bacterial growth is a *sine qua non* (without which, not) for optimal anti-T3SS candidate compound screening. Both *in vitro* (e.g., HeLa cells) and *in vivo* (murine/mammal models) tests must confirm that compounds are non-toxic for the host, the cells of the human body. Pharmacokinetics and -dynamics studies should assure that bacterial cell permeability and drug efflux pump hurdles are overcome. Moreover, T3SS-blockers must be NF-T3SS specific. Finally, structure-activity relationship evaluation will allow identification of the biological active functional groups of T3SS compounds and synthesis of more efficient drugs. This last step includes conducting a successful clinical trial.

Research aims and approach

- Summarise currently described T3SS virulence blockers according to the inhibitor screening detection method used to identify them, which are whole cell-based (WCB) HTSs or target-based (TB)-inhibition [6];
- identify double positives for both screening-methods allowing to perform a comprehensive examination on methodology cross-validation;
- highlight promising future directions for T3SS research.

Compounds detected via both approaches might appear to be the most promising virulence blockers. We hypothesise that the combination may counterbalance the deficiencies found on each of the approaches.

Methods

The division of this review into two screening approaches was inspired by the screening categorisation of Charro & Mota *et al.* [6]. Our review performs an updated and refined search of their general scheme, while looking to highlight positive cross-methodology compounds. The literature search was mainly conducted via PubMed and Academic Google Scholar. Query search included one or more combinations of key-words: "type three secretions system," "virulence blocker," "inhibitor screening," "effector protein," "transcription," "atomic characterization," "needle tip," and "ATPase." After screening for the earliest reviews, key experimental studies were filtered based on their abstracts. Upon filtering, experimental investigations on WCB-HTS and TB-approach-related papers were narrowed down to 19 and 37, respectively. Reference researches were eventually studied in detail, with a special focus on the methodology employed. All relevant articles found up to May 30th, 2019, were included.

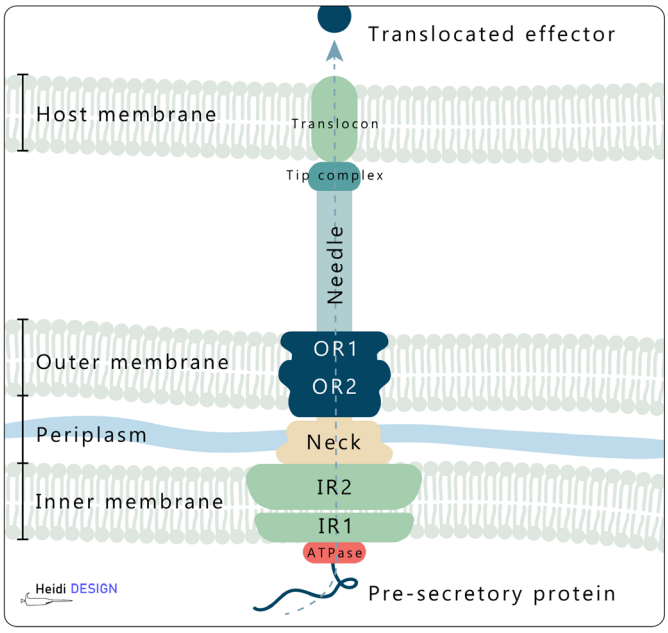

Figure 2: Schematic representation of type III secretion system of gram-negative bacteria

IR1/2: Internal ring 1 and 2; OR1/2: Outer ring 1 and 2; PG: peptidoglycan; C: chaperones.

Results

Whole cell-based, high throughput screening

Both for natural and synthetic compounds, WCB-HTS has been the primary approach for the study of T3SS virulence blockers. Considering the particular study design, this category has been further subdivided into three main read-outs (ROs).

RO1: Gene expression regulation of T3SS genes

Studies on the classic example of MDR and biological warfare strain, *Yersinia* spp., allowed the first detailed description of T3SS inhibitors discovered via WCB-HTS. These studies delivered the still extensively investigated presumed virulence blockers: salicylidene acylhydrazides (SAHs). In this study, the effector protein promoter of *Yersinia* spp. (Yop) was fused with *lux*AB, which encodes for a luciferase, to detect T3SS gene expression/inhibition (Figure 3A) [16]. Follow-up studies on SAHs allowed to characterise and confirm its inhibitory effects on other T3SS-carrying pathogenic bacterial genera, including *C. trachomatis*, *S. enterica*, and *S. flexneri* [17-20]. *S. flexneri*-T3SS vs SAHs elucidating research speculated that the detrimental effects of SAHs on the bacteria's T3SS could potentially be linked to needle assembly [19].

Notwithstanding SAHs promising discovery, their study design had some clear disadvantages [16]. The luciferase light reporter may have been suppressed at different levels: directly by selective binding to the luciferase enzyme causing its inhibition, by bacterial toxicity or, by blocking transcription factors and interfering on *lux*AB expression. Moreover, the bacterium secretes a negative regulator until it attaches to a eukaryotic cell. Thereby, Yop effector measurement is biased.

Subsequent experiments using SAHs compounds screened from this first WCB study overcame these observed limitations. Candidate inhibitors proved to specifically inhibit Yop as well as its translation after infection of HeLa cells [15]. Another pioneering study identified other non-SAHs *Yersinia* spp. T3SS inhibitors, using the same reporter gene: lux operon-luminescence [21]. Under low Ca^{2+}, *Yersinia* spp. bacterial growth is inhibited while T3SS expression is boosted. Taking advantage of this peculiar growth under low Ca^{2+}, luminescence was used to measure bacterial growth and T3SS inhibition. This approach was able to reject compounds that act indirectly on the inhibition of secretion by the toxic effects on bacteria [21].

T3SS-carrying phytopathogens studies are highly representative of the mode of action of T3SS-carrying human pathogens. Plants make use of phenolic compounds to combat the bacterial invasion. Studies on *Dickeya dadantii* showed how specific phenolic compounds-o-coumaric acid (OCA) and t-cinnamic acid (TCA) could upregulate phytopathogens' T3SS [22]. Furthermore, these discoveries are permitted to identify other potential T3SS repressors based on OCA and TCA isomers/analogues-like p-couramic acid, which is an isomer of OCA [23]. More recently, the T3SS of *Ralstonia solanacearum* was described to be induced by plant oleanic and ferulic acids [24, 25]. Investigations in phytopathogen *Erwinia amylovora* corroborated the broad T3SS inhibition capacity of SAHs as well as other virulence factors. Also, iron-scavenging showed to be fundamental in protecting bacteria from SAHs [26]. The transcriptional fusion of effector protein of *P. aeruginosa* with the GFP-encoding gene allowed us to screen a library of phenolic compounds as a reverse strategy to look for novel T3SS inhibitors. Since *P. aeruginosa* infects both humans and plants, plants may also include T3SS in their plant defence system (Figure 3A) [27].

RO2: *In vitro* T3SS effector protein absence/presence

HTS is performed to detect the absence/presence of the T3SS inhibitor derived effector protein, by using an enzyme-linked immunosorbent assay and using an anti-T3SS substrate (Figure 3B). Caminoside-A, a compound able to inhibit secreted proteins from specific bacteria, was the first T3SS inhibitor discovered when using this experimental approach. This particular screening design confirmed that growth was not inhibited [28]. Subsequent investigations in *Salmonella* spp. in the same study design detected two fungal analogues or compounds: Cytosporone B and fusaric acid [29, 30]. Both compounds allowed bacterial growth and showed no toxic effect on human cells. More studies on fusaric acid inferred that T3SS inhibition might be performed via effector protein (SicA) chaperone blockage [31].

RO3: T3SS secreted or translocated reporter proteins

Another WCB study subjected *Salmonella* spp. to HTS of small molecule libraries of natural and synthetic compounds. For that, based on a reporter construct of the effector (SipA) fused to the *Yersinia* phospholipase YplA, protein secretion *in vitro* was measured (Figure 3C). Upon cleavage of the supplied substrate, secreted SipA resulted in measurable fluorescence. This way, T3SS inhibitor 2-imino-5-arylidene thiazolidinedione was detected, which was subsequently demonstrated to also block *Yersinia* spp. T3SS [32]. Based on translational fusion of the effector protein with mature TEM-1-lactamase, translocation can be directly detected in living host cells, using fluorescent lactamase substrate. The application of this HTS allowed us to identify six *Yersinia* spp. specific translocation effector inhibitors (Figure 3C) [33].

Target-based inhibitor design

TB inhibitor design includes the rational design of inhibitors impeding the assembly of specific structures/effectors of T3SS (Figure 3). Inhibitors screening for T3SS components could be performed via rationally design WCB-HTS targeting specific T3SS components or HTS aided by structure-specific computer docking experiments.

Translocators of T3SS-(poly)peptides and proteins

In an attempt to isolate *Salmonella* spp. SipB effector protein inhibitors, in the form of exogenous purified polypeptides or polypeptides to

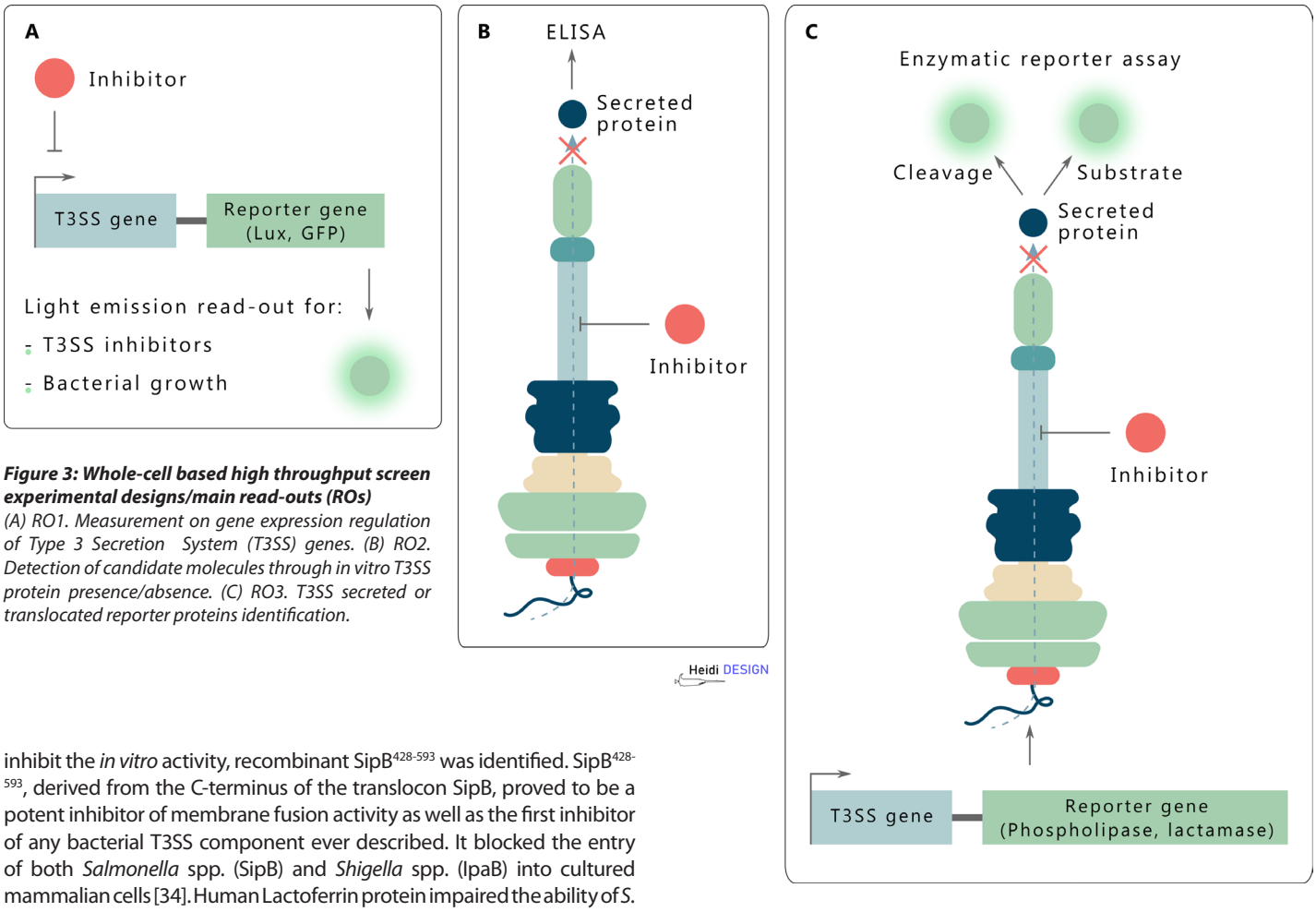

Figure 3: Whole-cell based high throughput screen experimental designs/main read-outs (ROs)
(A) RO1. Measurement on gene expression regulation of Type 3 Secretion System (T3SS) genes. (B) RO2. Detection of candidate molecules through in vitro T3SS protein presence/absence. (C) RO3. T3SS secreted or translocated reporter proteins identification.

inhibit the *in vitro* activity, recombinant SipB$^{428\text{-}593}$ was identified. SipB$^{428\text{-}593}$, derived from the C-terminus of the translocon SipB, proved to be a potent inhibitor of membrane fusion activity as well as the first inhibitor of any bacterial T3SS component ever described. It blocked the entry of both *Salmonella* spp. (SipB) and *Shigella* spp. (IpaB) into cultured mammalian cells [34]. Human Lactoferrin protein impaired the ability of *S. flexneri* to infect HeLa cells by inducing the degradation of its translocon proteins (IpaA and IpaB) [35]. More recently, CoilA and CoilB, 15 amino acid long peptides presenting coiled-coil regions of the translocon of EPEC (EspA), effectively inhibited EPEC T3SS-dependent hemolysis of red blood and mammalian cell invasion via EspA translocon blockage. Later translocation secretion studies on EHEC and *Citrobacter rodentium* reinforced and expanded CoilA and CoilB protection ability [36].

ATPase

As a highly conserved cytosolic protein, ATPase is an attractive target for broad-applicability anti-T3SS drugs [13]. Three prospective inhibitors were successfully identified via a computational screening of commercially available drug-like molecules against the active site of *Y. pestis* ATPase, followed by *in vitro* structure characterisation [37]. These three candidates were also effective against *Burkholderia mallei* together with *S. flexneri* ATPases, BsaS, and Spa47, respectively [37, 38]. Targeting ATPase might lead to concerns because of cross-reactivity with human enzymes. However, it has been argued that bacterial enzymes have less than 25% of identity to human ATPases and are significantly different, especially at the active sites [37].

AraC like transcription factor

AraC transcription factors (TFs) belong to the multiple adaptational response protein family. Inhibition of AraC-mediated activation of transcription of key virulence genes (including T3SS) has proven to be a promising and effective strategy. The screening of small molecules libraries for species-specific AraC-like TF *in vitro* aided to narrow down some candidate inhibitors. Benzimidazole, SE-1, and regacin were identified to prevent species-specific AraC-like TF and DNA binding in *Yersinia pseudotuberculosis* (LcrF), *S. flexneri* (VirF), and *C. rodentium* (RegA), respectively [39-41]. Benzimidazole and regacin had its efficacy further corroborated/ filtered *in vivo* [39, 41].

Effector protein

Chemical modifications and experimental screening of derivates of thiohydrazones, thiohydrazides, or oxamine acids allowed us to identify a potential chlamydial T3SS effector inhibitor: CL-55. CL-55 included all the requisites targeted: low host toxicity, highly selective with T3SS, good pharmacokinetic properties, and simple synthesis scheme. It inhibited chlamydial injection *in vitro* and selectively blocked the translocation of its effector protein, IncA [42]. A follow-up study successfully tested its application in a mice model [43]. Additional CL-55 *in vitro* assays and mice models both in *S. enterica* and *P. aeruginosa* corroborated its broad applicability [44, 45].

Needle tip complex

While it often remains enigmatic in which the T3SS component is inhibited, MBX2359, a class of phenooxyacetamide, is a notable exception. MBX2359 has shown to specifically target *P. aeruginosa* needle tip. Here, *P. aeruginosa* T3SS inhibitor-resistant mutant was used, and those mutation sites were then located via thorough sequencing [46]. This first study was further expanded by testing the efficacy of MBX2359 in a mouse model where *P. aeruginosa* abscess formation was alleviated, and the immune clearance of the mouse was boosted [47]. Computational modelling of *Salmonella* typhi SipD invasive needle tip protein combined with molecular docking experiments resulted in the detection of Caminoside-A. Interestingly, Caminoside-A matches the T3SS inhibitor described from a marine sponge 15 years ago via WCB-HTS [48].

Discussion

Overcoming limitations of WCB-HTS

The most pressing concern in the field of virulence blockers is the identification and characterisation of specific targets. Thereby, WCB phenotypic HTS are inherently biased and should be carefully evaluated to avoid false positives. SAHs are the most widely studied chemical compounds, all derived from WCB-HTS. Different studies have shown that SAHs mode of action could result in a synergistic effect. Observed T3SS inhibition could arise from diverse unspecific targets. These include other key conserved bacterial proteins/enzymes, iron chelation, heme metabolism pathways, or basic bacterial physiology, including flagellar T3SS-mediated swimming motility [19, 49-53]. In order to cope with these limitations, one study successfully chemically optimised SAHs and described more selective blockers [54]. In order to determine where T3SS inhibitors interrupt the T3SS process exactly, more general standardised orthogonal assays for HTS have been alternatively proposed [55]. In this regard, quantitative structure-activity relationship analysis, including statistical molecular design, forms a promising refined approach for the evaluation and smart search of putative T3SS inhibitors [56].

The good and the bad of TB approaches

TB is potentially superior to the phenotypic screens as the search is rational and directed against a very specific target. Thus, the TB approach translates into typically faster, cheaper, and more efficient inhibitors. In fact, they can be identified using less experimental screening and potentially lower off-target hits. Still, detected compound activity is often inactive on the cellular level and may lack cell permeability. For instance, some candidate TB approach derived T3SS ATPase and AraC-like TF inhibitors either have limited solubility or show non-specific inhibition at higher concentrations, respectively [37, 40]. Thus, in practical terms, not being useful for therapeutic applications. Here, state-of-the-art rational *in silico* design seems like a prospective bet, especially considering the major advances in the knowledge of the atomic structure of several injectisome components and the rapidly evolving computational docking experiments [13, 14]. Moreover, most T3SS proteins lack enzymatic activity, and this further hampers HTS.

Caminoside-A: positive on methodology cross-validation

Caminoside-A has been the only compound identified via both screening approaches WCB-HTS and TB inhibitor design. It constitutes a promising virulence blocker having proved its functionality in a real biological system (EPEC), together with its specific interaction with *S. enterica* needle tip complex, SipD [28, 48]. In other words, this ensures that uncertainties found in one approach are counterbalanced on the other.

Current status and next steps

The refinement of presented inhibitor design approaches, WCB and TB, would already allow for more advanced and encouraging studies. In this regard, developing cell-free systems to properly assess and screen for T3SS inhibitor *in vitro* is detrimental [6]. Still, further and clinic widespread use would face unique obstacles. For example, T3SS inhibitors do not affect bacterial replication outside the host. Thus, the standard minimum inhibitory concentration measurement will not be useful for assaying comparative drug activity. Instead, an effective dose of inhibitor will require animal models to predict appropriate dosing for humans [57]. Since most T3SS target a specific subset of pathogenic bacteria, accurate and fast diagnosis will be a prerequisite for treatment. For this purpose, point-of-care devices are currently developed [58].

Conclusion and outlook

T3SS is an essential and conserved virulence factor dispensable for bacterial growth. This review has illustrated that T3SS can also be viewed as an attractive target for the development of anti-bacterial drugs. Caminoside-A has been identified for the first time, to the best of our knowledge, as the only compound positive both for WCB and TB approaches. Critical steps for the near future should include the identification of the mode of action of known T3SS virulence blockers and *in silico* rational design of new T3SS inhibitors. These discoveries are expected to circumvent the increasing problems derived from MDR. Conversely, new drugs targeting T3SS of multiple pathogenic gram-negative bacteria will allow for a more sustainable therapeutic/prophylactic pathogen protection.

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EXAM QUESTIONS

As RAMS aims to enlighten both students and professionals, we would like to present you two exam questions. Find out if you can remember what you have learned during your bachelor's!

We challenge you!

Question 1

Antibodies against IL-17, the most relevant product of Th17-cells, are used to treat patients with psoriasis. While undergoing treatment with these antibodies, patients have an increased risk of developing …

A. Hay fever
B. Fungal infections
C. Viral infections

(Topic from Q4-2 MGZ Immune system, 2019)

Question 2

A physician suspects that their patient is infected with the Epstein-Barr virus (EBV). EBV serology was ordered to strengthen this hypothesis, and the following result were obtained: EBV-IgM positive, EBV-IgG negative. What conclusions can be made upon these laboratory results? The patient has a ...

A. Primary immune deficiency
B. Primary EBV-infection
C. Reactivation of a previous EBV-infection

(Topic from Q4-1 MGZ Immune system, 2017)

The answers to these questions can be found on page 30 in this journal.