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Targeting Bacterial Virulence: The Coming Out of Type VII Secretion Inhibitors

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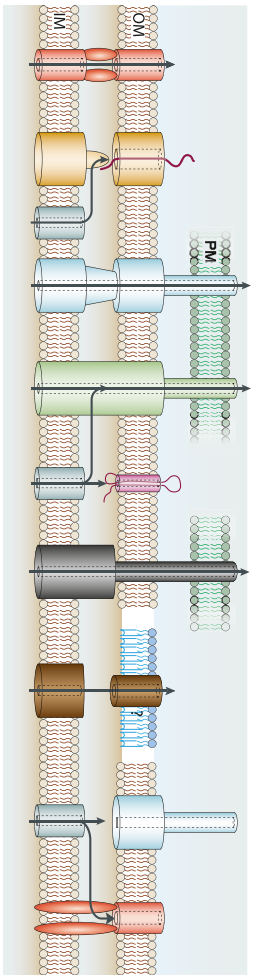
Type VII (ESX) secretion systems of pathogenic mycobacteria, such as *Mycobacterium tuberculosis*, are crucial for intracellular survival, host cell lysis, and the subsequent cell-to-cell spread. In this issue of *Cell Host & Microbe*, Rybniker et al. (2014) have used these characteristics to identify two classes of type VII secretion inhibitors.

Under the threat of the approaching "postantibiotic era," new strategies to combat bacterial infections are being explored. One of these strategies is to block virulence mechanisms of bacterial pathogens (Rasko and Sperandio, 2010). This strategy has several advantages, one of which is prevention of resistance development. Because compounds that block virulence mechanisms do not affect microbial growth directly, it is hypothesized that they are not subjected to (strong) selective pressure for resistance. Although this outlook is exciting, this hypothesis has not been thoroughly tested thus far. Another advantage is the rapid alleviation of disease symptoms by blocking toxins and, by extension, toxin delivery. This approach is already successfully used to treat active tetanus with human antitetanus immunoglobulins. Additionally, by using virulence factors as targets, new chemical classes that were overlooked previously could turn out to be very useful in the treatment of bacterial infections. A final and perhaps most important advantage is that the normal microflora is generally not affected by compounds acting against virulence mechanisms, which has obvious benefits for the patients, but also reduces the potential accumulation of resistance genes within the host microbiome.

Blocking bacterial virulence can be achieved in different ways: by blocking microbial attachment (adhesion blockers), by preventing biofilm formation, by disrupting the regulation of virulence factors, and by blocking the function of secreted toxins. In recent years special attention has been given to the so-called secretion inhibitors. These are compounds that block specialized secretion systems of bacterial pathogens. In order to transport crucial virulence effector proteins across the bacterial cell envelope and sometimes also across the host cell membrane, bacteria evolved different specialized secretion systems. In particular, Gram-negative bacteria and the mycolic acid-producing bacteria, both protected by a double membrane, are dependent on these specialized secretion systems. These secretion systems are generally known as type I to type IX secretion systems (Figure 1). The most enigmatic of these secretion systems is probably the type III secretion (T3S) system that resembles and functions like a small syringe, which is used to secrete a specific subset of effector proteins into host cells. Importantly, many of these T3S systems are unique for bacterial pathogens and crucial for bacterial virulence.

Different strategies have been successfully used for the isolation of secretion system inhibitors (overview in Figure 1). Most T3S inhibitors interfere with gene regulation required for the different stages of T3S system biogenesis (Marshall and Finlay, 2014). Other compounds block the assembly of the injection needle complex or the actual secretion process itself. Importantly, some of these inhibitors have a relatively broad range of activity and are active in several different pathogens. For instance, the salicylidene acylhydrazide compounds block T3S systems in both *Chlamydia trachomatis* and the Enterobacteriacaea. Although several of these secretion inhibitors are active in animal models, they have usually been administered at the same time as the pathogen itself. Therefore, more rigorous animal testing and pharmacokinetic/pharmacodynamic modeling of these compounds is required. The most promising secretion inhibitor is in fact an antibody fragment directed against the T3S system tip protein PcrV of *Pseudomonas aeruginosa*. A modified single-chain Fab version of these antibodies has been used with some success in clinical trials both for *P. aeruginosa* sepsis and cystic fibrosis patients (Milla et al., 2014). However, delivery of antibodies to the site of infection is often challenging, and small molecules might therefore be preferred.

The new kids on the block in this field are the type VII secretion (T7S) inhibitors. The T7S system is crucial for *M. tuberculosis*, the causative agent of tuberculosis and estimated to be responsible for the death of 1.3 million people each year. In the last decades, there has been a steady rise in the number of tuberculosis cases caused by multidrug-resistant and extensively drug-resistant *M. tuberculosis* strains. Therefore, the discovery of novel drugs to treat tuberculosis is a major priority. Fortunately, after a long period of near inactivity in this area, there has been a renaissance in tuberculosis drug development, which resulted in the identification of a number of promising new drugs (reviewed in Zumla et al., 2013). These recent breakthroughs clearly show that new tuberculosis drugs with a new mode of action can be identified.



secretion system	inhibitor	method of identification	mechanism	bacteria
T1SS				
T2SS	Yes	transcription reporter	inhibition of transcription	*V. cholerae*
T3SS	Yes	-transcription reporter -secretion reporter	-inhibition of transcription -impaired needle assembly	*Yersinia, Chlamydia, Pseudomonas, E. coli, Salmonella, Shigella*
T4SS	Yes	[1]bacterial two-hybrid [2]kinase assay [3]conjugation	[1]inhibit protein protein interactions [2]inhibit kinase activity [3]inhibit conjugation	[1]*Brucella* [2]*Helicobacter* [3]*E.coli*
T5SS				
T6SS				
T7SS	**Yes**	**-biological effect (cell lysis in tissue culture)**	**transcription**	***M. tuberculosis***
T8SS				
T9SS				

Figure 1. Overview of Inhibition of Bacterial Secretion Systems
Indicated are the availability of inhibitors, method of identification, method of action of the small molecule, and the species targeted (Baron, 2010; Rybniker et al., 2014).

M. tuberculosis has five T7S systems, designated ESX-1 through ESX-5 (Stoop et al., 2012). Three of these T7S systems are important for survival in the host; ESX-3 is responsible for the uptake of iron and zinc, and ESX-5 is responsible for the secretion of immunomodulatory effector proteins. However, the most crucial T7S system for virulence is ESX-1. ESX-1 was identified as the crucial virulence factor missing in the attenuated vaccine strain *Mycobacterium bovis* BCG. About a dozen different effector proteins have been identified that are secreted by ESX-1. Although the exact function of these different effector proteins still needs to be determined, together these proteins are required for the escape of *M. tuberculosis* from the phagosome of the macrophage into the cytosol. In addition, the lysis of host cells and the subsequent cell-to-cell spread is also dependent on ESX-1 effector proteins. Therefore, blocking ESX-1 is a promising strategy for the control of *M. tuberculosis*.

Rybniker et al. have taken up the challenge and developed a cell-based screen to identify ESX-1 inhibitors. Instead of macrophages, lung fibroblasts, which are highly susceptible to lysis by *M. tuberculosis* in an ESX-1-dependent fashion, were chosen to monitor ESX-1 secretion. Rybniker et al. developed a plate assay where they infected fibroblast with *M. tuberculosis* and measured cell viability after 72 hr of infection. Fifty-five putative ESX-1 compounds significantly increased fibroblast survival, without affecting bacterial growth in culture. Within this group the benzothiophenes and the benzyloxy benzylidene hydrazines were the two most prominent chemical classes, and from each entity one compound, BPT15 and BBH7, respectively, was chosen for further analysis. Both these compounds were active at low doses (IC_{50} of ~2 μM) and, as predicted, blocked the secretion of ESX-1 effector molecules. Interestingly, BPT15 seemed to be an ESX-1-specific inhibitor, whereas BBH7 also blocked the secretion of other, ESX-1-independent, proteins, as was determined by proteomic analysis. Perhaps because of this broader secretion defect, BBH7 also affected intracellular growth of bacteria.

Next, Rybniker et al. performed RNA-seq experiments to identify the mode of action for these compounds. Exposure to BBH7 resulted in the upregulation of many genes associated with a metal-ion overload. However, further research did not identify any causative relationship between metal-ion overload and reduced protein secretion; in fact, addition of zinc resulted in increased ESX-1 secretion. For the BTP15 compound, the analysis was more successful; Rybniker noticed that the addition of this compound affected a limited number of genes. Most of these affected genes belonged to both the hypoxia-induced DosR regulon and the MprA regulon. Subsequent experiments clearly showed that only *mprA* levels were affected. MprAB is a classical two-component signal transduction system, in which MprA is the response regulator in the cytosol and MprB sensor histidine kinase in the cytoplasmic membrane (He et al., 2006). MprAB is induced by different forms of cell envelope stress and upon intracellular growth within macrophages. Rybniker et al. showed in an in vitro kinase assay that BPT15 directly inhibits the MprB histidine kinase of the two-component signal transduction system. The link between MprAB and the ESX-1 system is the *espA* operon, which is tightly regulated by multiple response regulators, including MprA (Pang et al., 2013). EspA is not only a substrate of ESX-1, but also required for the secretion of most other effector molecules. Disruption of *espA* regulation usually results in reduced secretion of ESX-1 effectors. The next step will be to test whether these compounds are able to block mycobacterial virulence in an animal infection model.

Blocking the T7S system ESX-1 could be a highly efficient strategy to disarm *M. tuberculosis.* However, blocking multiple T7S systems is also an interesting option, because this pathogen does not seem to have horizontal gene transfer and acquires resistance usually by accumulating point mutations. By blocking multiple essential targets, we might confront the tubercle bacilli with an insurmountable task. Interestingly, BBH7 could be the first more general T7S blocker.

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