MINIREVIEW-PATHOGENS & PATHOGENICITY

SecA inhibitors as potential antimicrobial agents: differential actions on SecA-only and SecA-SecYEG protein-conducting channels

Jinshan Jin^{1,†}, Ying-Hsin Hsieh¹, Arpana S. Chaudhary², Jianmei Cui², John E. Houghton¹, Sen-fang Sui³, Binghe Wang² and Phang C. Tai^{1,*}

¹Department of Biology, Center for Biotechnology and Drug Design and Georgia State University, Atlanta, GA 30303, USA, ²Department of Chemistry, Center for Biotechnology and Drug Design and Georgia State University, P.O. Box 3965, Atlanta, GA 30303, USA and ³State Key Laboratory of Membrane Biology, Center for Structural Biology, School of Life Sciences, Tsinghua University, Beijing 100084, China

*Corresponding author: Department of Biology, Center for Biotechnology and Drug Design and Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, Georgia, 30303, USA; Tel: +1 404 413 5303; fax: +1 404 413 5301, E-mail: biopct@gsu.edu

[†]Present address: US Food and Drug Administration, National Center of Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079

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ABSTRACT

Sec-dependent protein translocation is an essential process in bacteria. SecA is a key component of the translocation machinery and has multiple domains that interact with various ligands. SecA acts as an ATPase motor to drive the precursor protein/peptide through the SecYEG protein translocation channels. As SecA is unique to bacteria and there is no mammalian counterpart, it is an ideal target for the development of new antimicrobials. Several reviews detail the assays for ATPase and protein translocation, as well as the search for SecA inhibitors. Recent studies have shown that, in addition to the SecA-SecYEG translocation channels, there are SecA-only channels in the lipid bilayers, which function independently from the SecYEG machinery. This mini-review focuses on recent advances on the newly developed SecA inhibitors that allow the evaluation of their potential as antimicrobial agents, as well as a fundamental understanding of mechanisms of SecA function(s). These SecA inhibitors abrogate the effects of efflux pumps in both Gram-positive and Gram-negative bacteria. We also discuss recent findings that SecA binds to ribosomes and nascent peptides, which suggest other roles of SecA. A model for the multiple roles of SecA is presented.

Keywords: SecA inhibitors; SecA-only channels; SecA-SecYEG channels; efflux pumps; Rose Bengal-thiouracil-triazole-pyrimidine analogs; SecA-ribosomes

INTRODUCTION

The role of SecYEG as a protein-conducting channel in Sec-dependent protein translocation in bacteria is well established by both genetics and biochemical studies (Davis and Tai 1980;

Danese and Silhavy 1998; Mori and Ito 2001; Papanikolaou et al. 2007; Driessen and Nouwen 2008). Details of the mechanism of function of the SecA-SecYEG-SecDF•YajC complex and the essential roles of SecA have also been reviewed extensively (Kusters and Driessen 2011; Lycklama and Driessen 2012; Bauer

et al. 2014; Chatzi et al. 2014). Although the SecYEG translocation pathway is homologous to the Sec61 pathway in mammals (Rapoport 2007; Park and Rapoport 2012), SecA is unique to bacteria (and chloroplasts in plants, Hu et al. 2007) and is essential for their cell growth; it is present in *E. coli* in both soluble and membrane-bound forms (Oliver and Beckwith 1981). X-ray structures of soluble SecA and SecA-SecYEG have been resolved (Hunt et al. 2002; Papanikou, Karamanou and Economou 2007; Zimmer, Nam and Rapoport 2008). Soluble SecA is an ATPase (intrinsic ATPase) that is regulated by its C-terminal domain, an activity that is increased by interaction with anionic lipids (lipid/membrane-ATPase), and further increased by precursors and membrane embedded with SecYEG (translocation ATPase) (Lill, Dowhan and Wickner 1990). The findings that (1) precursors of periplasmic alkaline phosphatase (proPhoA) and outer membrane OmpA (proOmpA) can be translocated post-translationally into membrane vesicles (Chen, Rhoads and Tai 1985), (2) ATP hydrolysis is essential for protein translocation (Chen and Tai 1985), and (3) SecA is required for protein translocation into membrane vesicles (Cabelli et al. 1988), paved the way for the complete reconstitution of anionic liposomes with purified SecA and SecYEG defining functional protein translocation complexes (Kusters and Driessen 2011; Lycklama and Driessen 2012; Bauer et al. 2014; Chatzi et al. 2014). Proton motive force, SecD-SecE-YajC, SecB and other chaperones, while not essential, contribute to the efficiency of the protein translocation process *in vitro* and in the cells. It should be noted that the reconstituted proteoliposome system has not addressed another important component, signal peptidase which cleaves the signal peptide from precursors.

SecA-DEPENDENT PROTEIN-CONDUCTING CHANNELS WITH OR WITHOUT SecYEG

E. coli SecA in solution is a dimer of 901 residues that binds to diverse partner ligands (Schmidt et al. 1988; Woodbury, Hardy and Randall 2002; Cooper et al. 2008). Most considerations of SecA-SecYEG translocation machinery view SecA as a peripheral motor that uses ATP hydrolysis to drive precursors through SecYEG translocons (Karamanou et al. 1999). SecA undergoes ATP-driven cycles of membrane insertion and de-insertion (Economou and Wickner 1994). However, SecA can integrate into membranes by itself. Thus, many SecA domains are exposed to the periplasmic side of membranes (Ramamurthy and Oliver 1997; Eichler and Wickner 1998; Jilaveanu and Oliver 2007). A significant fraction of SecA is permanently associated with the cytoplasmic membrane; it does not cycle on and off (Chen, Xu and Tai 1996). There are two asymmetric forms of SecA in the membrane (Tang et al. 2010) (Fig. 1b): SecA_S and SecA_M as revealed by the observations upon trypsin treatments, yielding 68 kD and 34 kD fragments (similar to treated soluble SecA) of the former, and 39 kD and 48 kD that are specific for membrane-bound SecA for the latter (Chen, Brown and Tai 1998). All four fragments are integrated into membranes as shown by their resistance to chemical treatments. SecA is present as a symmetric dimer in solution (Chen et al. 2008; Fig. 1a), and forms asymmetric SecA_S and SecA_M upon interaction with anionic phospholipids (You et al. 2013; Fig. 1b). Computing analyses predict that SecA can embed into membranes (Hu et al. 2007) and can bind to phospholipids at multiple sites (Keller 2011). The structural role of SecA has been shown by drastic conformational changes and formation of “ring-pore” structures by soluble SecA when it interacts with anionic phospholipids, as revealed by both Transmission-

Figure 1. Proposed multiple roles of SecA; (a) Soluble, symmetric dimer SecA (cryo-EM image; Chen et al. 2008); (b) Membrane-associated, asymmetric ring-pore structures (TEM image of SecA on the lipids, Wang et al. 2003); (c) Membrane-associated, SecA-only protein conducting channels, which are active for proteins with or without signal peptides (albeit inefficiently) in ion channel activity and protein translocation (Hsieh et al. 2011; You et al. 2013); (d) More efficient SecA-SecYEG-DFC channels require precursors with signal peptides, and can be converted from SecA-only channels (TEM image of SecA-SecYEG, Tang et al. 2011); (e) SecA binds to ribosomes (cryo-EM-based SecA-ribosome structures from Singh et al. 2014), presumably leading to (f) SecA-mediated co-translational translocation with the exiting nascent peptide providing the sole source for stable association of ribosome and membrane? (Smith et al., 1977, 1978; Herskovits and Bibi 2000; Halbedel et al. 2014; Rawat et al. 2015; Wang, Yang and Shan 2017)

Electronic-Microscopic and Atomic-Force-Microscopic observations (Wang et al. 2003). These ring-pore structures have diameters approximating 80–90 Å and pore diameters about 20–26 Å; they are active upon interaction with chaperone SecB (Chen, Tai and Sui 2007). Such ring-pore structures provide the morphological support for the observations that in the presence of ATP, SecA-liposomes alone form active ion channels and promote translocation of proOmpA (Hsieh et al. 2011; Fig. 1c). It has been suggested that, in addition to the SecA-SecYEG protein-conducting channel (Fig. 1d), there is a second SecA-only protein-conducting channel (You et al. 2013) (Fig. 1c). It should be noted that SecYEG is not active without SecA, but SecA-only channels can function without SecYEG (Hsieh et al. 2011). The two channels have different activities: the one (the SecA-SecYEG channel) is a high-affinity protein-conducting channel with considerable specificity and high efficiency, while the other (the SecA-only channel) is a primitive low-affinity channel with less specificity. In addition to SecA-dependent pathways (Chatzi et al. 2014), there are other high- and low-affinity transport systems in bacteria (Anderson and Oxender 1978).

The possible existence of a protein-conducting channel lacking SecYEG was from the original observations that certain precursors were efficiently translocated into reconstituted membrane vesicles deprived of SecY/PrfA, but still requiring SecA (Watanabe, Nicchitta and Blobel 1990; Watanabe and Blobel 1993). The membrane vesicles from cells depleted in SecE and SecY were also able to translocate certain precursor proteins, including proOmpA and proLpp (Yang, Lian and Tai 1997a; Yang, Yu and Tai 1997b), but not proPhoA. Moreover, Baars et al. (2008) showed that bacterial cells depleted in SecE and SecY could translocate and assemble OmpA, but not all

membrane proteins. These observations on the differential translocation of substrates through protein-conducting channels with or without SecYEG reveal fundamental differences between the two SecA-dependent translocation pathways. In most post-translational translocation studies the yardstick has been translocation of precursors inside membrane vesicles or proteo-liposomes as the translocated proteins become resistant to protease digestion. Blobel's group showed that *E. coli* membrane vesicles possess large, aqueous ion channels, whose activity is opened by signal peptides (Simon, Blobel and Zimmerberg 1989; Simon and Blobel 1992). To provide additional and alternative assays for protein-conducting channel activity, a semi-physiological assay of ion-channel activity was developed by injecting *E. coli* membranes into frog oocytes, in which SecA and functional precursors (or signal peptides) are also required (Lin et al. 2006). Reconstituted membrane vesicles deprived of SecYEG actively elicit both anion and cation channel activity, but have no signal peptide specificity (Lin et al. 2012). Both resistance to protease in liposomes and ion channel activity in oocytes are important assays that have established SecA-only channels as being functional protein translocators (Hsieh et al. 2011). As expected, chaperone SecB also enhances translocation activity (Hsieh et al. 2011). Even though ion channel activity relates only to channel opening, not protein translocation, measuring channel activity in semi-physiological oocytes has great advantages, as discussed (Lin et al. 2006; Hsieh et al. 2015).

In comparison with SecA-SecYEG (Fig. 1c,d), SecA-only channels (1) are smaller (Tang et al. 2011), (2) less efficient (about 35%), (3) require more ATP-Mg²⁺, (4) require SecA at an optimal concentration of 1–2 μ M (Hsieh et al. 2011), 10 times higher than SecYEG-containing membranes, though still in the physiological range (Mizushima, Tokuda and Matsuyama 1992; Moran, Phillips and Milo 2010); (5) lose signal-peptide specificity (like Prl mutants, Derman et al. 1993) and can translocate unfolded signal-peptide-less proteins (Hsieh et al. 2011); (6) lose ion-size selectivity (Lin et al. 2012 and unpublished data). The addition of SecYEG-SecDFC to lower-efficiency, SecA-only-liposome changes the shape and increases size of the pore structure (Tang et al. 2011) (Fig. 1d), and restores all activities (Hsieh et al. 2011; Hsieh et al. 2013). Unsurprisingly, although SecA-only channels can translocate OmpA, proOmpA or unfolded PhoA, proPhoA is translocated only by SecA-SecYEG channels (Zhang et al. 2013), consistent with observations that SecYEG-deficient membrane vesicles translocate proOmpA, but not proPhoA (Yang, Lian and Tai 1997a; Yang, Yu and Tai 1997b).

Although the molecular characterizations of the SecYEG protein-conducting channel have been quite extensive, little is known about the SecA-only channel. A model for SecA-only channel has been proposed (You et al. 2013, Fig. 1c). In this model, SecA functions as an asymmetric dimer in membranes SecA_M and SecA_S; (Chen, Brown and Tai 1998; Tang et al. 2011; You et al. 2013), not a monomer, as has been proposed in the SecA-SecYEG channel (Or et al. 2005). SecA_M is permanently embedded into the membrane (Chen, Xu and Tai 1996), providing the structural integrity of the channel and a similar protein translocating functions as the SecYEG channel, but lacks signal-peptide specificity-as in Prl suppressors (Derman et al. 1993; Huie and Silhavy 1995; Duong and Wickner 1999). Perhaps, SecA_M is a primitive counterpart to the SecYEG protein channel, acting as a protein-conducting core with less efficiency and specificity. SecA_S binds precursors and acts as the ATPase motor to drive the proteins through the SecA_M channel (Fig. 1c). In summary, SecA is an ATPase, an integral membrane protein capable of forming ring-pore structures, and a protein-conducting channel capable

of eliciting ion channel activity and promoting protein translocation. A recent study established these capacities by dissecting various domains for their intrinsic and lipid-stimulated ATPase, ring-like pore structure, ion channel activity, protein translocation activity and interactions with SecYEG-SecDF-YajC (Hsieh et al. 2017a). The existence of SecA-only channels partially explains why there are far more SecA proteins than SecYEG complexes in bacteria (Mizushima, Tokuda and Matsuyama 1992; Seoh and Tai 1997).

Development of simple assays for SecA-only channel activity makes it possible to measure the *in vitro* SecA activity of assorted bacterial species, which otherwise would be difficult to do with non-cogent SecYEG protein translocation assays (Zhang et al. 2013). Simplifying SecA-only channel assays also provides opportunities to evaluate for these inhibitors that target SecA and its physiological functions.

INHIBITORS TARGETING SecA

The need for new antibiotics/antimicrobials is widely recognized with the ever-increasing abundance of drug-resistant strains, including methicillin-resistant *Staphylococcus aureus* (MRSA) and multiple drug resistance (MDR), as reviewed extensively (Levy and Marshall 2004; Nikaido 2009; Rao et al. 2014; Chaudhary et al. 2015a). Most virulence factors and toxins from pathogenic bacteria are secreted through the Sec-transport system; consequently, inhibitors for their components would be logical antimicrobial candidates (Lanzetta et al. 1979; Stephens and Shapiro 1997; Rao et al. 2014). However, the inhibitors need to be specific and unique to bacteria to be useful for therapeutic application. Thus, inhibitors/antibiotics that affect the membrane, general proteases (Chen and Tai 1987a, Chen and Tai 1987a, 1989), and proteins with homologs to mammalian translocation systems are not of any practical application. For example, decatransin inhibits both SecYEG translocation and Sec61 translocation (Jenne et al. 2015). An extensive review of antibiotics/inhibitors targeting bacterial secretory pathway have been presented, including signal peptidase, and other secretory pathways (Rao et al. 2014). In recent years SecA has been widely recognized as a druggable target. Several excellent reviews have extensively delineated the needs, assays, chemical synthesis, properties and potency of specific SecA inhibitors (Chen et al. 2010; Segers and Anne 2011; Segers et al. 2011; Rao et al. 2014; Chaudhary et al. 2015a). This part of the mini-review on antibiotics/inhibitors will focus on more recent developments, structural classes of potent and selective small-molecule inhibitors, and the perspectives of the inhibitors as antimicrobials.

Different assays have been used for screening SecA inhibitors in the above reviews. To summarize, they are evaluated based on: (1) ATPase activity of various forms of intrinsic, unregulated or mutated SecA (Lanzetta et al. 1979; Lill, Dowhan and Wickner 1990; Mitchell and Oliver 1993; Gouridis et al. 2010); (2) ion channel activity of SecA-liposomes or membranes in the semi-physiological oocytes -with or without SecYEG (Lin et al. 2006; Hsieh et al. 2011; Lin et al. 2012); (3) protein translocation into membranes vesicles (Huang et al. 2012; De Waelheyns et al. 2015) and SecA-liposomes with or without SecYEG (Hsieh et al. 2011) and (4) efficacy in inhibiting bacterial growth combined with inhibition of the secretion of virulence factors and toxins (Jin et al. 2015). The first three assays determine the IC₅₀ (50% inhibition of activity) and differentiate activities of the SecA-only and SecA-SecYEG channels, while the inhibition of bacterial growth is expressed as MIC (minimal inhibitory

concentration). It is important to note that the roles of SecA and ATP hydrolysis in bacterial growth are not well understood. For example, a truncation of SecA by deletion of short N-terminal or C-terminal reduces both translocation ATPase and even protein translocation, but does not significantly affect the growth of *E. coli* (Floyd et al. 2014; Na et al. 2015). The rationales for these assays and summaries of structures of various SecA inhibitors have been described (Segers and Anne 2011; Rao et al. 2014; Chaudhary et al. 2015a). This review will briefly summarize the relevant literature, focusing more on biological effects.

Azide was the first known inhibitor of SecA, using an azide-resistant mutant of *E. coli* derived from a specific mutation in SecA (Oliver et al. 1990). Indeed, azide (at mM concentrations) does inhibit SecA translocation ATPase, protein translocation and the SecA-dependent ion current activity (Lin et al. 2006). Subsequently, however, azide resistant mutants were also found in SecY (PrIG mutants) as well as SecE (PrIG mutants) (Huie and Silhavy 1995; Li et al. 2007; Maillard et al. 2007). Consequently, azide appears to be more of a SecA-SecYEG translocation inhibitor and a more generic ATPase inhibitor than a specific inhibitor of SecA function (Bowler et al. 2006). There are several other inhibitors reported with various activity and MIC (Alksne et al. 2000; Parish et al. 2009; Jang et al. 2011; Segers et al. 2011; De Waelheyns et al. 2015). One natural fungal product CJ-21 056 has been found to inhibit translocation at 38.4 μ M, with an MIC of 12 μ M for MRSA and *Enterococcus faecalis* (Sugie et al. 2002). Using an ATPase assay to screen chemical libraries, the best indole derivative has an effective IC_{50} of 0.25 μ M; however, the reported MIC for *Agrobacterium tumefaciens* is at 0.76 mM (Akula et al. 2011). The large discrepancy may be due to its permeability into bacterial membranes, which may be circumvented to test its efficacy.

The Tai and Wang labs have undertaken multiple approaches, both in assay development and the design and synthesis of broad-spectrum, small molecule, SecA inhibitors against Gram-positive and Gram-negative bacteria, including MRSA and MDR pathogens. Three structural classes of small molecule compounds have been designed and evaluated (Fig. 2): Rose Bengal (RB) analogs (Huang et al. 2012; Cui et al. 2013; Jin et al. 2015), 5-cyano-6-thiouracil-pyrimidine analogs (Li et al. 2008; Chen et al. 2010; Chaudhary et al. 2015b) and triazole-pyrimidine analogs (Cui et al. 2016; Jin et al. 2016). Fluorescein dyes RB and erythrosine were found to effectively inhibit SecA activities (Huang et al. 2012). RB is the first sub-micromolar inhibitor of ATPase activity in the unregulated *E. coli* SecA (EcSecA); with EcSecA68N (EcSecA which lacks a C-terminus) having an IC_{50} of 0.5 μ M. In addition, RB showed an IC_{50} of 25 μ M against intrinsic SecA ATPase (*Bacillus subtilis* SecA at 7 μ M), 5 μ M against membrane/lipid ATPase, 0.9 μ M against translocation ATPase and 0.25 μ M against protein translocation. The antibacterial activity against Gram-positive *B. subtilis*, as measured in MIC, approximates 3.1 μ M. Using the NR698 mutant strain of Gram-negative *E. coli*, which has an outer-membrane deletion and thus increased permeability (Ruiz et al. 2005), the MIC is also at 3.1 μ M, though the MIC for the wild-type is over 1 mM. Permeability is apparently a major factor for the different sensitivity (see below). Molecular docking studies suggest that RB binds close to the ATP binding site, similar to CJ 21 058 (Huang et al. 2012). RB is a competitive inhibitor for ATP at low concentrations, but is a non-competitive inhibitor at high ATP concentrations (Hsieh et al. 2015). RB is relatively large (M_r 1017) and known to have other targets, such as F_1F_0 -ATPase (Huang et al. 2012), resulting in cytotoxicity. Nevertheless, RB was used as a lead compound for further optimization. The most active derivative of this group, SCA-50 (M_r 243), is a non-competitive inhibitor of ATP, active

Table 1. Summary of inhibition of SecA-only liposomes activities by SecA inhibitors. SecA of *E. coli* (EcSecA), *P. aeruginosa* (PaSecA), *S. aureus* (SaSecA1) and *B. anthracis* (BaSecA1) were tested. A. IC_{50} for ion channel activity of SecA-liposome determined in the oocytes; B. ProOmpA translocation into SecA-liposomes (From Hsieh et al. 2013; Jin et al. 2016. * indicates unpublished data; ND, not determined).

A. Inhibition on liposome ion-channel activity, IC_{50} (μ M)				
SecA protein	RB M_r : 1017	SCA-50 M_r : 298	SCA-15 M_r : 436	SCA-107 M_r : 472
EcSecA	0.4	2.3	*4.2	1.6
PaSecA	0.3	3.0	*3.2	1.3
SaSecA1	0.4	1.0	*2.3	0.6
BaSecA1	0.3	*1.0	*2.8	0.7
B. Inhibition on liposomes translocation activity, IC_{50} (μ M)				
	EcSecA	PaSecA	SaSecA1	BaSecA1
SCA-107	3.5	3.0	*2.0	*2.8
Rose Bengal	1.0	1.0	*1.0	ND

against SecA1 and SecA2 ATPase from *S. aureus* with an IC_{50} of 12 μ M for SecA1, and 1 μ M against SaSecA1 channel activity. SCA-50 inhibits *S. aureus* Mu50 toxin secretion, and is also active against *E. coli* NR698 and *B. subtilis* (Cui et al. 2013). Interestingly, SCA-50 is far more active against several MRSA strains (MIC 4 μ M) than RB (MIC 21–59 μ M) (Jin et al. 2015). In addition to the RB analogs, 5-cyano-6-thiouracil pyrimidine analogs (Li et al. 2008; Chen et al. 2010; Chaudhary et al. 2015b) and triazole-pyrimidine analogs (Cui et al. 2016; Jin et al. 2016) have also been developed and evaluated with the hope that different scaffolds may target different parts of SecA molecules (Figs. 2 and 3). Using antimicrobial assays as well as SecA-only ion channel and protein translocation assays, SCA-15 was identified as the most active compound from the thiouracil-pyrimidine series, and along with SCA-107 and SCA-112 from the triazole-pyrimidine series. They are all non-competitive inhibitors against ATP in SecA inhibition, and have a relatively low inhibition of ATPase activity, suggesting their effects on ATPase are allosteric for SecA in the lipid environment rather than binding directly to the ATP site (Jin et al. 2015; Jin et al. 2016). The lack of effects of these SecA inhibitors on other ATPases, such as H^+ -ATPase and SpuB, indicates a certain selectivity of the inhibitors for SecA functions. This is significant because by not targeting the high-affinity ATP site, it allows further optimization to minimize toxicity issues without concerns for off-target effects on other ATP-binding proteins. Indeed, molecular docking studies show that they bind different part of SecA, close to but not at the ATP binding (Fig. 3). Various SecA from other bacteria has also been evaluated in the SecA-only channel activity; the inhibitors are also very active against SecA from *P. aeruginosa*, *S. typhimurium* and *S. aureus*, and SecA1 from *S. pyogenes* (Zhang et al. 2013; Jin et al. 2016). The effects of these inhibitors on SecA-only channel activity, protein translocation and antimicrobial activity are summarized in Tables 1 and 2. SCA-107 is the most active inhibitor among these series of compounds.

The outer membrane of Gram-negative bacteria is a major permeability barrier, hindering the effectiveness of many antibiotics/antimicrobials. This obstacle can be circumvented by two outer membrane permeabilizers, PMBN and NAB7061. These compounds are polymyxin B derivatives that lack the fatty acid tail, have minimal toxicity, and allow antimicrobials to enter Gram-negative bacteria (Vaara et al. 2008; Vaara et al. 2010). In the presence of these permeabilizers, all classes of SecA inhibitors

Figure 2. Three classes of small molecule SecA inhibitors: Class A, RB analogs; Class B, Thiouracil-pyrimidine analogs and Class C, Triazole-pyrimidine analogs (From Jin et al. 2016).

Figure 3. Molecular modeling of three classes of small molecule SecA inhibitors. The program used is SYBYL 2.0 for file PDB ID- 2FSG with SecA dimers A and B. (A) Class A inhibitor (see Fig. 2), SCA-50, binds close to the ATP site of monomer B; (B) Class B inhibitor, SCA-15, binds at the interface of A and B; close to the ATP site of the B monomer and (C) Class C inhibitor, SCA-107, binds to the interface of A and B monomers (slightly closer to the ATP site of A, partially blocking the entrance to this ATP site). SCA-15 binds relatively closer to ATP site of the B monomer, followed by SCA-50, then the SCA-107. At the interface, SCA-15 binds to the B monomer, facing the A monomer. Lower panels: Inhibitors bindings relative to surrounding amino acid residues. (SCA-50 modified from Jin et al. 2015, SCA-15 from Chaudhary et al. 2015a, SCA-107, from Cui et al. 2016).

are active against several Gram-negative pathogens, including *P. aeruginosa*, *S. typhimurium*, *Shigella flexneri*, *A. baumannii* and *Klebsiella pneumonia* with low MIC (Table 2). These SecA inhibitors are broad spectrum bactericidal inhibitors against Gram-positive and Gram-negative pathogens, and are more effective than the last resort antibiotic, vancomycin (Jin et al. 2015; Jin et al. 2016). The results from the SecA-only ion channel activity and protein translocation assays parallel most closely those of the antimicrobial assays.

SecA functions as a membrane protein that spans the cytoplasmic membrane and forms a channel (Wang et al. 2003; You et al. 2013) (Fig. 1c). As a result, SecA inhibitors may access SecA directly from the extracellular matrix and exert their effects without entering inside cells (alternatively, SecA inhibitors may be poor substrates for efflux pumps). About 50% of *E. coli* SecA is in the cytoplasmic membrane, while all SecA1 of *S. pyogenes* is in the membranes (Rosch and Caparon 2004; Rosch and Caparon 2005). Consequently, targeting SecA may allow the

Table 2. MIC of SecA inhibitors to various bacterial strains, including some Gram-positive and Gram-negative MRSA and MDR pathogens. *With (+) or without (-) PMBN (Polymyxin B nanopeptide) which alone had no inhibition on growth ND, not determined (From Jin et al., 2015, 2016).

Strains, MIC (μ M)	PMBN*	RB Mr: 1017	SCA-50 Mr: 298	SCA-15 Mr: 436	SCA-107 Mr: 472	Vancomycin Mr: 1446
Gram-positive bacteria						
<i>S. aureus</i> Mu50	-	50.0	12.5	*5.0	1.6	5.5
<i>S. aureus</i> Newman	-	50.0	12.5	ND	1.6	ND
<i>B. anthracis</i> Sterling	-	ND	*6.3	6.3	3.1	ND
Gram-negative bacteria						
<i>E. coli</i> NR698	-	4.4	15.8	12.5	5.2	ND
<i>E. coli</i> MC4100	-	>1250	>1250	>1250	>1250	>150
<i>E. coli</i> MC4100	+	15.6	3.0	15.6	1.5	21.6
<i>P. aeruginosa</i> T15464	+	0.4	6.3	ND	0.4	21.6
<i>A. baumannii</i> ATCC9955	+	79.2	25.0	ND	2.1	172.5
<i>S. flexneri</i> ATCC12022	+	7.4	5.8	25.0	4.7	43.1

possibility of avoiding the effect of bacterial efflux transporters, which are responsible for MDR in highly pathogenic strains of bacteria including MRSA (Nikaido 2009; Jin et al. 2015; Jin et al. 2016). Indeed, SecA is required for the proper assembly of many efflux pumps with or without signal peptides. Even though *S. aureus* strains Mu50 and N315 are resistant to QacA efflux-mediated antiseptics, the levels of efflux pumps have no effects on the MIC and bactericidal nature of SecA inhibitors (Jin et al. 2015). Similar results were obtained with MDR strains of *P. aeruginosa* with MexJK-Opr-H pumps and *E. coli* with AcrAB pumps (Jin et al. 2016). These findings show that differing activities of the efflux pumps have no effect on the MIC of the SecA inhibitors, validating the ability for SecA inhibitors to abrogate the effects of efflux pumps in either Gram-positive or Gram-negative bacteria.

The biochemical specificity of inhibitors targeting SecA has been shown by the binding assays and immunoprecipitation pull-down study for *S. aureus* SecA1 and *E. coli* SecA (Jin et al. 2015; Jin et al. 2016). The critical genetic evidence is the identification of an *E. coli* azide-resistant strain carrying a single *seca-azi-9* mutation that is resistant to SCA-107 (Jin et al. 2016). This strain has an increased MIC of about 10–20 fold. The mutated *ecsecA* gene has been cloned, and identified as having single mutation resulting in SecAL515F. Complementation of the azide resistance of *seca-azi-9* in *E. coli* with *B. subtilis* SecA and subsequent increase in MIC further verifying the *seca-azi-9* resistance to SCA-107. Purified SecA-L515F protein, when assayed for channel activity and protein translocation, further confirms that the resistance is due to the singular SecA mutation (Jin et al. 2016), providing important genetic evidence of SecA being a key target for SCA-107 achieving its antimicrobial effect. Interestingly, this mutant is also partially resistant to SCA-50. Two recent articles that evaluated the effects of SecA inhibitors, based on thiouracil derivatives containing acyl thiourea or triazol-thiadiazole, reported that these inhibitors were also active against Gram-positive bacteria (Cui et al. 2017a, b). However, there are limited data on the correlation between SecA inhibition assays and MIC determination. Thus, it is hard to compare directly with other published SecA inhibitors.

The ultimate goal in searching for SecA inhibitors would be to test their efficacy in protecting the host from infection by bacterial pathogens. Indeed, SCA-107 protects mice against lethal infection by the *S. aureus* Newman strain (Eichenbaum, Tai, Wang, manuscript in preparation). This is encouraging and

warrants further investigation of these inhibitors. Further work in this field also needs to improve the solubility and the delivery of these inhibitors (Hu, Akula and Wang 2016), as well as overcoming the permeability hindrance of the outer membrane of Gram-negative bacteria. So far the best inhibitor is effective at high nM concentrations, which is better than many existing antibiotics, but may be optimized further. However, direct quantitative comparisons in efficacy of the different inhibitors are difficult to make when one is reminded of the fact that the SecA concentration in bacterial cells is around the 5–8 μ M range.

The presence of SecYEG alters the sensitivity of these inhibitors in both ion channel activity and protein translocation (Hsieh et al. 2013; Hsieh et al. 2017b). The IC₅₀ values of RB and SCA-107 are higher in the presence of SecYEG, and in membrane with wild-type SecYEG (Table 3A). This difference was observed in liposome reconstitution with or without SecYEG, or in membrane with or without SecYEG (Table 3A). Many *prlD/secA*, *prlA/secY* and *prlG/secE* suppressors are resistant to azide (Oliver et al. 1990; Osborne and Silhavy 1993; Huie and Silhavy 1995; Maillard et al. 2007). Their membrane vesicles exhibit a biphasic mode of inhibition in ion channel activity and in protein translocation, one with an IC₅₀ similar to wild-type, the other with a much higher IC₅₀ (Hsieh et al. 2017b). However, all bacterial strains have similar MIC despite vast differences in their IC₅₀ (Table 3B). It appears that SecA-only channels, which are more sensitive to SCA-107, have specific physiological functions in the cells that are distinct from those of SecA-SecYEG channels.

Along with all the rapid advances in molecular analysis of post-translational translocation, an understanding of co-translational secretion has not been pursued vigorously, even though extracellular labeling of nascent peptides on membrane-bound ribosomes, and the nascent peptides as the sole attachment of ribosome to membranes were reported early (Smith et al. 1977; Smith, Tai and Davis 1978; Davis and Tai 1980). Importantly, ATP is required, in addition to the translationally-specific GTP, for bacterial co-translational translocation, implying the involvement of SecA with membrane-bound ribosomes (Chen and Tai 1987b). Indeed, SecA is involved in co-translational translocation with or without signal peptides (Karamyshev and Johnson 2005; Huber et al. 2011) as well as post-translational translocation (Huber et al. 2011). Additionally, SecA binds ribosomes (Fig. 1e) and covers the nascent peptide emerging site with its N-terminal helix that is required for stable ribosome association (Kramer et al. 2009; Singh et al. 2014). SecA has also been shown

Table 3A. Changes of sensitivity to SecA inhibitors by SecYEG for ion channel activity in the oocytes. SecA-liposomes reconstituted with equal amount of SecYEG as in the MC4100 membranes (Memb). The SecYEG in MC4100 or PrlA4* membranes was removed by cholate (Watanabe, Nicchitta and Blobel 1990) to yield reconstituted membrane (RE-4100 Memb); and then similarly reconstituted with SecYEG-DFC (Re-MC4100 + SecYEGDFC) or RE-PrlA4 Memb. (Hsieh et al. 2011, 2017a).

A. Changes of ion channel sensitivity to SecA inhibitors by SecYEG

Channel activity IC ₅₀ (μM)	SecA-liposomes	SecA-lipo + YEGDFC	MC4100 Memb	RE-MC4100 Memb	RE-MC4100 + YEGDFC	PrlA4 Memb	RE- PrlA4 Memb
Rose Bengal	0.4	3.8	4.7	0.4	4.4	73	0.6
SCA-107	1.1	3.3	2.9	0.8	2.1	>90	2.5

Table 3B. Biphasic responses of PrlA/SecY membranes to inhibitor SCA-107. The IC₅₀ (μM) inhibition of PrlA membranes were extrapolated for low IC₅₀L and high IC₅₀H: Ion channel activity in oocytes, and in vitro pOmpA translocation into membrane vesicles (From Hsieh et al. 2017a). All suppressor strains are derivatives of MC4100 (Osborne and Silhavy 1993).

B. Biphasic responses of PrlA mutant membranes and MIC to SecA inhibitor SCA-107

	MC4100		PrlA4		PrlA666		Other PrlA, PrlG1	
Prl membranes	IC ₅₀	IC ₅₀ L	IC ₅₀ H	IC ₅₀ L	IC ₅₀ H	IC ₅₀ L	IC ₅₀ H	
IC ₅₀ on channel activity (μM)	3.9	3.4	125	1.7	154	1.4–6.1	>100	
IC ₅₀ on translocation activity (μM)	1.5	2.6	>50	2.7	>50	2.1–3.1	>50	
MIC of SCA-107 (μM)	1.5–5.5		3.1		0.8		1.6–3.1	

to interact with ribosomes in a mutually exclusive manner to SecYEG (Wu et al. 2012). Moreover, while membrane proteins may insert spontaneously into or across membranes (Engelman and Steitz 1981), the insertion of some proteins that require signal recognition particles has been demonstrated to be mediated by SecA (Qi and Bernstein 1999; Neumann-Haefelin et al. 2000), as have the co-translational insertion of membrane proteins without signal-peptides (Herskovits and Bibi 2000; Halbedel et al. 2014; Rawat et al. 2015). SecA mediates co-translational targeting and translocation of an inner membrane protein (Wang, Yang and Shan 2017), implicating a role for SecA-bound ribosomes in co-translational translocation (Fig. 1f). One intriguing question emerges: whether the 'efficient' biogenesis and integration of signal-peptide-less SecYEG into membranes also involve SecA. Energetically, the SecA-only channel is less efficient than the SecA-SecYEG channel, and extensive ATP hydrolysis is required for post-translational protein translocation, even through the SecB-SecA-SecYEG channels (Mao, Hardy and Randall 2009). It is possible that these post-translational pathways are less efficient than their co-translational counterparts, which perhaps can push nascent peptides by protein synthesis, or minimally save energy by translocating partially folded nascent peptides.

CONCLUSIONS AND PERSPECTIVES

Effective broad-spectrum inhibitors for SecA-dependent protein translocation have been developed, and may have therapeutic applications. These inhibitors are less effective against SecA-SecYEG channels than against the SecA-only channels, which translocate proteins with or without signal-peptides albeit with lower efficiency. It is interesting to note that when bacterial cells enter stationary phase, the amounts of SecA increase while the SecYEG levels decrease (Yang, Lu and Tai 2013), suggesting an important role for these SecA-only-channels in the secretion of proteins without signal-peptides (Tanji et al. 1991;

Yang et al. 2011). Since ribosomes can only attach to membranes via nascent peptides (Smith, Tai and Davis 1978), we propose that the interaction of SecA with membrane-ribosomes (Fig. 1f) may have special importance in the fundamental mechanism(s) of bacterial cell physiology, which SecA inhibitors may help to unravel. The interactions of SecA with membrane-bound ribosomes in co-translational secretion raise the intriguing prospect of combined application of both SecA inhibitors and ribosome antibiotics (Davis, Chen and Tai 1986).

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