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Development of a high-throughput screening assay for the discovery of small-molecule SecA inhibitors

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ABSTRACT

A major pathway for bacterial preprotein translocation is provided by the Sec-dependent preprotein translocation pathway. Proteins destined for Sec-dependent translocation are synthesized as preproteins with an N-terminal signal peptide, which targets them to the SecYEG translocase channel. The driving force for the translocation reaction is provided by the peripheral membrane ATPase SecA, which couples the hydrolysis of ATP to the stepwise transport of unfolded preproteins across the bacterial membrane. Since SecA is essential, highly conserved among bacterial species, and has no close human homologues, it represents a promising target for antibacterial chemotherapy. However, high-throughput screening (HTS) campaigns to identify SecA inhibitors are hampered by the low intrinsic ATPase activity of SecA and the requirement of hydrophobic membranes for measuring the membrane or translocation ATPase activity of SecA. To address this issue, we have developed a colorimetric high-throughput screening assay in a 384-well format, employing an *Escherichia coli* (*E. coli*) SecA mutant with elevated intrinsic ATPase activity. The assay was applied for screening of a chemical library consisting of ~27,000 compounds and proved to be highly reliable (average Z' factor of 0.89). In conclusion, a robust HTS assay has been established that will facilitate the search for novel SecA inhibitors.

The widespread use and overconsumption of antibiotics in human and veterinary medicine and their application as growth promoters are coresponsible for the emergence of drug-resistant bacteria. Despite this increasing problem of antibiotic resistance, antibiotic drug discovery programs did not keep pace, until recently, with this dramatic development. As a result, in the past 20 years only two novel antibiotic classes have been introduced, alongside several new derivatives of existing classes [1,2]. The emergence of superbugs (i.e., bacteria that are resistant to multiple antibiotics) has, however, rekindled interest in the field and one becomes more and more aware that new antibacterials are urgently needed. Special attention should be paid to compounds that may act via novel mechanisms of action to avoid cross-resistance with existing antibacterial drugs to meet the challenges posed by resistance today. In this framework, new antibiotic targets are currently being proposed and evaluated, including components of the bacterial protein secretion pathways [3]. The major route for the transport of bacterial proteins across or into the bacterial cytoplasmic membrane is provided by the Sec pathway [4]. Proteins destined for Sec-dependent translocation are synthesized as precursors with an N-terminal signal sequence which is required for targeting of the unfolded preprotein to the SecYEG translocase, a membrane-spanning channel consisting of the integral membrane proteins SecY, SecE, and SecG [5]. The driving force for the Sec-dependent transport of preproteins across the cytoplasmic membrane is provided by the peripheral membrane ATPase SecA [6,7]. In *Escherichia coli*, SecA is a large (MW = 102 kDa), dimeric, multifunctional protein that participates in nearly every step of the pathway. On binding of the preprotein to the SecYEG translocase, SecA undergoes a conformational change which "activates" the enzyme for high-affinity recognition of the SecB export chaperone, the signal peptide, and the mature domain of preproteins [8]. In addition, binding of SecA to SecYEG stimulates nucleotide exchange at SecA and results in a 2-fold stimulation of its intrinsic ATPase activity. Binding of a translocation-competent preprotein renders the Sec machinery fully active and results in maximal SecA ATPase activity known as "translocation ATPase" [9]. Through repeated cycles of ATP binding and hydrolysis, SecA undergoes conformational changes that drive the stepwise export of an unfolded

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preprotein through the translocase channel and across the bacterial membrane [10].

Because SecA is a conserved and essential protein in all bacteria but is absent in humans, it is considered as a promising antibacterial drug target [11–16]. Several approaches to discover SecA inhibitors have already been used, including virtual ligand screening [14], a SecA–LacZ reporter fusion system in *E. coli* [15], and a two-plate differential sensitivity antisense assay [16]. However, large-scale screening campaigns to identify SecA inhibitors are hampered by the low intrinsic ATPase activity of SecA and the complexity (i.e., the requirement of hydrophobic membranes and unfolded preproteins) of *in vitro* assays used for studying the SecA-catalyzed preprotein translocation reaction. To address this problem, we have developed a colorimetric high-throughput screening (HTS) assay in 384-well format, employing an *E. coli* SecA mutant with elevated intrinsic ATPase activity that is otherwise fully functional for protein translocation [17,18]. The present paper describes the development and optimization of the assay, together with its application to screen a diverse small-molecule compound collection.

Experimental procedures

General

Sodium chloride, sucrose, DMSO[1] and $MgCl_2$ were purchased from Merck (Darmstadt, Germany). Tris and EDTA were obtained from Promega (Madison, USA). Bovine serum albumin (BSA) was from Boehringer Mannheim (Germany). DNase and ATP were obtained from Roche Diagnostics (Mannheim, Germany). IPTG was obtained from Eurogentec (Liège, Belgium). Ampicillin, PMSF, potassium chloride, and the inorganic phosphate standard solution was supplied by Sigma (St. Louis, MO, USA). Nickel–nitrilotriacetic acid (Ni–NTA) agarose was purchased from Qiagen (St. Louis, Mo, USA). Biomol Green Reagent was obtained from Enzo LifeSciences (Plymouth, USA). Glycerol and imidazole were from Acros Organics (NJ, USA).

Protein expression and purification

Wild-type and W775A *E. coli* SecA were overexpressed in *E. coli* BL21.19 and purified as described [17]. Briefly, cell pellets were suspended in buffer A (50 mM Tris–HCl, 1 M NaCl, 10% glycerol, 5 mM imidazole, pH 8.0) containing 2.5 mM PMSF and 50 μg/ml DNase. After lysis by passing the cells three times through a French pressure cell (69 MPa), cell debris was removed by centrifugation. The cleared extract was applied to a nickel–nitrilotriacetic acid column preequilibrated with buffer A. After washing the column with 10 column volumes buffer A and 5 column volumes buffer B (50 mM Tris–HCl, 50 mM NaCl, 10% glycerol, 5 mM imidazole, pH 8.0), proteins were eluted by applying 10 column volumes of buffer C (50 mM Tris–HCl, 50 mM NaCl, 10% glycerol, 100 mM imidazole, pH 8.0). Peak fractions containing recombinant SecA were pooled and dialyzed against buffer D (50 mM Tris–HCl, 50 mM NaCl, 10% glycerol, 2 mM EDTA, pH 8.0). Protein samples were concentrated using Vivaspin centrifugal devices (Sartorius, Göttingen, Germany) with a MWCO of 3 kDa and dialyzed against buffer E (50 mM Tris–HCl, 50 mM NaCl, 50% glycerol, 2 mM EDTA, pH 8.0).

Enzyme preparations were aliquoted and stored at -80 °C until use.

[1] *Abbreviations used:* BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; IMV, inner membrane vesicle; IPTG, isopropyl β-D-1-thiogalactopyranoside; Ni–NTA, nickel–nitrilotriacetic acid; PMSF, phenylmethylsulfonyl fluoride.

The preprotein ProPhoA(Cys-) from *E. coli* was overexpressed in *E. coli* BL21.19 and purified as described [19,20]. Briefly, cell pellets were suspended in buffer F (50 mM Tris–HCl, 0.5 MNaCl, 5% glycerol, pH 8.0) containing 2.5 mM PMSF and 50 μg/ml DNase. After lysis bypassing the cells three times through a French pressure cell (69 MPa), ProPhoA(Cys-) was isolated as inclusion bodies and purified by Ni–NTA affinity chromatography under denaturing conditions. Peak fractions containing the recombinant preprotein (in 50 mM Tris–HCl, 50 mM NaCl, 5% glycerol, 100 mM imidazole, 6 M urea, pH 8.0) were pooled and dialyzed against 50 mM Tris–HCl, 50 mM KCl, 5% glycerol, 6 M urea, 1 mM EDTA, pH 8.0. Protein concentrations were estimated by the Bradford assay (Bio-Rad, München, Germany), using bovine serum albumin as the standard.

Isolation of inner membrane vesicles

Inner membrane vesicles (IMVs) were prepared from *E. coli* strain BL31(DE3) using pET610 [21] to overexpress SecYEG. Cells pellets (~30 g) were suspended in 30 ml 50 mM Tris–HCl, 20% glycerol, pH 8.0, containing 2.5 mM PMSF and 50 μg/ml DNase (buffer F). Cells were lysed by three passages through a French pressure cell (69 MPa), diluted with an equal volume of buffer F and centrifuged (4000*g*, 10 min, 4 °C) to remove cell debris. Membranes were collected from the supernatant by ultracentrifugation (35,000*g*, 90 min, 4 °C). The resulting pellet was resuspended in buffer F and loaded onto a five-step sucrose gradient consisting of 1.1, 1.3, 1.5, 1.7, and 1.9 M sucrose layers (in 50 mM Tris–HCl, pH 8.0). After ultracentrifugation (24,000*g*, 16 h, 4 °C), the brown fraction containing the IMVs was collected, diluted in 300 ml buffer F, and recollected by ultracentrifugation (35,000*g*, 90 min, 4 °C). Purified IMVs were suspended in 5 ml 50 mM Tris–HCl, 50 mM KCl, 5 mM MgCl2, pH 8.0 (buffer G). To inactivate endogenous SecA, IMVs were treated with 6 M urea (30 min, 4 °C). Urea-stripped IMVs were sedimented (35,000*g*, 35 min, 4 °C) through a sucrose cushion (0.2 M sucrose, 50 mM Tris–HCl, pH 8.0, 50 mM KCl), washed with buffer G, and after a second centrifugation step resuspended into 5 ml buffer G.

The suspension was homogenized with a homogenizer and the membrane suspension was passed through a lipid extruder for better homogenization. Protein concentrations were estimated by the Bradford assay, using bovine serum albumin as the standard. SecA-stripped IMVs were stored in small aliquots at -80 °C.

SecA ATPase activity measurements

The ATPase activity of SecA was quantified using the malachite green colorimetric method for the detection of free inorganic phosphate (P_i) [22]. Unless otherwise indicated, ATPase reactions (50 μl) were performed at room temperature (22 °C) in 50 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.4 mg/ml BSA, 0.5 μM (50 μg/ml) wild-type or ecSecA(W775A), 100 μM ATP, pH 8.0. ATPase reactions were stopped by adding 150 μl of the Biomol Green Reagent, incubated for 20 min at room temperature, and measured at 620 nm with a Tecan Infinite M200 microplate reader. ATP hydrolysis rates were calculated using a standard curve from known concentrations of inorganic phosphate solution (Sigma).

Screening of chemical library for SecA inhibitors

The chemical library (~27,000 small-molecules) of the Centre for Drug Discovery and Design (CD3) at KU Leuven was screened for inhibitors of *E. coli* SecA W775A. Molecules in the CD3 collection were purchased from various commercial vendors; these compounds were selected based on different parameters (e.g., Lipinski's rule of five, solubility, toxicophores, frequent hitters, unstable fraction) [23]. All compounds were dissolved in 100%

DMSO to a concentration of 5 mM and stored in 384-well master plates at −20 °C. Master plates were thawed at room temperature on the day of the screen and used to prepare 384-well daughter plates containing 1 mM compound stock solutions in 20% DMSO. Screening for SecA inhibitors was carried out in 384-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) using an Evo 200-based liquid handling robot system (Tecan Group Ltd., Männedorf, Switzerland). In each microplate, 32 positive (50 mM EDTA) and negative (no compound, 0.4% DMSO) control wells were included. ATPase reactions were performed in a total reaction volume of 20 μl, with the following final concentrations of reagents: 0.5 μM W775A SecA in 50 mM Tris–HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 0.4 mg/ml BSA, 0.4% DMSO, 20 μM compound, and 100 μM ATP.

After a 2-h incubation at room temperature (22 °C), ATPase reactions were stopped by adding 80 μl Biomol Green Reagent. Plates were incubated at room temperature (22 °C) for 30 min and absorbance was read at 620 nm in an Envision 2104 microplate reader (Perkin Elmer, Turku, Finland). Screening data were analyzed using the Pipeline Pilot platform (Accelrys, San Diego, CA, USA). The residual intrinsic SecA ATPase activity was analyzed on a plate-to-plate basis by comparing the A_{620} value per compound well with the plate-averaged control wells using the relationship

$$\%\ \text{residual ATPase activity} = \left(\frac{A_{620} - \mu_{pos}}{\mu_{neg} - \mu_{pos}}\right) \times 100, \quad (1)$$

where A_{620} is the well-specific absorbance value, and μ_{neg} and μ_{pos} are the plate-averaged negative and positive control values, respectively.

To assess the quality of the high-throughput screening (HTS) assay, the Z' factor for each assay plate was determined using the formula

$$Z' = 1 - \left(\frac{3\sigma_{neg} + 3\sigma_{pos}}{\mu_{neg} - \mu_{neg}}\right), \quad (2)$$

where σ_{neg} and σ_{pos} are defined as the calculated standard deviations in the plate-averaged negative and positive controls, and μ_{neg} and μ_{pos} are the plate-averaged negative and positive control values, respectively.

Inhibition of the intrinsic SecA(W775A) ATPase activity

Initial rates of intrinsic ATP hydrolysis were measured in duplicate as described above, either in the presence of vehicle (DMSO) or compound (0–200 μM). Following a preincubation for 10 min at 22 °C, 100 μM ATP was added to start the reactions. ATPase reactions were stopped by adding 150 μl of the Biomol Green Reagent, incubated for 20 min at room temperature, and read at 620 nm. From these values, the OD values obtained at each compound concentration without enzyme were subtracted for background correction. Data were expressed as the percentage of the rate of ATP hydrolysis in the absence of inhibitor. IC_{50} values were estimated by fitting the data by nonlinear regression analysis to a sigmoidal inhibition curve with variable slope using GraphPad Prism.

Inhibition of the translocation ATPase activity of wild-type SecA

Translocation ATP hydrolysis by WT ecSecA was measured at 37 °C in the presence of various inhibitor concentrations (0–200 μM) using the malachite green method as described before. Reaction mixtures (50 μl) containing WT ecSecA (50 μg/ml), urea-stripped IMVs (100 μg/ml total protein), the preprotein AlkProPhoA (300 μg/ml), and inhibitor or DMSO were preincubated for 10 min at 37 °C. ATPase reactions were started by the addition of 1 mM ATP and stopped by the addition of 150 μl Biomol Green Reagent. Data were expressed as the percentage of the rate of ATP hydrolysis in the absence of inhibitor. IC_{50} values were estimated by fitting the data by nonlinear regression analysis to a sigmoidal inhibition curve with variable slope using GraphPad Prism.

Inhibition of in vitro preprotein translocation

To investigate the effect of hit compounds on *in vitro* translocation, inhibitors were tested in a preprotein translocation assay, as described [20]. Briefly, translocation ATPase reactions (50 μl) were stopped after a 20 min incubation at 37 °C by placing the tubes on ice. Translocation of the preprotein AlkProPhoA was assayed by protease accessibility, using a final protease K concentration of 1 mg/ml (15 min digestion on ice). To control for *bona fide* translocation into the lumen of the IMVs, Triton X-100 (1% v/v final concentration) was added to one reaction prior to protease K digestion. After a 20 min incubation of the digestion mixture on ice, samples were precipitated with 12.5% (w/v) trichloroacetic acid, washed with ice-cold acetone, and solubilized in SDS sample buffer. Samples were analyzed by SDS–PAGE and Western blotting, using AlkProPhoA-specific antibodies.

Results

Assay optimization and validation

In the absence of ongoing translocation, the ATPase activity of *E. coli* SecA is suppressed by several intramolecular mechanisms which prevent futile ATP hydrolysis in the cytoplasm [18,24]. However, the ATPase activity of SecA is allosterically regulated and is stimulated by translocation ligands such as acidic phospholipid membranes, SecYEG, or preprotein [9,25]. Although several *in vitro* assays are available for measuring the intrinsic, membrane or translocation ATPase activities of *E. coli* SecA, these assays are not readily applicable for HTS applications. A major drawback of the membrane and translocation ATPase assays is the complexity of the assay system, which requires the use of inner membrane vesicles or proteoliposomes containing functional SecYEG complexes and also unfolded preproteins. Furthermore, the intrinsic ATPase activity of ecSecA is low ($k_{\text{cat}} \sim 5$ pmol P_i/pmol SecA protomer/min at 37 °C) which translates in a poor signal-to-background ratio. Because liquid handling systems used for automated HTS are usually not equipped with a temperature-controlled chamber, high-throughput screening reactions frequently must be performed at room temperature. At this temperature, however, the intrinsic activity of wild-type *E. coli* SecA is >10-fold lower (k_{cat} values) compared to the same activity at 37 °C, making it difficult to obtain high-quality screening data.

To tackle these issues, we have developed a high-throughput screening assay employing an ecSecA mutant (ecSecA(W775A)) with elevated intrinsic ATPase activity. The mutated residue, Trp^{775}, is located at the interface of the scaffold domain (SD) and the intramolecular regulator of ATPase activity domain 1 (IRA1), two subdomains of the C-domain of SecA (Fig. 1) [4,18]. The SD and IRA1 domains are important for the regulation of the ATPase activity of SecA as they serve as a molecular switch essential for coupling preprotein binding (which occurs at the preprotein binding domain; PBD) to ATP hydrolysis (catalyzed by the DEAD motor domain) [24]. In the absence of translocation ligands, the SecA ATPase activity is suppressed by intramolecular interactions between the C-domain and the DEAD motor domain. It has been demonstrated that Trp^{775} plays a key role in this interaction and that mutation of the bulky, hydrophobic Trp side chain for a short

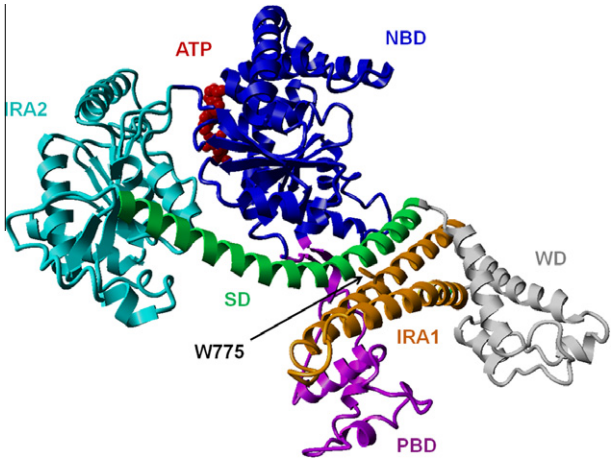

Fig.1. Ribbon representation of ecSecA mutant W775A. The figure was created with YASARA View (www.yasara.org), using the crystallographic coordinates of WT ecSecA (PDB code 2FSG). Color coding of SecA domains; NBD, dark blue; IRA2, cyan; PBD, magenta; IRA1, orange; SD, green; WD, gray. The side chain of Trp^{775} is shown as a stick model. The bound ATP molecule is shown in ball representation (red). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

alanine side chain results in a weakened C-domain/DEAD motor domain interaction and a ~5-fold increase in intrinsic ATPase activity compared to wild-type ecSecA [17,18,26].

The ecSecA(W775A) ATPase assay relies on the reaction of a molybdate–ascorbic acid complex with inorganic phosphate to produce an aggregate phosphomolybdate complex having a strong absorbance in the 600- to 700-nm range [22]. Initial experiments showed a linear relationship between the absorbance at 620 nm of the phosphomolybdate–malachite green complex and the P_i concentration from 0 to 2 nmol in a total assay volume of 20 µl (data not shown).

Because the Tecan liquid handling system used for HTS is not equipped with a temperature-controlled chamber, we decided to perform all screening reactions at room temperature (22 °C).

At this temperature, the rate of intrinsic ATP hydrolysis by ecSecA(W775A) was linear with incubation time and enzyme concentration in the full range tested (Figs. 2A and B).

Next, the kinetic parameters k_{cat} and K_m of ecSecA(W775A) for ATP were determined at 22 °C by measuring initial rates of intrinsic ATP hydrolysis at different substrate concentrations (from 0 to 2 mM ATP). As can be deduced from Fig. 3, the intrinsic ATPase activity of ecSecA(W775A) obeyed Michaelis–Menten kinetics. K_m and k_{cat} values obtained from fitting the data to the Michaelis–Menten equation were 45.6 µM and 1.36 min^{-1}, respectively. Based on the above observations, the ATP concentration for subsequent assays was fixed at 100 µM ATP and a 2-h incubation at 22 °C was used. Under these conditions, competition of ATP with small-molecule inhibitors for binding to the SecA active site is minimized, while still a good signal-to-background ratio in the absence of inhibitor is obtained.

In order to verify the assay's tolerance to DMSO, initial rates of ATP hydrolysis were measured in the presence of varying concentrations of DMSO (0–10%). At concentrations up to 2%, DMSO did not affect the stability of the color signal or the enzyme reaction (Fig. 4A).

Because no commercially available high-affinity SecA inhibitors have been described yet, we decided to use the metal chelator EDTA as a positive control (i.e., complete inhibition of the ATPase) in our HTS assay. It has been demonstrated that the ATPase activity of *E. coli* SecA is regulated by divalent metal ions such as Ca^{2+} and Mg^{2+} and that SecA-catalyzed ATP hydrolysis can be completely

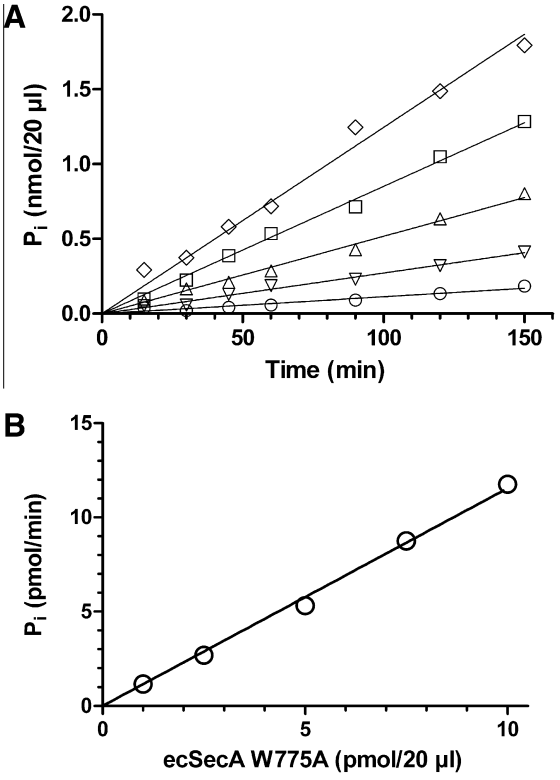

Fig.2. (A) Time courses of intrinsic ATP hydrolysis by ecSecA(W775A). EcSecA(W775A) (1–10 pmol) was incubated at room temperature (22 °C) with 1 mM ATP in 50 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.4 mg/ml BSA, pH 8.0) in a total reaction volume of 20 µl. At several time points after the start of the ATPase reaction, released P_i was quantified using the malachite green method as described under Experimental procedures. Data represent the average of three independent experiments, and standard deviations were less < 5%. Symbols used: ○, 1 µg SecA/20 µl; ▽, 2.5 µg SecA/20 µl; △, 5 µg SecA/20 µl; □, 7.5 µg SecA/20 µl; ◇, 10 µg SecA/20 µl. (B) Enzyme concentration dependence of the intrinsic ATPase activity of ecSecA(W775A). From the progress curves in Fig. 2A, initial rates of intrinsic ATP hydrolysis were determined by linear regression and plotted as a function of the enzyme concentration.

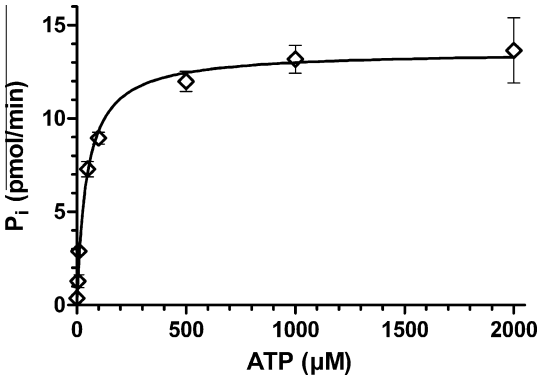

Fig.3. Michaelis–Menten plot of the intrinsic ATPase activity of ecSecA(W775A) at 22 °C. Initial rates of ATP hydrolysis by ecSecA(W775A) (10 pmol in 50 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.4 mg/ml BSA, pH 8.0) were measured at different ATP concentrations (0–2 mM) using the malachite green method as described under Experimental procedures. Values are the means of three independent experiments, with error bars representing the standard deviation.

abolished by adding EDTA to the reaction [27]. In order to check the minimal EDTA concentration required for total inhibition of the intrinsic ecSecA(W775A) activity, initial rates of ATP hydrolysis were measured in the presence of varying concentrations of EDTA. The minimal EDTA concentration required for complete inhibition of the intrinsic activity of ecSecA(W775A) was 50 mM (Fig. 4B).

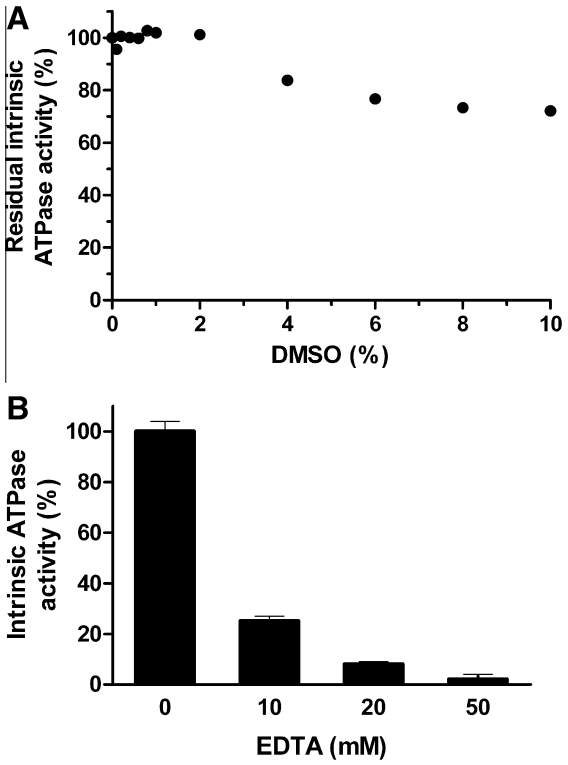

Fig.4. (A) Effect of DMSO on the intrinsic ATPase activity of ecSecA(W775A). Initial rates of ATP hydrolysis by ecSecA(W775A) (10 pmol in 50 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.4 mg/ml BSA, 100 μM ATP, pH 8.0) were measured in the presence of different DMSO concentrations using the malachite green method as described under Experimental procedures. Rates are expressed relative to the rate of ATP hydrolysis measured in the absence of EDTA. Data represent the average of three independent experiments, and standard deviations were less than 5%. (B) Effect of EDTA on the intrinsic ATPase activity of ecSecA(W775A). Initial rates of ATP hydrolysis by ecSecA(W775A) (10 pmol in 50 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.4 mg/ml BSA, 100 μM ATP, pH 8.0) were measured in the absence and presence of different EDTA concentrations (10, 20, and 50 mM) using the malachite green method as described under Experimental procedures. Rates are expressed relative to the rate of ATP hydrolysis measured in the absence of EDTA. Values are the means of three independent experiments, with error bars representing the standard deviation.

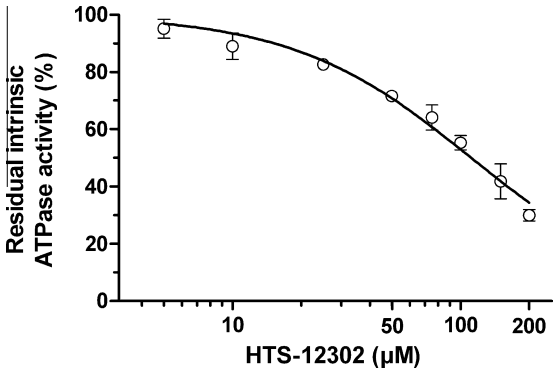

Fig.5. Inhibition of the intrinsic ATPase activity of W775A by compounds HTS12302. Initial rates of intrinsic ATP hydrolysis by ecSecA(W775A) (10 pmol/20 μl) were measured at 22 °C in the presence of 100 μM ATP and various concentrations (0–200 μM) of compound HTS12302. Rates were expressed relative to the rate of ATP hydrolysis measured in the absence of compound. IC_{50} values were determined by fitting the data by nonlinear regression analysis to a *one-phase* exponential *decay* curve, using GraphPad Prism. Values are the means of three independent experiments, with error bars representing the standard deviation.

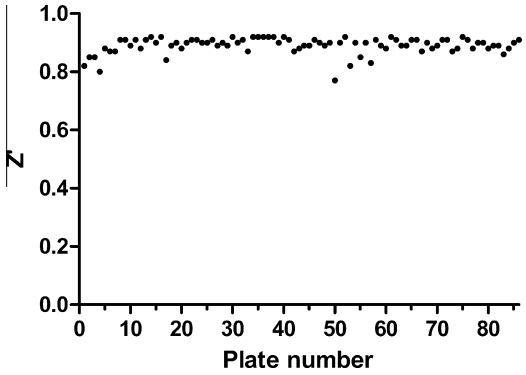

Fig.6. Assay performance of SecA HTS. For each screened 384-well plate (86 in total), the *Z′* factor was calculated as described under Experimental procedures. The average *Z′* factor was 0.89, indicating that the assay was adequately optimized for high-throughput screening.

To validate the optimized assay for inhibitor screening, we have evaluated the inhibitory activity of the known SecA inhibitor HTS12302, which was discovered by virtual ligand screening [14]. The IC_{50} value for inhibition of the intrinsic ATPase activity of ecSecA(W775A) by this compound was 116 μM (Fig. 5), in agreement with the reported IC_{50} of 100 μM against the DEAD motor domain of ecSecA [14].

High-throughput screening

The optimized ecSecA(W775A) ATPase assay in 384-well format was automated and used to screen the diverse compound library (~27,000 molecules) of the Centre for Drug Discovery and Design at KU Leuven, as described under Experimental procedures. The performance of the SecA screening assay was assessed using the *Z′* factor, which defines the difference between the positive and the negative controls of the dynamic signal being measured and the data variation of that signal (see Eq. (2)). Robust and reproducible HTS assays have a *Z′* factor ranging from 0.5 to 1 [28]. The average *Z′* value for the SecA assay was 0.89 (Fig. 6), consistent with a high-quality HTS assay.

Molecules showing more than 50% inhibition of the intrinsic ATPase activity of W775A ecSecA were considered as hits and further characterized in ATPase activity assays at 37 °C using wild-type ecSecA. The identified hit compounds did not only inhibit the intrinsic SecA ATPase activity but also SecA's membrane and translocation ATPase activity (Fig. 7A) and the *in vitro* translocation of the model preprotein AlkproPhoA (Fig. 7B). Remarkably, the inhibitory activity of hit compounds against W775A ecSecA was 5- to 7-fold higher compared to the activity against wild-type ecSecA (data not shown). Further characterization of hit molecules is in progress (data to be published).

Discussion

Drug resistance is an ever-increasing problem in the chemotherapy of bacterial infectious diseases. The *de novo* development and spread of drug-resistant bacteria and the horizontal transfer of resistance factors among bacteria have resulted in a dramatic increase in the incidence of drug-resistant infections, creating an urgent need to identify novel antibacterial targets and leading to new mechanisms of action. A promising target for antibacterial chemotherapy is protein secretion because the bacterial Sec secretion machinery is essential for viability, highly conserved among bacterial species, and has components with no eukaryotic counterparts.

A major pathway for protein translocation across and, if applicable, integration into the cytoplasmic membrane is provided by

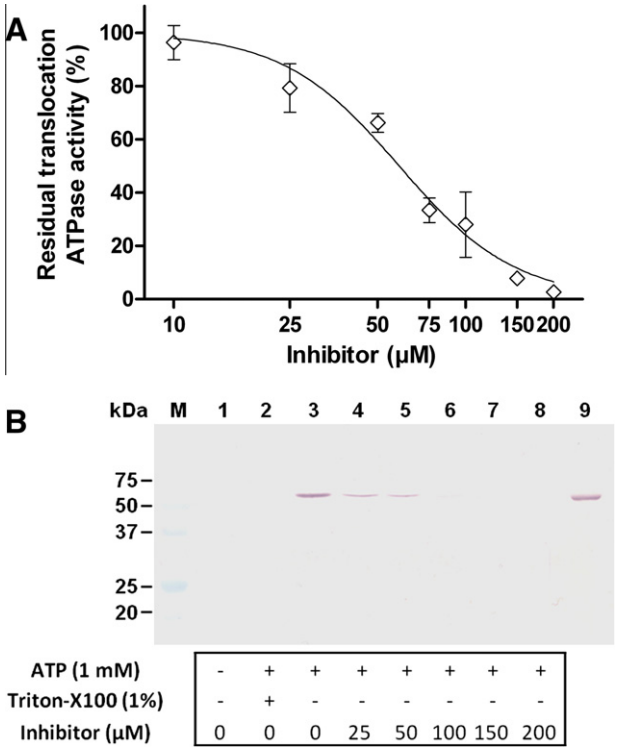

Fig.7. (A) Inhibition of the translocation ATPase activity of wild-type *E. coli* SecA by one of the hit compounds identified via HTS. Translocation ATP hydrolysis by WT ecSecA was measured at 37 °C in the presence of various compound concentrations (0–200 μM) as described under Experimental procedures. Rates were expressed relative to the rate of ATP hydrolysis measured in the absence of compound. (B) Inhibition of the *in vitro* preprotein translocation activity of wild-type *E. coli* SecA by one of the hit compounds identified via HTS. Translocation reactions were incubated at 37 °C in the presence of different concentrations of the hit compound (Lanes 3–8, 0–200 μM). After protease K digestion, samples were analyzed by SDS–PAGE and Western blotting with preprotein-specific antibodies. Lane 1, minus ATP control; Lane 2, Triton X-100 control; Lane 9, purified AlkProPhoA.

the Sec-dependent export pathway. Because the energy for the translocation reaction is provided by the peripheral membrane ATPase SecA, blocking of the SecA ATPase activity results in the accumulation of preproteins in the cytoplasm and subsequent cell death [29,30]. As a consequence, targeting the ATPase activity of SecA by small-molecule inhibitors provides an attractive approach for the discovery and development of antibiotics with a novel action mechanism.

A number of efforts have already been carried out to discover small-molecule SecA inhibitors [13–15,31]. Most of these studies, if not all, are small-scale screening campaigns of relatively small chemical libraries. One of the obvious difficulties in SecA drug discovery stems from the involvement of hydrophobic membranes in the Sec system, which makes the development of *in vitro* HTS assays cumbersome. For this reason, we have developed a colorimetric high-throughput assay employing a SecA mutant with elevated intrinsic ATPase activity, enabling the screening of large compound libraries in a lipid-free assay system. Screening of a chemical library consisting of ~27,000 compounds indicated that the assay was suitable for automation and robust, evidenced by an average Z' factor of 0.89. The screen has resulted in the identification of a number of small-molecule inhibitors of the intrinsic, membrane, and translocation ATPase activity of wild-type *E. coli* SecA (data to be published). Overall, the W775A ecSecA mutant was 5- to 7-fold more sensitive to inhibition compared to wild-type ecSecA. This may be explained by the W775A mutation which partially relieves the IRA1-mediated suppression of the intrinsic SecA ATPase activity. This hypothesis is further supported by the observation that *E. coli* SecA N68, which lacks the C-terminal 34-kDa regulatory domain (including the IRA1 domain) is about 10-fold more sensitive to inhibition by small-molecule inhibitors compared to wild-type SecA [13].

The identified molecules belong to different chemical classes, including the pyrrolopyrimidines and nipecotic acid derivatives (among others). These molecules may be useful as molecular tools for studying bacterial protein secretion or as ligands for crystallization studies, which will aid in the rational design and further structural optimization of SecA inhibitors with improved activity.

In conclusion, we have developed and validated a HTS assay in 384-well format for the identification of small-molecule SecA inhibitors. The developed HTS assay will facilitate the screening of large chemical libraries against SecA and aid in the discovery of new antibacterials with a novel mode of action.

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